Aus dem Institut für Hygiene und Infektionskrankheiten der Tiere
im Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Giessen

Immunmodulatorische Wirkung und pathogenetische Bedeutung der *Escherichia coli* Shigatoxine beim Rind

HABILITATIONSSCHRIFT

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Dr. med. vet. Christian Menge

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meiner Familie gewidmet

Abkürzungsverzeichnis

AP-1	*activator protein 1*
Apaf-1	*apoptotic protease activating factor*
B23	Nukleoplasmin
Bak	*Bcl-2 homologous antagonist/killer*
Bax	*Bcl-2 associated X protein*
Bcl-2	*B cell lymphomal leukemia-2*
BID	*BH3-interacting domain death agonist*
cAMP	zyklisches Adenosinmonophosphat
CAD	*Caspase activated DNAse*
Caspase	*cystein protease cleaving an aspartic acid residue*, Kaspase
CD	*Cluster of Differentiation*
CD_{50}	zytotoxische Dosis 50 %
Cer	zeramide, Zeramid
DC	*dendritic cell*, dendritische Zelle
EHEC	enterohämorrhagische *Escherichia coli*
ER	endoplasmatisches Retikulum
ERK	*extracellular signal-regulated kinase*
FCS	*fetal calf serum*, fötales Kälberserum
Gal	Galaktose
GalNAc	N-Azetyl-Galaktosamin
Gb_2	Galabiosylzeramid
Gb_3	Globotriaosylzeramid
Gb_4	Globotetraosylzeramid
Glc	Glukose
GRO-α	*growth-related oncogene alpha*
GTP	Guanosintriphosphat
HC	*hemorraghic colitis*, Hämorrhagische Kolitis
Hsp70	Hitzeschockprotein 70
HUS	*hemolytic uremic syndrome*, Hämolytisch-Urämisches Syndrom
IL	Interleukin
IFN	Interferon
IFNAR	Interferon Typ I-Rezeptor
IκBα	*inhibitor of κB*, NF-κB-Inhibitor
IP-10	*10 kDa Interferon Inducible Protein*
JNK	*jun N-terminal kinase*

Kaspase	siehe Caspase
kDa	Kilodalton
lee	*locus of enterocyte effacement*
LPS	Lipopolysaccharid
MALDI-TOF-MS	*matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*
MAPKp38	Mitogen-aktivierte Proteinkinase p38
MEKK1	MAPK-Kinase-Kinase 1
MHC	*major histocompatibility complex*, Haupthistokompatibilitätskomplex
MCP-1	*monocyte chemoattractant protein 1*
mpk-1	Gen für die *mitogen-activated protein kinase phosphatase*
NAD	Nikotinamidadenindinukleotid
NF-κB	nuclear factor κB
PARP	poly(ADP-Ribose)-Polymerase
PBMC	*peripheral blood mononuklear cells*, mononukleäre Zellen des peripheren Blutes
PHA-P	Phytohämagglutinin P
PKC	Proteinkinase C
p.n.	post natum
RANTES	*regulated upon activation, normal T cell expressed, and secreted*
real-time RT-PCR	*real-time reverse transcriptional polymerase chain reaction*, revers-transkriptionale Polymerase-Kettereaktion in Echtzeit
ROS	*reactive oxygen species*, reaktive Sauerstoffmetaboliten
rStxB1	rekombinant gewonnene B-Untereinheit des Shigatoxins 1
SAPK	Stress-aktivierte Proteinkinase
SEK1/MMK4	*stress-activated protein kinase/extracellular signal-regulated kinase kinase 1/4*
STEC	Stx-bildende *Escherichia coli*
Stx	Shigatoxin, syn. Verotoxin
StxB	B-Untereinheit des Shigatoxins
StxA	A-Untereinheit des Shigatoxins
tBID	*truncated BID*, gespaltenes BID
TGF-β	*transforming growth factor beta*
TLR	„Toll-like“ Rezeptor
TNF-α	Tumor-Nekrose-Faktor alpha
vs.	versus

1. EINLEITUNG

Bakterien, die die Darmschleimhaut von Säugetieren über längere Zeiträume kolonisieren, sind in Koevolution mit dem mukosalen Immunsystem entstanden. Dabei haben die Bakterien Pathogenitätsmechanismen entwickelt, die es ihnen erlauben die Immunabwehr ihrer Wirte zu modulieren. Zu den am besten charakterisierten bakteriellen Immunmodulatoren gehören die hitze-labilen Enterotoxine (LT) von *Escherichia coli* und das Choleratoxin (CT) von *Vibrio cholerae*, die zu den Toxinen mit einer 5B-plus-A-Grundstruktur zählen. Diese Holotoxine sind zwar wirksame Adjuvanzien, ihre Untereinheiten besitzen aber zum Teil gegenteilige immunologische Wirkungen. So stimulieren die Rezeptor-bindenden B-Untereinheiten die Sekretion pro-inflammatorischer Zytokine (98). Die rezeptor-vermittelte Endozytose der enzymatisch aktiven A-Untereinheit kann dagegen die Hemmung der Antigen-Prozessierung (301) und die Induktion anti-inflammatorischer Zytokine nach sich ziehen (98). Obwohl auch eine Bindung an „Toll-like“-Rezeptoren diskutiert wird (99), wirken diese bakteriellen Immunmodulatoren vor allem über bestimmte Glykolipid-Rezeptoren (Ganglioside, v.a. GM1) der eukaryontischen Zellmembran (301).

Shigatoxine, die von den sogenannten Shigatoxin-bildenden *E. coli* (STEC; *syn.* enterohämorrhagische *E. coli*, EHEC) sezerniert werden, ähneln in ihrem Aufbau LT und CT (303). Aufgrund der hohen enzymatischen Aktivität der A-Untereinheit, die eine Hemmung der zellulären Proteinbiosynthese katalysiert, werden die Shigatoxine vor allem zu den zytoletalen Toxinen gerechnet (234). Sie können aber unabhängig von ihrer enzymatischen Aktivität eine Vielzahl anderer zellulärer Reaktionen auslösen (333) und durch die Stimulierung dendritischer Zellen auch als Adjuvanz wirken (243). Dabei dienen den Shigatoxinen ebenfalls Glykolipide (Glykolipide der Globo-Serie) als zelluläre Rezeptoren (179).

Als das wichtigste Reservoir für STEC werden asymptomatisch, aber persistent infizierte Rinder angesehen. Die molekularen Grundlagen der dauerhaften Kolonisation der bovinen Darmschleimhaut durch STEC sind bislang nur ungenügend aufgeklärt (227, 304, 315). In der vorliegenden Arbeit wurde deshalb der Frage nachgegangen, ob Shigatoxine bei der bovinen STEC-Infektion als mukosale, kolonisationsfördernde Immunmodulatoren wirken können. Da bakterielle Glykolipid-Rezeptor-Agonisten als mukosale Adjuvanzien auch beim Rind Verwendung finden (368), dienen die Untersuchungen mit Stx auch dem grundlegenden Verständnis der Wirkung dieser Substanzen auf das Schleimhaut-assoziierte Immunsystem.

2. SCHRIFTTUM

2.1 Shigatoxine von *Shigella dysenteriae* und *Escherichia coli*

2.1.1 Toxinvarianten

Die Familie der Shigatoxine (Stx) ist nach einem seit Anfang des letzten Jahrhunderts bekannten Zytotoxin von *Shigella dysenteriae* Typ 1 benannt. Wie andere bakterielle Toxine (Choleratoxin von *Vibrio cholerae*, hitzelabiles Enterotoxin von *Escherichia coli*), gehört Stx zu den heteromeren Protein-Toxinen, die aus einer aktiven (StxA-; 32 kDa) und fünf rezeptorbindenden (StxB-; 7,7 kDa) Untereinheiten bestehen (140). 1977 wurde von Konowalchuk *et al.* (165) das Zytotoxin eines *E. coli* Isolates beschrieben, das, aufgrund seiner Toxizität für Vero-Zellen, als Verotoxin bezeichnet wurde. O'Brien und LaVeck bestätigten seine nahe Verwandtschaft zum Shigellen-Toxin (235). Inzwischen werden mehrere von *E. coli* produzierte und mit dem Shigellen-Toxin verwandte Zytotoxine unterschieden, die sich in zwei Gruppen aufteilen lassen. Zur ersten Gruppe sind das Toxin von *Shigella dysenteriae* selbst und das Stx1 von *Escherichia coli* zu rechnen. Beide Toxine sind auf Proteinebene bis auf eine Aminosäure in der A-Untereinheit identisch (319). Zusätzlich konnten noch die Varianten Stx1c (372) und Stx1d (27) nachgewiesen werden, die eine 91-95 %ige Nukleotidsequenzhomologie zu Stx1 aufweisen. Die zweite Gruppe, deren Aminosäuresequenz nur ca. 56 % homolog zu Stx1 ist, umfasst Stx2 mit den Varianten Stx2c (294), Stx2d (205), Stx2e (350), Stx2f (293) und Stx2g (173). Antiseren gegen die Varianten, die untereinander eine Aminosäurehomologie von 84-99 % aufweisen, zeigen zumindest eine partielle Kreuzneutralisation, nicht jedoch gegenüber der Stx1-Gruppe (78). Stx2e besitzt zu den anderen Toxinvarianten der Stx2-Gruppe hinsichtlich Aminosäuresequenz und biologischer Aktivität die geringste Ähnlichkeit. Stx2e bindet auch bevorzugt an einen anderen Rezeptor auf Zielzellen und stellt das ursächliche Agens für die *Ödemkrankheit* des Ferkels dar (51, 79). Die Gene für Stx1, Stx2 und die Stx2-Varianten kommen im Genom der Shigatoxin-bildenden *E. coli* (STEC) als Teil des Genoms lambdoider Bakteriophagen vor (292). Die Bakterien können zum Teil mehr als einen Toxintyp bilden, da sie mehrere *stx*-konvertierende Phagen enthalten können. Die Phagen ermöglichten die horizontale Übertragung der *stx*-Gene sowohl zwischen verschiedenen *E. coli*-Stämmen als auch auf *Citrobacter freundii*- und *Enterobacter cloacae*-Stämme (108).

2.1.2 Struktur

Die *stx*-Gene kodieren für zwei Polypeptidketten entsprechend der Toxinuntereinheiten. Die Translationsprodukte umfassen jeweils eine Peptidkette für die reife Untereinheit, der eine N-terminale Signalsequenz aus 22 (StxA) bzw. 19-20 (StxB) Aminosäuren vorausgeht (50, 131, 294). Bei der Ausschleusung der Peptidketten in den periplasmatischen Raum von *E. coli* werden diese Signalsequenzen durch die membranständige Signalpeptidase I abgespalten (131).

Die 5 B-Untereinheiten des Shigatoxins 1 lagern sich spontan, ohne Ausbildung kovalenter Bindungen, zu einem pentameren Ring zusammen (266). Jede einzelne Untereinheit besteht aus 69 Aminosäuren mit einer internen Disulfid-Brücke zwischen den Zystein-Resten in Position 4 und 57 (**Tab. 2.1**) (298). Die StxB1-Sekundärstruktur weist 2 dreisträngige, antiparallele β-Faltblätter auf, die zur Aussenseite des Pentamers gerichtet sind (312). Zwei dreisträngige Faltblätter benachbarter Monomere formen ein sechssträngiges β-Faltblatt. Das β2-Faltblatt eines Monomers interagiert dabei über Wasserstoff-Brücken mit dem β6-Faltblatt des nächsten Monomers. Zwischen den Monomeren bildet sich eine Tasche, in der eine der bis zu drei möglichen Rezeptor-Bindungsstellen lokalisiert ist (11). Jedes Monomer enthält weiterhin eine α-Helix, die zum Zentrum des pentameren Rings hin ausgerichtet ist und mit den α-Helices der anderen 4 Monomere eine zentrale 11 Å-weite Pore auskleidet (**Abb. 2.1**) (312). Während die zentralen α-Helices bei pH-Änderungen, wie sie bei der Endozytose des Toxins im Endosom auftreten, eine Konformationsänderung aufweisen, sind die aussen gelegenen, die Rezeptor-Bindungsstelle bildenden β-Faltblätter gegen Veränderungen relativ stabil (278).

Tabelle 2.1: Funktion der Aminosäurereste in den B-Untereinheiten der Shigatoxine

Aminosäure in Toxin:			Funktion:	Referenz:
Stx1	Stx2 [1]	Stx2e		
(-20)-(-1)	(-19)-(-1)	(-19)-(-1)	Signalsequenz	(50, 131, 294)
	Ala1		Wasserstoffbrücke zu Ser53; Teil der Rezeptor-Bindungsstelle **II**	(76)
3-8			β1-Faltblatt	(312)
Cys4	Cys3		Disulfid-Brücke zum Cys57 (essentiell für Rezeptorbindung); Teil der Rezeptor-Bindungsstelle **II**	(76, 132, 298)
9-14			β2-Faltblatt, Wechselwirkung mit dem β6-Faltblatt des nächsten Monomers (Rezeptor-Bindungsstelle **I** [2])	(312)
Lys13			Teil der Rezeptor-Bindungsstelle I [2]	(182)
10-20			hydrophile Domäne: Rezeptorbindung, Bindung neutralisierender Antikörper, hochkonserviert bei allen Stx (antigene Domäne ?)	(298)
Asp16			Rezeptorbindung	(339)
Asp17			Teil der Rezeptor-Bindungsstelle **I** [2]	(182, 339)
		Asn17	Rezeptorspezifität für Gb_4	(339)
Asp18	Asp17		Rezeptorspezifität für Gb_3/CD77	(132)
20-24			β3-Faltblatt	(312)
Thr21			Teil der Rezeptor-Bindungsstelle **I** [2]	(182, 233)
27-31			β4-Faltblatt	(312)
Glu28			Teil der Rezeptor-Bindungsstelle **I** [2]	(182, 233)
Phe30			zwischen Rezeptor-Bindungsstelle **I** und **II** gelegen; essentiell für beide Bindungsstellen	(37)
Asn32			Teil der Rezeptor-Bindungsstelle **II**	(233)
Arg33		Arg32	Teil der Rezeptor-Bindungsstelle **II** und **III**	(76, 233, 249)
Trp34	Trp33		Teil der Rezeptor-Bindungsstelle **III**	(11, 76, 183)
Asn35	Asn34		Teil der Rezeptor-Bindungsstelle **III** ?	(76)

Tabelle 2.1: Fortsetzung

Aminosäure in Toxin:			Funktion:	Referenz:
Stx1	Stx2 [1]	Stx2e		
36-46			α-Helix, bildet mit den α-Helices der anderen 4 B-Untereinheiten die zentrale Pore des Pentamers und hat Kontakt mit der antiparallelen α-Helix des A_2-Fragmentes; Konformationsänderung bei niedrigem pH-Wert (Bedeutung für die Translokation des A_2-Fragmentes)	(75, 278, 312)
Ala43		Ala42	Rezeptorbindung	(249)
49-53			β5-Faltblatt	(312)
Lys53		Lys52	Rezeptorbindung (Beteiligung an Rezeptorspezifität ?)	(132)
	Ser53		Wasserstoffbrücke zu Ala1; Teil der Rezeptor-Bindungsstelle **II**	(76)
Cys57	Cys56		Disulfid-Brücke zum Cys4 (essentiell für Rezeptorbindung); Teil der Rezeptor-Bindungsstelle **II**	(76, 132, 298)
Gly60		Gly59	Teil der Rezeptor-Bindungsstelle I [2]	(182, 249)
65-68			β6-Faltblatt, Wechselwirkung mit dem β2-Faltblatt des vorhergehenden Monomers (Rezeptor-Bindungsstelle **I** [2])	(312)
Glu65			Rezeptorspezifität für Gb_3; Teil der Rezeptor-Bindungsstelle **I** [2]	(182, 233)
		Gln64	Lokalisation des Toxins in *E. coli*	(132)
			Rezeptorspezifität für Gb_4	(339)
		Lys66	Rezeptorspezifität für Gb_4	(132, 339)
Phe68	Phe67	Phe67	Rezeptorbindung	(250)
Arg69	Asn68	Asn68	Rezeptorbindung	(250)
C-terminale 4 (-5)			Rezeptorbindung	(339)

Legende: [1] bezogen auf StxB2 mit 68 Aminosäuren

[2] Ausbildung von H-Brücken zwischen polaren und sauren Seitenketten der B-Untereinheit und polaren Gruppen der Kohlenhydrate (182, 312)

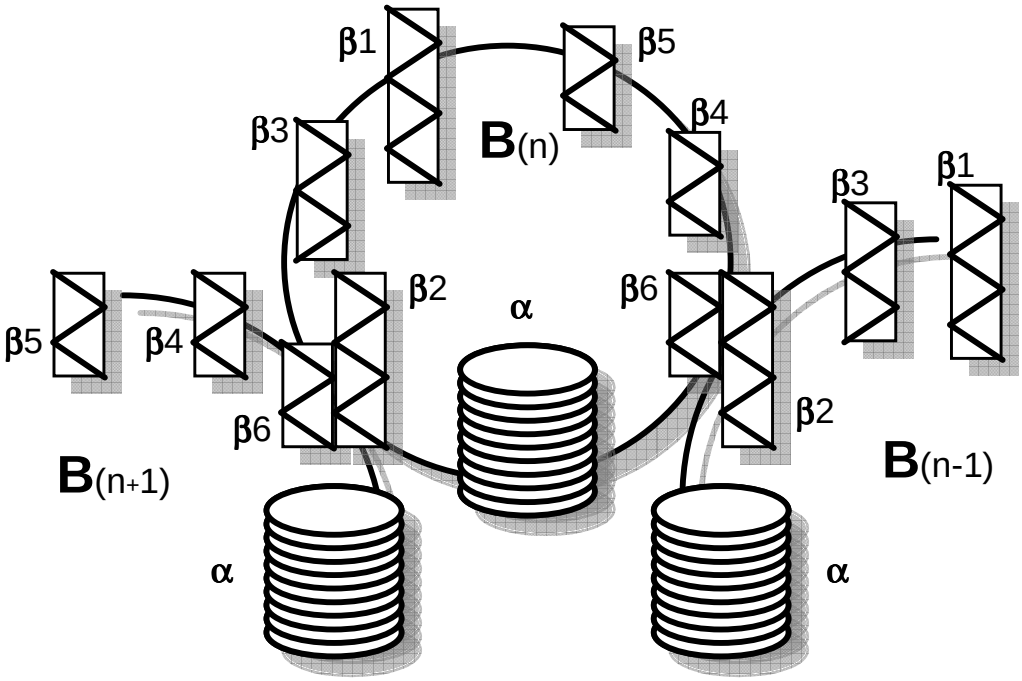

Abb. 2.1: Vereinfachtes Strukturmodell der B-Untereinheit des Shigatoxins 1 als Bestandteil eines Pentamers (Details siehe Text)

Die A-Untereinheit des Shigatoxins 1 besteht aus 293 Aminosäuren. Zwei Zystein-Reste in Position 242 und 261 sind über eine Disulfid-Brücke miteinander verbunden (50). Die dazwischen gelegenen Arginin-Reste in Position 248 und 251 sind Teil einer trypsin-sensitiven Schnittstelle. Die proteolytische Spaltung trennt das enzymatisch aktive 27 kDa A_1-Fragment von dem für die Holotoxin-Struktur verantwortlichen 6 kDa A_2-Fragment (7, 50). Neun Aminosäuren (Aminosäurerest 279-287) des C-Terminus des A_2-Fragmentes bilden eine α-Helix aus, die antiparallel zu den 5 α-Helices der B-Untereinheiten gelagert ist, die sie im Holotoxin umgeben (75, 76, 95). Die α-Helix des A_2-Fragmentes ragt dadurch 11 Å tief in die 20 Å-tiefe zentrale Pore des B-Pentamers (75, 76). Der Rest der A-Untereinheit liegt dem pentameren Ring auf (**Abb. 2.2**). Jeweils 2 β-Faltblätter des A_1- und des A_2-Fragmentes bilden dabei einen nicht-kovalenten und trotz der Symmetrie des Pentamers asymmetrischen Kontakt zum Ring aus (75, 76). Insbesondere den Aminosäuren in Position 277-278 und 288-289, die der α-Helix des A_2-Fragmentes unmittelbar N- und C-terminal benachbart sind, kommt dabei eine Bedeutung zu. Sie bilden Wechselwirkungen mit geladenen oder aromatischen Aminosäuren ausserhalb der Pore auf der planen Fläche des B-Pentamers aus (95), so dass die A-Untereinheit im Holotoxin mit 3 der 5 B-Untereinheiten in Verbindung steht (75, 76).

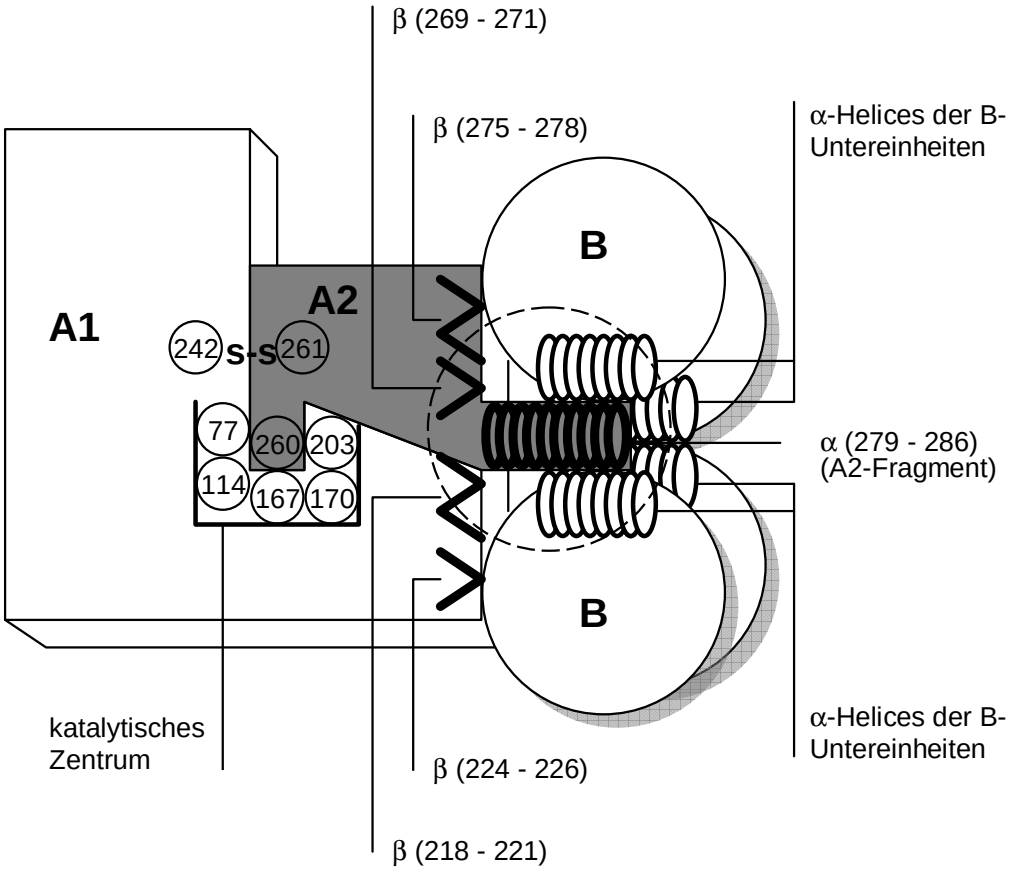

Abb. 2.2: Strukturmodell des Shigatoxin 1 Holotoxins (Zahlen in Klammern geben die an der Ausbildung der entsprechenden Sekundärstruktur beteiligten Aminosäuren an)

Die Toxine der Stx2-Gruppe setzen sich aus einer A-Untereinheit mit 296-297 Aminosäuren und B-Untereinheiten aus 68-70 Aminosäuren zusammen (94, 294, 330). Die intramolekulare Disulfidbrücke in der A-Untereinheit des Shigatoxins 2 wird zwischen Zysteinresten in den Positionen 241 und 260 ausgebildet und der Carboxyterminus der A-Untereinheit bildet nach Durchtritt durch die zentrale Pore des B-Pentamers eine kurze α-Helix aus (76). In der zu StxA1 nur zu 55 % homologen Aminosäuresequenz des StxA2 sind die für die Konformation essentiellen Aminosäuren zwar hochkonserviert (130), trotzdem weisen die Toxine weitere strukturelle Unterschiede auf. So ist im Gegensatz zu Stx1, bei dem die enzymatische Grube im Holotoxin durch den Methioninrest in Position 260 des A_2-Fragmentes blockiert wird (75), die Grube im Stx2 für Wassermoleküle zugänglich (76). Auch durch die Position des Tyrosinrestes in Position 77 unterscheidet sich das katalytische Zentrum des Shigatoxins 2 von dem des Shigatoxins 1 (76). Dessen ungeachtet ist der molekulare Mechanismus der enzymatischen Aktivität aller Shigatoxine nahezu identisch (siehe **2.3.1.4**). Für die Unterschiede in der Art und dem Umfang von Gewebeschäden, die durch Stx1- bzw. Stx2-bildende *E. coli* beim Menschen verursacht werden, werden vor allem

Unterschiede in den Rezeptor-Bindungsstellen der B-Untereinheiten verantwortlich gemacht, die Unterschiede in der Affinität der jeweiligen Toxine für den Stx-Rezeptor Gb_3/CD77 nach sich ziehen (76).

2.2 Globotriaosylzeramid (Gb_3/CD77)

Globotriaosylzeramid (Gb_3) ist der funktionelle Rezeptor für die meisten Shigatoxine. Da Gb_3 auch auf Immunzellen präsent ist, wurde es als CD77 in die Liste der Leukozyten-Antigene aufgenommen. Als funktioneller Rezeptor für Stx2e, die schweinepathogene Toxinvariante, wurde Globotetraosylzeramid (Gb_4) identifiziert.

Es gibt jedoch auch Hinweise auf die Existenz mehrerer Bindungsstellen auf Zellen mit unterschiedlicher Affinität für Stx (248, 339). Somit ist es denkbar, dass zusätzlich zu Gb_3/CD77 bzw. Gb_4 noch nicht-funktionelle Rezeptoren vorhanden sind (176). Allerdings stellt Gb_3/CD77 kein chemisch einheitliches Molekül dar. Vielmehr handelt es sich hierbei um eine Gruppe von Glykosphingolipiden, die eine identische Kohlenhydratgruppe tragen, sich aber in der Zusammensetzung ihrer Fettsäurekomponenten unterscheiden. Die Fettsäurekomponenten beeinflussen die Affinität der Toxin-Rezeptor-Bindung und den Weg der Toxin-Internalisierung. Werden auf Zellen Toxin-Bindungsstellen mit unterschiedlicher Affinität identifiziert, so könnten sich dahinter auch unterschiedliche Gb_3/CD77-Spezies verbergen, die ungeachtet ihrer Affinität als funktionelle Rezeptoren fungieren können (248).

2.2.1 Struktur, Synthese und Regulation der Expression

Bei Gb_3/CD77 und Gb_4 handelt es sich um neutrale Glykosphingolipide der Globo-Serie. Die Lipidkomponente dieser Moleküle wird von Zeramiden, Amidverbindungen zwischen einem Sphingosinmolekül und einer Fettsäure, gebildet. Neutrale Glykosphingolipide tragen an der endständigen Hydroxylgruppe des Sphingosins β1-glykosidisch gebundene Oligosaccharide, die durch Enzyme des Golgiapparates hinzugefügt werden (238). So entsteht durch Kopplung von Glukose und Galaktose an das Zeramid Laktosylzeramid (Galβ1-4Glcβ1-1Cer), das Vorläufermolekül für die Stx-Rezeptoren (129). Die UDP-Galaktose:Laktosylzeramid α1-4-Galaktosyltransferase (Gb_3-Synthetase) fügt der Saccharidkette einen weiteren Galaktoserest hinzu, wodurch Gb_3/CD77 (Galα1-4Galβ1-4Glcβ1-1Cer) entsteht (**Abb. 2.3**) (213, 238, 327). In einem weiteren Syntheseschritt entsteht

durch die Addition eines N-Azetyl-Galaktosaminrestes durch die N-Azetyl-Galaktosyltransferase das Gb_4-Molekül (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer) (180).

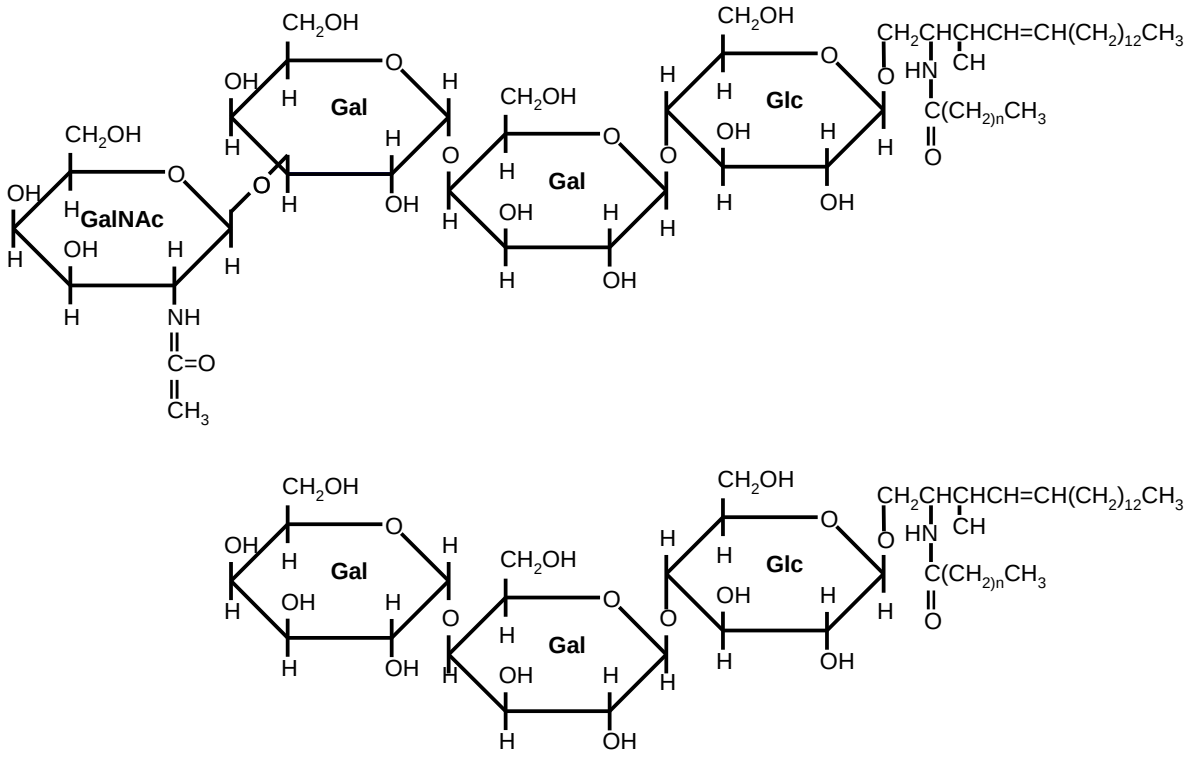

Abb. 2.3: Molekulare Struktur des Globotetraosylzeramids Gb_4 (oben) und des Globotriaosylzeramids Gb_3 (unten)

Für die meisten Toxin-empfindlichen Zellen wurden etwa $1x10^6$ bis $1x10^7$ Rezeptormoleküle pro Zelle errechnet (176, 244, 287). Die Expression hängt jedoch in vielen Zellen und Geweben von mehreren Faktoren ab. Eine aktive Zellteilung scheint die wichtigste Voraussetzung für eine maximale Empfindlichkeit der Zielzellen zu sein (237), denn die Empfindlichkeit ist von der Phase des Zellzyklus´ abhängig, in der sich die Einzelzelle befindet. Pudymaitis *et al.* (262) berichteten, dass Vero-Zellkulturen an der Grenze zwischen G1-Phase und S-Phase maximal sensibel sein sollen, bevor eine 10fache Reduktion der Empfindlichkeit eintritt. In der G1-Phase geht eine maximale Synthese von Gb_3/CD77-Rezeptoren und ihre Präsentation auf der Zelloberfläche einher mit einer maximalen Bindung von Toxinmolekülen durch die Zellen. Da der Gb_3/CD77-Gehalt der Kulturen den gesamten Zyklus hindurch nahezu konstant war, soll an der Phasengrenze eine erhöhte Oberflächenexposition und ein erhöhter Umsatz des Rezeptors den Toxineintritt erleichtern (262). Dem widersprachen allerdings Majoul *et al.* (191), die durch Untersuchungen auf

Einzelzellebene zeigten, dass die Synthese und die Oberflächenexpression von Gb_3/CD77 in der G2 und der M-Phase ein Maximum erreicht.

Von Glykosphingolipiden ist darüberhinaus bekannt, dass sie zwar in Zellen chemisch nachweisbar sein können, aber trotzdem nicht auf der Oberfläche zur Ligandenbindung zur Verfügung stehen. Ihre Exposition ist von der Zellfunktion abhängig und sie können leicht von längeren Kohlenhydratketten oder Proteinen überdeckt werden, die die kleinen und nahe der Membran gelegenen Köpfe der Glykolipide maskieren (24, 262). Entsprechend kann die Verfügbarkeit von Gb_3/CD77-Rezeptoren durch Trypsinierung erhöht werden (262).

Die oberflächliche Gb_3/CD77-Expression hängt auch vom Differenzierungsgrad der Zellen oder Gewebe ab. Während undifferenzierte THP-1-Zellen Stx-sensibel sind, führt die Differenzierung *in vitro* durch Stimulation mit Phorbolestern, IFN-γ oder GM-CSF zu einer zunehmenden Resistenz gegenüber dem Toxin, die mit einer reduzierten Rezeptorexpression einhergeht (265). Dagegen steigt bei Kaninchen die Aktivität der Gb_3-Synthetase in Ileumzellen am 18. Lebenstag sprunghaft an, während die Aktivität der Galaktosidase stark abnimmt (212). Infolgedessen ist erst ab dem 20. Lebenstag Gb_3/CD77 auf den Zellen nachweisbar und die Anzahl der Moleküle steigt bis zum 24. Tag auf das Niveau der Zellen adulter Tiere an. Dies wird begleitet von einem Anstieg der Empfänglichkeit der entsprechenden Darmabschnitte für die enterotoxische Wirkung des Shigatoxins (211).

Das Gleichgewicht zwischen dem Syntheseschritt von Laktosylzeramid zu Gb_3/CD77 durch die Gb_3-Synthetase und dem Abbau durch die α-Galaktosidase reguliert die Gb_3/CD77-Expression auch auf HeLa-Zellen (24, 129). In Daudi-Zellen haben die Enzymaktivitäten dagegen keinen Einfluss auf die Gb_3/CD77-Expression, so dass noch weitere Regulationsmechanismen existieren müssen (261).

LPS verstärkt die Wirkung der Shigatoxine auf humane Nabelschnur-Endothelzellen (HUVEC) durch eine Erhöhung der Anzahl der entsprechenden Rezeptoren auf der Zelloberfläche um das 10fache (187, 341). Ähnlich wie LPS, verstärkt auch der unter LPS- sowie Stx-Einwirkung aus Makrophagen freigesetzte Tumor-Nekrose-Faktor-alpha (TNF-α) in additiver und synergistischer Weise die Wirkung der Shigatoxine an Endothelzellen. Durch Vorinkubation von Endothelzellen mit TNF-α läßt sich die Anzahl der Stx-Bindungsstellen um das bis zu 100fache steigern (341). Diese Erhöhung der Rezeptorexpression beruht auf einer gesteigerten *de novo*-Synthese der Rezeptoren durch eine Proteinkinase C-vermittelte Aktivitätssteigerung sowohl der Gb_3-Synthetase (340) als auch der Enzyme die die Synthese der Vorläufermoleküle katalysieren (318). Unter Einwirkung von LPS oder TNF-α setzt die

Erhöhung der Rezeptorzahl nach 6-8 Stunden ein und hält 48 Stunden an (340). IL-1β, das wie TNF-α unter LPS- und Stx-Einwirkung aus Makrophagen freigesetzt wird, sensibilisiert Endothelzellen mit der gleichen Kinetik und in gleichem Maße gegenüber Stx wie TNF-α (146). Auch IL-1β induziert in den Endothelzellen über den Transkriptionsfaktor NF-κB die Produktion von Enzymen, die vermehrt Sphingomyelin-Moleküle zu Zeramiden abbauen, die dann als Substrat für die Gb_3/CD77-Synthese dienen (146).

Im Gegensatz zu HUVEC werden humane renale mikrovaskuläre Endothelzellen (HRMEC) und glomeruläre Endothelzellen („glomerular capillary endothelial cells“, GCEC) *in vitro* durch Toxinkonzentrationen geschädigt, die bei HUVEC keine Effekte auslösen können (238, 345). Bereits 5 bis 6 Stunden nach Zugabe von Stx1 zu GCEC-Kulturen nimmt die Proteinbiosynthese der Zellen ab. Nach 10 Stunden manifestiert sich ein zytotoxischer Effekt (345). Entsprechend weisen diese Nierenendothelzellen, verglichen mit HUVEC, einen 50fach höheren Gb_3/CD77-Gehalt und damit eine Rezeptordichte wie Vero-Zellen auf (238). Die Heterogenität der Reaktion der Endothelzellen auf Shigatoxine (238) manifestiert sich auch in der unterschiedlichen Bedeutung von Zytokinen. Während Zytokine bei Nierenendothelien *in vitro* weder einen Einfluß auf die Rezeptorexpression noch auf die zytotoxische Wirkung der Shigatoxine besitzen (238, 345), steigern TNF-α und IL-1β die Rezeptorexpression bei mikrovaskulären Endothelzellen aus dem menschlichen Gehirn erheblich (61, 264).

Experimentell lässt sich in Zellen die Synthese des Rezeptors auch durch Buttersäure, die die Differenzierung von Zellen auslöst, induzieren (148, 287). Da Buttersäure ein Stoffwechselprodukt der anaeroben Darmflora darstellt, ist es denkbar, dass sie *in vivo* eine ähnliche Wirkung auf Darmepithelzellen hat.

2.2.2 Verteilung auf Zellen und in Geweben

Gb_3/CD77 und Gb_4 wurden auf verschiedenen Tumorzelllinien, primär kultivierten Zellen und in Geweben verschiedener Spezies nachgewiesen. Dabei unterscheiden sich verschiedene Tierarten erheblich in der Verteilung der Stx-Rezeptoren (**Tabelle 2.2**). Der Nachweis von Gb_3/CD77 bzw. Gb_4 korreliert meist mit der Empfänglichkeit der Zellen oder Gewebe gegenüber einer zytotoxischen Wirkung der Shigatoxine. So erklären die Rezeptorspezifitäten von Stx2e bzw. den anderen Shigatoxinen zusammen mit den Unterschieden im Vorkommen von Gb_4 bzw. Gb_3/CD77 auf Zellen und in Geweben zum Teil die Unterschiede in den

klinischen Symptomen nach Infektionen mit Stx2e oder Stx1/2-bildenden *E. coli* (20, 51, 105, 280, 339).

Obwohl Shigatoxine ursprünglich als zytoletale Toxine beschrieben wurden, ist inzwischen deutlich, dass die zellulären Wirkungen der Toxine ein breites Spektrum umfassen, wobei bestimmte Wirkungen häufig spezifisch für bestimmte Zelltypen sind. Diesen verschiedenartigen, meist eher modulierenden Wirkungen kommt in der Pathogenese von STEC-bedingten Erkrankungen wahrscheinlich eine grössere Bedeutung zu als der zytoletalen Wirkung (259). Dabei hängt die Zellspezifität der Stx-Wirkung wesentlich von der Lokalisation des Gb_3/CD77 in der Zellmembran ab (69). So ist Gb_3/CD77 in HeLa-Zellen, die empfindlich für die zytoletale Wirkung der Shigatoxine sind, Bestandteil von Detergentien-unlöslichen, Glykosphingolipid-reichen Mikrodomänen der Zellmembran, die auch als „lipid rafts“ bezeichnet werden (69). Hier lokalisiertes Gb_3/CD77 kann sowohl die Endozytose und den retrograden Transport der Shigatoxine zum biosynthetischen/sekretorischen Vesikeltransportweg vermitteln (69) als auch die Aktivierung von Signalkaskaden von der Zelloberfläche aus (145). Die biochemische Zusammensetzung der Gb_3/CD77-enthaltenden „lipid rafts“ ist derzeit noch unklar. In polarisierten Caco-2-Zellen (168) und in HeLa-Zellen (69) ist Gb_3/CD77 strikt mit dem Gangliosid GM_1, einem gebräuchlichen Marker für „lipid rafts“ kolokalisiert, jedoch existieren in Vero-Zellen zu verschiedenen Phasen des Zellzyklus´ unterschiedliche „lipid rafts“, die entweder GM_1 oder Gb_3/CD77 enthalten (191).

Im Gegensatz dazu befindet sich Gb_3/CD77 bei humanen Monozyten und Makrophagen, die gegenüber der Proteinbiosynthese-inhibierenden Wirkung des Shigatoxins 1 resistent sind, aber auf das Toxin mit einer veränderten Zytokingen-Transkription reagieren (343), ausserhalb von „lipid rafts“ (69). Während Caco-2-Zellen nach Desintegration der „lipid rafts“ das gebundene Stx nicht mehr internalisieren können (168), sind humane Monozyten dazu noch in der Lage, transportieren das Stx aber überwiegend zu späten Endosomen, in denen es vor dem Übertritt in das Zytosol degradiert wird (69).

Tabelle 2.2: Übersicht über die zelluläre Verteilung von Gb_3/CD77-artigen Stx-Rezeptoren[1] bei verschiedenen Spezies

Zellart:	Mensch	Maus	Kaninchen	Schwein	Rind	Schaf	Ziege
Intestinale Epithelzellen	(+) nur für Stx2 (296) + nur Zelllinien	+ nur distales Kolon (120)	+ (149, 211)	? (260)	+ (110); (diese Studie)		
Endothelzellen							
in grossen Gefässen	+ (188, 214, 242, 341, 369)				+ (17); - (242)		
in der Mikrovaskulatur	+ (66, 240, 242, 254)						
intestinal	+ (128)		+ (270)		- (260)		
in renalen Glomeruli	+ (30, 186, 345)			+ (361)	- (260)		
im ZNS	+ (61, 264)		+ (270)	+ (361)			
in der Lunge		+ (274)					
Nervenzellen				? (20)			
Nierentubulusepithelzellen	+ (30, 32, 112, 157, 180)	? (119)	- (373)	+ (361)	+ (110, 260)		
Renale glomeruläre Epithelzellen	+ (115)		- (373)				
Fibroblasten					+ (diese Studie)		
Intestinale Myofibroblasten	+ (296)						
Monozyten / Makrophagen	(+) (343) + (162)	+ (335)		+ (361)	+ (diese Studie)		
Mesangiumzellen	(+) (344) + (272, 344)		- (373)		- (110)		
Gewebsmakrophagen				+ (208, 361)	+ (diese Studie)		

Tabelle 2.2: Fortsetzung

Zellart:	Mensch	Maus	Kaninchen	Schwein	Rind	Schaf	Ziege
Lymphozyten				- (361)	+ (diese Studie)		
B-Zellen	+ (40, 198, 199)	+ (93)			+ (diese Studie)		
αβT-Zellen	- (40, 198)	- (119, 161)			+ (diese Studie)		
γδT-Zellen					+ (diese Studie)		
Granulozyten	(+) (332)			+ (361)	- (diese Studie)	+ (diese Studie)	+ (diese Studie)
Erythrozyten	+ (16, 231)			- (20)			
Plättchen	+ (41, 143)						

Legende: [1] Stx2e-Bindungsstellen / Gb_4 wurde nicht berücksichtigt

+ = Gb_3/CD77 und/oder Stx-Bindungsstellen nachgewiesen; - = Gb_3/CD77 und/oder Stx-Bindungsstellen nicht nachweisbar;

(+) = von Gb_3/CD77 abweichende Stx-Bindungsstellen nachweisbar; ? = Hinweise auf das Vorhandensein von Stx-Bindungsstellen

2.2.3 Interaktionen mit Stx

2.2.3.1 Affinität und Kinetik der Toxin-Rezeptorbindung

Die Bindung des Shigatoxins an Gb_3/CD77 als Bestandteil einer Zellmembran ist mit einer Assoziationskonstanten von ca. $1x10^{10}M^{-1}$ hochaffin (59, 211, 244). Die hohe Avidität beruht im wesentlichen auf der Multivalenz der Rezeptorbindung über bis zu 15 Rezeptor-Bindungsstellen je Holotoxinmolekül. Die Assoziationskonstante des Shigatoxins an einzelne, lösliche Gb_3/CD77-Moleküle ist mit $1x10^{3}M^{-1}$ erheblich niedriger (310). Daneben existieren auf HeLa-Zellen aber auch niederaffine Bindungsstellen mit hoher Bindungskapazität. Im Gegensatz zu den hochaffinen Bindungsstellen ist die Kapazität der niederaffinen Bindungsstellen jedoch nicht proportional zur Empfindlichkeit der Zellen gegenüber Stx (59, 126, 176). Da die Affinität vom Lipidanteil des Gb_3/CD77-Moleküls abhängt, könnte die Ursache für die biphasischen Bindungskinetiken in unterschiedlichen Lipidanteilen liegen (248). Auch beeinflussen andere Membranlipide ("Hilfslipide") die Rezeptoraffinität (152). Für Stx2 wurde unter experimentellen Bedingungen eine 10fach niedrigere Rezeptoraffinität als für Stx1 ermittelt. Dies könnte die höhere Aktivität von Stx1 in Zytotoxizitätstests erklären (105).

Die Stx-Bindung besitzt eine Temperaturabhängigkeit mit einer maximalen Bindung bei 37°C (176, 286). Jedoch auch bei 0°C, bei der Toxin nicht endozytiert wird, erfolgt sie sehr rasch. Bei 4°C werden bereits nach 5 Minuten mehr als 50 % der maximal möglichen Toxinbindung, nach 15 Minuten das Maximum erreicht (126). Die Konstante für die Bindungsrate beträgt etwa $1,5x10^{6}\ M^{-1}s^{-1}$ (59). Wegen der hohen Bindungsaffinität kommt es kaum zu einem Austausch zwischen freiem und gebundenem Toxin. Nur 20 % des zellgebundenen Stx1 lösen sich nach 20 Stunden Inkubation bei 0°C wieder von den Zellen ab (59). Die Toxinbindung durch Zellen ist pH-abhängig mit einem breiten Plateau zwischen pH 5-8 (126). Erst unter einem pH-Wert von 3,5 nimmt Affinität des Toxins für Gb_3/CD77 deutlich ab (278).

2.2.3.2 Der Kohlenhydratanteil des Rezeptors

Mit Ausnahme von Stx2e binden alle Shigatoxine spezifisch an die terminale Galabiose (Galα1-4Gal) des Gb_3/CD77 in der Membran eukaryontischer Zellen (176, 181, 280, 346). Unter künstlichen Bedingungen binden die Toxine aber auch an isolierte Di- oder Trisaccharide (280). Durch Vorinkubation mit Galabiose lässt sich die zytoletale Wirkung

von Stx auf HeLa-Zellen drastisch reduzieren. Eine Hemmung findet bereits in äquimolaren Konzentrationen statt (24, 176, 346). Eine Vorbehandlung von Zellen oder Geweben mit α-Galaktosidase senkt deren Toxin-Bindungsvermögen (51, 181, 346). Aber auch Gb_2 (Galabiosylzeramid, Galα1-4Galβ1Cer) scheint auf HeLa-Zellen und Daudi-Zellen als funktioneller Rezeptor zu fungieren (39, 280). Ausserdem binden die Toxine an das P1-Blutgruppenantigen, das ebenfalls über eine endständige Galabiose verfügt (176).

Die Variante Stx2e bindet bevorzugt an Gb_4, in dessen Kohlenhydratkette die Galabiose subterminal zu einem N-Azetyl-Galaktosaminrest steht (51). Da eine Deazetylierung dieses Kohlenhydrates die Bindung von Stx2e nicht beeinflusst, ist vermutlich nur die terminale β1-3 Galaktosestruktur für die Bindung verantwortlich (51). Eine solche Struktur kommt auch in Galaktosylglobotetrasylzeramid (Gb_5) und im Blutgruppenantigen Forssman vor. An diese Membranbestandteile kann die Stx2e-Variante ebenfalls binden (51, 280).

2.2.3.3 Der Lipidanteil des Rezeptors

Stx bindet bevorzugt an Kohlenhydratstrukturen, wenn das Oligosaccharid an ein Lipid oder ein Protein gekoppelt ist (176). In Säugetieren wurde bisher eine Galabiosestruktur am terminalen, nicht reduzierenden Ende von Sacchariden nur bei Glykolipiden gefunden (24). Die Struktur des Lipidanteils ist für die Präsentation der Galabiosestruktur auf der Zelloberfläche essentiell. So bindet Stx nicht an Digalaktosyldiglyzeride, sondern nur an Oligosaccharide, die an ein Zeramid gekoppelt sind (181, 248). Die Zeramidanteile der Stx-Rezeptoren bestehen aus einem Sphingosin- oder Dihydrosphingosin-Molekül, an das über eine Amidbindung eine langkettige Fettsäure gebunden ist (248). In Gb_3/CD77-Molekülen aus dem Kaninchendarm oder der Muttermilch finden sich hydroxylierte und unhydroxylierte Fettsäuren (149, 230). Im Gegensatz dazu weisen die Gb_3/CD77- und Gb_4-Moleküle aus humanen Nieren nur unhydroxylierte Fettsäuren auf (248).

Auf die räumliche Orientierung der Kohlenhydratanteile (**Abb. 2.4**) hat insbesondere die Länge der Fettsäuren des Lipidanteils einen Einfluss. Shigatoxine erkennen bevorzugt Rezeptoren mit C12- bis C24-Fettsäuren (248). Während die Toxine unter experimentellen Bedingungen auch an Rezeptoren mit kurzkettigen Fettsäuren binden, scheint es in der Lipidumgebung einer Membran eine untere Grenze der Fettsäurelänge zu geben, die für eine Toxinbindung erforderlich ist (248). Bei der dünnschichtchromatographischen Auftrennung von Glykolipiden aus humanen Nieren lassen sich 2 Banden von Gb_3/CD77-Molekülen unterscheiden, die jeweils eine Mischung aus Glykolipiden mit Fettsäuren unterschiedlicher

Länge darstellen. Eine Bande wird dominiert von Gb_3/CD77-Molekülen mit einer C24:1-Fettsäure und bindet Stx1 mit niedriger Affinität, aber hoher Kapazität. Die zweite Gruppe wird dominiert von Gb_3/CD77-Molekülen mit einer C16:1-Fettsäure und bindet Stx1 mit hoher Affinität und niedriger Kapazität. Die Affinität und Kapazität der Bindung von Stx1 an beide Banden ist dabei höher als bei einer Bindung an die jeweiligen semisynthetischen Rezeptoranaloge. Eine Mischung von Rezeptormolekülen mit unterschiedlichem Fettsäureanteil in physiologischen Membranen begünstigt damit vermutlich die Stx-Bindung. Möglicherweise kommt es durch die Mischung von unterschiedlichen Rezeptoren und zusätzlichen "Hilfslipiden" zur Ausbildung einer unebenen Oberfläche, die die Bindung des StxB-Pentamers fördert (248).

Die Affinität verschiedener Shigatoxine zu Gb_3/CD77-Molekülen mit Fettsäuren einer bestimmten Länge ist jedoch nicht immer gleich. Während Stx1 bevorzugt an Gb_3/CD77-Moleküle mit C20:0- oder C22:1-Fettsäuren bindet, zeigt Stx2c eine Präferenz für Rezeptoren mit C18:0- oder C18:1-Fettsäuren. Da sich die beiden Toxine nur schlecht gegenseitig von ihren jeweils bevorzugten Rezeptoren verdrängen, binden sie vermutlich an unterschiedliche, aber überlappende Kohlenhydratepitope, die aufgrund der unterschiedlichen Fettsäureanteile in den Gb_3/CD77-Molekülen in der Membranumgebung unterschiedlich präsentiert werden (152).

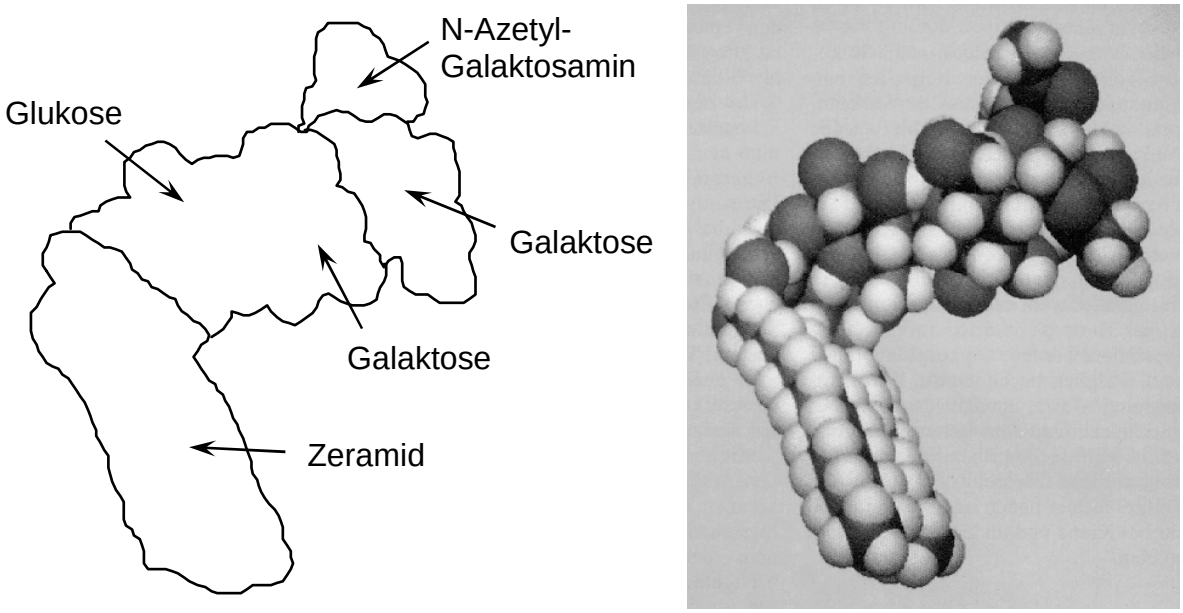

Abb. 2.4: 3D-Modell des Globotetraosylzeramid (Gb_4) (nach (100))

2.2.3.4 Die Rezeptorbindungsdomäne der Toxine

Für die Stx-Bindung an Rezeptoren ist das Pentamer der B-Untereinheiten verantwortlich (105, 124, 349). Die A-Untereinheit liegt nach Bindung des Toxins an den Rezeptor auf der membranabgewandten Seite (312) und ist zumindest bei Stx1 an der Bindung nicht beteiligt (125). Die Rezeptorbindung scheint insbesondere durch die Aminosäuren Asp16, Asp17, Arg33, Trp34, Ala43, Lys53, Gly60 der reifen StxB1 vermittelt zu werden (105, 132, 183, 249, 250). Ausserdem ist die Disulfidbrücke zwischen den Aminosäuren 4 und 57 für die Bindung essentiell (132). Die Bindung wird darüberhinaus vom C-terminalen Ende der B-Untereinheit stabilisiert. Führt eine Entfernung der letzten beiden Aminosäuren (Phe68 und Arg69) bereits zu einer reduzierten Bindung von Stx1 an den Rezeptor, so findet nach Entfernung der letzten 4 Aminosäuren keine Rezeptorerkennung mehr statt (250). Trotz z.T. widersprechender Befunde nahm man ursprünglich an, dass die Tertiärstruktur der Shigatoxine hoch konserviert ist und bei den Toxinen der Stx2-Gruppe für die Rezeptorbindung die zu Stx1 homologen Aminosäuren verantwortlich sind (130, 250). Jedoch weiss man inzwischen, dass pro B-Untereinheit bis zu drei unabhängige Rezeptor-Bindungsstellen existieren, die sich wiederum zwischen den verschiedenen Toxinen unterscheiden können.

Die **Rezeptor-Bindungsstelle I** wird von 2 β-Faltblättern in Form einer Tasche ausgebildet (**Abb. 2.5**). Die Faltblätter gehören jeweils zu einer von zwei im Pentamer benachbarten B-Untereinheiten. Die polaren und sauren Seitenketten der Aminosäuren Asp16-17 der β-Faltblätter können Wassserstoffbrücken zu den polaren Gruppen der Kohlenhydrate in den Rezeptoren ausbilden (233). Konservierte aromatische Ringe des Phe30 legen sich ausserdem an die Zuckerringe (233, 312). Die Rezeptorspezifität für Gb_3/CD77 wird hauptsächlich durch Asp18 im Stx1-Molekül bzw. Asp17 in den Molekülen der Stx2-Toxine bedingt. Bei Stx2e befindet sich an dieser Stelle ein Asparaginrest. Darüberhinaus stabilisieren im Stx2e-Molekül die Aminosäuren Gln64 und Lys66 vermutlich über eine Wechselwirkung mit dem N-Azetyl-Galaktosaminrest die Bindung an Gb_4 (105, 339).

Obwohl die Rezeptor-Bindungsstelle von zwei benachbarten Monomeren gebildet wird, ist die Affinität der Bindung der Monomere an den Rezeptor identisch mit der des Pentamers (266). So kompetitieren StxB1 und das Stx1-Holotoxin in äquimolaren Mengen um die Bindung an Gb_3/CD77 (105). Dieser scheinbare Widerspruch lässt sich dadurch erkären, dass nur eine Seite der Tasche die Affinität vermittelt während die andere nur die Bindung stützt. Bei einem Vergleich der Aminosäuresequenz von CD19, einem Gb_3/CD77-bindenden

Molekül auf B-Zellen (siehe **2.2.4.1**), und den B-Untereinheiten der Shigatoxine zeigt sich eine etwa 50 %ige Identität. Diese umfasst bei den B-Untereinheiten die Aminosäuren, die für die Ausbildung der Rezeptor-Bindungsstelle zwischen zwei B-Untereinheiten im Pentamer verantwortlich sind. Dabei liegen die meisten übereinstimmmenden Aminosäuren im (n+1) B-Monomer (**Abb. 2.1**). Dies deutet daraufhin, dass diese Seite der Rezeptor-Bindungsstelle allein ausreicht, um die Glykolipid-Bindungsspezifität zu definieren, während die andere Seite den Zugang zum Rezeptor nur unterstützt (195).

Während Lingwood *et al.* (182) aufgrund von kristallographischen Untersuchungen die Stelle I als wichtigste Rezeptor-Bindungsstelle des Shigatoxins 1 betrachten, zeigten Kitova *et al.* mit Hilfe der Resonanz-Massenspektrometrie, dass eine Bindung von Gb_3-homologem P^K-Trisaccharid an Stelle I erst nach Absättigung von 5 anderen Bindungsstellen im StxB1-Pentamer stattfindet (155). Diese **Rezeptor-Bindungsstelle II** befindet sich bei StxB1 auf der anderen Seite des Phe30 und wird durch eine Glycin-Schleife (Gly60-62) sowie Asn32 und Arg33 gebildet (233). Die Energie der Interaktion zwischen isoliertem Gb_3/CD77 und Stelle II beträgt etwa 50 % der Energie der Wechselwirkung zwischen Gb_3/CD77 und Stelle I (233). Allerdings sind die Affinitätsunterschiede deutlich geringer, wenn Gb_3/CD77 Bestandteil einer Lipidmembran ist (307), da die Umgebung des Rezeptors einen grossen Einfluss auf sein Bindungsverhalten besitzt (siehe **2.2.3.3**). In der B-Untereinheit des Shigatoxins 2 hat die Disulfidbrücke zwischen den Zysteinresten 3 und 56 eine andere Konformation als die zwischen den Zysteinresten 4 und 57 in Stx1 (76). Eine Bindung von Gb_3/CD77 in die Rezeptor-Bindungsstelle II des Shigatoxins 2 in der gleichen Art wie in Stx1 würde eine Konformationsänderung dieser Disulfidbrücke erfordern, da sonst die Kohlenhydratkette des Rezeptors mit dem Ser54 kollidieren würde (76). Die Stelle II wird allerdings von Stx2c bevorzugt, da dieses Toxin an der dem Asp17 des Shigatoxins 1 homologen Position einen Asparaginrest besitzt, der nicht in der Lage ist Wasserstoffbrücken zum Kohlenhydratanteil des Gb_3/CD77 auszubilden (182, 233). Diese Unterschiede könnten unter anderem die unterschiedlichen Rezeptoraffinitäten von Stx1, Stx2 und Stx2c erklären und Auswirkungen auf die biologische Wirkung der Shigatoxine im Gewebe haben (76).

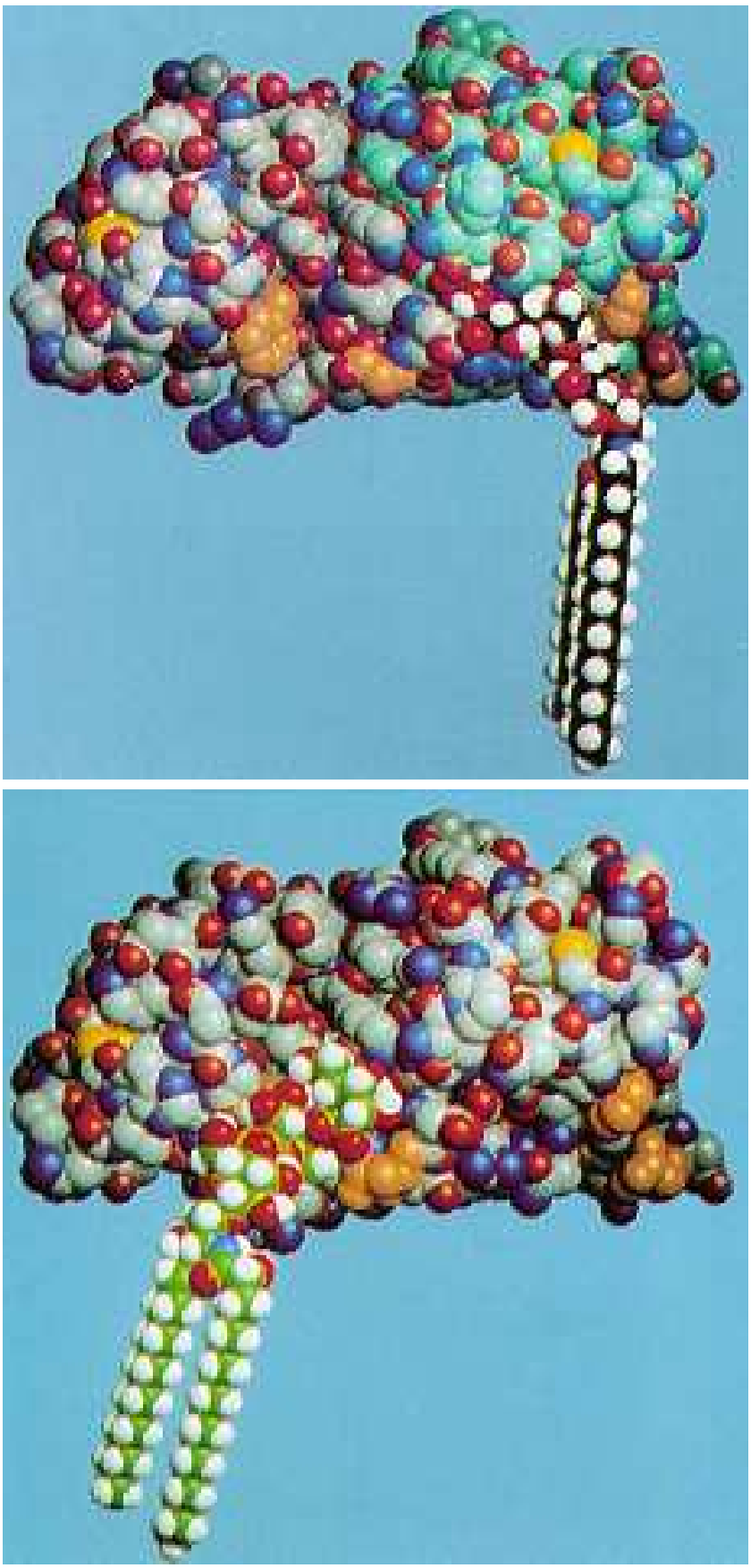

Abb. 2.5: 3D-Darstellung der Gb_3-Bindungsstellen I (oben) und II (unten) in zwei benachbarten B-Untereinheiten des Shigatoxins 1 (aus (182))

Darüberhinaus wird auch die Existenz einer niedrigaffinen **Rezeptor-Bindungsstelle III** am N-Terminus der StxB α-Helix und damit an der Basis des B-Pentamers beschrieben, wo der C-Terminus des A_2-Fragmentes aus der zentralen Pore austritt (11, 76, 177). Jedoch scheint dieser Bindungsstelle bei der Vermittlung der zytoletalen Aktivität des Shigatoxins 1 nur eine untergeordnete Bedeutung zuzukommen (363). In Stx2 haben, anders als bei Stx1, die 5 Trp33 jeder B-Untereinheit keine einheitliche Ausrichtung (76). Um Gb_3/CD77 an dieser Stelle binden zu können, müssen aber möglicherweise alle 5 Tryptophanreste so beweglich sein, dass sie sich gemeinsam mit den benachbarten Asparaginresten in Position 34 zur Bindungskonformation drehen können (76).

2.2.4 Wechselwirkung mit physiologischen Liganden

2.2.4.1 humanes CD19

CD19 ist ein 95-kDa Protein mit einer extrazellulären Region, die drei potentielle, über Disulfidbrücken verbundene Domänen umfasst. Die Expression von CD19 ist das erste Anzeichen, dass sich hämatopoetische Zellen zu B-Zellen differenzieren. CD19 geht erst wieder bei der terminalen Differenzierung der B-Zellen zu Plasmazellen verloren. Auf der Zelloberfläche ist CD19 an der Bildung eines Komplexes mit CD21, CD81 und Leu-13 beteiligt. Eine Ligandenbindung an CD19 führt zu einer Signaltransduktion und dadurch zur Aktivierung der Integrin-abhängigen Adhäsion, zur Proliferation und Differenzierung im Rahmen der B-Zell-Reifung (195), aber auch zur Apoptose der Zelle (150).

Der Vergleich der Aminosäuresequenzen zwischen CD19 und StxB1 ergibt eine fast 50 %ige Übereinstimmung. Diese betrifft bei CD19 Bereiche der extrazellulären Domäne, bei StxB1 die Aminosäuren, die im Pentamer für die Ausbildung der Rezeptor-Bindungsstelle I verantwortlich sind. Dadurch bindet CD19 auf der Oberfläche von B-Zellen an das ebenfalls dort vorhandene Gb_3/CD77 (195). Nur im Komplex mit Gb_3/CD77 wird CD19 retrograd über das ER bis hin zur Kernmembran transportiert (150). Bei Mutanten, denen Gb_3/CD77 fehlt, liegt CD19 in einer Form vor, die sich durch eine herabgesetzte Affinität gegenüber Gb_3/CD77 auszeichnet und eine Integrin-abhängige Anheftung nicht mehr aktivieren kann (195). Deshalb wird eine Wechselwirkung zwischen Gb_3/CD77 und CD19 auf der Oberfläche von B-Zellen vermutet, die mehrere Ereignisse umfasst. CD19 wird auf B-Zellen wahrscheinlich als unreifes Protein ohne Disulfidbrücken exprimiert. Auf der Oberfläche der Zellen bindet CD19 dann an Gb_3/CD77-Moleküle, die auf der gleichen Zelle vorhanden sind. Dadurch kommt es zur Annäherung von Thiolgruppen im CD19-Molekül, zur Bildung von

Disulfidbrücken und damit zur Ausreifung des Moleküls. Das ausgereifte CD19-Molekül ist an der Ausbildung von zwei Formen der Zell-Zell-Adhäsion beteiligt, die *in vivo* vermutlich eine essentielle Rolle beim Homing von B-Zellen und bei der Ausbildung von Keimzentren in den lymphatischen Organe spielen. Einerseits interagieren CD19 und Gb_3/CD77 auf benachbarten Zellen, wodurch B-Zellen untereinander, aber auch an ebenfalls CD19 exprimierende follikuläre dendritische Zellen binden können. Andererseits bindet CD19 an Gb_3/CD77 der gleichen Zelle und erfährt dadurch eine Konformationsänderung. Diese Änderung ist Voraussetzung für die Signalübertragung, die vom CD19/CD21/CD81-Komplex ausgeht und zur starken Zelladhäsion durch Integrine führt. Über diese beiden Adhäsionsformen werden die B-Zellen in den Keimzentren verankert. Wird zum Abschluss der B-Zell-Differenzierung Gb_3/CD77 und möglicherweise auch CD19 herunterreguliert, geht diese Anheftung verloren und die B-Zellen werden aus den Keimzentren ins System ausgeschwemmt. In der Tat tragen nur Zellen in den Keimzentren Gb_3/CD77 und terminal differenzierten Plasmazellen fehlt CD19 (195). Bindet Stx an Gb_3/CD77 auf B-Zellen, stehen die Gb_3/CD77-Moleküle CD19 nicht mehr zur Bindung zur Verfügung. Dies könnte sich einerseits über die Auslösung von Apoptose durch Stx1 hinaus gravierend auf die B-Zell-Reifung und -Differenzierung auswirken, andererseits aber auch die Auslösung von Apoptose durch Vernetzung von CD19-Molekülen auf der Zelloberfläche verhindern (150).

2.2.4.2 Untereinheit 1 des Interferon-α Rezeptors (IFNAR-1)

Auch IFNAR-1 weist auf Aminosäureebene Homologien zu den Bereichen der StxB auf, die für die Bindung des Pentamers an Gb_3/CD77 verantwortlich sind. Diese Homologie betrifft besonders das humane und das bovine IFNAR-1 (86). Obwohl die Sequenzhomologie im Falle des IFNAR-1 nicht so hoch ist wie bei CD19 (195), kann auch der Interferon-Rezeptor auf der Zelloberfläche an Gb_3/CD77 binden (86). Diese Wechselwirkung mit Gb_3/CD77, aber auch mit Gb_2 und eventuell mit Gb_4, führt zu einer Konformationsänderung des Rezeptors, der dadurch von der niederaffinen in die hochaffine Form überführt wird (183). Auf diese Weise modulieren die Glykolipide den Interferon-Rezeptor ohne seine Expression zu beeinflussen. Nur die Bindung von Interferon an den hochaffinen Rezeptor kann eine biologische Wirkung auslösen (86). Stx hat, vermutlich aufgrund der pentameren Struktur der B-Untereinheiten, zu bestimmten Isoformen des Gb_3/CD77 eine höhere Affinität als der Interferon-Rezeptor (183). Inkubiert man Daudi-Zellen mit Stx1, wird die Bindungskapazität der Zellen für Interferon-α (IFN-α) deutlich gesenkt. Die Kapazität

entspricht dann etwa der von Daudi-Zell-Mutanten, die kein Gb_3/CD77 exprimieren (86). In diesen Mutanten ist die Interferon-abhängige Aktivierung zytosolischer Transkriptionsfaktoren (86) und dadurch sowohl die proliferationsinhibierende (194) als auch die antivirale Wirkung des IFN-α deutlich reduziert (151). Entsprechend kann Stx1 in Zellen mit physiologischer Gb_3/CD77-Expression den antiinvasiven Effekt von IFN-α auf die Invasion von Bakterien in HEp-2-Zellen aufheben (26). Auch werden Stx-produzierende *Shigella flexneri*-Keime durch Interferone nicht an der Invasion gehindert (26). Für eine effiziente Verdrängung des Interferon-Rezeptors von Gb_3/CD77 reicht die isolierte B-Untereinheit des Toxins aus (151). Obwohl die Verdrängung an der Zelloberfläche stattfindet und eine Endozytose des Toxins / der B-Untereinheit nicht erforderlich ist (151), könnte die Hemmung der Proteinbiosynthese die Blockade der Interferon-Wirkungen noch weiter verstärken (26). Allerdings werden offensichtlich die verschiedenen Wirkungen des IFN-α durch Interferon-Rezeptoren vermittelt, die mit verschiedenen Isoformen des Gb_3/CD77 verbunden sind. So verbleiben Gb_3/CD77-Isoformen, die langkettige Fettsäuren enthalten, bevorzugt an der Plasmamembran und vermitteln von dort nach Wechselwirkung mit IFNAR-1 die antivirale Aktivität des IFN-α. Gb_3/CD77-Isoformen mit kurzkettigen Fettsäuren werden dagegen bevorzugt internalisiert und vermitteln dadurch nicht nur den zytoletalen Effekt der Shigatoxine, sondern auch die IFNAR-1-abhängige proliferationsinhibierende Wirkung des IFN-α (151).

2.2.4.3 Haupthistokompatibilitätskomplexe der Klasse II (MHC-II-Moleküle)

Durch Sequenzvergleiche *in silico* wurde eine dem Stx ähnliche Aminosäuresequenz in der β-Kette von humanen und murinen MHC-II-Molekülen entdeckt (83). Im Gegensatz zu CD19 ist die Oberflächlichen-Expression von MHC-II bei Gb_3/CD77-defizienten Daudi-Zell-Mutanten jedoch unverändert, auch wenn diese Zellen eine erniedrigte Gesamtmenge an MHC-II-Protein aufweisen. Es wurde vermutet, dass eine Bindung von Gb_3/CD77 an die MHC-Moleküle deren Peptid-bindenden Eigenschaften modifizieren könnte (83).

Das Vorhandensein von Stx-ähnlichen Gb_3/CD77-Bindungsstellen in CD19, dem Interferon-Rezeptor und MHC-II könnte für den Verlauf von STEC-Infektionen auch insofern Bedeutung haben, als das Immunsystem eventuell eine spezifische humorale Antwort gegen Stx unterdrückt, um einer Autoimmunreaktion gegen identische Epitope in körpereigenen Molekülen vorzubeugen. Dies könnte in Verbindung mit der Schädigung von B-Zellen durch

Stx erklären, warum Menschen nach Stx-Exposition nur niedrige Antikörpertiter gegen die Toxine entwickeln (195, 268).

2.3 Wirkungsmechanismen der Shigatoxine

2.3.1 Internalisierung und enzymatische Aktivität

2.3.1.1 Rezeptor-vermittelte Endozytose

Die Shigatoxine sind die ersten bekannten Liganden, die Glykolipid-Rezeptoren zur Endozytose durch Clathrin-ummantelte Vesikel benutzen (130). Nach Bindung an die in „lipid rafts“ befindlichen Gb_3/CD77-Moleküle (69) kommt es nach 10 bis 15 Minuten durch Seitwärtsbewegung innerhalb der Membran zu einer Anreicherung dieser Rezeptoren in Clathrin-ummantelten Einziehungen der Membran (286). Der zugrundeliegende Mechanismus ist noch nicht bekannt. Da Gb_3/CD77 aber kein transmembranes Molekül darstellt, könnte die Wechselwirkungen mit Membranproteinen, wie CD19 (150), IFNAR-1 (86) oder MHC-II Molekülen (83), den Rezeptor nach Vernetzung durch das multivalente Toxin in den Membraneinziehungen zu halten (289). Zusätzlich scheint eine hohe Dichte von bestimmten Gb_3/CD77-Isoformen auf der Zelloberfläche die Zusammenziehung von gebundenen Stx-Molekülen in „lipid rafts“ zu begünstigen (69).

Zum Zeitpunkt der Anreicherung in bestimmten Membranbereichen ist das Toxin bereits in tubulären und vakuolären Endosomen nachweisbar (286). Nach der Fusion der Endosomen mit sauren Vesikeln werden 5-10 % des endozytierten Toxins bei 37°C innerhalb von 60 Minuten zu den trans-Zisternen des Golgiapparates transportiert (286, 289). Der zugrundeliegende Sortiermechanismus, der das Toxin spezifisch weiterleitet, wird durch die Länge der Fettsäuren im Gb_3/CD77-Molekül bestimmt (5, 288) und ist unabhängig von Rab9 (136) oder Rab5 (232). Vielmehr sind γ-Adaptin (192), SNARE-Proteine (193), Syntaxin 5 (328) und der Phopsphoinositol-bindende Clathrin Adaptor EpsinR (276) an diesem cAMP-abhängigen Prozess beteiligt (288). Während der Rezeptor vermutlich binnen Minuten zur Zelloberfläche zurückkehrt (88), verbleibt das Toxin in der Zelle. Das Toxin wird nicht wieder zur Zelloberfläche transportiert, auch wenn es nicht in das Zytosol übertritt (286).

Obwohl Stx kein KDEL-Motiv besitzt (87), erreicht Stx vom Golgi-Apparat aus über einen speziellen retrograden Transportweg, der abhängig von der kleinen GTPase Rab6a ist (193, 353), anders als die endozytierte Flüssigkeit das endoplasmatische Retikulum (ER) und sogar die Kernmembran (283). Offensichtlich wird das Beschreiten dieses Transportweges

wesentlich durch die spezifischen Eigenschaften des Gb_3/CD77 verursacht. So wird auch humanes CD19 nur im Komplex mit Gb_3/CD77 retrograd bis zur Kernmembran transportiert (150). Für die Ausbildung der Zytotoxizität ist der Transport des Toxins zum ER essentiell, da anscheinend nur hier die erforderliche Translokation in das Zytosol stattfinden kann (286). Hemmt man in Zellen die Endozytosevorgänge durch Entzug von ATP, Hemmstoffe des Zytoskeletts, Absenkung des intrazellulären pH-Wertes oder Temperaturen unter 20°C werden die Zellen gegenüber dem Toxin resistent (24, 127, 286). Für die Endozytose spielt die Polarität von Zellen keine Rolle, da das Toxin sowohl vom apikalen als auch vom basolateralen Pol mit gleicher Effizienz aufgenommen wird (204, 287).

Allerdings binden auch viele toxinresistente Zelllinien das Toxin mit hoher Affinität (59). Man vermutet, dass die unterschiedliche Empfindlichkeit von der langsameren Internalisation, einer ineffizienten intrazellulären Prozessierung oder einer niedrigeren Sensitivität des Proteinsynthese-Mechanismus abhängen könnte. In der Tat existiert in manchen Zellen ein zweiter Endozytoseweg für Stx, der durch Gb_3/CD77-Moleküle vermittelt wird, die sich ausserhalb von „lipid rafts“ in der Zellmembran befinden (69, 232). Dieser Endozytoseweg ist Clathrin-unabhängig (232) und transportiert Stx zum Teil zu späten Endosomen, in denen das Toxin degradiert wird (69). Stx kann zwar über diesen Weg auch effizient zumindest bis zum Golgiapparat transportiert werden (232), allerdings sind die entsprechenden Zellen ca. 1000fach weniger sensibel als Zellen, die das Toxin zum ER und zur Kernmembran transportieren (5). Die grosse Variabilität in der Empfindlichkeit von Zellen gegenüber den Shigatoxinen ist damit sowohl durch den Lipidanteil der jeweils experimierten Gb_3/CD77-Isoform(en) und seinen Einfluss auf die intrazellulären Transportrouten als auch durch den relativen Anteil Clathrin-abhängiger und -unabhängiger Endozytose bedingt (285).

2.3.1.2 Prozessierung der Toxine in der Zielzelle

Die enzymatische Funktion der Shigatoxine ist mit einem enzymatischen Spaltprodukt der A-Untereinheit assoziiert (236). Durch Spaltung der A-Untereinheiten mit Trypsin am Argininrest 248 oder 251 entstehen zwei Fragmente von 27 und 6 kDa, die durch eine Disulfidbrücke verbunden bleiben. Nach deren Reduktion wird das enzymatisch aktive 27 kDa A_1-Fragment freigesetzt (234). Die proteolytische Spaltung der StxA wurde sowohl in Bakterienlysaten als auch in Vero-Zellen nachgewiesen (279). Im Rahmen der Intoxikation von Zellen findet sie aber wahrscheinlich in den Endosomen oder dem trans-Golgi-Apparat statt. Hier wird StxA bei einem pH-Wert von 5-6 durch die lösliche Form von Furin, einer

Kalzium-sensitiven Serinprotease mit einer Spezifität für Arg-X-Arg/Lys-Arg-Motive, gespalten (81, 284). Alternativ kann Stx, wenn auch mit niedrigerer Effizienz, durch die zytosolische Protease Calpain gespalten werden (81). Im zellfreien System erweist sich das A_1-Fragment des Shigatoxins 1 in gereinigter Form als 3fach effektiver als enzymatisch vorbehandeltes Toxin und 6fach effektiver als Holotoxin (267). Die Ursache für diesen Aktivitätsunterschied liegt in der Struktur des A_2-Fragmentes, dessen Methionin in Position 260 in der enzymatisch aktiven Tasche des A_1-Fragmentes liegt und damit eine Substratbindung verhindert (**Abb. 2.2**). Erst durch die Abspaltung des A_2-Fragmentes wird das A_1-Fragment aktiv (75). Hemmt man die Fusion von Endosomen mit Lysosomen und inhibiert den proteolytischen Abbau der endozytierten Proteine, schützt man die Zellen vor der Wirkung des Toxins (127). Ein weitergehender Abbau des Toxins findet in sensibilisierten MDCK-Zellen auch 2 Stunden nach der Toxinzugabe nicht statt (287).

2.3.1.3 Translokation der A-Untereinheit in das Zytosol

Die Translokation des Toxins (seiner A_1-Untereinheit) in das Zytosol der Zelle ist eine essentielle Vorraussetzung für die Intoxikation von Zellen (129, 285). Nach bisherigem Kenntnisstand tritt das A_1-Fragment der Toxine vom ER aus durch die Vesikelmembran in das Zytosol über. Ob für die Prozessierung des Toxins oder seinen Durchtritt durch die Membran eine vorherige Fusion der Endosomen mit sauren Lysosomen notwendig ist, wird von einigen Autoren bestritten (282).

Die StxB1 erfährt *in vitro* bei niedrigen pH-Werten Konformationsänderungen (278). Bei pH 4,5 treten deutliche, reversible Veränderungen am Tryptophan in Position 34 auf. Dieses Tryptophan bildet den Eingang in einen von den α-Helices der 5 B-Untereinheiten gebildeten zentralen Kanal des Pentamers. Möglicherweise wird diese Veränderung durch Protonierung einer Aspartatseitenkette des Aspartatrestes 16 oder 18 und der dadurch bedingten Aufhebung einer Salzbrücke zum benachbarten Argininrest 33 hervorgerufen. Die Folge ist eine Destabilisierung des N-Terminus der α-Helix oder eine Beeinflussung der Polarität in der Tryptophan-Umgebung. Sinkt der pH-Wert weiter ab, ist auch die α-Helix selbst von einer Konformationsänderung betroffen. *In vivo* treten diese Veränderungen u.U. schon bei höheren pH-Werten auf, wenn die B-Untereinheiten an den Rezeptor gebunden und mit StxA assoziiert sind (278). Da die von der Konformationsänderung betroffenen Aminosäurenreste in der B-Untereinheit für die Rezeptorbindung und -spezifität des Holotoxins verantwortlich

sind (105, 183, 249, 330), führen die Änderungen möglicherweise zu einer Ablösung des Holotoxins vom Rezeptor (88).

Beim Choleratoxin, einem Toxin mit ähnlicher multimerer Struktur, bilden die α-Helices der 5 B-Untereinheiten ebenfalls eine zentrale Pore, deren Durchmesser dem des StxB1-Pentamers entspricht. Diese Pore soll Modellvorstellungen zur Folge als transmembraner Kanal dienen, den das A_1-Fragment, unterstützt vom A_2-Fragment, durchquert und somit auf die zytoplasmatische Seite der Membran gelangt (278). Darüberhinaus liegt das Choleratoxin im Bereich der ER-Membran mit dem Sec61p Proteinkomplex assoziiert vor, der während des normalen Zellwachstums sowohl neu synthetisierte Proteine in das ER-Lumen einschleust als auch unkorrekt gefaltete Proteine retrograd wieder in das Zytosol zur Degradierung durch Proteasomen zurücktransportiert (324).

Darüberhinaus gibt es deutliche Hinweise darauf, dass auch die B-Untereinheit selbst in das Zytosol der Zelle transloziert wird. So gelingt es durch Erzeugung von Fusionsproteinen mit der B-Untereinheit exogene Antigene in den MHC-I-Präsentationsweg einzuschleusen (96, 97, 171).

2.3.1.4 Proteinbiosynthesehemmung durch Ribosomeninaktivierung

Stx zählt zu den potentesten Inhibitoren der Proteinbiosynthese eukaryotischer Zellen (105). Die Grundlage dieser Wirkung ist eine Inaktivierung der 60S-Untereinheit eukaryotischer Ribosomen. Die Nukleotidsequenz der ribosomalen 28S RNA, wichtigster funktioneller Bestandteil der 60S Untereinheit, ist im Bereich von Position 4320-4329 in eukaryotischen Zellen hochkonserviert (63). Eine verwandte Struktur in der 23S rRNA von *E. coli* ist an der Bindung von Elongationsfaktoren beteiligt (210). Dieser Bereich liegt als "Haarnadelstruktur" vor (236), in deren Schleife sich in Position 4324 ein Adeninrest befindet (**Abb. 2.6**). In toxinbehandelten Zellen wie auch in zellfreien Systemen wird dieser Adeninrest spezifisch vom enzymatisch aktiven A_1-Fragment der Toxine nicht-phosphorolytisch abgespalten (63, 241). Diese N-Glykosidase-Aktivität ist unabhängig von verschiedenen Kofaktoren (NAD, ATP, NADP, NADPH, Elongationsfaktoren, Aminoazyltransferasen) (267) und allen Shigatoxinen gemeinsam (130). Durch Vergleiche zwischen den Aminosäuresequenzen des Shigatoxins 1 und des Rizins wurden drei Homologie-Regionen identifiziert, die die Aminosäuren 51-55, 167-171 und 202-207 des Shigatoxins 1 umfassen (367). Überträgt man diese Homologien auf die bekannte Kristallstruktur von Rizin A, liegen die beiden letztgenannten Bereiche in einer vermutlich

enzymatisch aktiven Tasche des Moleküls. Den Boden dieser Tasche bilden Glu167 und Arg170. Die obere Seitenwand wird von den phenolischen Ringen der Tyrosinreste 77 und 114 gebildet. Die Ringe des Trp203 schliessen die Tasche zur anderen Seite hin ab (52).

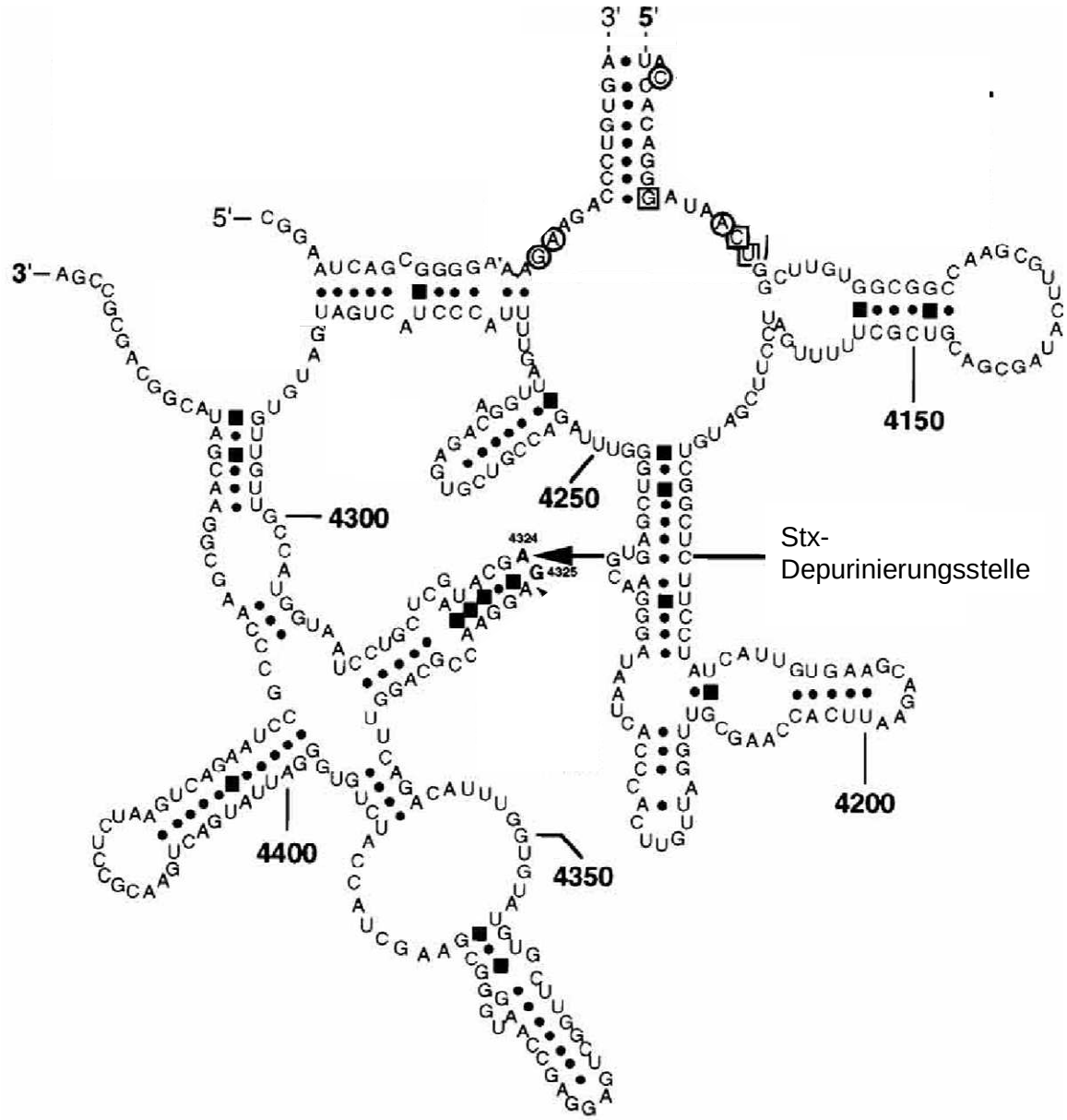

Abb. 2.6: Sekundärstruktur der von Stx in eukaryontischer 28S rRNA erkannten Zielstruktur (modifiziert nach (123)). Watson-Crick Paarungen sind durch schwarze Punkte markiert, non-Watson-Crick Paarungen durch schwarze Quadrate.

In Analogie zu Rizin A wurde für den Ablauf der durch Stx katalysierten Reaktion folgendes Modell vorgeschlagen (**Abb. 2.7**) (52):

1. Arg170 bindet über ionische Wechselwirkungen das Ribose-Phosphat-Rückrat der 28S rRNA. Die Tyrosine 77 und 114 und Trp203 stabilisieren mit ihren aromatischen Ringen diese Bindung und richten den Adeninrest 4324 der rRNA aus.
2. Tyr77 übergibt ein Proton an ein Stickstoffatom im Adeninring und schwächt damit die Bindung zwischen C1 der Ribose und N9 des Adeninrestes.
3. Das protonierte Adenin dissoziiert und hinterlässt ein positiv geladenes Oxocarboniumion im Ribosering, das durch Glu167 stabilisiert wird.
4. Abschliessend attackiert ein Wassermolekül das Oxocarboniumion, hydroxyliert die Ribose und stellt den Protonendonator Tyr77 wieder her.

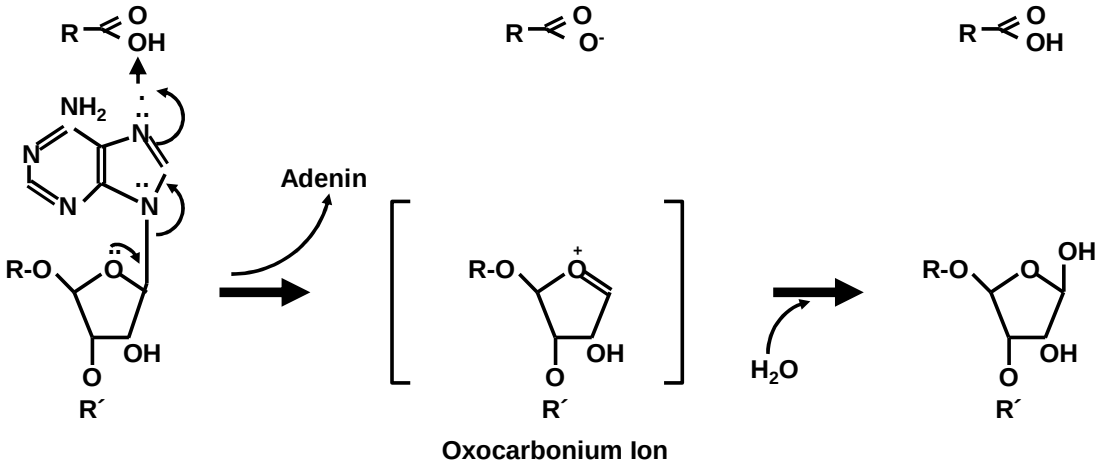

Abb. 2.7: Vermuteter Mechanismus der N-Glykosidase-Wirkung des Shigatoxins (nach (52))

Die Abspaltung des Adeninrestes führt vermutlich zu einer Konformationsänderung der 28S rRNA und dadurch zu einer Abnahme der Affinität der Ribosomen zum eukaryotischen Elongationsfaktor 1 (eEF1), messbar in einer deutlichen Senkung der eEF1-abhängigen GTPase-Aktivität (239). Als Folge dieser Konformationsänderung kommt es zu einem drastischen Abfall der eEF1-abhängigen Bindung von Aminoazyl-tRNA an die Ribosomen (239). Weitere Reaktionen der Proteinbiosynthese (Aminoazetylierung von tRNA, Initiation, Peptidyltransferase-Reaktion, Translokation) sind von der Toxinwirkung nicht betroffen (239). Trotzdem kommt es zu einer vollständigen und irreversiblen Inaktivierung der

Ribosomen (267). Von dieser Inaktivierung sind insbesondere Ribosomen betroffen, die zum Zeitpunkt der Toxineinwirkung an mRNA gebunden und biosynthetisch aktiv sind, da die Toxine 60S Untereinheiten nur dann inaktivieren können, wenn diese Bestandteil eines vollständigen Ribosoms sind (267). Damit stoppt die Proteinbiosynthese der betroffenen Ribosomen im Stadium der Elongation mit Fixierung der Polysomenstruktur (267), wodurch die betroffene mRNA der Reaktion mit gegebenenfalls noch vorhandenen intakten Ribosomen entzogen wird. Die Reaktionsgeschwindigkeit der Inaktivierung wurde im zellfreien System auf 40 Ribosomen pro Minute pro A_1-Fragment geschätzt (267). In HeLa-Zellen wurde aus der CD_{50} und der verwendeten Zellzahl ein Verhältnis von 1000 Ribosomen je Toxinmolekül abgeleitet (25). Ein einzelnes Rizinmolekül soll 1500 Ribosomen pro Minute inaktivieren können (135).

2.3.1.5 Kerntransport und intranukleäre Wirkungen

Obwohl der Übertritt der A_1-Untereinheit in das Zytosol wahrscheinlich auf Höhe des endoplasmatischen Retikulums stattfindet, ist in manchen Zellsystemen der retrograde Transport bis hin zur Kernmembran für den zytoletalen Effekt des Toxins erforderlich (5). Darüberhinaus lässt sich in den Zellen sogar eine Akkumulation der StxB innerhalb des Nukleus beobachten (5). Dieser Transportweg ist so effizient, dass es sogar gelingt mit Hilfe von chimeren Proteinen aus StxB1 und DNA-bindenden Proteinen DNA-Fragmente gezielt in den Zellkern einzuschleusen (67).

Erstaunlicherweise findet ein Transport der StxB1 bis zu den Nukleoli auch in solchen Zellen statt, die gegenüber der Hemmung der Proteinbiosynthese durch Stx resistent sind (68). Der zugrundeliegende Transportweg, der in humanen Makrophagen vorkommt, ist zwar noch nicht aufgeklärt, aber definitiv unabhängig vom retrograden Beschreiten des biosynthetisch/sekretorischen Transportweges über das ER (68). Es wäre denkbar, dass der Transport innerhalb des Zytosols stattfindet, da eine Anhebung des endosomalen pHs, der für die Translokation der Shigatoxine in das Zytosol essentiell ist, den Transport blockiert (68). Dies würde allerdings die Translokation auch der B-Untereinheit voraussetzen, wie sie von Nakagawa *et al.* postuliert wurde (224). In der Tat interagiert StxB1 mit BiP, einem im ER lokalisierten Chaperon, das mit dem retrograden Transport von Proteinen in das Zytosol in Verbindung gebracht wurde (68). In permeabilisierten Zellen diffundiert StxB auch passiv in den Nukleus, jedoch erfordert der Transport in nativen Zellen die Anwesenheit von ATP (68). Nach der Translokation in den Kern bindet StxB1 im Verhältnis 1:1 an das nukleäre Protein B23 (Nukleoplasmin), wobei beide Isoformen (B23.1 und B23.2) gleichermaßen erkannt

werden (68). B23 stellt ein multifunktionales Protein dar, dass auch am Zusammensetzen der Ribosomen beteiligt ist. Durch die Interaktion der StxB1 mit B23 wird möglicherweise auch das Stx-Holotoxin gezielt zu den Nukleoli, dem Bildungsort seiner molekularen Zielstruktur, geleitet (68).

Die Inaktivierung von Ribosomen hat den grössten Anteil an der zytoletalen Aktivität der Shigatoxine, jedoch besitzt Stx1 auch eine Adenin-spezifische N-Glykosidase-Aktivität für einzelsträngige DNA (21). *In vitro* bindet das A_1-Fragment an die DNA und gleitet an ihr bis zur Erkennung der Zielstruktur entlang (21). Obwohl Stx1 selbst keine DNAse-Aktivität entfaltet, führt die Entfernung zahlreicher Adenine zu einer Schwächung des Zucker-Phosphat-Rückrates der DNA und zu Strangbrüchen (21), die in Endothelzellen bereits nach der Hemmung der Proteinbiosynthesse durch die ribosomale Wirkung des Shigatoxins 1, aber mehrere Stunden vor der Induktion von DNAsen des Apoptoseprogramms (siehe **2.3.2.2**), auftreten (22).

2.3.2 Induktion von Zelltod

2.3.2.1 Folgen der Proteinbiosynthese-Hemmung

Die irreversible Hemmung der Proteinbiosynthese durch Stx führt nicht zum sofortigen Untergang der entsprechenden Zelle (290). Obwohl die Proteinbiosynthese in hochsensitiven Zellen bereits nach 30 Minuten abnimmt und nach 45 Minuten vollständig sistiert, nehmen die Zellen noch über mehrere Stunden markiertes Uridin für die RNA-Synthese auf (25, 267). Das Polysomenprofil vergifteter Zellen bleibt intakt (267). Die Zellen sind noch 90 Minuten nach Toxinzugabe endozytotisch aktiv und halten über 120 Minuten ihren intrazellulären Kaliumspiegel konstant. In dieser Zeit zerstört Stx also weder die Membranintegrität noch beeinflusst es die oxidative Phosphorylierung (25). Erst 4 Stunden nach Beginn der Toxineinwirkung zeigt die DNA von HeLa-Zellen Anzeichen von Fragmentierung (77).

Die Manifestation der funktionellen und später auch morphologischen Veränderungen der Zellen als Folge der Intoxikation benötigt mehrere Stunden, in denen zelleigene Mechanismen in Gang gesetzt werden. Elektronenmikroskopisch zeigen sich nach 6 Stunden im Kern geschädigter Vero-Zellen scharf umrissene Chromatinmassen (290). Das Zytosol weist in Kernnähe zahlreiche Vakuolen auf. Diese stellen zum Teil Lipidtröpfchen aus Abschnürungen des Kerns dar, zum Teil handelt es sich um autophagische Vakuolen, die durch den Gehalt membranösen Materials gekennzeichnet sind (290). Die Hemmung der Autophagie durch

spezifische Wirkstoffe schützt die Zellen vor Lyse, auch zu einem Zeitpunkt, an dem das Toxin die Proteinbiosynthese der Zellen bereits zerstört hat (290).

Die morphologischen Veränderungen und der DNA-Abbau sind charakteristisch für Apoptose (290). Obwohl es sich bei der Apoptose um eine aktive Form des Zelltodes handelt, für den in vielen Zellsystemen eine intakte Proteinbiosynthese Voraussetzung ist (6), können auch verschiedene Inhibitoren der Translation Apoptose auslösen (33). Möglicherweise führt der Mangel an Apoptose Inhibitor-Protein dann zum Ablauf des genetischen Programms und zum Zelltod (33). So geht der durch Stx1 oder Stx2 in humanen Endothelzellen induzierten Apoptose eine deutlich erniedrigte Expression an Mcl-1, einem Mitglied der anti-apoptotischen Bcl-2-Familie, voraus (65). Die DNA-Fragmentierung als Folge der Stx-Wirkung kann zwar nicht in allen Zellsystemen beobachtet werden und Hemmstoffe der Apoptose können die Zellen nicht immer vor Lyse schützen (290). Die Hemmung der Proteinbiosynthese durch Stx stellt aber einen wichtigen Kofaktor bei der Auslösung von Apoptose dar, wenn die Zellen bereits durch einen anderen Stimulus sensibilisiert worden sind (122). Dieser Stimulus kann dabei durchaus von Stx selbst ausgehen, da die Toxine auch unabhängig von ihrer Proteinbiosynthese-inhibierenden Wirkung Apoptose auslösen können (siehe **2.3.2.3**). Darüberhinaus sensibilisiert Stx1 humane Endothelzellen gegenüber LPS-induzierter Apoptose durch die Hemmung der Expression des anti-apoptotischen Proteins FLIP, eines Kaspase 8-Inhibitors (66).

Lichtmikroskopisch sind an den Zellen über 12 Stunden keinerlei morphologische Veränderungen feststellbar (267). Nach diesem Zeitraum verlieren sie jedoch ihre Membranintegrität (267, 290). Die Fähigkeit der Toxine Zelllyse zu induzieren erlaubt es den Molekülen nicht nur solche Zellen zu schädigen, in denen sie initial die Proteinbiosynthese inhibieren. Während der Zelllyse und der damit verbundenen Freisetzung von Zellinhalt greifen die freigesetzten proteolytischen Enzyme unter Umständen auch Nachbarzellen an und unterstützen damit die Ausbildung eines toxischen Effektes im Gesamtorganismus (290).

Die Wirkung der Toxine führt nicht immer zur vollständigen Zerstörung der Zellen. In weniger empfindlichen Zellen, wie z.B. konfluenten Endothelzellen ohne Zytokin-Stimulation, wird die Proteinbiosynthese durch das Toxin nur auf 60 % des Ausgangswertes reduziert und die Zellen bleiben bis zu 48 Stunden unvermindert vital (237). Gerade diese Endothelzellen werden aber beim Menschen als das Hauptziel der Toxinwirkung *in vivo* angesehen, so dass in der Pathogenese der Stx-assoziierten Erkrankungen auch eine subletale Schädigung dieser Zellen eine Rolle spielen dürfte (237).

2.3.2.2 direkte Aktivierung des Apoptoseprogramms

Neben der indirekten Aktivierung des Apoptoseprogramms durch Hemmung der Proteinbiosynthese können die Shigatoxine auch direkt Apoptose in Zellen auslösen. Es hat sich gezeigt, dass dabei zwei unterschiedliche Signalwege existieren (336). Der eine Weg nimmt seinen Ursprung von der Bindung und Kreuzvernetzung des Gb_3/CD77 auf der Zelloberfläche (siehe **2.3.2.3; Abb. 2.9**), während ein anderer Weg die Internalisierung der enzymatisch aktiven A-Untereinheit in das Zytosol voraussetzt (**Abb. 2.8**).

Die Depurinierung der 28S rRNA translationell aktiver Ribosomen durch StxA führt zu strukturellen Veränderungen in kritischen Regionen der rRNA und damit zu funktionellen Beeinträchtigungen während der Translation (123). Diese Störungen dienen offensichtlich als Erkennungssignal für die Aktivierung Stress-aktivierter Proteinkinasen (SAPK/JNK) und induzieren eine „ribotoxic stress response“ (123). Diese hat die Induktion der Expression verschiedener Chemokingene (siehe **2.3.3**) ebenso zur Folge wie die Aktivierung von Kaspasen (305). Entsprechend können Inhibitoren der Mitogen-aktivierten Proteinkinase p38 (MAPK38) wie SB202190 und SB203580 Zellen auch vor Stx-induziertem Zelltod schützen (118, 305). Obwohl das Ausbleiben entzündlicher Erscheinungen normalerweise ein typisches Merkmal der Apoptose darstellt, sind somit bei der Wirkung der Shigatoxine die Signalwege, die zur Expression pro-inflammatorischer Gene bzw. zur Apoptose führen, eng miteinander verbunden (305).

Die molekulare Verbindung zwischen der „ribotoxic stress response“ und der Aktivierung der Kaspase-Kaskade ist noch nicht aufgeklärt (35) scheint jedoch sowohl von CD95 als auch von TNF-Rezeptoren unabhängig zu sein (156, 172). Von zentraler Bedeutung für die Auslösung der Apoptose ist in Burkitts Lymphomzellen (336), Hep2-Zellen (35) und HeLa-Zellen (77) die Aktivierung der Kaspase 8. Aktivierte Kaspase 8 spaltet und aktiviert die zentrale Effektor-Kaspase 3 (77, 156, 336). Kaspase 3 ihrerseits spaltet sowohl den nukleären Faktor „Acinus“ (275) als auch Kaspase 6 (77, 164), die wiederum Lamin A spaltet und aktiviert (329). Darüberhinaus scheint ein positiver Rückkoppelungsmechanismus zu existieren, bei dem Kaspase 6 direkt Kaspase 8 aktiviert (172). Die Aktivierung von „Acinus“ und Lamin A führt zur Kondensation des Chromatins (275) und der Zerstörung der internen Struktur des Zellkerns (329). Ausserdem inaktiviert die Kaspase 3 sowohl einen Inhibitor Kaspase-abhängiger DNAsen (CAD) (77) als auch die poly(ADP-Ribose)-Polymerase PARP (35), ein DNA-Reparaturenzym. Die Aktivierung der CAD bei gleichzeitiger Hemmung von DNA-Reparaturenzymen resultiert in der Fragmentierung der DNA durch internukleosomale

Spaltung. Zusätzlich treten vermehrt Strangbrüche als Folge der Depurinierung einzelsträngiger DNA auf, die von der StxA direkt im Zellkern katalysiert wird (21). Durch Behandlung von Zellen mit Curcumin, das die Expression von Hitzeschockprotein Hsp-70 auslöst, kann die DNA-Fragmentierung verhindert und die Zellen vor der zytoletalen Wirkung der Shigatoxine geschützt werden (308).

In THP-1-Zellen kann nach Einwirkung von Stx auch eine Aktivierung von Kaspase 2 und 10 (164), in Burkitts Lymphomzellen auch eine Aktivierung von Kaspase 7 (156) beobachtet werden, jedoch ist die Bedeutung dieser Kaspasen noch unklar.

Bei der Induktion von Apoptose durch die Stx-Holotoxine hat die Aktivierung des Kaspase 8-abhängigen Signalweges in Burkitts Lymphomzellen die grösste Bedeutung (336). Allerdings spaltet in Stx-behandelten HeLa- und Hep2-Zellen die Kaspase 8 auch BID, ein proapoptotisch wirkendes Mitglied der, in der äusseren Mitochondrien-Membran lokalisierten Familie der Bcl-2-Faktoren (35, 77). Aktiviertes BID (gespaltenes („truncated“) BID, tBID) unterstützt die Oligomerisierung der proapoptotischen Proteine Bax und Bak. Die resultierende Erhöhung in der Permeabilität der Mitochondrienmembran lässt das Membranpotential zusammenbrechen und Zytochrom C in das Zytosol austreten (77). Zytosolisches Zytochrom C komplexiert mit Apaf-1 („apoptotic protease activating factor“) und aktiviert Kaspase 9 (35, 77, 80, 164). Die Aktivierung von Kaspase 9 verstärkt die durch Kaspase 8 direkt induzierte Spaltung und Aktivierung von Kaspase 3 (77, 164).

Interessanterweise findet sich in der Polypeptidkette der A-Untereinheit des Shigatoxins 2 ein Sequenzmotiv (NWGRI, Aminosäurereste 223-227) mit Homologie zur BH1-Domäne des antiapoptotischen Bcl-2 (322). Diese Domäne ist für die antiapoptitische Wirkung des Bcl-2 unabdingbar und dient normalerweise zur Interaktion mit anderen Bcl-2-Molekülen bzw. mit Bax und Bak. Über die BH1-homologe Domäne kann sich StxA2 nach Translokation zu den Mitochondrien mit Bcl-2 zu Komplexen verbinden, Bax und Bak verdrängen und dadurch ebenfalls deren Oligomerisierung induzieren (322). Obwohl auch in Stx1 eine Sequenz (NWGRL, Aminosäurereste 234-230) mit grosser Ähnlichkeit zur Bcl-2 BH1-Domäne existiert, interagiert nur Stx2, nicht aber Stx1, mit Bcl-2 über NWGRI *in vitro* und *in vivo* (322). Dies könnte erklären, warum dem mitochondrialen Signalweg, der in der Aktivierung von Kaspase 9 mündet, bei der Wirkung des Shigatoxins 1 auf Burkitts Lymphomzellen keine Bedeutung zukommt (156). Entsprechend schützt die ektope Expression von Bcl-2 in diesen Zellen zwar vor Gb_3/CD77-vermittelter Apoptose durch StxB1 (siehe **2.3.2.3**), nicht aber vor der Wirkung des entsprechenden Holotoxins (90).

Auch oxidativer Stress durch einen intrazellulären Anstieg reaktiver Sauerstoff-Metaboliten (ROS) kann zu einer Permeabilitätserhöhung der Mitochondrienmembran führen (80). Bei der Intoxikation von Zellen durch Stx kann dieser Stress aus einer verminderten Expression zellulärer Katalase resultieren (118, 202). Zusätzlich zu ihrer Wirkung auf Mitochondrien verursachen die ROS eine Erhöhung der Konzentration zytosolischen NADs und öffnen dadurch an der Zellmembran NAD-aktivierte Ca^{++}-Kanäle (109). Der unkontrollierte Einstrom von Ca^{++}-Ionen in Stx-behandelte Zellen bewirkt die Phosphorylierung der Mitogen-aktivierten Proteinkinase p38 (MAPKp38) (118), wodurch eine positive Rückkoppelung zur Aktivierung der „ribotoxic stress response“ entsteht. Zumindest in bestimmten Zellsystemen scheint der Ca^{++}-Einstrom eine essentielle Rolle zu spielen, da Ca^{++}-Kanalblocker wie Verapamil Vero- und HeLa-Zellen vor der zytoletalen Wirkung der Shigatoxine schützen können (282).

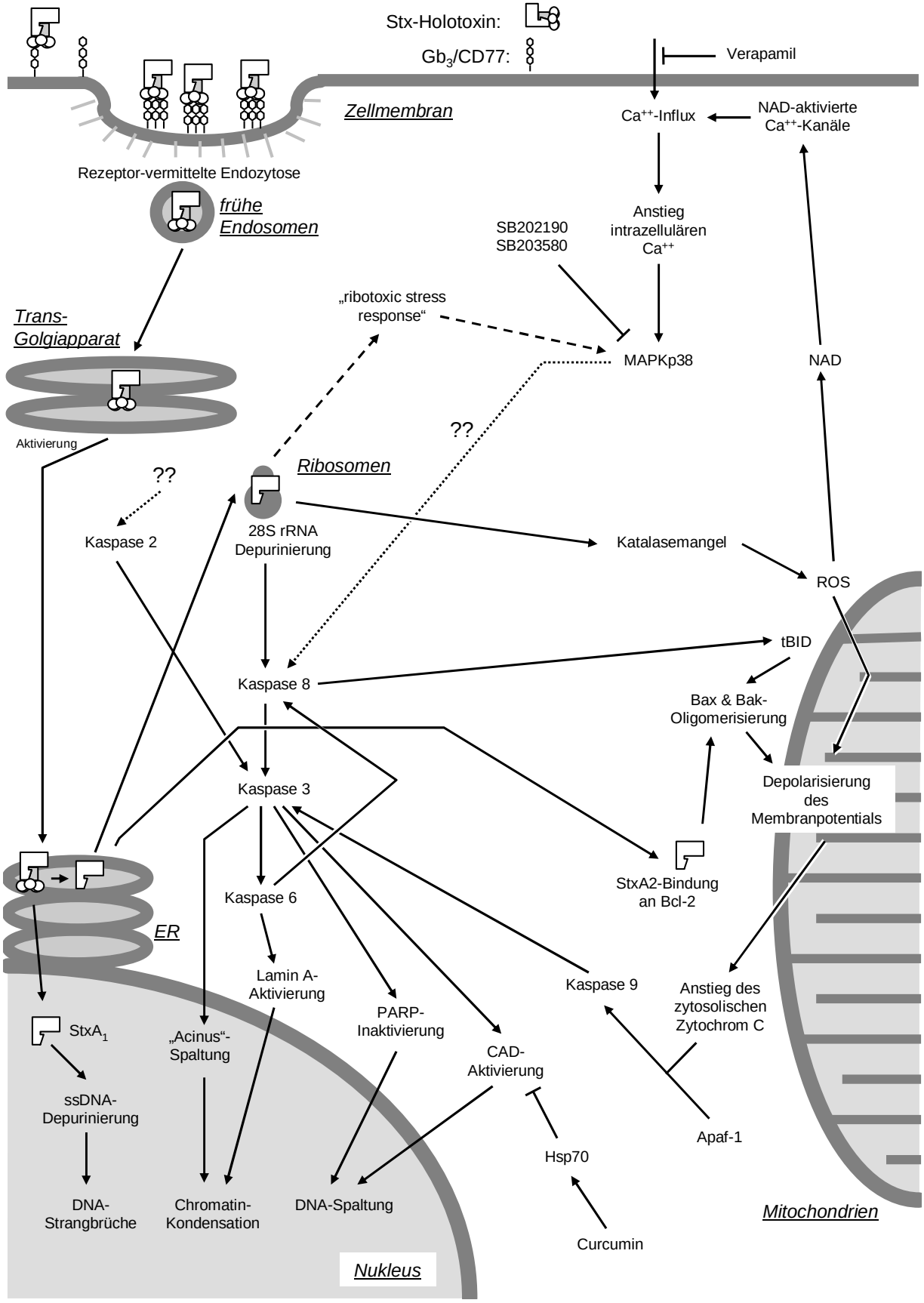

Abb. 2.8: Modell der Aktivierung intrazellulärer Signalwege und der Auslösung von Apoptose durch Stx-Holotoxine (Details siehe Text)

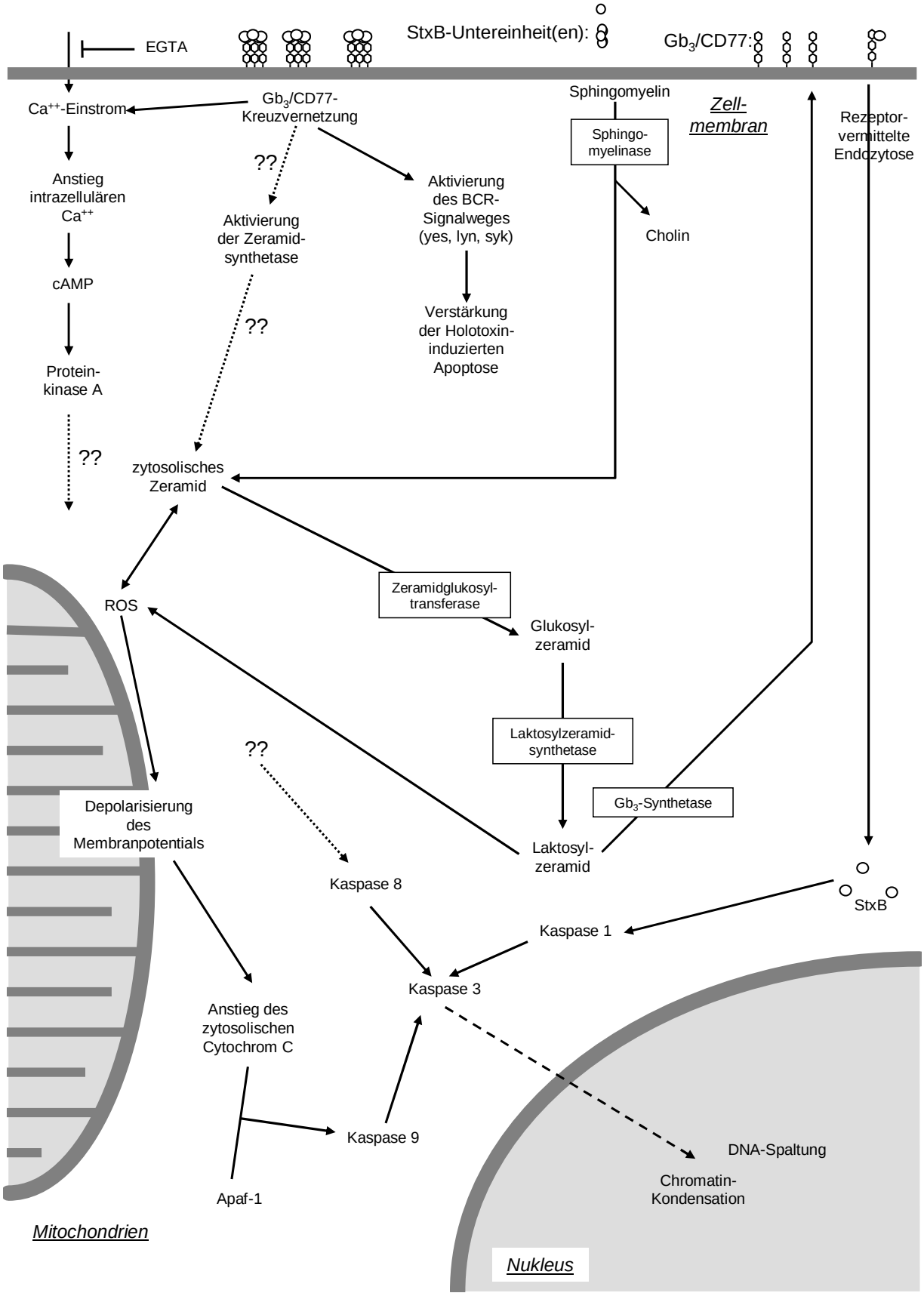

Abb. 2.9: Modell der Aktivierung intrazellulärer Signalwege und der Auslösung von Apoptose durch die B-Untereinheiten der Shigatoxine (Details siehe Text)

2.3.2.3 Aktivierung von Gb_3/CD77-abhängigen Signalwegen

Eine Reihe von Tumorzelllinien ist resistent gegenüber einer Apoptoseinduktion durch die isolierte B-Untereinheit der Shigatoxine (77, 164, 359). Dagegen löst vor allem bei Burkitts Lymphomzellen (144, 197, 326, 336), aber auch bei der humanen Nierentubuluszelllinie ACHN (144, 145), die Kreuzvernetzung von Gb_3/CD77 auf der Zelloberfläche durch einen gegen CD77 gerichteten Antikörper oder durch die Bindung der StxB verschiedene intrazelluläre Signale aus, die den Prozess der Apoptose initiieren.

In diesen sensitiven Zellen ist Gb_3/CD77 in „lipid rafts“ in räumlicher Nähe zu den Src-Kinasen Yes und Lyn sowie Syk lokalisiert (144, 145, 216). Bei Einwirkung von Stx werden binnen zehn Minuten die Tyrosinreste der in den „rafts“ vorhandenen Proteine hyperphosphoryliert. Dabei löst die Bindung von Stx an Gb_3/CD77 initial eine Anreicherung von Yes und Lyn in den „lipid rafts“ aus. Nach Aktivierung entfernen sich Yes und Lyn aus den „lipid rafts“, bleiben aber mit der Zellmembran assoziiert und folgen nicht der Internalisierung der Stx-Gb_3/CD77-Komplexe (144, 145). In B-Zellen wie Burkitts Lymphomzellen ist die Apoptose-auslösende, von Gb_3/CD77 ausgehende Signalkaskade somit eng mit der des B-Zell-Rezeptors verknüpft (216). Die Aktivierung der Signalkaskade durch die oberflächliche Bindung der Shigatoxine verstärkt die Apoptoseinduktion, die durch die zytosolischen Wirkungen des Stx-Holotoxins ausgelöst wird (144).

Bereits 30 Sekunden nach Kreuzvernetzung von Gb_3/CD77 lässt sich ausserdem ein massiver Einstrom von extrazellulären Ca^{++}-Ionen feststellen, der nach 120 Sekunden sein Maximum erreicht und dem innerhalb weniger Minuten eine Erhöhung der intrazellulären cAMP-Konzentration und die Aktivierung der Proteinkinase A nachfolgt (326).

Nach 30 Minuten steigt der Gehalt der Zellen an zytosolischem Zeramid an, dessen Ursprung derzeit noch unklar ist (326). Obwohl Agonisten wie TNF-α oder IL-1β die Freisetzung von Zeramid aus Sphingomyelin durch Aktivierung einer endogenen Sphingomyelinase induzieren können, lösen uropathogene *E. coli*, die mit ihren P-Fimbrien ebenfalls an Gb_3/CD77 binden können, in Epithelzellen (A498) nur eine geringgradige Aktivitätssteigerung der Sphingomyelinase und keine messbare Sphingomyelinhydrolyse aus (106). Auch in Stx-behandelten Burkitts Lymphomzellen geht der Anstieg zytosolischen Zeramids nicht mit einer Abnahme des Sphingomyelins einher (326). Da auch der zelluläre Gehalt an Gb_3 konstant bleibt, scheidet auch ein Abbau des Stx-Rezeptors nach Ligandenbindung als Quelle aus (326). Die Ursache für den Anstieg des Zeramidgehaltes

könnte in der Aktivierung der Zeramid-Synthetase liegen, wie es nach Stimulation von bovinen γδT-Zellen über das WC1-Antigen beobachtet werden konnte (154).

Zytosolisches Zeramid ist Teil eines Signalweges, der insbesondere Apoptose auslöst, aber auch das Wachstum und die Differenzierung von Zellen moduliert oder die Sekretion von Zytokinen stimuliert (106). Somit könnte hier eine weitere Querverbindung zwischen der Stx-induzierten Apoptose und der Expression von Mediatoren des Immunssystems bestehen. Ein erhöhter intrazellulärer Zeramidspiegel, wie er durch Behandlung mit einer bakteriellen Sphingomyelinase in humanen Endothelzellen herbeigeführt werden kann, induziert eine vermehrte Expression von Enzymen des Gb_3/CD77-Syntheseweges und eine vermehrte Synthese und oberflächliche Expression von Gb_3/CD77 (240). Da daraus eine Sensibilisierung der Zellen gegenüber Stx resultiert (240), ist es denkbar, dass zumindest in entsprechend sensitiven Zellen die Bindung bereits weniger Stx-Moleküle an Gb_3/CD77 ausreicht, um im Sinne eines positiven Regelkreises die Rezeptorexpression zu verstärken und der Aufnahme grösserer Toxinmengen durch die Zellen Vorschub zu leisten. Eine vermehrte Synthese von Gb_3/CD77 führt auch zu einer Zunahme einer Synthesevorstufe des Gb_3/CD77, des Laktosylzeramids, in den Zellen (240).

Zeramid und Laktosylzeramid ist gemeinsam, dass sie an den Mitochondrien die vermehrte Generierung von ROS auslösen können (4, 80). Dies aktiviert den mitochondrialen Signalweg der Apoptoseauslösung, der bei der Aktivierung des Gb_3/CD77-abhängigen Signalweges im Vordergrund steht (90, 336). Allerdings kann nach Kreuzvernetzung von Gb_3/CD77 auf Burkitts Lymphomzellen auch eine Aktivierung von Kaspase 8 beobachtet werden (156). Der zugrundeliegende Mechanismus ist nicht bekannt, jedoch zeigt die Aktivierung der Kaspase 8, dass es auch über Gb_3/CD77 zu einer gewissen Aktivierung des extrinsischen, Kaspase-abhängigen Apoptoseweges kommt. Interessanterweise kann auch StxB1, obwohl es selbst keine proteolytische Aktivität besitzt, bei artifizieller Expression innerhalb eukaryontischer Zellen die Aktivierung von Kaspase 1 und 3 auslösen und Apoptose einleiten (224).

2.3.3 „ribotoxic stress response“ und Induktion der Expression proteinartiger Mediatoren

Die durch die Depurinierung der 28S rRNA induzierte „ribotoxic stress response“ (123) ist nicht spezifisch für StxA, da sie auch durch andere Toxine, die wie Rizin oder α-Sarzin die Haarnadelstruktur der rRNA zwischen Position 4320-4329 modifizieren, ausgelöst wird

(123). Sind die Ribosomen zum Zeitpunkt der Toxineinwirkung biochemisch aktiv, resultieren die strukturellen Veränderungen im Peptidyltransferase-Zentrum der Ribosomen möglicherweise in einer Aktivierung kleiner GTP-bindender Proteine, die ihrerseits eine Proteinkinase-Kaskade aktivieren, die über MEKK1 und SEK1/MKK4 in der Aktivierung der stress-aktivierten Proteinkinasen SAPK/JNK1 münden (123). Die Depurinierung der rRNA an Position A4324 ist bereits 15 min nach Zugabe von Rizin A zu Zellen nachweisbar (123). Ihr folgt nach 30 min eine über Stunden anhaltende Phosphorylierung von SEK1/MKK4 und Aktivierung von SAPK/JNK1 (123). Die Aktivierung dieser Kaskade ist unabhängig von der Hemmung der Proteinbiosynthese und findet auch unter Bedingungen statt, unter denen die Proteintranslation nur geringgradig beeinflusst wird (123). Dies erklärt, warum die Zellen trotz der Einwirkung von Rizin oder Stx noch in der Lage sind mit einer deutlich gesteigerten Transkription der „immediate early"-Gene *c-fos* und *c-jun* zu reagieren, die nach 60 min apparent wird (123, 305, 337). In manchen Zellen scheint die vermehrte Transkription von *c-fos* und *c-jun* auch Folge der Aktivierung der MAPKp38 zu sein (28, 305, 337). Die durch Stx induzierte *c-jun* Transkription zieht einen erhöhten Gehalt der Zellen an c-Jun-Protein nach sich, das dann auch verstärkt in der phosphorylierten Form vorliegt (163). Interessanterweise korreliert der Anstieg phosphorylierten c-Juns mit der gleichzeitig verstärkten Expression von *mpk-1* („mitogen-activated protein kinase phosphatase 1"), obwohl diese Phosphatase sowohl JNK als auch MAPKp38 inaktivieren kann (163).

Zusätzlich zur Induktion der SAPK/JNK1- und MAPKp38-Signalwege stimuliert Stx auch eine Proteinkinase C- (PKC-) Aktivität, jedoch ist noch nicht abschliessend geklärt um welche der verschiedenen PKC-Isoformen es sich dabei handelt (74). Allerdings kann in Stx-behandelten humanen Monozyten auch eine erhöhte Aktivität von Kinasen nachgewiesen werden, die sich im Signalweg stromabwärts von atypischen Isoformen der PKC befinden und durch extrazelluläre Signale reguliert werden („extracellular signal-regulated kinases"; ERK) (29). Dies lässt vermuten, dass die Stx-induzierte PKC-Aktivität von atypischen Isoformen der PKC ausgeht, die zur Aktivierung weder Kalzium noch Lipide benötigen und z.B. an der Signaltransduktion bei LPS beteiligt sind (74). Die Involvierung dieses Signalweges wie auch die Tatsache, dass eine Stx-abhängige Aktivierung von SAPK/JNK1 und MAPKp38 auch in humanen Monozyten beobachtet werden kann (28), könnte darauf hindeuten, dass die Shigatoxine die Expression proteinartiger Mediatoren auch unabhängig von ihrer ribotoxischen Wirkung induzieren können. Dafür spricht auch, dass ein Anstieg der intrazellulären Zeramid-Konzentration, wie er von Stx1 unabhängig von der enzymatischen Wirkung durch die Bindung an Gb_3/CD77 erzeugt werden kann (326), bereits mit einer

vermehrten Zytokin-Genexpression in Verbindung gebracht wurde (106). Auch die von Nakagawa *et al.* (224) beschriebene Induktion von Kaspase 1 durch intrazytoplasmatische Expression von StxB1 führt zu einer vermehrten Synthese von IL-1β und TNF-α. Gegen diese Vermutung spricht jedoch, dass mehrere Untersuchungen gezeigt haben, dass isolierte B-Untereinheiten der Shigatoxine oder enzymatisch inaktive Holotoxine exogen keine Genexpression induzieren können (104, 163, 277, 305, 365, 366).

Unklar ist bislang, ob der Aktivierung der Expression von Mediatoren-Genen neben der Induktion der Transkriptionsaktivatoren *c-jun* und *c-fos* durch Stx auch eine Translokation des Transkriptionsaktivators NF-κB zugrunde liegt. NF-κB-Bindungsstellen finden sich in den Promotorregionen vieler pro-inflammatorischer Gene. Sakiri *et al.* (277) zeigten in der Tat, dass in THP-1-Zellen, einer humanen monozytären Zelllinie, nach Einwirkung von Stx1 erhöhten Konzentrationen von TNF-α-spezifischer mRNA die nukleäre Translokation von NF-κB und AP-1 („activator protein-1“) ebenso vorausgeht wie die Degradation des NF-κB-Inhibitors IκBα. Auch in ACHN-Zellen induziert Stx2 eine vermehrte *tnf-α*-Transkription über eine Aktivierung der cAMP-abhängigen Proteinkinase A, MAPKp38 und NF-κB (225). In Vero- bzw. T84-Zellen können diese Effekte durch gereinigtes Stx dagegen nicht ausgelöst werden, sondern nur in Verbindung mit einem anderen, nicht identifizierten Faktor (28) bzw. nach Infektion der Zellen mit einem STEC-Stamm (45). Vermutlich wird die Translokation von NF-κB im Verlaufe der STEC-Infektion überwiegend durch Flagellin über den „Toll-like“-Rezeptor 5 verursacht, da *stx*- und *eae*-negative *E. coli*-Stämme diesen Effekt nicht, gereinigtes Flagellin dagegen schon hervorrufen kann (13).

Als Folge der Toxineinwirkung auf die Ribosomen steigt in vielen Zellsystemen der intrazelluläre Gehalt an mRNA an, die spezifisch für primäre Antwortgene („primary response genes“) ist. Die Induktion dieser Gene ist refraktär gegenüber einer translationalen Blockade. Shigatoxine verstärken insbesondere die mRNA-Induktion durch andere Agonisten, ein Phänomen, das als Superinduktion bezeichnet wird (104, 338). Obwohl andere Inhibitoren der Proteinbiosynthese wie Cycloheximid den zellulären Gehalt an bestimmten mRNA-Spezies sowohl durch Steigerung der Aktivität transkriptioneller Aktivatoren als auch durch Stabilisierung der mRNA erhöhen, scheint bei Stx dem letztgenannten Mechanismus die erheblich grössere Bedeutung zuzukommen (338). Trotz Einsetzen der Proteinsynthese-Hemmung bereits nach wenigen Stunden führt die erhöhte Gesamtmenge an spezifischer mRNA auch zu einer verstärkten Neusynthese der kodierten Proteine, die nach 12 Stunden messbar wird und bis zu 24 Stunden anhält (338). Dabei ist die Stärke der allgemeinen Proteinsynthese-Hemmung in den Zellen sogar negativ mit der Translationsaktivität für

bestimmte Proteine korreliert (337). Allerdings steht einer massiven Steigerung der mRNA-Menge oft eine nur erheblich geringere Zunahme des entsprechenden Proteins gegenüber (103, 337, 365). Dies wird als Auswirkung des partiellen Translationsblocks interpretiert (337, 365). Welche Mediatoren von Stx-behandelten Zellen verstärkt exprimiert und sezerniert werden, hängt von der Art und Herkunft der Zelle ab und ist in **Tabelle 2.3** zusammengefasst.

2.3.4 Induktion der Synthese von Arachidonsäure-Metaboliten

Die Entdeckung von Zhang *et al.* (370), dass sich die Stx-induzierte TNF-α-Expression in humanen Monozyten durch Behandlung der Zellen mit einem synthetischen Antagonisten des Plättchen-aktivierenden Faktors PAF hemmen lässt, könnte darauf hindeuten, dass noch ein weiterer Stx-induzierter Signalweg existiert. Tatsächlich führt die Behandlung von Monozyten mit PAF in Verbindung mit LPS zu einer Aktivierung der Zeramid-Synthetase und einer Steigerung des intrazellulären Zeramidspiegels (8). Letzteres kann auch nach Einwirkung von Stx auf Burkitts-Lymphomzellen beobachtet werden, auch wenn hier die Quelle des Zeramids noch unbekannt ist (326). Allerdings kann auch die B-Untereinheit des Choleratoxins die zelluläre Synthese von Arachodinsäure-Metaboliten steigern (251), so dass ein von Glykolipid-Rezeptoren in „lipid rafts“ ausgehendes Signal für die Steigerung des Zeramidspiegels verantwortlich sein könnte. Ein erhöhter Zeramidgehalt stimuliert die sekretorische Phospholipase A_2 ($sPLA_2$) (8), deren Transkription und Translation zusammen mit der der zytosolischen PLA_2 ($cPLA_2$) durch Stx1 auch in primären humanen glomerulären Epithelzellen induziert wird (291). In der Folge steigt die Produktion von Arachidonsäure, was in Verbindung mit einer Aktivitätssteigerung der Cyclooygenasen in einer vermehrten Synthese von Prostacyclin (PGI_2) und Thromboxan A_2 (TxA_2) resultiert (291). Freie Arachidonsäure-Metaboliten können u.a. Proteinkinase C und MAPK aktivieren und über die Modifikation der Gentranskription entzündliche Reaktionen hervorrufen. Da PGI_2 und TxA_2 aber zum Teil gegenteilige Effekte hervorrufen, ist die Bedeutung dieser Mediatoren bei der Wirkung der Shigatoxine noch ungeklärt (291). Tatsächlich wird die Stx1-induzierte *tnf-α*-Expression in humanen Monozyten zwar von einer vermehrten Synthese des Prostaglandins E_2 (PGE_2) begleitet, die Superinduktion von PGE_2 durch Anisodamin hemmt jedoch die Stx1-induzierte *tnf-α*-Expression (371).

Tabelle 2.3: Übersicht über die durch Shigatoxine oder deren Untereinheiten induzierten Zytokine und Chemokine

Zellart /-linie	Spezies	Toxin	Induktion von		Referenz
			mRNA [1]	Protein [1]	
Intestinale Epithelzellen					
Hct-8	Mensch	Stx1	IL-8, GRO-α, GRO-β, GRO-γ, ENA-78	IL-8, GRO-α	(338)
		Stx1 + 2	IL-8	IL-8	(337)
Caco-2	Mensch	Stx1 + 2	IL-8, MCP-1, MIP-1α, MIP-1β, TNF-α	IL-8	(365)
Renale Epithelzellen					
Glomerula, primär	Mensch	Stx1	IL-1, IL-6, TNF-α	IL-1, IL-6, TNF-α	(113)
Tubulus, primär	Mensch	Stx1	IL-1, IL-6, TNF-α	IL-1, TNF-α	(114)
Endothelzellen					
Gehirn~, primär	Mensch	Stx1	IL-1β, IL-, 6, TNF-α	IL-6, IL-8	(62)
Aorta~, primär	Rind	Stx1 (+2)	Preproendothelin-1	Endothelin	(17)
Fibrozyten /-blasten					
NIH3T3	Maus	StxB1	IL-1β, TNF-α	IL-1β, TNF-α	(224)

Legende: [1] Die Diskrepanz zwischen den Angaben zur mRNA- und Proteininduktion beruht in der Regel darauf, dass in vielen der zitierten Publikationen nicht alle der Mediatoren auf mRNA-Ebene und auf Proteinebene quantifiziert wurden.

Tabelle 2.3: Fortsetzung

Zellart /-linie	Spezies	Toxin	Induktion von		Referenz
			mRNA [1]	Protein [1]	
Monozyten/Makrophagen-artige					
THP-1, differenziert	Mensch	Stx1 + 2	TNF-α	TNF-α	(74)
THP-1, differenziert	Mensch	Stx1	IL-1β, TNF-α	IL-1β	(104)
THP-1, differenziert	Mensch	Stx1	IL-8, MIP-1α, MIP-1β, GRO-α, IL-1β, TNF-α	IL-8, MIP-1α, MIP-1β, GRO-α	(103)
THP-1, differenziert	Mensch	Stx1		TNF-α	(363)
THP-1, undifferenziert	Mensch	Stx1		IL-1β, TNF-α	(265)
THP-1	Mensch	Stx1	TNF-α	TNF-α	(277)
Blut~, primär	Mensch	Stx1 + 2		GM-CSF, TNF-α	(29)
Blut~, primär	Mensch	Stx1 + 2	IL-8, IL-1β, TNF-α	IL-8, IL-1β, TNF-α	(370, 371)
Blut~, primär	Mensch	Stx1		IL-1β, TNF-α	(265)
Blut~, primär, non-adhärent	Mensch	Stx1	IL-6	IL-8, IL-1β, IL-6, TNF-α	(343)
Peritonealexsudat~	Maus	Stx2		TNF-α	(10)
Peritonealexsudat~	Maus	Stx1 + 2	TNF-α	IL-1α, IL-6, TNF-α	(335)
Mesangiumzellen, primär	Mensch	Stx1	MCP-1		(302)

2.4 Shigatoxin-bildende *Escherichia coli* als humanpathogene Erreger

Bestimmte STEC-Stämme, die auch als enterohämorrhagische *E. coli* (EHEC) bezeichnet werden, lösen beim Menschen hämorrhagische Kolitiden (HC) aus, die sich zu lebensbedrohlichen Erkrankungen wie dem Hämolytisch-Urämischen Syndrom (HUS) entwickeln können (140). Die Erkrankungen nehmen ihren Ausgang von der enteralen Besiedelung und Vermehrung der Erreger. Auch bei systemischen Manifestationen im Rahmen der Erkrankungen konnten die Keime extraintestinal nicht nachgewiesen werden (203). Vielmehr kommt den Shigatoxinen eine zentrale Rolle in der Pathogenese zu. Dabei wird derzeit von folgendem Modell ausgegangen (102, 259):

Viele STEC verfügen neben dem Stx-Bildungsvermögen über die Eigenschaft die enterale Mukosa unter Ausbildung von „attaching and effacing"-Läsionen zu besiedeln. Diese Veränderungen entstehen primär unabhängig von der Wirkung der Shigatoxine (170). Bei der enteralen Besiedlung und Vermehrung werden die Shigatoxine jedoch von den Keimen in grossen Mengen produziert und freigesetzt (271), was vermutlich die Läsionen der Darmwand weiter verstärkt. Das histologische Bild der Darmschädigung, die besonders das Zäkum und Kolon betrifft, ist gekennzeichnet durch eine fokale, sehr enge Anheftung der Bakterien an die Epithelzellen der Zottenspitzen. Die Mikrovilli des Bürstensaums sind verdickt, miteinander verbunden oder vollständig verstrichen. Unter der Anheftungsstelle der Bakterien befinden sich intrazellulär massive Aggregate von Zytoskelettkomponenten. Die regelmässige Zellanordnung ist gestört, stellenweise kommt es zur Ulzeration (170). Der Verlust reifer Epithelzellen wird z.T. durch unreife Epithelien ausgeglichen. In der Submukosa ist eine Fibrinexsudation und/oder Hämorrhagie festzustellen. Häufig findet sich eine neutrophile Infiltration der geschädigten Darmwand (20, 101, 147, 269).

Durch die vorgeschädigte Epithelzellschicht (253), die Transmigration von Granulozyten (117) und aufgrund aktiver, Gb_3/CD77-unabhängiger Transportvorgänge (2, 116, 252) gelangt Stx in das subepitheliale Gewebe der Darmwand (2). Hier löst es eine thrombotische Mikroangiopathie der Kapillaren und Arteriolen aus. Die Schwellung der Endothelzellen, verbunden mit einer Aufweitung des Subendothelialraumes, geht mit einer Verengung des Gefässlumens einher. Dieses ist häufig durch Thromben verschlossen. Auch die glatten Muskelzellen der *Tunica media* sind von einer Nekrose betroffen. Die Gefässumgebung ist durch Ödematisierung und/oder Hämorrhagie gekennzeichnet (269). Diese Schädigungen werden für den hämorrhagischen Charakter der HC verantwortlich gemacht.

Über die Blutbahn wird das Toxin, gebunden an Erythrozyten (16) oder Granulozyten (332), in den gesamten Organismus transportiert. Gleichzeitig tritt auch eine Endotoxämie auf (166). In der Folge kommt es zur Ausbildung von Organschäden ausserhalb des Magen-Darm-Traktes. Von einer direkten Schädigung durch die Shigatoxine sind vor allem die Endothelzellen der Niere und des Gehirns betroffen (269). In der Folge bildet sich an den Kapillaren der entsprechenden Organe eine Mikroangiopathie aus wie sie für die Darmwandkapillaren beschrieben wurde (269). Diese Mikroangiopathie bewirkt eine Ödematisierung und Hämorrhagie des betroffenen Organs sowie ischämische Schäden. Dies hat beim HUS in der Niere die Nekrose von Glomerula und Tubuli zur Folge (19, 167, 269).

Neben der direkten Schädigung von Endothelzellen durch die Shigatoxine gibt es auch Hinweise auf eine direkte Wirkung auf Nerven- und Immunzellen (40, 358).

2.5 Shigatoxin-bildende *Escherichia coli* beim Rind

Rinder sind das Reservoir für STEC bzw. EHEC (219). Der Mensch kann sich vor allem durch die orale Aufnahme von mit Rinderkot verunreinigten Nahrungsmitteln infizieren (219). Da es keine spezifische Therapie für das EHEC-bedingte HUS des Menschen gibt, hat die Verhinderung der Infektion grösste Priorität. Eine Expositionsprophylaxe ist aber schwierig, da weltweit bis zu 82 % der Rinder STEC mit dem Kot ausscheiden (85, 219). Rinder infizieren sich schon im Kälberalter durch geringste Infektionsdosen (15). Nach initialer Vermehrung im Zäkum und Kolon kommt es zur Etablierung einer persistierenden Infektion mit monatelangem Ausscheidertum (91). Während sich die als besonders humanpathogen geltenden Stämme des Serovars O157:H7 bevorzugt über den Lymphfollikeln (228) und auf dem Plattenepithel (256) in der rekto-analen Übergangszone absiedeln, kolonisieren STEC anderer Serovare gleichmässig die Dickdarmschleimhaut (228). In Zeiten niedriger Exposition (Weideperiode) kann zwar die Ausscheidungsrate vorübergehend unterhalb der Nachweisgrenze für die Bakterien liegen (139), jedoch kann derselbe STEC-Klon über lange Zeiträume innerhalb einer Herde nachgewiesen werden (85, 175). Obwohl die STEC bei Kälbern länger ausgeschieden werden als bei adulten Tieren (44) und bei letzteren Stx-spezifische Antikörper nachgewiesen werden können (255), schützt eine vorherige Infektion nicht vor Reinfektion mit dem gleichen STEC-Stamm (44).

3. VORARBEITEN UND ZIELSETZUNG

3.1 Hemmung der Transformation und Proliferation boviner Lymphozyten durch Shigatoxin 1

Obwohl persistent infizierte Wiederkäuer seit geraumer Zeit als das wichtigste Reservoir für STEC angesehen werden (219), war lange unbekannt, ob den Shigatoxinen eine Bedeutung als Pathogenitätsmerkmal auch bei STEC-Infektionen von Rindern und Schafen zukommt. Epidemiologische Untersuchungen (354) und experimentelle Infektionen (31, 47) weisen zwar darauf hin, dass STEC bei Kälbern blutige Durchfälle auslösen können, die Pathogenität wird jedoch anderen bakteriellen Faktoren zugeschrieben. (134, 220). Bei HC und HUS des Menschen und der Ödemkrankheit der Schweine steht eine Stx-vermittelte Schädigung von Endothelzellen im Zentrum der Pathogenese (259, 269, 341). Das Fehlen von Stx-Rezeptoren auf bovinen Endothelien, zumindest im Bereich der kleinen Gefässe in der Darmwand, führte zu der Vermutung, dass Rinder resistent gegenüber Stx sein könnten (260).

Allerdings stellen Zellen des Immunsystems ebenfalls wichtige Zielzellen für Stx dar. Beim Menschen sind vor allem IgG- und IgA-bildende B-Zellen hochgradig sensibel für die zytoletale Wirkung des Shigatoxins 1 (40, 197). Auch Infektionen gnotobiotischer Ferkel mit Stx1-bildenden *E. coli* (STEC1) lösen eine Immunsuppression aus (36). Dieser lymphotropen Wirkung der Shigatoxine wird eine Bedeutung in der Pathogenese STEC-induzierter Erkrankungen zugeschrieben (40). Jedoch verhindert die Immunsuppression langfristig nicht die Bildung Stx-spezifischer Antikörper. So wiesen Wieler *et al.* Stx2e-spezifisches IgG bei an Ödemkrankheit erkrankten Schweinen nach (355) und Reymond *et al.* (268) zeigten, dass selbst beim Menschen Stx-spezifische Antikörper im Serum auftreten können.

Seren und Kolostren von Kühen enthalten häufig neutralisierende Antikörper gegen Stx1 und zum Teil auch gegen Stx2 (137, 255). Rinder sind somit nicht nur häufig mit STEC infiziert, sondern die Keime sind auch in der Lage unter den Bedingungen im Darm des Rindes Stx zu bilden. Dieses intestinale Stx wirkt offensichtlich auf bovine Lymphozyten nicht nur als Antigen. In eigenen, orientierenden Untersuchungen induzierte affinitäts-chromatographisch gereinigtes Stx1 bei Zellen einer bovinen, mit Bovinem Leukosevirus (BLV) -infizierten B-Lymphomzelllinie (BL-3) Apoptose, wenn die Zellen durch Inkubation mit B-Zell-Mitogenen sensibilisiert wurden (207). Unstimulierte oder mit T-Zell-Mitogenen inkubierte Zellen waren dagegen resistent gegenüber Stx1 (207). Inzwischen konnte die Arbeitsgruppe von Carolyn Hovde, Moscow, Idaho, USA zeigen, dass diese zytoletale

Aktivität der Shigatoxine die Permeabilisierung der Zellmembran der Zielzellen durch die Replikation von BLV voraussetzt (12, 71). Auf diese Weise sollen die Shigatoxine gezielt virus-infizierte Zellen eliminieren (70). Allerdings ergaben eigene Untersuchungen, dass das Wirkungspektrum des Shigatoxins 1 bei bovinen Lymphozyten mehr als nur die Wirkung auf virus-infizierte B-Zellen umfasst. Bei Primärkulturen boviner PBMC (mononukleärer Zellen aus dem peripheren Blut adulter, leukosefreier Rinder) löste Stx1 zwar weder Nekrose noch Apoptose aus, Stx1 blockierte aber bereits im Nanogramm-Konzentrationsbereich den Mitogen-induzierten Anstieg der metabolischen Aktivität dieser Zellen (207). Dabei hing die Wirkung des Shigatoxins 1 auf bestimmte Lymphozyten-Subpopulationen wiederum massgeblich von einer adäquaten Stimulation der Zellen ab. Während unstimulierte Lymphozyten resistent gegenüber Stx1 waren, führte die Stimulation mit einem T-Zell-Mitogen (PHA-P) zur Transformation und Proliferation insbesondere der $CD8\alpha^+$ T-Zellen und diese Wirkung wurde durch Stx1 blockiert. Unter Stimulation mit B-Zell-Mitogenen reduzierte Stx1 dagegen den Anteil lebender B-Zellen. Die Modulation der Expression von Aktivierungsmarkern (CD25, CD71) durch Stx1 deutete an, dass Stx1 die Proliferation verhinderte, indem es den Aktivierungsprozess der Zellen in einer frühen Phase blockierte (**Abb. 3.1**). Auch Rinder sind somit nicht als resistent gegenüber der Wirkung dieses potenten Zytotoxins anzusehen. Der Wirkung von Stx auf bovine B- und auch T-Lymphozyten könnte vielmehr eine entscheidende Bedeutung in der Wechselwirkung zwischen STEC und ihrem Reservoirwirt zukommen.

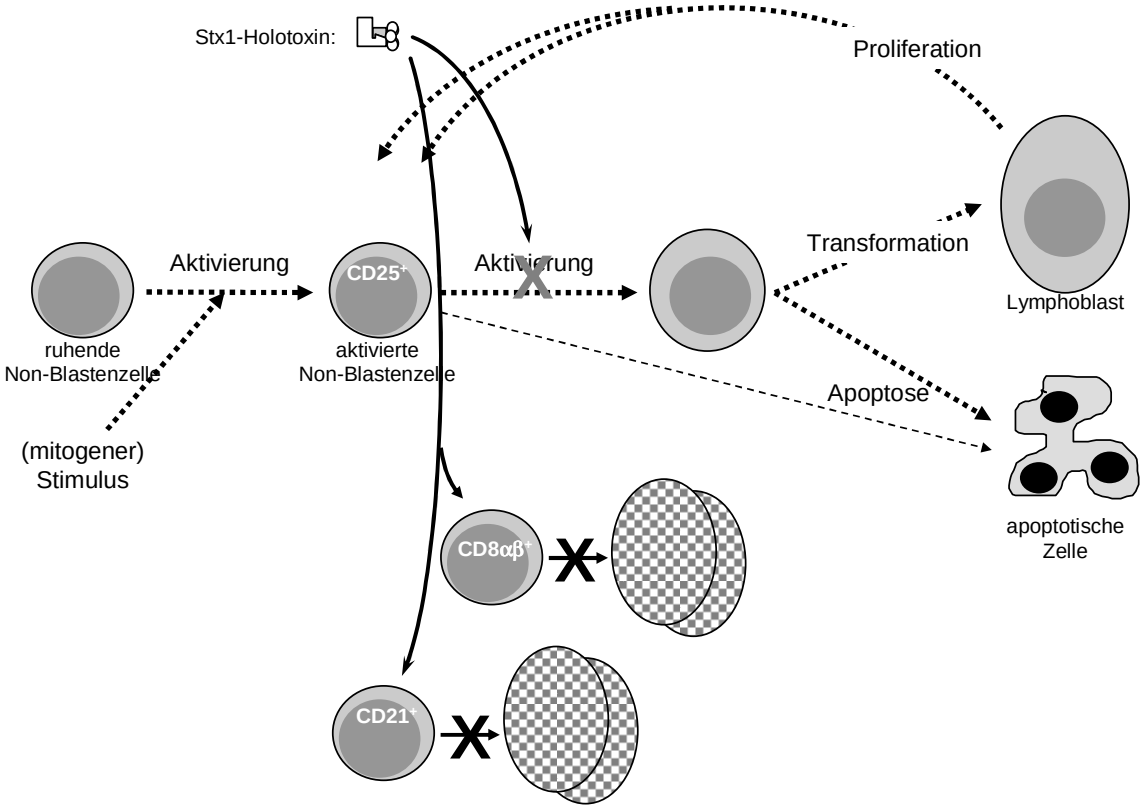

Abb. 3.1: Modell der Wirkung von Stx1 auf die Transformation und Proliferation aktivierter, peripherer Lymphozyten des Rindes (Details siehe Text)

3.2 Zielsetzung

Die molekularen Mechanismen, die der Fähigkeit der STEC zugrunde liegen ihren Wirt über längere Zeit kolonisieren zu können, sind nur unzureichend verstanden. Bei experimentellen Infektionen von Kälbern ist das auf dem *locus of enterocyte effacement* (*lee*) kodierte Intimin für die Ausbildung von „attaching and effacing"-Läsionen bei der Kolonisierung der Schleimhaut essentiell (49). Intimin sowie Bestandteile des vom *lee* kodierten Typ III-Sekretionsapparates tragen, möglicherweise durch die Induktion der sehr engen Adhäsion der Bakterien an Epithelzellen, zur Persistenz von STEC O157:H7 und O26:H$^-$ bei Rindern und Schafen bei (43, 57, 342).

Da bei STEC-Infektionen adulter Rinder nach bisherigem Kenntnisstand aber keine intestinale Entzündung auftritt, wurde spekuliert, dass sich die STEC als Kommensalen an das Milieu im Darm des Rindes angepasst haben könnten (304). Allerdings zeichnet sich die

intestinale Mukosa auch unter normalen Bedingungen durch einen Zustand „physiologischer Entzündung“ aus, der durch die Anwesenheit zahlreicher Leukozyten in intraepithelialen und subepithelialen Kompartimenten gekennzeichnet ist (72). Bovine STEC-Isolate verfügen über eine Reihe von Pathogenitätsmerkmalen und Virulenzfaktoren, die es ihnen erlauben in vielfältiger Weise mit intestinalen Epithelzellen zu interagieren (13, 49, 85, 356). Es erscheint daher unwahrscheinlich, dass nicht wenigstens einer dieser Faktoren von der Mukosa als Signal erkannt wird, das Mechanismen der angeborenen oder erworbenen Immunabwehr aktiviert, die „physiologische Entzündung“ verstärkt und dadurch die Kolonisierung der Schleimhaut durch STEC auch im Rind terminiert (300, 313). Auch der häufige Nachweis von Stx-spezifischen Antikörpern beim Rind (18, 137, 255) spricht dagegen, dass die Persistenz der bovinen STEC-Infektion aus einem Unvermögen des lokalen Immunsystems resultiert auf STEC und ihre Produkte zu reagieren. Demnach müssen die STEC eine Strategie entwickelt haben, die aktiv die Immunantwort limitiert, die intestinale Homöostase erhält und die dauerhafte Kolonisierung erlaubt.

Lymphostatin, das Genprodukt des *lifA* bei enteropathogenen *E. coli*, das bei nicht-O157 STEC-Stämmen fast identisch als *efa-1* vorkommt, inhibiert die Proliferation humaner (158-160) und boviner Lymphozyten und scheint bei STEC O5- und O111-Stämmen auch die intestinale Kolonisation beim Rind zu begünstigen (316). Die Genprodukte homologer Gene bei STEC O157:H7 (*toxB* und eine trunkierte Form von *efa-1*) jedoch haben auf die intestinale Kolonisation bei Schafen und Rindern keinen Einfluss (314).

Stx ist damit das einzige bislang identifizierte Pathogenitätsmerkmal, das allen STEC-Stämmen gemeinsam ist und beim Rind immunsuppressiv wirkt (207). Zumindest bei Schafen ist die Fähigkeit der STEC zur Persistenz in der Tat mit der Fähigkeit zur Bildung der Stx assoziiert (42). Im Hinblick auf die Entwicklung einer Bekämpfungsstrategie gegen STEC-Infektionen im Rind waren deshalb die Ziele der hier beschriebenen Arbeiten:

1. Kenntnisse über den Wirkungsmechanismus des Shigatoxins 1 auf bovine periphere Lymphozyten zu gewinnen,
2. die Übertragbarkeit der gewonnenen Erkenntnisse auf Lymphozyten der Darmschleimhaut zu überprüfen sowie
3. weitere potentielle, nicht-lymphozytäre Zielzellen für Stx im Darm des Rindes zu identifizieren.

4. METHODIK

Für die vorliegenden Arbeiten sind die in den entsprechenden Veröffentlichungen (siehe Abschnitt 6) beschriebenen Methoden und Verfahren angewandt worden.

- Affinitätschromatopraphische Aufreinigung von Proteinen und deren Analyse mit Hilfe der SDS-PAGE und des Immunoblots,
- Präparation und Kultivierung mononukleärer Zellen aus dem peripheren Blut (PBMC) von Rindern,
- Nachweis boviner Leukozytenoberflächenantigene mit Hilfe der Durchflusszytometrie,
- Quantifizierung der Proliferation boviner PBMC,
- Quantifizierung der Synthese zytokin- und chemokin-spezifischer mRNA,
- Präparation und Kultivierung polymorphkerniger Granulozyten (PMN) aus dem peripheren Blut von Rindern und Schafen,
- Quantifizierung der Phagozytoseleistung und der Aktivierbarkeit von bovinen und ovinen PMN mit Hilfe der Durchflusszytometrie,
- Quantifizierung der Migrationsaktivität boviner PMN,
- Präparation und Kultivierung boviner Kolonepithelzellen,
- Extraktion und biochemische Analyse von Membranlipiden durch MALDI-TOF Massenspektrometrie und Dünnschichtchromatographie.

Für eine detaillierte Beschreibung einer Auswahl von Methoden zur Untersuchung der Wirkung von Shigatoxinen auf mononukleäre Zellen sei insbesondere auf die Veröffentlichung **6.4** verwiesen.

Die Kapitel über Epithelzellen und Makrophagen-artige Zellen enthalten bislang unveröffentlichte Originaldaten. Diejenigen Methoden, die diesen Arbeiten zugrundeliegen und deren Beschreibung nicht Bestandteil einer der angefügten Publikationen ist, sind im Folgenden dargestellt.

4.1 Untersuchung primärer boviner Kolonepithelzellen

4.1.1 Gewinnung und Kultivierung

In Anlehnung an die Methode von Föllman *et al.* (73) wurden am Schlachthof Giessen jeweils etwa 30 cm lange Abschnitte des proximalen Kolon ascendens von gesunden, 18 bis 24 Monate alten Rindern verschiedener Rasse und beiderlei Geschlechts kurz nach deren Tötung entnommen. Nach dem Ausstreifen des Darminhaltes erfolgte ein mehrmaliges Waschen mit steriler, 4°C kalter NaCl-Lösung (0,9 %). Sichtbares Fettgewebe wurde mit einer Schere abgesetzt, der Darm längs eröffnet und Farbe sowie Zustand der Mukosa kontrolliert. Nach weiteren (2- bis 3mal) Waschungen mit steriler, 4°C kalter NaCl-Lösung wurde der Mukus vorsichtig mit Hilfe eines Glasobjektträgers abgeschabt und das Darmstück nochmals gespült. Der Transport zum Labor erfolgte auf Eis in sterilem Transportmedium (PBS mit 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2,5 µg/ml Gentamycin, 2,5 µg/ml Amphoterizin B, 4 mM L-Glutamin und 0,2 % Glukose).

Unter sterilen Bedingungen wurde dann mittels zweier Glasobjektträger die Mukosa von dem auf einem Eisbett gelagerten Darmstück abgeschabt, in mit eiskaltem HBSS gefüllte Petrischalen überführt und mit einer Rasierklinge in feine Stücke zerschnitten. Die zerkleinerte Mukosa wurde in mehreren 50 ml Röhrchen (Fa. Greiner) mit eiskaltem HBSS gewaschen (130 x , 5 min, 4°C, gut vorgekühlt). Überstand und Mukusschicht wurden vorsichtig mit einer Pasteurpipette abgesaugt, die Röhrchen wieder mit eiskaltem HBSS aufgefüllt und die Waschschritte so lange wiederholt, bis die Mukusschicht weitestgehend vom Zellpellet entfernt worden war.

Danach wurden 30 ml der vereinigten Pellets zu 120 ml vorgewärmte (37°C) Verdau-Lösung (60 ml DMEM, 60 ml HBSS, 2 mM L-Glutamin, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2,5 µg/ml Amphoterizin B, 2,5 µg/ml Gentamycin, 150 U/ml Kollagenase 1 CLS) gegeben und bei 100 U/min, 37°C und 8 % CO_2 auf einem Magnetrührer für 45 Minuten inkubiert. Nachdem die resultierende Zellsuspension zweimal durch 0,6 x 25 mm Kanülen gepresst wurde, wurde sie für weitere 10 Minuten wie beschrieben inkubiert. Danach wurden die Krypten bei 202 x g, 4°C, für 7 Minuten pelletiert und unter vorsichtigem Schwenken mit 50 ml Sorbitol-Lösung (2 % in HBSS, 4°C) resuspendiert. Nach Zentrifugation (50 x g, 5 Minuten, 4°C) wurden die aus den Epithelzellkrypten gebildeten Pellets erneut in Sorbitol-Lösung aufgenommen und die Dichtegradienten-Zentrifugation wie beschrieben solange wiederholt, bis im Überstand bei mikroskopischer Kontrolle Krypten sichtbar wurden. Schließlich wurden die Pellets vereinigt und mit Kulturmedium (s.u.)

versetzt. In 25 cm^2 grosse Kulturflaschen (Fa. Corning Costar), die am Vortag mit Kollagen beschichtet (2,8 µl/cm^2, KollagenR, Fa. Serva, eingesetzt 1:10 verdünnt) worden waren, wurden zwischen 7500 und 10000 Krypten ausgesät und bei 37°C und 8 % CO_2 kultiviert.

Als Kulturmedium diente „Dulbecco`s Modified Eagles-Medium“ (DMEM) mit den in **Tabelle 4.1** aufgeführten Zusätzen.

Tabelle 4.1: Mediumzusätze für die Anzüchtung boviner Epithelzellen

Substanz	Konzentration	Bezugsquelle
L-Glutamin	4 mM	PAA-Laboratories
Penicillin	100 U/ml	PAA-Laboratories
Streptomycin	100 µg/ml	PAA-Laboratories
Gentamycin	2,5 µg/ml	Biochrom AG
Amphoterizin B	2,5 µg/ml	PAA-Laboratories
bovines Transferrin	5 µg/ml	GIBCO™, Invitrogen Corporation
bovines Insulin	10 µg/ml	Biochrom AG
nichtessentielle Aminosäuren	0,15 mM	Biochrom AG
D-(+)-Glukose	2,7 mg/ml	Sigma-Aldrich Chemie GmbH
Hydrocortison	1 µg/ml	Sigma-Aldrich Chemie GmbH
EGF (epidermal growth factor)	30 ng/ml	A.F. Schützdeller GmbH, Tübingen

Dieses initiale Kulturmedium wurde für die ersten 24 Stunden der Anzucht intestinaler Epithelzellen aus Kolonkrypten mit 10 % fötalem Kälberserum (FCS) versetzt. An Tag 1 erfolgte ein Mediumwechsel zur Entfernung der zahlreichen Zelltrümmer. Das für die weitere Kultivierung verwendete Medium wurde mit 2 % FCS und 0,5 % Rinderhypophysenextrakt (c.c.pro, Neustadt) ergänzt und am 3. Tag nach Gewinnung der Krypten erneut ausgetauscht. Am 4. Inkubationstag war in 85-95 % der adhärenten Zellen durchflusszytometrisch und fluoreszenzmikroskopisch das für Zellen epithelialen Ursprungs charakteristische Zytokeratin nachweisbar.

4.1.2 Fluoreszenzmikroskopische Untersuchung

Zur Charakterisierung der Zellen mit Hilfe des Fluoreszenzmikroskopes am 4. Inkubationstag wurden die gleichen Antikörperkombinationen verwendet wie bei der durchflusszytometrischen Analyse (**Tabelle 4.2**). Alle Arbeitsschritte wurden bei Raumtemperatur in einer feuchten Kammer bei Lichtausschluß durchgeführt. Die Probenvorbereitung zum Nachweis von Gb_3/CD77 und eines Zytoskelettproteins folgte folgendem Schema:

1. Entfernen des Mediums aus dem Zellkulturgefäss durch vorsichtiges Abkippen,
2. zweimaliges Waschen des Zellrasens mit PBS,
3. Fixation der Zellen in 2 % PFA für 30 min,
4. zweimaliges Waschen des Zellrasens mit PBS,
5. Permeabilisation mit Digitonin (Sigma-Aldrich Chemie, 0,005 % in PBS; 10 min),
6. zweimaliges Waschen des Zellrasens mit PBS,
7. Ausstanzen der Präparate aus den Böden der Kulturgefässe mit Hilfe einer erhitzten Kupferstanze und sofortiges Überführen in PBS-gefüllte Vertiefungen einer 12-Loch-Zellkulturplatte,
8. Begradigen der Präparatekanten mit Hilfe eines Skalpells,
9. Entfernen des PBS durch Absaugen, anschliessend Inkubation der Zellen mit je 25 µl Gebrauchslösung des anti-CD77-Antikörpers bzw. mit 50 µl Gebrauchslösung eines Antikörpers gegen Vimentin oder Zytokeratin,
10. zweimaliges Waschen des Zellrasens mit PBS,
11. Entfernen des PBS durch Absaugen, anschließend Inkubation der Zellen mit je 50 µl Gebrauchslösung des Fluoreszenz-markierten anti-Ratte-IgM und des Fluoreszenz-markierten anti-Maus-IgG für 1 Stunde,
12. zweimaliges Waschen des Zellrasens mit PBS,
13. Eindecken der Präparate auf einem Objektträger mit 60 µl Mowiol-Mounting-Medium.

Die Vorbereitung der Präparate zum Nachweis von Bindungsstellen für rStxB1 erfolgte analog und gemäss der in **6.4** und **6.6** beschriebenen Verfahren.

4.2 Immortalisierung und Charakterisierung primärer boviner Kolonzellen

Zellen aus primären Kulturen boviner Kolonzellen wurden am 4. Inkubationstag von Dr. M. König, Institut für Virologie, durch Transfektion mit dem Plasmid pSV3-neo nach der Methode von Pauly *et al.* (247) immortalisiert. Nach Klonierung der Zellen mit erhaltenener Proliferationsfähigkeit wurden die gewonnenen Zelllinien durchflusszytometrisch (zur Methodik siehe **6.4**) und fluoreszenzmikroskopisch (siehe **4.1.2**) unter Verwendung der in **Tabelle 4.2** aufgeführten Antikörper analysiert.

Tabelle 4.2: Aufstellung der zur Charakterisierung boviner Kolonzellen verwendeten Antikörper

Zielantigen	Verwendeter Antikörper					Gebrauchs-lösung[1]
	Wirts-spezies	Isotyp	Farb-stoff	Name	Bezugsquelle	
CD11a (bovin)	Maus	IgG2a		IL-A99	Dirk Werling, London	unverdünnt
CD11b (bovin)	Maus	IgG1		IL-A15	Jan Naessens, Nairobi	unverdünnt
CD11c (bovin)	Maus	IgG1		IL-A16	Jan Naessens, Nairobi	unverdünnt
CD14 (bovin)	Maus	IgG1		CC-G33	Dirk Werling, London	unverdünnt
CD77	Ratte	IgM		38.13	Beckman-Coulter	1:10 in PBS
CD172a (bovin)	Maus	IgG1		IL-A24	Jan Naessens, Nairobi	unverdünnt
MHC-I (bovin)	Maus	IgG2a		IL-A88	Jan Naessens, Nairobi	unverdünnt
MHC-II (bovin)	Maus	IgG1		J-11	Jan Naessens, Nairobi	unverdünnt
Vimentin	Maus	IgG1		3B4	DAKO A/S	1:13 in PBS
Pan-Zytokeratin	Maus	IgG1	FITC	C-11	Sigma-Aldrich Chemie	1:100 in PBS
(Maus)IgG(γ)			FITC		Medac	1:200 in PBS
(Maus)IgG			PE		Sigma-Aldrich Chemie	1:50 in PBS
(Ratte)IgM(μ)			PE		Beckman-Coulter	1:200 in PBS
(Ratte)IgM			FITC		Dianova	1:100 in PBS

[1] unverdünnt = unverdünnter Hybridomzellkulturüberstand

4.3 Funktionelle Untersuchungen an bovinen Kolonzellen

4.3.1 Zytotoxizitätsbestimmungen

Zur Bestimmung möglicher zytotoxische Effekte des Shigatoxins 1 auf primäre Kolonepithelzellen und immortalisierte Kolonzelllinien wurden in Anlehnung an Gentry & Dalrymple (82) 2×10^4 Zellen pro Vertiefung in 96-Loch-Mikrotiterplatten mit flachem Boden (Fa. Nunc) mit gereinigtem Stx1 in Anwesenheit und Abwesenheit eines StxB1-spezifischen monoklonalen Antikörpers (13C4) (320) inkubiert. Einige Ansätze wurden zusätzlich mit LPS (O55; 25 µg/ml; Sigma-Aldrich Chemie GmbH) supplementiert. Für die Untersuchung von Epithelzellen wurden die Vertiefungen der Mikrotiterplatten durch Kollagenisierung (s.o.) vorbereitet. Nach Inkubation bei 37°C, 5 % CO_2 und Wasserdampfsättigung über 72 Stunden und mikroskopischer Auswertung wurde die Stoffwechselaktivität der Zellen durch Zusatz von MTT (3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazoliumbromid; Sigma-Aldrich Chemie GmbH) wie in **6.4** beschrieben quantifiziert und im Verhältnis zu den mitgeführten Positiv- und Negativ-Kontrollen berechnet.

Alternativ wurde nach Inkubation mit Stx1 der Anteil apoptotischer Epithelzellen über die durchflusszytometrische Quantifizierung M30-Antigen-positiver Zellen bestimmt . Dazu fand ein kommerzieller Testkit (M30 ZytoDEATH, Fluorescein; Roche) nach Herstellerangaben Anwendung. Dieser Test basiert auf der Verwendung eines fluorescein-konjugierten monoklonalen Antikörpers, der ein kaspase-aktiviertes Epitop des Zytokeratins 18 erkennt, das nur im Zytoplasma apoptotischer Zellen vorkommt.

4.3.2 Quantifizierung der Expression ausgewählter Zyto- und Chemokine

Der Einfluss von Stx1 auf den Gehalt primärer Kolonepithelzellen und immortalisierte Kolonzelllinien an mRNA mit Spezifität für ausgewählte Zyto- und Chemokine wurde in Zusammenarbeit mit Dr. Anja Taubert, Institut für Parasitologie, wie in **6.10** beschrieben bestimmt. Dazu wurde 4 Tage nach Gewinnung der Krypten (Epithelzellen) bzw. nach der letzten Passagierung (Zelllinien) das Kultivierungsmedium durch mit Stx1, anti-StxB1 und/oder LPS supplementiertem Medium ersetzt und für weitere 4 bzw. 24 Stunden kultiviert. Nach Gewinnung der mRNA wurden für die Quantifizierung mittels „real-time“-PCR zusätzlich zu den in **6.10** aufgeführten weitere Primer und Sonden verwendet (**Tabellen 4.3 und 4.4**).

Tabelle 4.3: Basenfolge der in der „real-time“-PCR verwendeten Primerpaare zur Quantifizierung Zytokin- und Chemokin-spezifischer mRNA

Primer	Basenfolge 5`bis 3`Ende
GAP-RANTES-forward	-GGC GTG AAC CAC GAG AAG TAT AA-
GAP-RANTES-reverse	-CCC TCC ACG ATG CCA AAG T-
bo-GROalpha-128-forward	-CGC CTG TGG TCA ACG AAC T-
bo-GROalpha-210-reverse	-CAC CTT CAC GCT CTG GAT GTT-
bo-RANTES-148-forward	-CCC TGC TGC TTT GCC TAT ATC T-
bo-RANTES-225-reverse	-GCA CTT GCT GCT GGT GTA GAA A-
bo-TGFbeta-748-forward	-GGC CCT GCC CTT ACA TCT G-
bo-TGFbeta-821-reverse	-CGG GTT GTG CTG GTT GTA CA-
bo-IL12-p40-677-forward	-GCA GCT TCT TCA TCA GGG ACA T-
bo-IL12-p40-713-reverse	-CCT CCA CCT GCC GAG AAT T-

Tabelle 4.4: Basenfolge der in der „real-time“-PCR verwendeten Sonden zur Quantifizierung Zytokin- und Chemokin-spezifischer mRNA

Sonde	Basenfolge 5`bis 3`Ende
GAP-RANTES	-ATA CCC TCA AGA TTG TCA GCA ATG CCT CCT-
GRO-α	-CCA GTG CCT GCA GAC CTT GCA GG-
RANTES	-CCC GCA CCC ACG TCC AGG AGT-
TGF-β	-CCT GGA TAC ACA GTA CAG CAA GGT CCT GG-
IL-10	-AGC CTG TGG CAT CAC CTC TTC CAG GTA A-
IL-12	-CAC CCA AGA ACC TGC AAC TGA GAC CAT TAA-

Die Quantifizierung der von bovinen Kolonepithelzellen freigesetzten für Granulozyten attraktiven Substanzen efolgte im Migrationstest wie in **6.10** beschrieben.

5. ERGEBNISSE UND DISKUSSION

5.1 Charakterisierung des Shigatoxin-Rezeptors boviner Lymphozyten

Gb_3/CD77 ist der Rezeptor für die B-Untereinheit der Shigatoxine, der die Endozytose vermittelt und der A-Untereinheit Zutritt zum Zytosol der Zelle verschafft (179, 261, 285). Stx1 und StxB1 können aber auch nach Bindung an Gb_3/CD77 ohne Internalisierung intrazelluläre Signale auslösen (197). Alternative Stx-bindende Strukturen sind dagegen vermutlich nicht in der Lage eine Wirkung der Shigatoxine zu vermitteln (332). Die Kenntnis molekularer Vorgänge im Kontext der Rezeptorbindung ist damit von entscheidender Bedeutung für das Verständnis der Stx-Wirkungen beim Rind und war deshalb Ziel initialer Untersuchungen.

(Vorbemerkung:
Bei den im Folgenden beschriebenen Studien wurde Gb_3/CD77 sowohl durch Immundekoration mit einem CD77-spezifischen Antikörper als auch durch Bindungsstudien mit der B-Untereinheit des Shigatoxins 1 nachgewiesen. Bei Gb_3 handelt es sich jedoch nicht um eine einzelne biochemisch definierte Struktur, sondern um eine Gruppe von Glykosphingolipiden, denen nur der Kohlenhydratanteil gemeinsam ist. Die beiden zum Nachweis verwendeten Liganden erkennen unterschiedliche Isoformen des Gb_3 (32). Deshalb wird im Folgenden sprachlich unterschieden zwischen a.) CD77(-Antigen) als der mit dem Antikörper 38.13 nachweisbaren Struktur, b.) Stx-Rezeptor als Stx-bindende Struktur und c.) Gb_3 als der Summe aller Isoformen. Die Doppelbezeichnung Gb_3/CD77 wird nur angewandt, wenn diese Unterscheidung nicht möglich war.)

5.1.1 Einführung von CD77 als aktivierungsabhängig exprimiertes Oberflächenantigen

Neutrale Glykosphingolipide (GSL), zu denen Gb_3 gehört, sind lipidartige Bestandteile der Zellmembranen aller Säugetierzellen und die Kohlenhydratanteile vieler GSLs werden auf der Zelloberfläche präsentiert (297). Allerdings haben GSLs eine charakteristische Zusammensetzung in funktionell unterschiedlichen Zelltypen. So stellen Gb_3 und Gb_4 bei humanen Lymphozyten stadienspezifische Markerantigene dar, die eine Subpopulation terminal differenzierter B-Zellen in Keimzentren kennzeichnen (327). Obwohl generell die Quantifizierung von Aktivierungsmarkern wichtige Einblicke in die Mechanismen der Lymphozytenregulation erlaubt und auch bei bovinen Lymphozyten Anwendung findet (206, 263, 352), war bislang die Expression von GSLs bei bovinen Lymphozyten noch nicht untersucht worden.

5.1.1.1 CD77-Expression durch bovine B-Lymphomzellen (BL-3)

Durch beispielhafte Untersuchungen an BL-3-Zellen konnte unter Verwendung eines gegen das humane CD77-Antigen gerichteten Antikörpers erstmals CD77 bei bovinen Lymphozyten nachgewiesen werden (siehe **6.3**). Allerdings exprimierten in unstimulierten Kulturen nur eine Minderheit der Zellen das Antigen auf der Zelloberfläche; bei den meisten Zellen war es nur intrazellulär nachweisbar. Die oberflächliche Expression beschränkte sich fast ausschliesslich auf Zellen mit veränderter Morphologie und eingeschränkter Vitaliät („subvitale Zellen“). Dies könnte erklären, warum unstimulierte BL-3-Zellen gegenüber der apoptotischen Wirkung des Shigatoxins 1 resistent sind (207). Erst der Zusatz von B-Zell-Mitogenen zum Kulturmedium, der auch die Zellen für Stx1 sensibilisiert (207), resultierte in einem verstärkten Nachweis von CD77-Molekülen auf der äusseren Zellmembran auch bei Zellen mit erhaltener Vitalität (siehe **6.3**).

5.1.1.2 Kinetik der CD77-Expression in Primärkulturen

Untersuchungen mit Primärkulturen boviner PBMC bestätigten die Übertragbarkeit dieser Befunde auf nicht-transformierte Zellen. PBMC waren zwar unmittelbar nach der Gewinnung nicht in der Lage den CD77-Antikörper zu binden (siehe **6.3**), dies änderte sich jedoch bereits am ersten Inkubationstag. Obwohl auch ohne Stimulation CD77 auf der Oberfläche exprimiert wurde, steigerte der Zusatz von Mitogenen zum Medium die Expression erheblich. Der Anteil CD77-exprimierender Zellen an allen Zellen der Kultur stieg im weiteren Verlauf der Inkubation kontinuierlich an, wobei die CD77-Expression bei Zellen unterschiedlicher Morphologie allerdings erheblich differierte. Der höchste Anteil $CD77^+$-Zellen war in der Population der Blasten zu verzeichnen, die insbesondere nach mitogener Stimulation aus Non-Blasten-Zellen durch Grössenzunahme hervorgegangen waren (zur durchflusszytometrischen Definition der Zellpopulationen gemäss ihrer morphologischen Eigenschaften siehe Abb. 3 in **6.3**). Dabei erreichte der Anteil der $CD77^+$-Blasten am 3. bis 4. Tag der Kultur sein Maximum während der Anteil subvitaler $CD77^+$-Zellen immer weiter anstieg. Die Populationen der Blasten, Non-Blasten und subvitalen Zellen unterschieden sich auch in der Zahl der oberflächlich exprimierten CD77-Moleküle. Bei den Blasten und auch bei den wenigen $CD77^+$-Non-Blasten variierte die Fluoreszenintensität für den Nachweis des Antigens über einen weiten Bereich. Im Gegensatz dazu waren bei den subvitalen Zellen von Anbeginn der Kultur Zellen mit sehr starker CD77-Expression nachweisbar und diese $CD77^{\text{“bright“}}$-Zellen akkumulierten in der subvitalen Population bis zum 8. Tag. Subvitale

Zellen unterschieden sich von den übrigen Populationen vor allem durch ihre, den apoptotischen Zellen ähnliche Morphologie (198). Es bestanden jedoch keine Korrelationen zwischen der CD77-Expression und verschiedenen Anzeichen von Zelltod.

Auch die Tatsache, dass CD77 auf der Oberfläche boviner Lymphozyten *in vitro* insbesondere nach mitogener Stimulation exprimiert wurde, deutet daraufhin, dass die CD77-Expression einen intermediären Aktivierungszustand der Zellen anzeigte. Anscheinend waren die ruhenden Lymphozyten, die zuerst die Population der Non-Blasten bildeten, zunächst CD77-negativ oder CD77$^{\text{“dim“}}$. Nach (mitogener) Aktivierung steigerte sich die CD77-Expression soweit, dass 3 bis 4 Tage nach Initiierung der Kultur bis zu 60% der Blastenzellen CD77-Moleküle in mässigem Umfang (CD77$^{\text{“moderate“}}$) exprimierten. Das Verschwinden der CD77$^{\text{“moderate“}}$-Zellen aus der Blastenpopulation zum 6. bis 8. Tag der Kultivierung ging einher mit einer Kumulation von subvitalen CD77$^{\text{“bright“}}$-Zellen. Vergleichbare Muster der CD77-Expression liessen sich auch bei BL-3-Zellen zeigen. Offensichtlich verlief die CD77-Expression bis zur moderaten Expression (CD77$^{\text{“moderate“}}$) parallel zum Aktivierungsprozess der Zellen. An diesem Punkt der Aktivierung überlebten die Zellen weiterhin, teilten sich, nahmen dadurch an Grösse ab und erschienen wiederum als CD77$^{\text{“dim“}}$ Non-Blastenzellen. Alternativ leiteten die Zellen die Apoptose ein, was mit einer weiteren Steigerung der oberflächlichen CD77-Expression (CD77$^{\text{“bright“}}$) einherging (**Abb. 5.1**).

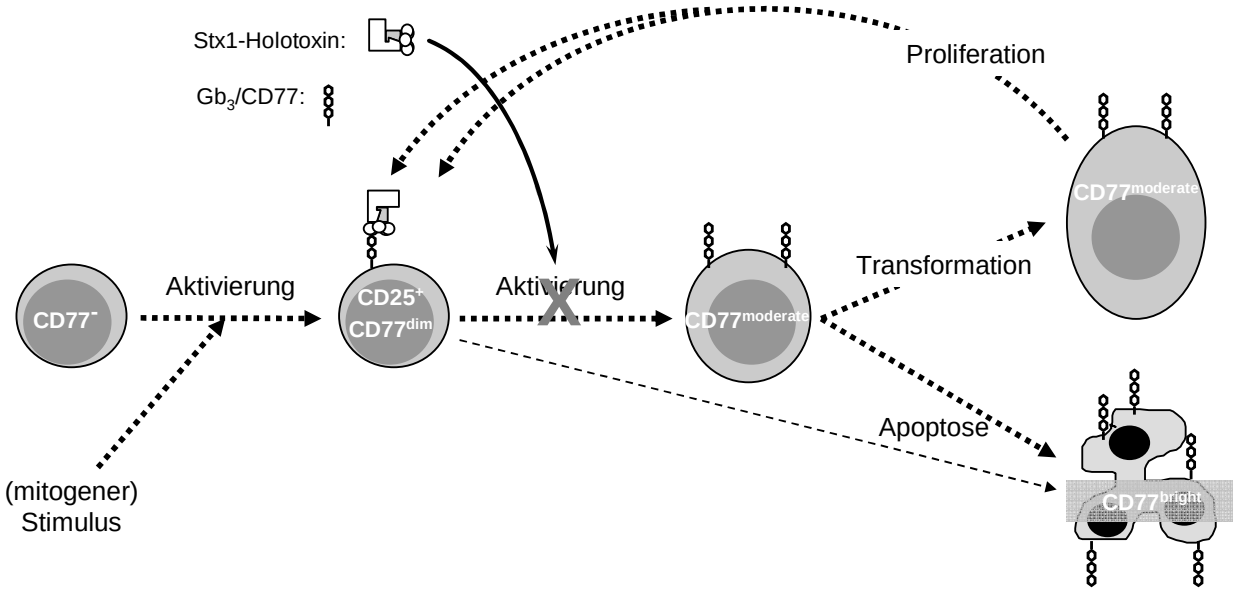

Abb. 5.1: Modell der aktivierungsabhängigen Expression von Gb_3/CD77 auf peripheren Lymphozyten des Rindes (Details siehe Text)

Diese Art der CD77-Expression ähnelt der durch humane B-Zellen. $CD77^+$ B-Zellen besitzen den Phänotyp aktivierter Zellen, sind jedoch negativ für die klassischen Aktivierungsmarker CD23, CD25 und CD71 und weisen Charakteristika apoptotischer Zellen auf (198). Dabei sind die Zellen jedoch nicht auf den Zelltod fixiert, sondern können durch Behandlung mit IL-4 und CD40-Liganden gerettet werden (199). Überlebende B-Zellen regulieren die Expression von CD77 wieder vollständig herunter, werden dafür aber positiv für CD23 (199). Obwohl bovine $CD77^+$-Lymphozyten teilweise CD25 koexprimierten (siehe **6.6**), stellt CD77 auch auf bovinen Immunzellen, die *in vitro* (siehe **6.3**) oder *in vivo* (siehe **5.3.2** und **5.3.3**) eine Stimulation erfahren haben, einen bislang unbekannten Aktivierungsmarker dar, der für bestimmte Phasen im Aktivierungsprozess dieser Zellen charakteristisch ist.

5.1.1.3 CD77-Expression durch Lymphozyten-Subpopulationen

Unter mitogener Stimulation exprimierten verschiedene Subpopulationen (γδT-Zellen, $CD4^+$ T-Zellen, $CD8\alpha^+$ T-Zellen, B-Zellen) das CD77-Antigen auf der Oberfläche (siehe **6.3**), jedoch unterschieden sich die Subpopulationen in ihren Expressionmustern. Während fast alle $CD8\alpha^+$ T-Zellen, die besonders empfindlich für Stx1 sind (207), CD77 mit hoher Dichte koexprimierten, war sowohl die Zahl positiver Zellen als auch die Antigendichte auf der Oberfläche der weniger sensitiven $CD4^+$ T-Zellen und der B-Zellen deutlich niedriger.

Das CD77-Antigen besitzt damit bei bovinen Lymphozyten eine weite zelluläre Verbreitung und ist nicht auf B-Zellen beschränkt. Bei humanen Lymphozyten war die CD77-Expression ursprünglich mit der onkogenen Transformation von Burkitt-Lymphomzellen assoziiert worden (357). In gesunden Probanden kennzeichnet CD77 dagegen eine Subpopulation von Zellen in den tonsillären Keimzentren (198). $CD77^+$-Zellen koexprimieren hier sIgM, aber nicht sIgD, und sind positiv für CD10, CD38, LFA-1, LFA-3 und CD44 und eine Reihe von B-Zellmarkern wie CD19, CD20, CD21, CD22 und CD40 (197, 198). Während bei mehreren lymphoiden prä-B- und T-Zelllinien sowie myeloiden Zelllinien ursprünglich kein CD77 nachgewiesen werden konnte (40, 197), bestätigten Ramegowda und Tesh (265) Ergebnisse von Kniep *et al.* (162), die bereits 1985 Gb_3-Moleküle auch bei myeloiden Zellen des Menschen in späten Differenzierungsstadien chemisch nachgewiesen hatten. Die hier erstmalig gefundene Expression von CD77 durch T-Zellen stellt damit unter den bislang untersuchten (Säugetier-)Spezies eine Besonderheit boviner Lymphozyten dar.

5.1.2 Biochemische Charakterisierung zellulärer Glykolipide

Bislang konnten nur wenige Differenzierungsmarker beim Rind aufgrund der Kreuzreaktivität monoklonaler Antikörper gegen die homologen humanen Antigene identifiziert werden (309). Bei Vergleichen zwischen Proteinen verschiedener Säugetiere finden sich die niedrigsten Sequenzhomologien regelmässig bei Liganden und Rezeptoren, die in Wirtsabwehrmechanismen involviert sind. Entsprechend erkennt nur ein geringer Teil der Antikörper gegen humane Antigene das entsprechende Homolog auf bovinen Lymphozyten (222). Im Gegensatz dazu sind GSLs weit weniger spezies-spezifisch (297). Trotzdem galt es zu klären, ob das, unter Verwendung eines gegen das humane CD77-Antigen gerichteten Antikörpers, bei bovinen Lymphozyten detektierte Antigen auch biochemisch mit Globotriaosylzeramid identisch ist.

Nach Lipidextraktion war bei bovinen PBMC nach 4-tägiger mitogener Stimulation eine deutlich grössere Menge neutraler Glykolipide nachweisbar als bei unstimulierten Zellen unmittelbar nach der Gewinnung (siehe **6.3**). Parallel zur mitogen-induzierten CD77-Expression auf der Oberfläche war der Anstieg des Glykolipidgehaltes vor allem auf eine Zunahme der Zeramid-Trihexoside zurückzuführen. Während die MALDI-TOF-MS-Analyse neutraler Glykolipide unstimulierter PBMC vor allem Zeramid-Monohexoside und nur Spuren von -Di- und -Trihexosiden nachwies, enhielt die entsprechende Präparation stimulierter PBMC Zeramid-Mono- und -Trihexoside in äquivalenten Mengen. Der Kohlenhydratanteil der jeweils nachgewiesenen Zeramid-Monohexoside bestand ausschliesslich aus Glukose. Dagegen enthielt der Kohlenhydratanteil der -Trihexoside Galaktose und Glukose im Verhältnis 2:1. Die weitergehende Analyse mittels α-Galaktosidaseverdau und Methylierungsanalyse bestätigte schliesslich, dass es sich bei den Zeramid-Trihexosiden um Gal(α1-4)Gal(1-4)Glc(1-1)Cer und damit um Globotriaosylzeramid, Gb_3, handelte. Das aus bovinen PBMC gewonnene Gb_3 wurde im ELISA durch den CD77-Antikörper 38.13 erkannt.

Der Lipidanteil aller bei bovinen PBMC nachgewiesenen Zeramid-Mono- und -Trihexoside enthielt ausschliesslich C18-Sphingosin (siehe **6.3**). Allerdings unterschieden sich unstimulierte und stimulierte PBMC hinsichtlich des Fettsäureanteils ihrer GSLs. Zwar war bei allen Zeramid-Mono- und -Trihexosiden eine C16:0 Fettsäure die dominierende Fettsäurespezies, jedoch konnten auch grössere Anteile an C22:0- und C24:0-Fettsäuren nachgewiesen werden. Zeramid-Trihexoside stimulierter PBMC zeichneten sich insbesondere dadurch aus, dass bei ihnen der Anteil von Molekülen mit Fettsäuren mit mehr als 20 Kohlenstoffatomen deutlich erhöht war.

Die aktivierungsabhängige Expression von Gb_3/CD77 auf der Oberfläche der Lymphozyten könnte demnach von mehreren Faktoren abhängen. Der biochemische Nachweis von Gb_3 in unstimulierten Lymphozyten, auf deren Oberfläche keine Bindungsstellen für anti-CD77 nachweisbar waren, wie auch die erheblich stärkere Nachweisbarkeit des CD77-Antigens in intrazellulären Kompartimenten von BL-3-Zellen (siehe **5.1.1.1**), lässt sich vermutlich durch eine spezifische Eigenschaft der GSLs erklären, die als „*crypticity*“ bezeichnet wird. So berichteten Pudymaitis und Lingwood (262), dass, abhängig von bestimmten zellulären Funktionszuständen, Gb_3 in Zellen biochemisch in signifikanter Menge nachweisbar ist, aber trotzdem auf der Zelloberfläche nicht zur Ligandenbindung zur Verfügung steht. Dies könnte auf dem Vorhandensein von Gb_3/CD77 ausschliesslich in intrazellulären Kompartimenten beruhen oder auf einer Maskierung der relativ kleinen extrazellulären Anteile der GSLs durch intrinsische Membranproteine. Zusätzlich zu möglichen Veränderungen in der zellulären Verteilung von Gb_3/CD77 führte die Stimulation boviner Lymphozyten sowohl zu einer mehrfachen Zunahme der Gesamtmenge an Zeramid-Monohexosiden und Gb_3 als auch zu einer Veränderung in den Fettsäuren ihrer Lipidanteile. Dies impliziert, dass die Induktion der oberflächlichen Gb_3/CD77-Expression überwiegend auf einer *de novo*-Synthese dieser GSLs beruht. Eine transiente Aktivierungen der entsprechenden Glykosyltransferasen wurde bereits als Ursache für Verschiebungen in der Glykolipidzusammensetzung während der Differenzierung humaner B-Zellen identifiziert (327).

Derzeit ist bei Wiederkäuern die Zahl von Aktivierungsmarkern, für die definierte Antikörper existieren, noch sehr begrenzt (46, 217, 221, 223). GSLs sind wenig speziesspezifisch, aber in ihrer zellulären Expression ausreichend restringiert, um als Differenzierungsmarker verwendet werden zu können (297). Das hier erstmalig bei Lymphozyten des Rindes nachgewiesene CD77-Antigen ist das erste GSL-artige Leukozytenantigen, dessen Existenz beim Rind nachgewiesen wurde. Über die Möglichkeit hinaus nun CD77-Antikörper zur Untersuchung des bovinen Immunsystems einsetzen zu können könnte die bevorzugte Überprüfung von Antikörpern gegen GSL anderer Säugerspezies weitere Kreuzreaktivitäten aufdecken und die Auswahl der für das Rind verfügbaren Aktivierungsmarker vergrössern.

5.1.3 Untersuchungen zur Shigatoxin-Bindung

Der Umfang der CD77-Expression auf der Oberfläche korrelierte nur teilweise mit der Empfindlichkeit boviner Lymphozyten gegenüber Stx1. Die Zellen schienen vielmehr nur in einer frühen Phase der Aktivierung, die durch eine niedrige bis moderate CD77-Expression gekennzeichnet ist, transient gegenüber Stx1 empfindlich zu sein (siehe **5.2.1** und **6.6**). Überdies schloss die breite zelluläre Verteilung von CD77 auch die $CD4^+$ T-Zellen ein, deren Proliferation nur geringfügig durch Stx1 beeinflusst wird (207). Deshalb galt es zu klären, ob und unter welchen Bedingungen Gb_3/CD77 bei bovinen Lymphozyten als Stx-Rezeptor fungiert.

5.1.3.1 Bindung von Stx1 und rStxB1

In Übereinstimmung mit der Beobachtung, dass die Lymphozyten unmittelbar nach der Isolation kein anti-CD77 banden, besassen die Zellen zu diesem Zeitpunkt auch keine nachweisbaren Bindungsstellen für das Stx1-Holotoxin oder die rekombinante B-Untereinheit (rStxB1). Wie schon zum Nachweis von anti-CD77-Bindungsstellen war eine mitogene Stimulation erforderlich, um einen grösseren Anteil der Zellen in die Lage zu versetzen Stx1 oder rStxB1 zu binden (siehe **6.5**). Die Bindung war bei allen untersuchten Lymphozyten-Subpopulationen nach viertägiger Stimulation nachweisbar. Dabei spiegelte das Bindungsverhalten durchaus die Empfindlichkeit der Subpopulationen für Stx1 wider: während die Hälfte der $CD8\alpha^+$ T-Zellen und zwei Drittel der B-Zellen rStxB1 in grösserem Umfang banden, war eine geringfügige Bindung lediglich bei einem Drittel der $CD4^+$ T-Zellen und einem Viertel der γδT-Zellen zu beobachten.

Nach Extraktion von neutralen GSLs aus stimulierten PBMC erkannte rStxB1 spezifisch eine Doppelbande von Zeramid-Trihexosiden (siehe **6.5**). Hinweise dafür, dass bovines Gb_3/CD77 auch in der physiologischen Membranumgebung rStxB1 binden kann, ergaben sich aus dem Nachweis von rStxB1/anti-CD77-doppelt positiven Zellen. Allerdings war die rStxB1-Bindung nicht strikt mit der Fähigkeit der Zellen korreliert den Antikörper 38.13 zu binden. So war zu Beginn der Aktivierung (2. Inkubationstag) eine CD77-negative Zellpopulation nachweisbar, die rStxB1 binden konnte. Im weiteren Verlauf der Aktivierung (bis zum 6. Inkubationstag) trat dann verstärkt eine Population von Zellen auf, die zwar anti-CD77, aber nicht rStxB1 zu binden vermochte. Nähere Untersuchungen am 4. Inkubationstag, an dem die Populationen der $CD77^+$-Zellen mit und ohne rStxB1-Bindungsvermögen etwa gleich gross waren, zeigten, dass rStxB1 die nachfolgende Bindung des CD77-Antikörpers an

die Zellen partiell blockieren konnte. Bemerkenswerterweise konnten hohe Konzentrationen an rStxB1 den CD77-Antikörper auch wieder von seiner Bindungsstelle verdrängen, wenn rStxB1 nach anti-CD77 zu den Zellen gegeben wurde. Offensichtlich exprimierte zu diesem Zeitpunkt der Aktivierung ein grosser Teil der Lymphozyten Gb_3-Moleküle mit einer für rStxB1 erheblich höheren Affinität als für anti-CD77 38.13. Andererseits waren auch bei hohen rStxB1-Konzentrationen noch $CD77^+$-Zellen nachweisbar, was auf eine zweite Population von Zellen hindeutet, deren Gb_3-Moleküle eine besonders hohe Affinität für anti-CD77 38.13 besitzen. Hinweise auf eine dritte Population von Zellen mit Gb_3-Molekülen, die eine vergleichbare Affinität für rStxB1 und anti-CD77 besitzen, ergaben sich aus der Beobachtung, dass im mittleren getesteten Konzentrationsbereich von rStxB1 eine Kompetition der Liganden um Bindungsstellen nachweisbar war.

Sowohl rStxB1 als auch der CD77-Antikörper 38.13 erkennen spezifisch den terminalen Galabioserest (Gal(α1-4)Gal) der Gb_3-Moleküle (176, 357). Allerdings zeigten Chark *et al.* (32) kürzlich, dass sich die von StxB1 bzw. dem monoklonalen Antikörper erkannten Kohlenhydratepitope geringfügig unterscheiden. Darüberhinaus wird die Bindung der Liganden aber auch durch die Membranumgebung des Gb_3 sowie dessen Lipidanteil beeinflusst (32). Der Nachweis von drei Populationen boviner Lymphozyten, die Gb_3-Moleküle mit unterschiedlicher Affinität für die Liganden exprimieren, beruht in der Tat vermutlich auf unterschiedlichen Fettsäuremustern in den Gb_3-Molekülen. So fanden Pellizzari *et al.* (248) in menschlichen Nieren Gb_3-Moleküle, die C16-Fettsäuren enthielten und eine deutlich höhere Affinität für Stx1 besassen als andere Zeramid-Trihexoside. Auch die Fettsäureanalyse boviner Gb_3-Moleküle ergab, dass C16-Fettsäuren nur in einer sehr frühen Phase der Lymphozytenaktivierung dominierten (siehe **5.1.2** und **6.3**). Dies deckt sich mit der Tatsache, dass rStxB1-bindende Lymphozyten vor allem zu Beginn der Inkubation nachweisbar waren (siehe **6.3**) und erklärt, warum die Zellen nur in dieser Phase besonders sensibel für Stx waren (siehe **5.2.1** und **6.6**).

5.1.3.2 Internalisierung von Stx1 und rStxB1

Die Zielstrukturen für die enzymatische Wirkung der Shigatoxine sind die Ribosomen (63), die von den Toxinen nur erreicht werden können, wenn sie über Clathrin-ummantelte Membraneinziehungen nach Gruppierung sonst zufällig verteilter Stx-Bindungsstellen internalisiert wurden (127, 286). Obwohl der Anteil boviner Lymphozyten, die Gb_3-Moleküle mit hoher Affinität für Stx exprimierten, vor allem zu Beginn der Aktivierung der Zellen am

grössten war, war noch am 4. Inkubationstag ein Teil der nachweisbaren Gb_3-Moleküle in der Lage innerhalb von 30 min die Internalisierung des Shigatoxins 1 zu vermitteln (siehe **6.5**). Dies belegt, dass auch beim Rind prinzipiell ein an Gb_3/CD77 gekoppelter Translokations-mechanismus existiert. Allerdings liessen sich nicht nur 30 min nach Zugabe der rStxB1 (siehe **6.5**), sondern auch noch nach mehrtägiger Inkubation (nicht gezeigt) an die Oberfläche der Lymphozyten gebundene rStxB1-Moleküle nachweisen. Möglicherweise exprimieren Lymphozyten des Rindes 2 Populationen von Stx-Rezeptoren wie sie bereits von Lindberg *et al.* (176) für andere Zellsysteme postuliert und inzwischen durch Studien zur Funktion des humanen Stx-Rezeptors bestätigt wurden (178). Demnach ist eine Isoform des Rezeptors nur zur Bindung des Liganden in der Lage, während eine andere, möglicherweise in sehr viel geringerer Zahl vorhandene Isoform die Internalisierung des Toxins vermittelt.

5.1.3.3 Einfluss Stx-spezifischer Antikörper auf Bindung und Internalisierung von rStxB1

Auch bei den Tieren, von denen für die nachfolgend beschriebenen Untersuchungen Lymphozyten gewonnen wurden, waren wie bei vielen adulten Rindern (255) Antikörper nachweisbar, die die Stx1-Wirkung auf Vero-Zellen neutralisieren konnten. Beim Vergleich der Grenztiter der Seren und dem Ausmass der Stx1-Wirkung auf verschiedene Lymphozyten-Subpopulationen in PBMC-Kulturen dieser Tiere (siehe **5.2.1**, **5.2.3** bzw. **6.6**) ergab sich jedoch kein signifikanter Zusammenhang (siehe **6.5**). Da präparationsbedingt die PBMC von anderen Komponenten des Blutes inkl. der Immunglobuline getrennt untersucht worden waren, stellt die Empfindlichkeit gegenüber Stx1 also eine intrinsische Eigenschaft der Lymphozyten dar und ist unabhängig vom Immunstatus eines einzelnen Tieres.

Allerdings ergaben sich Hinweise auf einen möglichen Einfluss der Wirtsantwort durch die widersprüchliche Wirkung des murinen anti-StxB1-Antikörpers 13C4, der als Modell für Stx1-spezifische Immunglobuline verwendet wurde. So führte die Präinkubation von Stx1-Holotoxin mit einem molaren Überschuss des Antikörpers zwar zu einer vollständigen Blockade sowohl der nachweisbaren Bindung als auch der Wirkung des Shigatoxins 1 auf bovine Lymphozyten (siehe **6.5**). Die Präinkubation von rStxB1 mit geringeren als equimolaren Mengen des Antikörpers führte aber zu einer verstärkten Bindung von rStxB1 an die Oberfläche der Zellen. Dieser Effekt beruhte zumindest zum Teil auf dem Erhalt der Bindungfähigkeit von rStxB1/Antikörper-Komplexen und war nicht durch die Bindung an Fc-Rezeptoren bedingt. Sich anschliessende Internalisierungsversuche belegten, dass auch diese

rStxB1/Antikörper-Komplexe noch von den Zellen endozytiert werden konnten. Überraschenderweise verhinderte der Antikörper die Internalisierung jedoch vollständig, wenn die Antigen-Antikörper-Reaktion erst stattfand, nachdem rStxB1 bereits an seinen Rezeptor gebunden hatte (siehe **6.5**).

Erfolgt eine aktive oder passive Immunisierung gegen Stx vor einer experimentellen Infektion mit STEC, können in einer Reihe von Infektionsmodellen die Versuchstiere vor klinischen Symptomen als Folge der Infektion geschützt werden (54, 189, 347). Stx-spezifische Antikörper beugen auch beim Menschen der Entwicklung des HUS vor (141, 174). Allerdings ist in der Literatur immer noch umstritten, ob der protektive Effekt der Antikörper auf der Verhinderung der Bindung oder der Verhinderung der Internalisierung der Shigatoxine beruht. Nakao *et al.* (226) berichteten, dass Stx2-spezifische monoklonale Antikörper die Rezeptorbindung blockieren. Sowohl Lindberg *et al.* (176) als auch Eiklid & Olsnes (59) zeigten, dass Zellen durch die Zugabe von Antiserum nicht mehr geschützt werden können, wenn die Rezeptorbindung bereits stattgefunden hat. Im Gegensatz dazu konnten Sandvig *et al.* (286) aber Zellen zumindest teilweise schützen, wenn das Antiserum bis zu 15 min nach Toxinbindung zugegeben wurde. Ähnliche Beobachtungen wurden auch an humanen mikrovaskulären Endothelzellen gemacht (128). Da der murine monoklonale anti-StxB1 zumindest in hohen Antikörper/Antigen-Verhältnissen die rStxB1-Bindung effizient verhinderte, könnten die hohen Stx-spezifischen Antikörpertiter, die bei Rindern beschrieben wurden (18, 137, 255), durchaus protektiv sein. Allerdings scheinen die Antikörper die Wechselwirkung zwischen Stx und seinem Rezeptor zu beeinflussen. So konnten rStxB1/anti-StxB1-Komplexe, die sich in Lösung gebildet hatten, von den Zellen auch endozytiert werden, während solche, die sich erst nach der Rezeptorbindung der rStxB1 ausbildeten, auf der Zelloberfläche verblieben. Da bovines Gb_3/CD77 in verschiedenen Isoformen vorkommt und eine einzelne B-Untereinheit über bis zu drei unabhängige Gb_3/CD77-Bindungstellen verfügt (11), könnte die Erklärung für diese Unterschiede in der Benutzung einer alternativen Bindungsstelle im rStxB1 oder einer anderen Rezeptorisoform liegen. Durch StxB1-spezifische Antikörper verursachte Störungen der Ligand/Rezeptor-Interaktionen könnten auch in einer Veränderung der biologischen Wirkung des Shigatoxins 1 resultieren, da die drei Rezeptor-Bindungsstellen z.B. des Shigatoxins 1 mit unterschiedlichen biologischen Wirkungen in Verbindung gebracht wurden (363). Ausserdem würde die durch subinhibitorische Konzentrationen von StxB1-spezifischen Antikörpern verstärkte Bindung des Shigatoxins 1 an bovine Lymphozyten die Maskierung oberflächlicher Gb_3/CD77-Moleküle verstärken. Obwohl der physiologische Ligand für Gb_3/CD77 beim Rind noch

unbekannt ist, darf vermutet werden, dass diese Maskierung die Wechselwirkungen zwischen Immunzellen und von Lymphozyten mit anderen Zellen im Gewebe *in situ* erheblich beeinträchtigt (195). Der Nachweis Stx-spezifischer Antikörper im Serum von Rindern ist damit nicht mit einem effizienten Schutz vor der immunsupprimierenden Wirkung der Shigatoxine gleichzusetzen. In der Tat korrelieren die Antikörpertiter weder mit der Elimination des Erregers noch mit einem Schutz vor Reinfektion (137).

5.2 Mechanismus der Wirkung des Shigatoxins 1 auf periphere Lymphozyten des Rindes

5.2.1 Wirkung von Stx1 auf die Proliferation $CD77^+$-Lymphozyten

Eine Reihe von Studien, die sowohl experimentelle Infektionen als auch Kulturen lymphoblastoider Zellen umfassten, konnten immunmodulatorische Effekte der Shigatoxine aufzeigen (36, 40, 321). Der diesen Effekten zugrundeliegende Mechanismus ist jedoch bislang an Primärkulturen kaum untersucht worden. Bei der induzierbaren Expression von CD77 auf der Oberfläche boviner Lymphozyten stieg der Anteil $CD77^+$-Zellen in der Population der Non-Blasten und vor allem in der Population der Blasten kontinuierlich an, um am 4. Inkubationstag sein Maximum zu erreichen. Funktionelle Untersuchungen mit gereinigtem Stx1 zeigten, dass bereits die Anwesenheit von Stx1 im Nanogramm-Konzentrationsbereich (im Verozell-Test ermittelte 200 CD_{50}/ml; 1 verozytotoxische Dosis 50% entspricht 0,4-0,8 pg/ml gereinigten Shigatoxins (244)) die Zahl $CD77^+$-Zellen in der Blasten und Non-Blasten-Population drastisch reduzierte (siehe **6.6**).

Allerdings wurden nicht alle $CD77^+$-Zellen aus den Kulturen eliminiert. Noch nach achttägiger Inkubation mit Stx1 waren vitale $CD77^+$-Lymphozyten nachweisbar (siehe **6.6**). In der frühen Phase der Kultivierung exprimierten in Anwesenheit von Stx1 sogar signifikant mehr lebende Non-Blasten das CD77-Antigen als in Kulturen mit Stx1 und anti-StxB1 13C4. Da die Zugabe von Stx1 auch die durchschnittliche Zahl an CD77-Molekülen auf der Oberfläche der verbleibenden Zellen reduzierte, könnte Stx1 einen zytoletalen Effekt in den Kulturen ausüben und selektiv die Gb_3/$CD77^{\text{“high“}}$-Zellen eliminieren. Eine Korrelation zwischen der Zahl der oberflächlichen Gb_3/CD77-Moleküle und der Empfindlichkeit von Zellen für Stx1 wurde bereits für Zelllinien beschrieben (59). Allerdings geht in Primärkulturen boviner Lymphozyten die signifikante Reduktion der zellulären Stoffwechselaktivität durch Stx1 nicht mit einen Anstieg der Zahl apoptotischer oder nekrotischer Zellen einher (207). Weiterhin reduzierte Stx1 den Anteil $CD77^+$-Zellen auch in

der Population der Non-Blasten, die sich während der gesamten Inkubation nur durch eine niedrige bis moderate CD77-Expression auszeichneten (siehe **5.1.1**). Da bovine Lymphozyten hochaffine Stx-Rezeptoren nur transient in einer frühen Phase der Aktivierung exprimierten (siehe **5.1.3**), wirkte das Stx1 vermutlich nur auf eine vergleichsweise kleine Zahl von Zellen zwischen den CD77$^{\text{“low“}}$- und CD77$^{\text{“moderate“}}$-Stadien der Aktivierung. Die betroffenen Zellen verharrten dann entweder in diesem Zustand oder leiteten die Apoptose ein, wobei sie vorübergehend CD77 auf hohem Niveau exprimierten. Dies könnte die erhöhte Zahl CD77^{+} Non-Blasten in Stx1-supplementierten Kulturen erklären (siehe **6.6**). Für eine Wirkung des Shigatoxins 1 auf wenige, CD77 nur moderat exprimierende Lymphozyten spricht auch, dass sich der Effekt des Shigatoxins 1 weder qualitativ noch quantitativ signifikant anders darstellte, wenn die Zugabe des Shigatoxins 1 erst am 3. Inkubationstag zu vorstimulierten PBMC-Kulturen erfolgte, in denen viele Zellen einen CD77$^{\text{“high“}}$-Phänotyp aufwiesen. Auch unter diesen Versuchsbedingungen waren noch tagelang CD77^{+}-Zellen in Stx1-supplementierten Kulturen nachweisbar. Da sich auch die Struktur der Gb_3/CD77-Moleküle im Verlauf der Aktivierung veränderte (siehe **5.1.2**), wurden bovine Lymphozyten offensichtlich wieder refraktär gegenüber Stx1, wenn sie den Aktivierungsprozess über CD77$^{\text{“low“}}$- und CD77$^{\text{“moderate“}}$-Stadien bis zum CD77$^{\text{“high“}}$-Stadium durchlaufen haben.

Unabhängig von der Empfindlichkeit verschiedener Lymphozyten-Subpopulationen gegenüber Stx1 betraf die Reduktion der Zahl der CD77^{+}-Zellen alle Subpopulationen gleichermaßen. Das bovine Immunsystem zeichnet sich damit durch eine breite zelluläre Verteilung der Empfindlichkeit gegenüber Stx1 aus. Dagegen beschränkt sich bei humanen Lymphozyten die Wirkung des Shigatoxins 1 auf das B-Zell-Kompartiment (40, 198). Andererseits fehlen Rindern im Gegensatz zum Menschen Rezeptoren für Stx in der Mikrovaskulatur (260). Möglicherweise stellt also bei Rindern das Immunsystem das wichtigste Zielorgan für Stx dar. Bedingt durch die nur transiente Empfindlichkeit boviner Lymphozyten blockieren STEC durch die Sekretion der Shigatoxine eher die Entstehung einer adaptiven Immunantwort, als eine vorhandene Immunantwort wieder zu unterdrücken. Dies ist aus Sicht der Keime sehr effizient, da für die Hemmung einer dann noch kleinen Zahl an Zielzellen geringe Toxinmengen ausreichen würden.

5.2.2 Wirkung der B-Untereinheit

Obwohl der B-Untereinheit des Shigatoxins 1 die enzymatische Aktivität des Holotoxins fehlt, hatte sie auf bovine PBMC-Kulturen einen dem Stx1-Holotoxin vergleichbaren Effekt. Noch in einer Konzentration von 20 ng / ml war ein reduzierter Anteil der $CD77^+$-Blasten nachweisbar. Die Reduktion beruhte damit nicht auf einer Maskierung der CD77-Moleküle (siehe **6.5**). Über eine von den B-Untereinheiten der Shigatoxine induzierte und von der Zelloberfläche ausgehende Signaltransduktion wurde bereits wiederholt von anderen Arbeitsgruppen berichtet (5, 138, 145). Allerdings spricht die Tatsache, dass anti-StxB1-Antikörper unter bestimmten Bedingungen die Bindung von rStxB1 an bovine Lymphozyten erhöhten (siehe **5.1.3.3**) ohne die biologische Wirkung der rStxB1 zu verstärken (Daten nicht gezeigt) dagegen, dass ein Signal ausschliesslich von der Bindung der rStxB1 ausgeht. Da selbst die B-Untereinheit zytoletal wirken kann, wenn sie innerhalb von transfizierten eukaryontischen Zellen exprimiert wird (224), ist die Aufnahme der rStxB1 in bovine Lymphozyten möglicherweise zwingend erforderlich. Allerdings waren für die rStxB1-Wirkung deutlich höhere Konzentrationen erforderlich als bei Verwendung des Stx1-Holotoxins. In anderen *in vitro*-Systemen sensibilisiert Stx durch die Hemmung der Proteinbiosynthese die Zellen gegenüber Signalen von der Zelloberfläche. Diese Signale können dann noch durch geringste Mengen der B-Untereinheit ausgelöst werden und starten das Apoptoseprogramm (144, 197). Stx1 induzierte bei bovinen Lymphozyten zwar keine Apoptose, vor der vollständigen Hemmung der Proteinbiosynthese schädigt Stx1 aber die 28S rRNA, worauf einige Zellen mit einer „ribotoxic stress response“ reagieren (337). Signale, die von der Bindung der B-Untereinheit (oder ihres Pentamers) an oberflächliches Gb_3/CD77 ausgehen, würden damit mit der Induktion von Genen der Sofortantwort nach Internalisierung des Stx-Holotoxins zusammenwirken. Beides könnte zum supprimierenden Effekt des Shigatoxins 1 auf bovine Lymphozyten beitragen (**Abb. 5.2**).

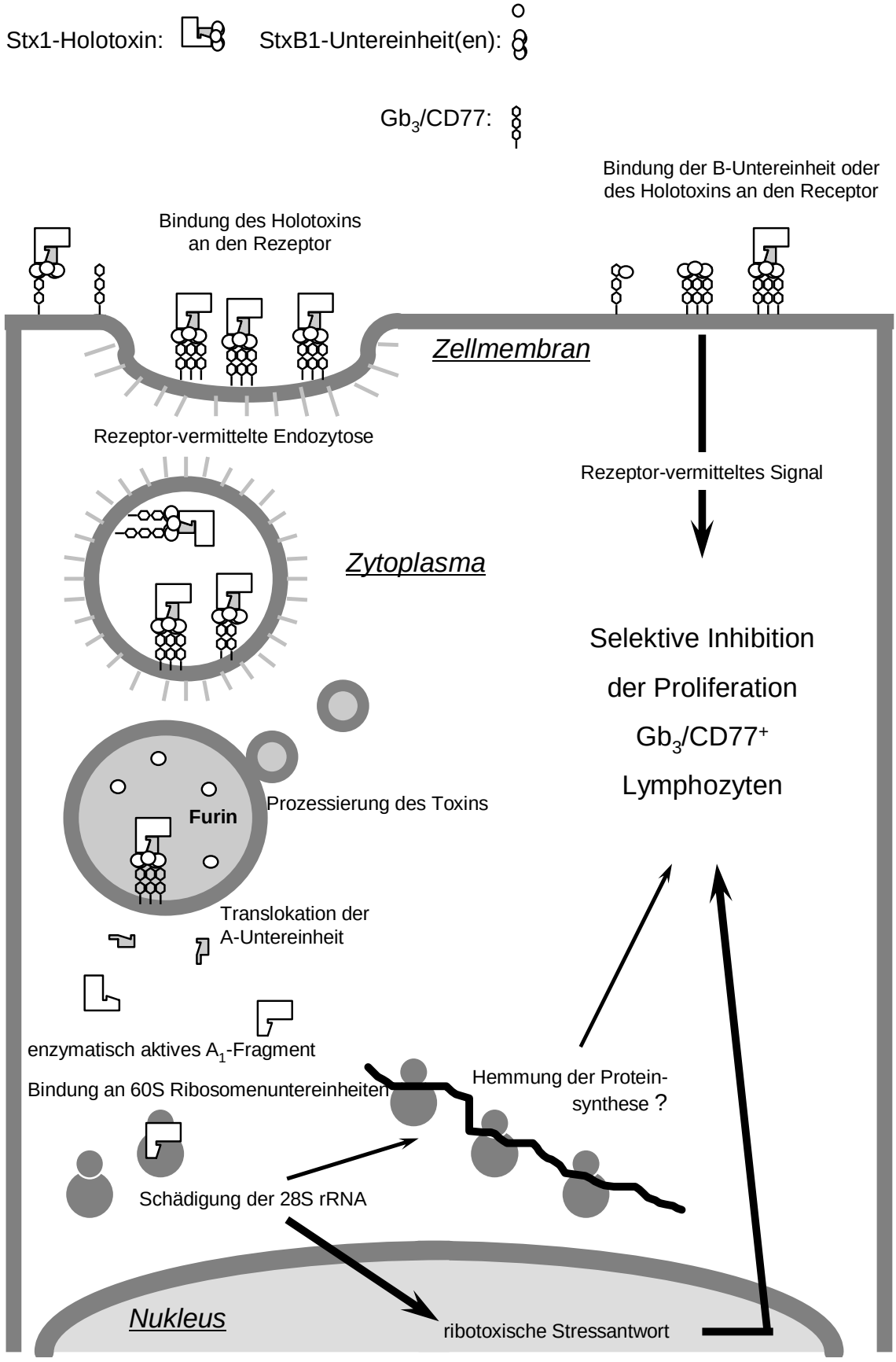

Abb. 5.2: Hypothetisches Modell zum Wirkungsmechanismus von Stx1 auf periphere Lymphozyten des Rindes (Details siehe Text)

5.2.3 Wechselwirkung mit ausgewählten Zytokinen

Die letale Wirkung von Exotoxinen auf Zellen des Immunsystems zählt zu den Mechanismen mit denen sich pathogene Bakterien innerhalb ihres Wirtes einen Überlebensvorteil sichern (33). Allerdings haben viele bakterielle Exotoxine, die ursprünglich über ihre zytoletalen Effekte definiert wurden, zusätzlich auch modulierende Eigenschaften (299). Die Fähigkeit die Synthese oder Sekretion pro- oder anti-inflammatorischer Zytokine anzuregen, kann dabei für die Pathogenese der induzierten Erkrankung eine ähnlich grosse Bedeutung haben wie die eigentliche zytoletale Wirkung (107). So spielt zwar in der Pathogenese des HUS die Stx-bedingte Schädigung von Endothelzellen eine wichtige Rolle (246, 259), der Umfang der Organschädigungen hängt aber wesentlich von der lokalen Induktion verschiedener Zytokine und Chemokine in den toxingeschädigten Geweben ab (246, 259, 333, 334). Da Stx1 die Mitogen-induzierte Proliferation boviner Lymphozyten inhibierte, aber keinen zytoletalen Effekt auslöste, sollte der Einfluss ausgewählter Zytokine auf die Proliferation in Anwesenheit und Abwesenheit von Stx1 aufgeklärt werden.

TNF-α wird von humanen Monozyten als Reaktion auf eine Stimulation mit Stx freigesetzt und sensibilisiert benachbarte Endothelzellen gegenüber der zytoletalen Wirkung der Shigatoxine (341, 343). Da Präparationen boviner PBMC bis zu 15 % Monozyten enthalten (207), wäre denkbar, dass die Stx1-induzierte Hemmung der Lymphozyten-proliferation nur die indirekte Folge der Freisetzung von TNF-α aus Monozyten war. Allerdings verstärkte die Zugabe von rekombinantem bovinem TNF-α weder die CD77-Expression der Lymphozyten noch konnte es den Effekt des Shigatoxins 1 nachahmen (siehe **6.6**). IFN-α bindet an seinen Rezeptor (IFNAR) nur dann, wenn dieser in der hochaffinen Form komplexiert mit Gb_3/CD77 vorliegt (86). Wegen der partiellen Homologie zwischen dem bovinen IFNAR und StxB1 (86) erschien es denkbar, dass IFN-α und Stx1 sich gegenseitig in ihrer Wirkung durch Stabilisierung bzw. Destabilisierung des Gb_3/CD77-IFNAR-Komplexes beeinflussen könnten. Allerdings zeigte sich, dass rhuIFN-α den inhibitorischen Effekt des Shigatoxins 1 konzentrationsabhängig noch weiter verstärkte. Dies spricht dafür, dass beide Substanzen getrennte Signaltransduktionswege benutzen. Da Stx1 auch die Expression des bovinen IL-2-Rezeptors (CD25) verstärkt und verlängert (207), könnte der Effekt des Shigatoxins 1 auch auf einer Blockade der parakrinen boIL-2-Sekretion beruhen, die z.B. bei humanen Lymphozyten durch das Produkt des *lifA*-Gens enteropathogener *E. coli* ausgelöst wird (159, 160). Exogenes rboIL-2 konnte jedoch den inhibitorischen Effekt des Shigatoxins 1 auf bovine Lymphozyten nicht aufheben.

5.3 Identifizierung und Charakterisierung boviner intestinaler Zielzellen für Shigatoxin 1 *in vitro*

5.3.1 Granulozyten

Erst in jüngerer Zeit wurde die lokale Bedeutung der Granulozyten im Darm bei der Pathogenese der humanen HC und des HUS deutlich (259). So verzögert Stx das Einsetzen der Apoptose in humanen Granulozyten (184) und beschleunigt die Bildung reaktiver Sauerstoffmetaboliten, die dann zum Gewebsschaden in der intestinalen Mukosa beitragen können (153). Darüberhinaus induziert Stx aber auch die Freisetzung chemoattraktiver Substanzen aus Epithelzellen (338, 365) und verstärkt die Transmigration von Granulozyten durch die Epithelzellschicht (117). Beides trägt nicht nur zur Entwicklung der intestinalen Entzündung bei, sondern ist vermutlich die Voraussetzung für die Resorption signifikanter Mengen der Shigatoxine aus dem Darmlumen (259). Es gibt sogar Anhaltspunkte dafür, dass die Granulozyten nach Durchtritt der Toxine durch die Darmschranke für den Transport der Shigatoxine zu den sensitiven Organen wie den Nieren verantwortlich zeichnen (332).

In erster Linie sind Granulozyten jedoch Effektorzellen des angeborenen Immunsystems, die zur Begrenzung der mukosalen Kolonisation durch Bakterien beitragen (325). Granulozyten infiltrieren die Kolonmukosa STEC-infizierter Kälber (295), doch ihre Bedeutung für den Verlauf der STEC-Infektion ist noch unklar. Das Fehlen intestinaler Entzündungserscheinungen bei STEC-infizierten adulten Wiederkäuern könnte darauf hindeuten, dass die Granulozyten hier nicht in die Mukosa gelockt und durch Stx aktiviert werden. In der Tat ist die Entzündung, die durch bestimmte STEC-Stämme im Darmligatur-test bei Kälbern ausgelöst wird, unabhängig von der Expression des Shigatoxins 1 (313). Demnach könnten sich die Granulozyten der Wiederkäuer grundsätzlich von denen des Menschen unterscheiden, indem erstere gegenüber Shigatoxinen resistent sind.

5.3.1.1 Stx-Rezeptor-Expression durch Granulozyten von Rindern und Schafen

In Übereinstimmung mit dieser Hypothese zeigten beispielhafte Untersuchungen mit Granulozyten aus dem peripheren Blut, dass Granulozyten beim Rind keine Stx-Rezeptoren exprimierten (siehe **6.9**). Die Zellen waren weder in der Lage anti-CD77 noch rStxB1 zu binden. Auch die Stimulation mit LPS, die die Expression anderer Oberflächenantigene wie CD11b auf bovinen Granulozyten verstärkt (53), konnte keine CD77-Expression induzieren. Eine mögliche Altersabhängigkeit wie für Darmepithelzellen des Kaninchens beschrieben

(211) konnte durch die Untersuchung der Granulozyten von 1-3 Wochen alten Kälbern ebenfalls ausgeschlossen werden. Auch Granulozyten, die durch die Blut/Milch-Schranke migriert waren und sich dadurch in einer Reihe zellulärer Funktionen von Blutgranulozyten unterscheiden (306), exprimierten kein CD77.

Im scharfen Gegensatz dazu konnten Granulozyten aus dem Blut von Schafen, die vergleichend untersucht wurden, sowohl den anti-CD77-Antikörper als auch rStxB1 binden (siehe **6.9**), ohne dass dafür eine weitere Stimulation der Zellen notwendig war. Die rStxB1 vermochte nicht nur die nachfolgende Bindung des Antikörpers vollständig zu verhindern, sondern konnte bereits die von den Zellen gebundenen Antikörper wieder verdrängen. Auch alle Granulozyten in der Milch von Schafen exprimierten CD77 *ex vivo*. Die Struktur des ovinen CD77 ist biochemisch noch nicht untersucht, aber es scheint bei den Granulozyten nur eine Klasse von Stx-Rezeptoren vom Gb_3/CD77-Typ vorzukommen. Damit unterscheiden sich die Granulozyten dieser Spezies nicht nur von denen des Rindes, sondern auch von denen des Menschen. Letztere besitzen einen bislang nicht näher charakterisierten niedrig affinen Stx-Rezeptor, der biochemisch nicht mit Gb_3/CD77 identisch ist (332).

5.3.1.2 Einfluss von Stx1 auf granulozytäre Funktionen

Die Inkubation boviner Granulozyten mit Stx1 *in vitro* für bis zu 18 Stunden hatte keinerlei Einfluss auf die Vitalität der Zellen, ihre phagozytäre Aktivität oder Stimulierbarkeit (siehe **6.9**). Vor dem Hintergrund der Bedeutung einer Stx-bedingten Aktivierung humaner Granulozyten für die Pathogenese des HUS, hilft die offensichtliche Resistenz boviner Granulozyten für das Toxin zu erklären, warum STEC-Infektionen bei adulten Rindern keine klinischen Erscheinungen hervorrufen.

Auch bei Granulozyten von Schafen führte die Inkubation mit Stx1 nicht zu einer Aktivierung der Zellen (siehe **6.9**). Zwar war die Phagozytoseaktivität der Zellen etwas erhöht, nachdem sie 18 Stunden dem Holotoxin ausgesetzt waren, jedoch reduzierte diese Behandlung gleichzeitig das Vermögen der Zellen Sauerstoffmetaboliten zu produzieren. Den deutlichsten Effekt hatte Stx1 in diesem Zeitraum auf die Vitalität der Zellen, indem es den Anteil frühapoptotischer Zellen verdoppelte, während rStxB1 keine nachweisbare Wirkung besass. Der ovine Gb_3/CD77-artige Rezeptor schien dabei die Wirkung des Toxins auf Granulozyten anders zu vermitteln als der niedrig affine Rezeptor auf humanen Granulozyten. Während bei ovinen Zellen die Wirkung offensichtlich von der enzymatischen Wirkung des Toxins abhing, die eine Internalisierung des Toxins voraussetzte, wird bei humanen Granulozyten die Superoxyd-Bildung bereits durch die Bindung der StxB1 gestört (153).

Auch kann bei humanen Granulozyten die Verzögerung des Einsetzens der Apoptose durch Stx2 durch Proteinkinase-Inhibitoren blockiert werden (184). Dies impliziert die Existenz eines Signalweges, der von den Stx-Rezeptoren auf der Zelloberfläche ausgeht und bei humanen B-Lymphomzellen mit Gb_3/CD77 assoziiert ist (326), bei Granulozyten des Schafes aber offensichtlich fehlt. Zweifelhaft ist, ob die Apoptose-Induktion durch Stx einen unmittelbaren Vorteil für die STEC bei der Kolonisation im Schafdarm darstellt. Ein antiphagozytärer Phänotyp könnte zwar zur Persistenz der Infektion beitragen, indem er die antimikrobielle Aktivität der Granulozyten auf der Schleimhaut reduziert oder verhindert, dass die Bakterien durch M-Zellen zu Strukturen des darmassoziierten Schleimhaut-Immunsystems transportiert werden (64). Allerdings trat eine signifikante Wirkung des Shigatoxins 1 auf ovine Granulozyten erst nach 18-stündiger Einwirkung auf (siehe **6.9**). Die Phagozytose von Bakterien durch Granulozyten benötigte jedoch nur wenige Minuten (siehe **6.1**) und enteropathogene *E. coli* können die Phagozytose durch Makrophagen bereits innerhalb von 120 min durch Produkte des *lee*-kodierten Typ III-Sekretionsapparates inhibieren (89). Wenn diese molekularen Wechselwirkungen auch nach Kontakt zwischen *lee*-positiven STEC-Stämmen und phagozytierenden Zellen des Schafes ablaufen, ist der protrahierte Effekt des Shigatoxins 1 wahrscheinlich irrelevant.

5.3.2 Lymphozyten in mesenterialen Lymphknoten

Beim Rind ist die Expression von CD77 durch Lymphozyten des systemischen Teils des Immunsystems nicht auf *in vitro*-Bedingungen beschränkt. So war dieses Antigen auf vielen B-Zellen aus den Keimzentren von Mesenteriallymphknoten ebenso nachweisbar wie auf $CD4^+$ sowie $CD8\alpha^+$ T-Zellen aus diesen Organen (siehe **6.3**).

5.3.3 Intraepitheliale Lymphozyten

Bei der lokalen Wirkung der Stx auf intestinale Immunzellen des Rindes dürfte allerdings intraepithelialen Lymphozyten (IEL) eine entscheidende Bedeutung zukommen, da sie die ersten Immunzellen sind, die luminal gebildetes Stx erreichen kann.

5.3.3.1 Stx-Rezeptorexpression durch Subpopulationen

Nach Gewinnung der Zellen aus der Mukosa des Ileums adulter Rinder war CD77 auf der Oberfläche aller untersuchten Lymphozyten-Subpopulationen einschliesslich der αβT-Zellen

und der γδT-Zellen *ex vivo* nachweisbar (siehe **6.7**). Im Durchschnitt exprimierten ca. 15 % der IEL CD77, jedoch unterschieden sich die Subpopulationen hinsichtlich ihrer Expressionstärke erheblich. Der überwiegende Teil der CD77$^{\text{“high“}}$-IEL stellte sich als aktivierte, reife T-Zellen dar, die CD3, CD6, ACT-2 und CD8α koexprimierten. Auch CD8β$^+$ T-Zellen und Subpopulationen von γδT-Zellen (WC1$^+$, TcR1-N7$^+$) zeichneten sich durch eine starke Oberflächenexpression von CD77 aus. Allerdings koexprimierten ca. 40 % der CD8α$^+$ T-Zellen CD77 aber nur ca. 18 % der CD8β$^+$ T-Zellen. Obwohl die durchschnittliche Nachweisstärke für CD77 zwischen verschiedenen IEL-Präparationen (d.h. verschiedenen Spendertieren) schwankte, zeichneten sich CD4$^+$ T-Zellen und B-Zellen (CD21$^+$, membranständiges IgM$^+$) durchgängig durch eine nur geringradige CD77-Expression aus. Wie schon bei peripheren Lymphozyten konnten auch bei IEL drei Populationen von Zellen nachgewiesen werden, die entweder anti-CD77, rStxB1 oder beide Liganden binden konnten. Die überwiegende Expression anti-CD77 bindender Isoformen des Gb_3, weist auf einen aktivierten Zustand der Zellen hin. In der Tat war der Aktivierungsmarker ACT-2, der von Waters *et al.* (348) zur Bestimmung des aktivierten Phänotyps boviner IEL verwendet wurde, bei ca. 40 % de IEL nachweisbar. Jedoch korrelierte die CD77-Expression nicht mit der Expression verschiedener anderer Aktivierungsmarker (siehe **6.7**). CD77 war vor allem auf den TcR1-N7$^+$ und WC1$^+$ Subpopulationen der γδT-Zellen vorhanden. Eine weitere Analyse dieser Zellen ergab, dass CD8α von ca. 30 % aller γδT-Zellen (TcR1-N12$^+$) koexprimiert wurde, wohingegen nur ca. 18 % der TcR1-N12$^+$-Zellen CD8β koexprimierten. Demnach stellten vermutlich CD8αα Homodimer-positive γδT-Zellen, die bereits von Wyatt *et al.* (364) beschrieben wurden, und möglicherweise andere CD8α$^+$ IEL die Subpopulationen mit den höchsten Anteilen CD77-positiver Zellen dar. Bei Mäusen und Menschen besteht die Hauptfunktion CD8αβ$^+$ αβT-IEL in der Lyse infizierter Zellen, während γδT-IEL (und möglicherweise CD8αα$^+$ αβT-IEL) die Regeneration der Epithelzellschicht stimulieren. Obwohl die Funktionen boviner IEL bislang wenig untersucht sind, lässt sich spekulieren, dass die STEC durch eine Stx-vermittelte Schädigung dieser Zellart sowohl auf lokale Immunreaktionen als auch auf die stetige Erneuerung der Epithelzellschicht Einfluss nehmen. Tatsächlich wurden beide Aspekte bereits mit dem persistenten Charakter boviner STEC-Infektionen in Verbindung gebracht (siehe **5.4.4**) (190).

5.3.3.2 Wirkung von Stx1 auf die Aktivierung

Trotz ihres aktivierten Phänotyps sprechen IEL allgemein schlecht auf eine mitogene Stimulation *in vitro* an (218). Trotzdem gelang es durch verschiedene Stimuli in Kulturen boviner IEL die Transformation zu Blasten zu induzieren (siehe **6.7**). Vergleichbar mit den bei peripheren Lymphozyten beobachteten Wirkungen inhibierte Stx1 auch hier die Transformation und reduzierte den Anteil von Zellen mit Blasten-artiger Morphologie binnen 72 Stunden. Die Reduktion betraf vor allem den Anteil $CD77^+$-Zellen innerhalb der Blasten, aber auch $CD77^+$ Non-Blasten.

Bemerkenswerterweise unterschied sich die zelluläre Verteilung von CD77 bei *in vitro* stimulierten IEL erheblich von der *ex vivo* beobachteten Verteilung. Nach Inkubation exprimierten vergleichbare Anteile der $CD8\alpha^+$- und $CD8\beta^+$-IEL das CD77-Antigen. Der höchste Anteil CD77-positiver Zellen fand sich nun jedoch bei den $CD4^+$ T-Zellen und den $CD21^+$ B-Zellen. Demnach ist prinzipiell jede Subpopulation boviner IEL zur CD77-Expression in der Lage.

5.3.3.3 „Natürliche Killerzell-Aktivität“ in Anwesenheit von Stx1

Um die Auswirkungen des Shigatoxins 1 auf die mukosale Immunantwort besser verstehen zu können, wurden die IEL auch funktionell charakterisiert. Unter Verwendung einer bovinen B-Lymphomzelllinie als Zielzellen konnte dabei erstmals gezeigt werden, dass IEL des Rindes über eine MHC-unabhängige zytotoxische Aktivität verfügen. Diese „Natürliche Killerzell-Aktivität“ wurde durch Inkubation der Zellen mit Stx1 jedoch nicht beeinflusst (siehe **6.7**).

5.3.3.4 Expression von Chemokingenen in Anwesenheit von Stx1

In bovinen IEL waren Transkripte für eine Reihe von Chemokin-Genen (IL-8, IP-10, MCP-1) *ex vivo*, aber auch nach Kultivierung der Zellen, nachweisbar (siehe **6.10**). Migrationsstudien mit bovinen Granulozyten belegten, dass IEL auch chemoattraktive Substanzen synthetisieren und sezernieren können. Damit ist erstmalig gezeigt, dass auch beim Rind den IEL eine aktive Rolle bei der Rekrutierung von Immunzellen in die intestinale Mukosa zukommt.

Allerdings war Stx1 *in vitro* weder in der Lage die Transkription von *il-8*, *ip-10* oder *mcp-1* noch die Sekretion chemoattraktiver Substanzen zu beeinflussen (siehe **6.10**). Dieser

Befund sowie die Tatsache, dass auch bovine Kolonepithelzellen durch Stx1 nicht zur Sekretion granulozytotroper Chemokine angeregt werden (siehe **5.3.4**), steht im Einklang mit Ergebnissen von *in vivo* Studien, die zeigten, dass Stx1 nicht an der Entstehung intestinaler Entzündungserscheinungen bei STEC-infizierten Kälbern beteiligt ist (313). Dies darf als weiterer Beleg dafür gelten, dass, im Gegensatz zu EHEC-Infektionen des Menschen, Stx1 bei STEC-Infektionen des Rindes nicht als Virulenzfaktor mit pro-inflammatorischen Eigenschaften fungiert.

5.3.3.5 Wirkung von Stx1 auf die Transkription von Zytokingenen

Auch eine Reihe von T_H-Prototyp-Zytokinen (IL-2, IFN-γ, IL-4, IL-10 und TGF-β) wurde von bovinen IEL aus dem Ileum transkribiert. Untersuchungen mittels quantitativer "real-time RT-PCR" zeigten allerdings, dass Stx1 *in vitro* keinen Einfluss auf den Gehalt der Zellen an mRNA für die pro-inflammatorischen T_H1-Zytokine IL-2 und IFN-γ oder das anti-inflammatorische T_H3-Zytokin TGF-β hatte (siehe **6.10**). Die Behandlung mit Stx1 veränderte auch nicht den Gehalt an *il-10*-spezifischen Transkripten, auch wenn diese insgesamt nur in sehr geringer Zahl nachweisbar waren. In deutlichem Gegensatz dazu induzierte Stx1 eine starke Zunahme an mRNA für das T_H2-Zytokin IL-4. Ein solcher Effekt, der 6 bis 8 Stunden nach Kontakt des Shigatoxins 1 mit den IEL sein Maximum erreichte, war bereits nach 4 Stunden nachweisbar. Obwohl sich IEL-Präparationen von mehreren Tieren in ihrer Empfindlichkeit für Stx1 unterschieden, reichten bereits sehr geringe Konzentrationen (66, 22 und sogar 7 verozytotoxische Dosen 50 % pro ml) für eine Induktion aus. Die Wirkung des Shigatoxins 1 war so stark, dass *il-4*-spezifische Transkripte zur häufigsten mRNA-Spezies in den IEL wurden. Die Zahl der *il-4*-Transkripte überstieg nach 6 Stunden sogar um das 10-fache die Zahl der *tgf-β*-Transkripte, die in Toxin-freien Kulturen die dominierende mRNA darstellten. Da Grogan *et al.* (92) für murine T-Zellen zeigen konnten, dass die Aktivierung der Zellen binnen Stunden zu einer verstärkten Transkription von *il-4* führt, könnte auch die Erhöhung der *il-4*-mRNA in bovinen IEL das Resultat einer durch Stx1 erhöhten Transkriptionsaktivität sein. Allerdings kann Stx1, vermutlich über Stimulierung des p38 MAP-Kinase Signalweges (337, 362), durch eine Verlängerung der Halbwertszeit auch die Akkumulation bestimmter Zytokin- und Chemokin-mRNAs induzieren (104, 338). Da die Translations-abhängige mRNA-Degradierung in Säugerzellen Transkript-spezifisch abläuft (133), bleibt noch zu klären, ob Stx1 in bovinen iIEL die *de novo* mRNA-Synthese verstärkte oder die Degradierung vorhandenen Transkripte verzögerte. Interessanterweise war die

Wirkung des Shigatoxins 1 auf die Menge Zytokin-spezifischer Transkripte spezifisch für IEL, da bei PBMC ein solcher Effekt nicht nachweisbar war.

Allerdings war die Stx1-Wirkung nicht von einem messbaren Anstieg der Synthese des IL-4-Proteins begleitet (siehe **6.10**). So war nur bei einem kleinen Anteil boviner IEL die Bildung von IL-4 *in vitro* nachweisbar. Der Anteil wurde durch Zugabe von Stx1 zu den Kulturen nicht weiter gesteigert. Da sogar eine Stx1-induzierte hundertfache Steigerung von mRNA-Gehalten in einer sehr viel kleineren Steigerung der nachweisbaren Menge an entsprechendem Protein resultieren kann (338), kann nicht ausgeschlossen werden, dass Stx1 doch die *il-4*-Expression bis auf die Proteinebene steigert. Auch könnte die nachgewiesene Bildung von IFN-γ in den IEL-Kulturen eine rasche reziproke Herunterregulation der T_H2-Aktivierung bewirkt haben, wodurch ein bereits sehr niedriger IL-4-Spiegel weiter reduziert (273) und der Effekt des Shigatoxins 1 maskiert wurde.

Unklar ist bislang noch die zelluläre Herkunft der induzierten *il-4*-mRNA. IL-4 wird charakteristischerweise von der T_H2-determinierten Unterpopulation der $CD4^+$ T-Zellen produziert. Obwohl Stx-Rezeptoren des Gb_3/CD77-Typs beim Rind vor allem von $CD8\alpha^+$ T IEL *ex vivo* exprimiert werden, konnten *in vitro* auch $CD4^+$ T-IEL zur Gb_3/CD77-Expression aktiviert und damit gegenüber Stx1 sensibilisiert werden (siehe **6.7**). Jedoch korrelierte die Menge der nach Stx1-Einwirkung vermehrt nachweisbaren IL-4 mRNA nicht mit dem Anteil von $CD4^+$-IEL in den untersuchten Zellpräparationen (siehe **6.10**). Möglicherweise sind bovine $CD4^+$-IEL nicht die Hauptquelle der Stx1-induzierten IL-4 mRNA. Bei humanen Lymphozyten stimulieren andere Ribosomen-inaktivierende Toxine wie Mistellektine die intrazelluläre Expression von IL-4 sowohl in $CD4^+$ als auch in $CD8^+$ T-Zellen in gleichem Umfang (311). Die Induktion der *il-4*-Transkription ist dann mit dem verstärkten Autreten von Apoptosemarkern assoziiert. Deshalb wird vermutet, dass verschiedene Apoptose-induzierende Toxine einen gemeinsamen Signalpfad aktivieren, in dem intrazelluläres IL-4 eine Bedeutung stromabwärts der Apoptose zukommt (311).

5.3.3.6 Mechanismus der Induktion der *il-4*-Transkription durch Stx1

Die Stx1-induzierte Vermehrung der *il-4*-Transkripte in bovinen IEL-Kulturen ist jedoch eindeutig unabhängig von der Induktion von Apoptose. Stx1 löste keine nachweisbare Erhöhung der Apoptoserate aus und ein Apoptoseinduktor, Actinomycin D, löste keine Vermehrung der *il-4*-mRNA aus (siehe **6.10**). Diese Ergebnisse zeigen, dass die Stx1-induzierte Vermehrung der *il-4*-mRNA kein Nebeneffekt einer Apoptoseinduktion war.

Vielmehr verbleiben die bovinen IEL, die auf Stx1 reagieren, bei STEC-Infektionen vermutlich funktionell eingebunden in das mukosale immunologische Netzwerk. Interessanterweise hemmt IL-4 spezifisch die Stimulierbarkeit humaner $CD8^+$-IEL ohne gleichzeitig die Proliferation peripherer $CD8^+$ T-Zellen zu beeinflussen (58). Demnach könnte die Depletion von $CD8^+$-IEL im Verlaufe experimenteller STEC-Infektionen bei Kälbern (siehe **6.8**) zumindest zum Teil auf einer Stx1-induzierten Produktion von IL-4 beruhen.

Durch die Blockade der Stx1-Wirkung durch Brefeldin A wurde deutlich, dass die Aufnahme und Prozessierung des Holotoxins unabdingbar war (siehe **6.10**). Auch war die enzymatische Aktivität des Stx1-Holotoxins für die *il-4* mRNA-Induktion zwingend erforderlich, da rStxB1 alleine diesen Effekt nicht auszulösen vermochte. Der Gehalt boviner IEL an *il-4*-Transkripten wurde selbst durch sehr niedrige Konzentrationen (7 CD_{50}/ml) an Stx1 noch beeinflusst. Trotzdem war die Fähigkeit der Zellen zur Synthese von IFN-γ selbst bei deutlich höheren Stx1-Konzentrationen (200 CD_{50}/ml) noch vollständig erhalten. Bitzan *et al*. (17) hatten bereits beobachtet, dass Stx1 die Menge bestimmter Transkripte in bovinen Endothelzellen in Toxinkonzentrationen erhöht, die unter denen liegen, die für eine messbare Hemmung der Proteinbiosynthese erforderlich sind. Demnach lässt sich die Diskrepanz zwischen erhöhter Menge an *il-4*-mRNA und unveränderter IL-4-Synthese in bovinen IEL nicht durch eine Blockade der Proteinbiosynthese durch Stx1 erklären.

5.3.4 Epithelzellen

Nach Optimierung einer von Föllmann *et al.* (73) beschriebenen Methode gelang es bovine Kolonepithelzellen zu gewinnen und zu kultivieren. Mithilfe der Durchflusszytometrie und Fluoreszenzmikroskopie konnten sowohl CD77-Antigene als auch Bindungsstellen für rStxB1 auf der Oberfläche einer Subpopulation epithelialer Zellen nachgewiesen werden (**Abb. 5.3**). Der überwiegende Teil der CD77-Moleküle war jedoch intrazellulär im kernnahen Bereich lokalisiert. Wie von Hoey *et al.* (111) beschrieben, waren die Zellen resistent für eine zytoletale Wirkung des Shigatoxins 1 (**Abb. 5.4**).

In vitro-Untersuchungen lassen vermuten, dass bei humanen STEC-Infektionen das im Darmlumen gebildete Stx bei Epithelzellen zur Freisetzung einer Vielzahl von Chemokinen führt (338). Bei primären Kulturen boviner Kolonepithelzellen waren signifikante Mengen an mRNA für die Chemokine GRO-α, IL-8 und RANTES nachweisbar. Allerdings wurde weder die Transkription der Gene (**Abb. 5.5 A**) noch die in den Überständen nachweisbare chemoattraktive Aktivität für Granulozyten durch Stx1 verändert (**Abb. 5.6**).

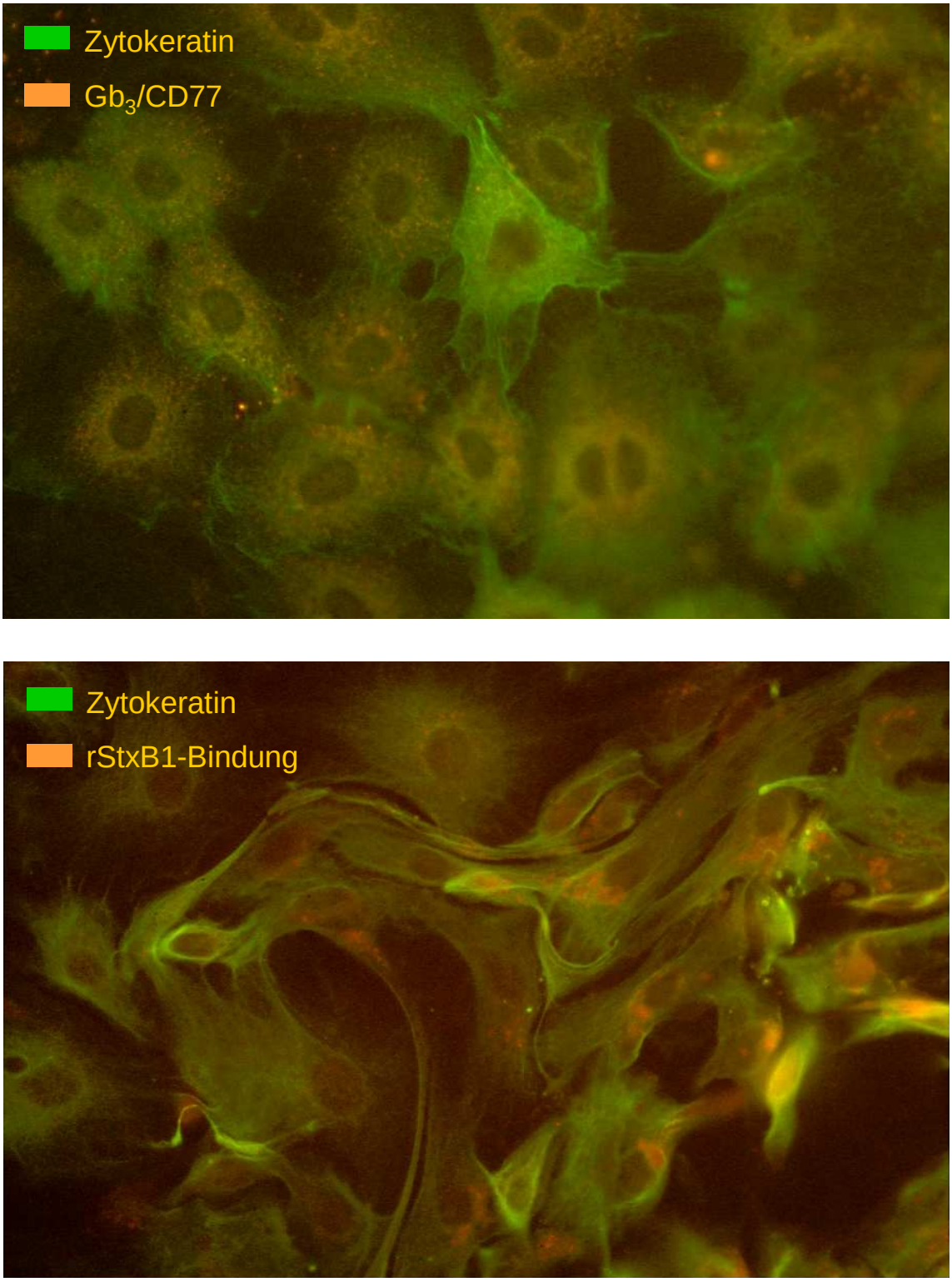

Abb. 5.3: Fluoreszenzmikroskopischer Nachweis von Zellen mit Gb_3/CD77-Antigen bzw. rStxB1-Bindungsstellen in Primärkulturen boviner Kolonzellen.

Vier Tage nach Gewinnung aus Kolonkrypten wurden die Zellen fixiert, permeabilisiert und immundekoriert. Zytokeratin-positive Zellen (grün) sind epithelialen Ursprungs.

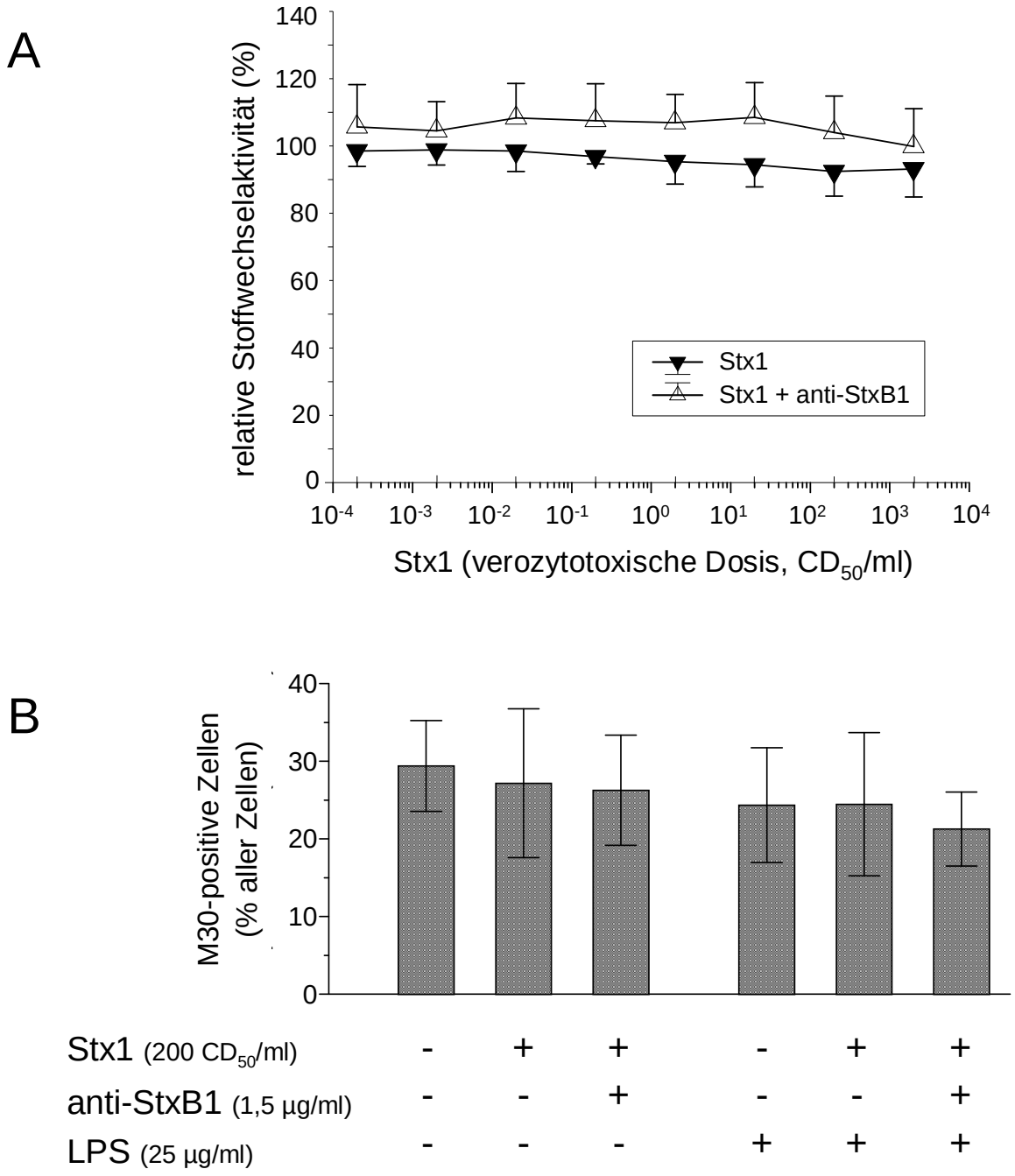

Abb. 5.4: Einfluss von Stx1 auf die Stoffwechselaktivität (A) und den Anteil apoptotischer Zellen (B) in Primärkulturen boviner Kolonepithelzellen.

Dargestellt sind (A) Mittelwerte und Standardabweichungen aus 21 Bestimmungen mit 7 unabhängigen Epithelzellpräparationen (c(anti-StxB1): 1,5 µg/ml) bzw. (B) die Mittelwerte und Standardabweichungen von 6 Bestimmungen mit 3 unabhängigen Zellpräparationen nach jeweils 72stündiger Inkubation. M30 bezeichnet eine Antigen, das nur in apoptotischen Epithelzellen nachweisbar ist.

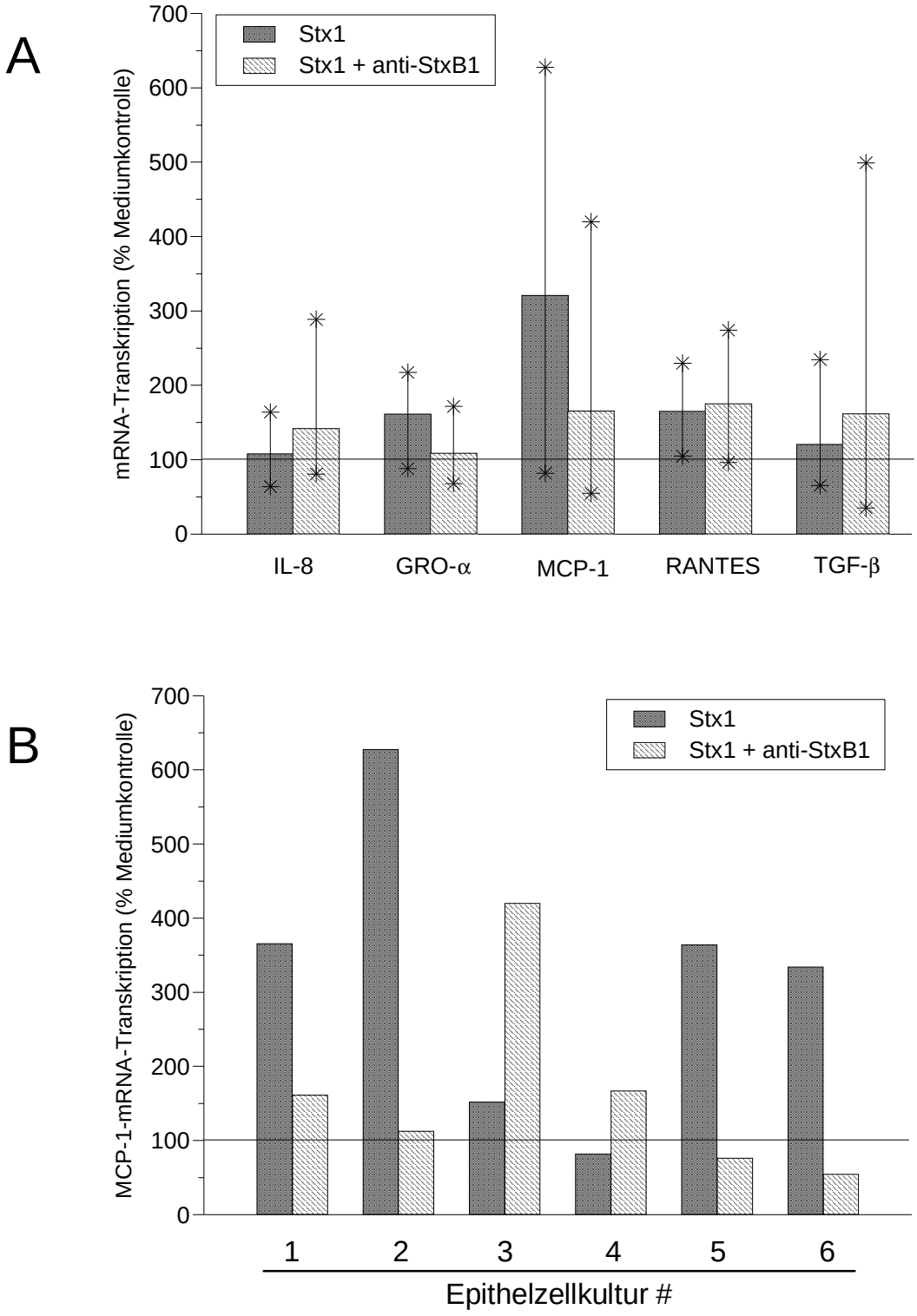

Abb. 5.5: Effekt von Stx1 auf die Transkription ausgewählter Gene in primären bovinen Kolonepithelzellen.

Dargestellt sind Mittelwerte, Minima und Maxima (A) bzw. Einzelwerte (B) der Untersuchung sechs unabhängiger Zellpräparationen nach 4stündiger Inkubation; c(Stx1): 200 CD_{50}/ml, c(anti-StxB1): 1,5 µg/ml.

Interessanterweise erhöhte Stx1 in 4 von 6 untersuchten Kolonepithel-Zellkulturen innerhalb von 4 Stunden die Menge an Transkripten für das mono- und lymphozytotrope MCP-1 (**Abb. 5.5 B**). Dies widerspricht der Vermutung von Hoey *et al.* (111), dass bovine Kolonepithelzellen resistent gegenüber Stx1 sind, da sie das Toxin nach Aufnahme in Lysosomen degradieren würden. Auch humane Makrophagen transportieren Stx nach Gb_3/CD77-vermittelter Endozytose in lysosomale Kompartimente (69) und sind gegenüber der zytoletalen Wirkung des Shigatoxins 1 resistent (335). Die Zellen reagieren aber trotzdem auf Stx1 mit der vermehrten Expression pro-inflammatorischer Zytokine (335, 343). Auch wenn die Beurteilung der Bedeutung für die bovine STEC-Infektion weitere Untersuchungen erfordert, sind dies die ersten Hinweise darauf, dass auch beim Rind intestinale Epithelzellen Zielzellen für Shigatoxine darstellen könnten.

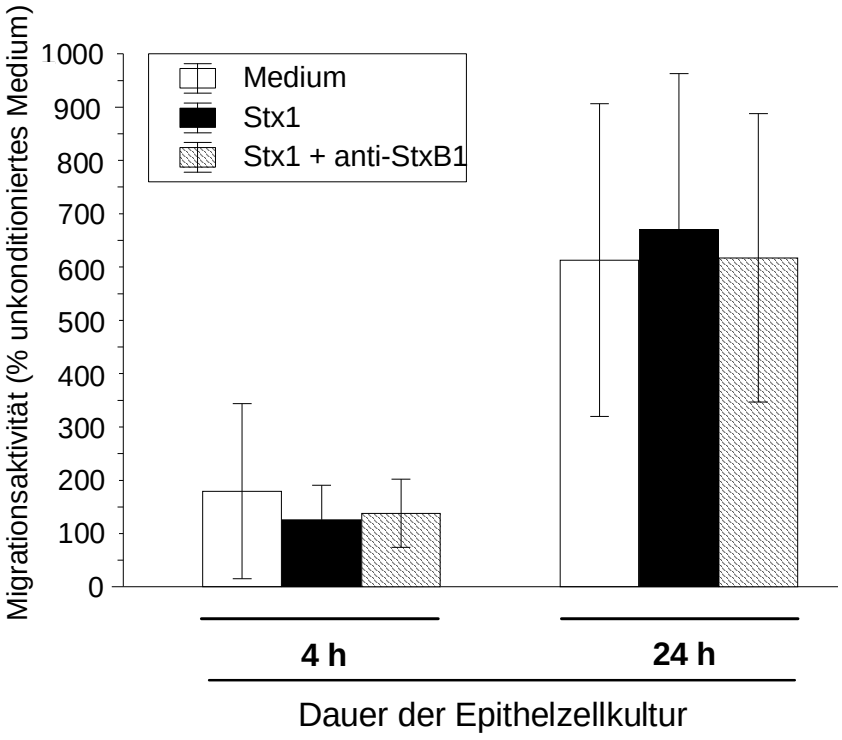

Abb. 5.6: Freisetzung Granulozyten-attraktiver Substanzen durch Stx1-behandelte primäre bovine Kolonepithelzellen.

Dargestellt sind Mittelwerte und Standardabweichungen der Ergebnisse mit konditionierten Epithelzell-Kulturüberständen (jeweils n = 5-6; C); c(Stx1): 200 CD_{50}/ml, c(anti-StxB1): 1,5 µg/ml.

5.3.5 Makrophagen-ähnliche Zellen

In Kulturen aus Kryptenpräparationen kamen auch fibroblastenartige Zellen vor, die sich durch eine starke Oberflächenexpression von CD77 auszeichneten und besonders empfindlich gegenüber Stx1 waren (**Abb. 5.7**). Um sie für funktionelle Untersuchungen in Reinkultur darstellen zu können, wurden sie in Zusammenarbeit mit Dr. Matthias König (Institut für Virologie, Justus-Liebig-Universität Giessen) mit dem Plasmid pSVneo3 transfiziert und immortalisiert (247). Einer der erhaltenen Zellklone exprimierte CD77. Diese Zelllinie entsprach auch insofern den in Primärkulturen beobachteten Zellen, als sie auf die Zugabe von Stx1 zum Kulturmedium mit einer Umverteilung von oberflächlichem CD77 in intrazelluläre Kompartimente reagierte (nicht gezeigt). Die Zellen der Linie wiesen ebenfalls eine fibroblastenartige Morphologie auf. Bei Gewebsfibroblasten handelt es sich jedoch nicht ausschliesslich um Zellen des primären Mesenchyms (245). Diese Zellen können sich auch aus Fibrozyten des Blutes entwickeln, die ihrerseits aus $CD14^+$ Monozyten entstanden sind (1). Die nähere Charakterisierung der Zelllinie ergab, dass sie neben MHC-I auch CD14 und CD172a, nicht jedoch MHC-II, CD11b oder CD11c exprimierte (**Abb. 5.8**). Bovine Monozyten, die aus dem peripheren Blut über Gelatine-Adhärenz angereichert wurden, koexprimierten *in vitro* ebenfalls CD172a und CD77 (nicht gezeigt). Somit könnte es sich bei der etablierten Zelllinie um makrophagen-artige oder dendritische Zellen (DC) handeln.

Bei Stimulation mit LPS reagierte die Zelllinie nicht mit einer vermehrten *il-12*-Transkription wurde aber gegenüber der Wirkung von Stx1 sensibilisiert. Nur bei gleichzeitiger Behandlung der Zellen mit LPS und Stx1 war binnen 4 Stunden eine erhöhte Menge *il-10*-spezifischer mRNA nachweisbar. Diese Wirkung des Shigatoxins 1 hielt für 24 Stunden an (**Abb. 5.9**). Bovine Makrophagen und DCs unterscheiden sich deutlich in der Reaktion auf TLR-Agonisten. Während bovine DCs vermehrt *il-12*-spezifische mRNA bilden, bilden Monozyten vermehrt *il-10*-mRNA (351). Vermutlich geht die etablierte Zelllinie damit auf Gewebsmakrophagen zurück. Entsprechend der Fähigkeiten von Gewebsmakrophagen abhängig vom Aktivierungs- und Differenzierungsgrad verschiedene Chemokine zu sezernieren und damit eine zentrale Rolle in der Steuerung der Leukozyten-migration zu spielen (200) waren auch in den immortalisierten Zellen signifikante Mengen an *gro-α-*, *il-8-*, *mcp-1-* und *rantes*-spezifischen Transkripten nachzuweisen, die durch Stx1 bis zu 70-fach gesteigert wurden. Weitere Untersuchungen müssen nun zeigen, inwieweit eine mögliche Störung des von Gewebsmakrophagen sezernierten „stromal address codes“ (245)

oder der antigen-präsentierenden Funktion dieser Zellen zur Stx-induzierten Immunmodulation beim Rind beiträgt.

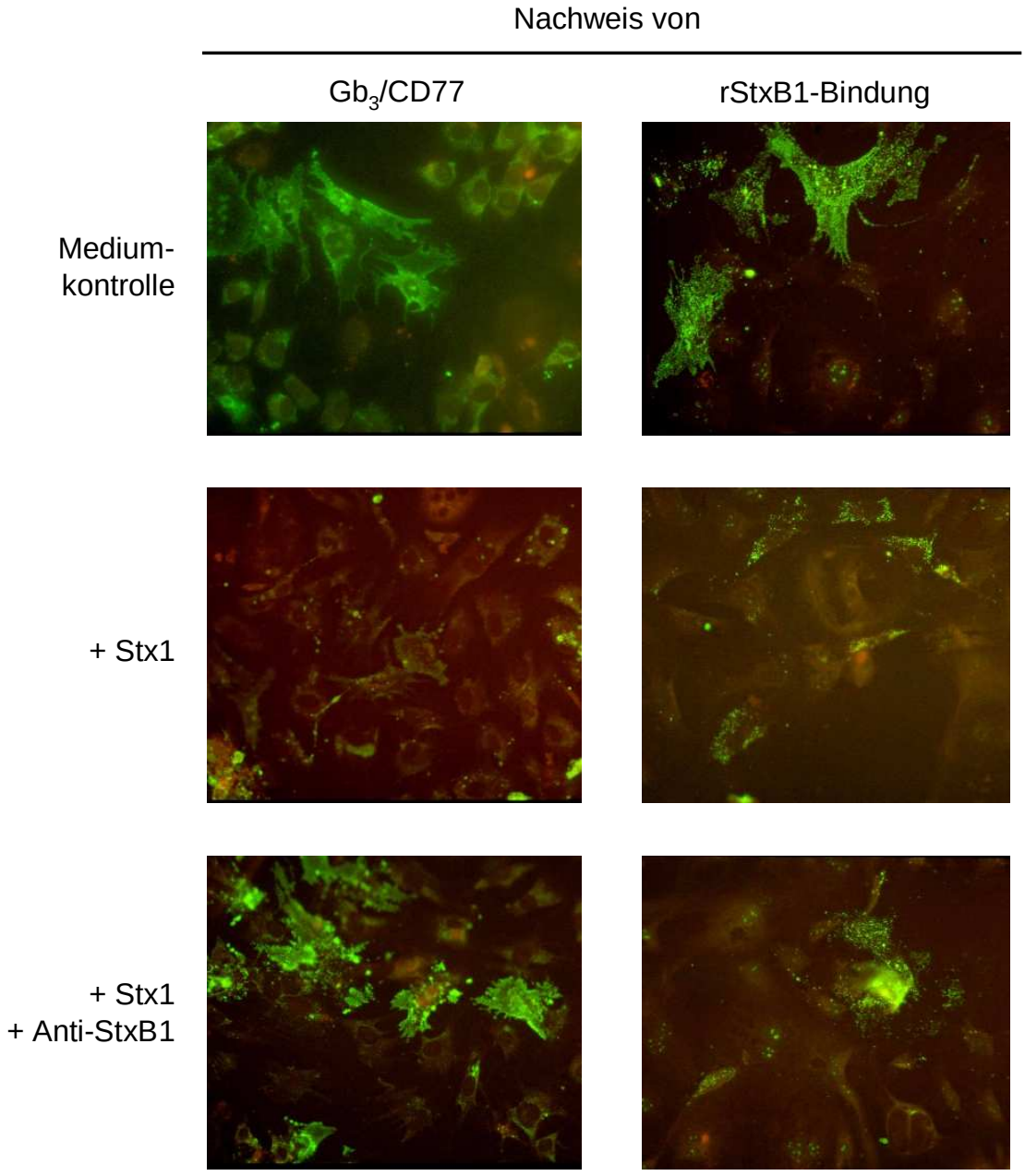

Abb. 5.7: Fluoreszenzmikroskopischer Nachweis von Zellen mit Gb_3/CD77-Antigen bzw. rStxB1-Bindungsstellen in Primärkulturen boviner Kolonzellen nach Inkubation in der Anwesenheit oder Abwesenheit von Stx1.

Vier Tage nach Gewinnung aus Kolonkrypten wurde das Zellkulturmedium mit Stx1 (200fache verozytotoxische Dosis 50 % pro ml) bzw. anti-StxB1 monoklonaler Antikörper 13C4 (1,5 µg/ml) supplementiert. Nach weiteren 72 Stunden Kultivierung wurden die Zellen fixiert, permeabilisiert und immundekoriert.

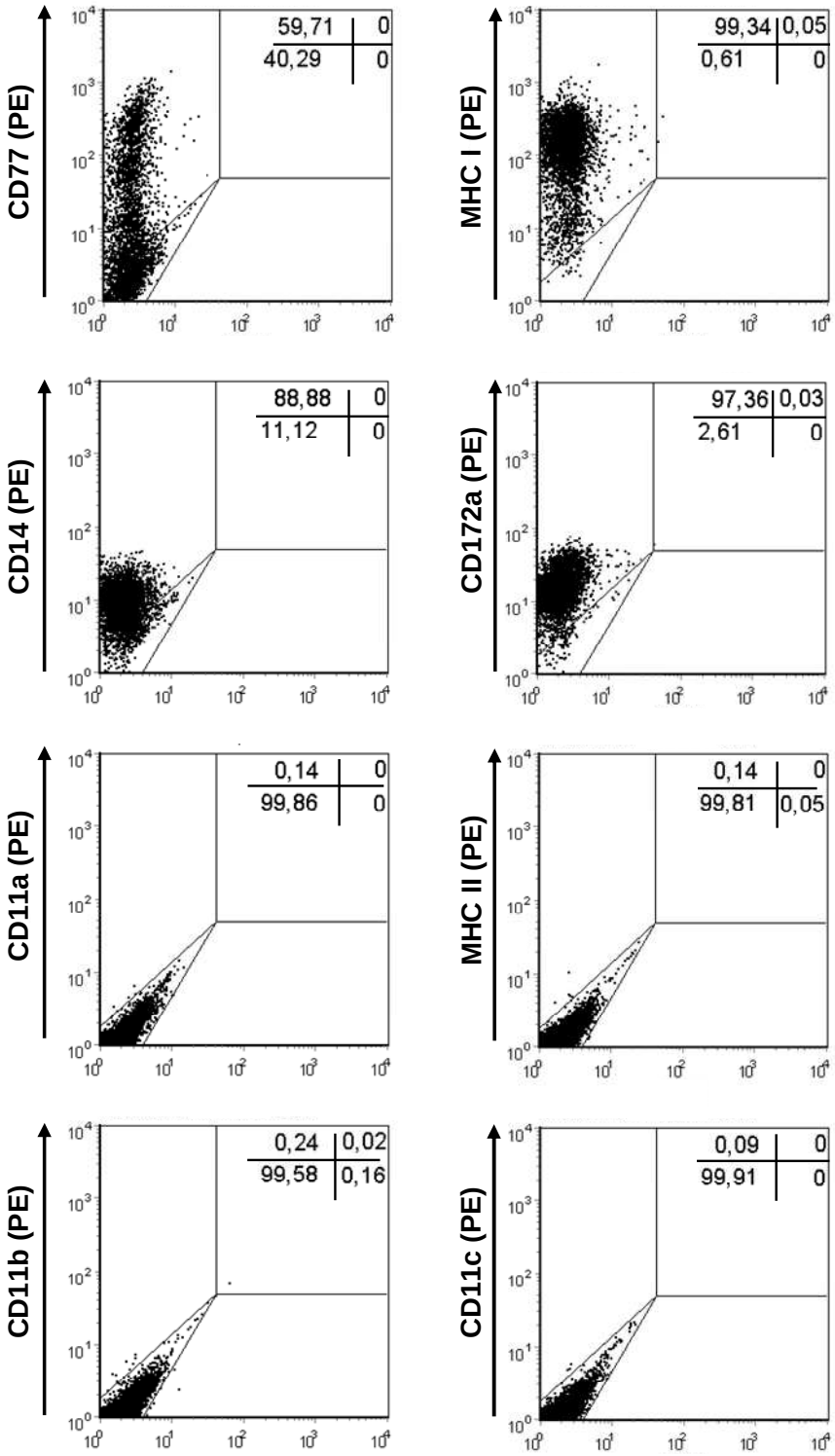

Abb. 5.8: Durchflusszytometrische Analyse der Expression von Oberflächenantigenen bei immortalisierten nicht-epithelialen Zellen aus dem Kolon des Rindes

Repräsentative Ergebnisse aus bis zu 3 unabhängigen Versuchen mit Doppelbestimmungen, Zahlen geben den Anteil der Zellen im jeweiligen Teil der Dot-plot-Darstellung an den vitalen Zellen der Kultur an.

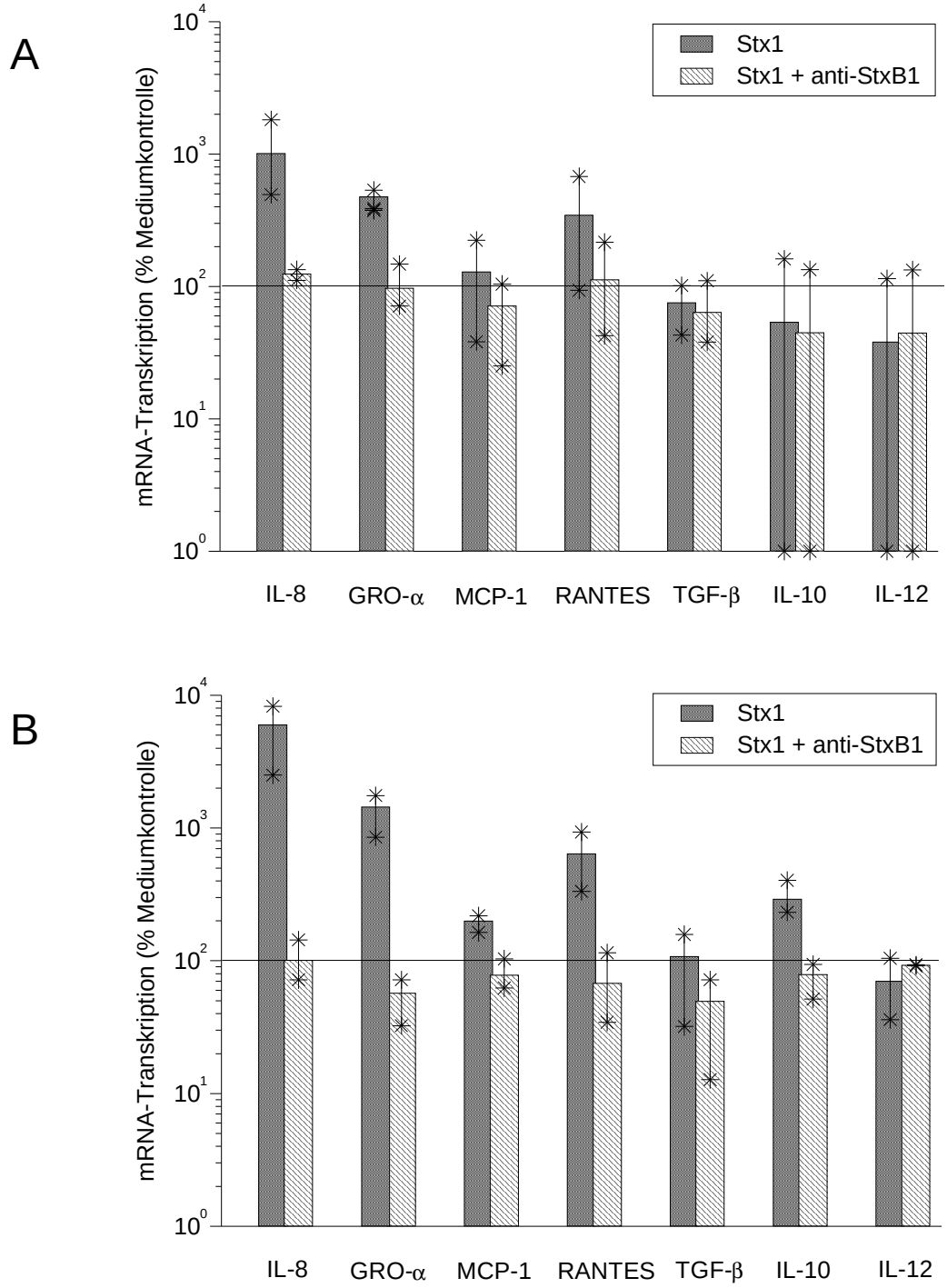

Abb. 5.9: Effekt von Stx1 auf die Transkription ausgewählter Gene bei einer immortalisierten, nichtepithelialen Zelllinie aus dem Kolon des Rindes.

Dargestellt sind die Mittelwerte, Minima und Maxima von drei unabhängigen Experimenten nach 24stündiger Inkubation in Abwesenheit (A) oder in Anwesenheit (B) von LPS; c(Stx1): 200 CD_{50}/ml, c(anti-StxB1): 1,5 µg/ml, c(LPS): 25µg/ml.

5.4 Implementierung eines Tiermodells

Für *in vivo*-Untersuchungen zur Aufklärung der Interaktion zwischen STEC und ihrem Wirt bietet sich zur Reduktion des tierexperimentellen Aufwandes die Verwendung junger Kälber an. Auch unter natürlichen Bedingungen findet die Erstinfektion mit STEC vermutlich bereits im frühen Kälberalter statt (15). Im Gegensatz zu adulten Tieren können sich aber bei neugeborenen Kälbern infolge der Infektion histologische Veränderungen ausbilden (47, 295). Diese Alterunterschiede könnten mit den Besonderheiten des Immunsystems in der Neugeborenenphase in Verbindung stehen. Die Entwicklung und die Reife zellulärer Abwehrfunktionen bei neugeborenen Kälbern war deshalb, zunächst getrennt von der Frage nach der Wirkung der Shigatoxine, Gegenstand erster Untersuchungen.

5.4.1 Phänotypische Charakterisierung peripherer Lymphozyten beim neugeborenen Kalb

Im Blut neugeborener Kälber stehen im Vergleich zu älteren Tieren hohe absolute Granulozytenzahlen leicht reduzierten Lymphozytenzahlen gegenüber (169). Wilson *et al.* (360) stellten auch erhebliche Unterschiede zwischen neugeborenen Kälbern und adulten Tieren bezüglich der Zusammensetzung der T-Zellen fest. Dies konnte durch eigene Untersuchungen, bei denen vergleichbare Anteile der wichtigsten Lymphozytenpopulationen im Blut 1 Stunde nach der Geburt und in der 3. bis 9. Lebenswoche ermittelt wurden, nicht bestätigt werden (siehe **6.2**). Allerdings fanden sich im Blut der Neonaten ca. 70% weniger B-Zellen als bei den älteren Tieren.

Obwohl sich innerhalb der ersten Lebensstunden (1 h post natum (p.n.) vs. 4 h p.n.) weder die absoluten Leukozytenzahlen noch das Differentialblutbild signifikant veränderten, waren innerhalb der mononukleären Zellen Verschiebungen nachweisbar (siehe **6.2**). So nahm unabhängig von der Kolostrumaufnahme der Anteil $CD8\alpha^+$ T-Zellen signifikant ab, während der Anteil an Monozyten entsprechend anstieg.

5.4.2 Entwicklung der Phagozytosekompetenz beim neugeborenen Kalb

Phagozytose durch Granulozyten ist zwar ein erster und unverzichtbarer Abwehrmechanismus gegen bakterielle Erreger, bei neugeborenen Kälbern sind mehrere Schritte dieses vielstufigen Prozesses aber noch funktionell eingeschränkt (374). So können, z.T. bedingt durch die niedrige Immunglobulin-Konzentration (3), die Seren von neugeborenen Kälbern Bakterien nur schlecht opsonieren (185). Selbst opsonierte Partikel werden aufgrund einer reduzierten granulozytären Expression von F_c-Rezeptoren nur schlecht erkannt (374). Zusätzlich zu einer reduzierten Phagozytoseaktivität (169) ist auch die Bildung von Sauerstoffmetaboliten („oxidative burst“) und die Myeloperoxidase-Aktivität eingeschränkt (56, 374). Um diese in Einzeluntersuchungen ermittelten Erkenntnisse im Zusammenhang interpretieren zu können, wurden in den ersten Stunden p.n. phagozytäre Funktionen und die opsonierende Serumaktivität im Vollblut in Abhängigkeit von der Kolostrumaufnahme quantifiziert und mit Werten aus der 3. bis 9. Lebenswoche verglichen. Besonderes Augenmerk wurde dabei auf die separate Analyse von Granulozyten und Monozyten gelegt, da letztere zwar den Übergang von angeborener zu erworbener Immunität repräsentieren, aber ihre Funktionen in diesem Lebensalter bislang nicht untersucht worden waren.

Durch den direkten Vergleich zeigte sich, dass bei neugeborenen Kälbern lediglich bestimmte Komponenten der phagozytären Abwehr eingeschränkt waren. Vermutlich wurden diese Einschränkungen durch die höhere Aktivität anderer Komponenten kompensiert (siehe **6.1**). So liess sich durch Analyse der Phagozytose auf Einzelzellebene nachweisen, dass unter limitierenden Versuchsbedingungen die Phagozytose von *E. coli* durch Granulozyten von Kälbern unmittelbar nach der Geburt zwar verzögert, aber in ihrer Kapazität nicht eingeschränkt war. Die Verzögerung der Phagozytose beruhte vermutlich auch auf humoralen Faktoren, da sie durch Opsonierung der *E. coli* mit dem Plasma adulter Tiere partiell aufgehoben werden konnte. Im deutlichen Gegensatz dazu hatte die Opsonierung der Bakterien keinerlei Einfluss auf die Phagozytose durch Monozyten, die bei den neugeborenen Kälbern sogar eine signifikant höhere Phagozytose-Kapazität aufwiesen. Auch reagierte bei den Neugeborenen ein grösserer Anteil an Monozyten und Granulozyten auf Stimulierung mit der Synthese reaktiver Sauerstoffmetaboliten (ROS). Bereits früher wurde eine verkürzte Reaktionszeit der ROS-Synthese bei den Granulozyten neugeborener Kälber beschrieben (56). Die hier vorgestellten Ergebnisse relativieren jedoch die Vorstellung anderer Autoren, dass, aufgrund einer eingeschränkten Proteinkinase C-Aktivität (55), die ROS-Synthese der Granulozyten in Kälbern dieser Alterstufe generell eingeschränkt ist (185). Während die Gesamtmenge der gebildeten ROS bei Granulozyten-Kulturen von Neugeborenen durchaus

niedriger sein kann als bei denen von älteren Tieren (185), könnte eine erhöhte Zahl reaktiver Zellen (siehe **6.1**), gemeinsam mit der höheren monozytären Phagozytoseleistung, dazu beitragen die reduzierte granulozytäre Phagozytose-Leistung zu kompensieren.

Die Feststellung, dass die Einschränkung der granulozytären Phagozytoseleistung auch auf humoralen Faktoren beruht, liess sich durch die Untersuchung der Phagozytenfunktionen nach der Kolostrumaufnahme bestätigen (siehe **6.1**). Nach der Aufnahme war insbesondere die Phagozytose nicht-opsonierter *E. coli* durch Granulozyten beschleunigt. Ähnliches wurde bereits für die Phagozytose von Hefen bei Lämmern (14) und von *E. coli* bei Kälbern (185) beschrieben. Allerdings fand nach der Kolostrumaufnahme auch die Phagozytose von *E. coli*, die mit homologem Plasma adulter Tiere voropsoniert worden waren, beschleunigt statt. Vermutlich wirkt sich somit das Kolostrum auch innerhalb weniger Stunden bereits direkt auf die zelluläre Aktivität der Phagozyten aus. Die Existenz einer die Phagozytose unterstützenden Substanz im Kolostrum wurde bereits von mehreren Autoren postuliert, jedoch ist ihre chemische Natur noch unbekannt (185). So enthält humanes Kolostrum zwar Konzentrationen an TNF-α, die geeignet sind das Immunsystem der Neugeborenen zu beeinflussen (317), bei bovinen Granulozyten wird die Phagozytose von *Staphylococcus aureus* durch TNF-α jedoch nicht verstärkt (34).

Der Vorstellung von einer generellen Einschränkung phagozytärer Funktionen bei neugeborenen Kälbern muss somit widersprochen werden. Da regelmässig im Blut von Tieren dieses Alters sehr hohe relative und absolute Zahlen an Granulozyten gefunden werden (siehe **6.1**) (169), ist die gesamte Kapazität zur Elimination von Mikroorganismen vermutlich sogar um ein Vielfaches höher als bei älteren Kälbern und adulten Tieren.

5.4.3 Untersuchungen zur Immunmodulation durch STEC *in situ* im Darmligaturtest bei Kälbern

Nachdem die Wirkung von Stx1 auf Primärkulturen verschiedener boviner Zellen *in vitro* detailliert untersucht worden war, wurden zur Abschätzung der Bedeutung der erhobenen Befunde *in vivo* zwei tierexperimentelle Ansätze verfolgt (siehe auch **5.4.4**). In einem ersten Ansatz wurden Studien in separierten Darmschlingen durchgeführt, in denen STEC das Epithel unter Ausbildung von „attaching and effacing"-Läsionen kolonisieren (281, 313). Die Eignung solcher Darmschlingen für die Bearbeitung immunologischer Fragestellungen wurde unlängst von Gerdts *et al.* bewiesen (84). Bei den im Folgenden beschriebenen Untersuchungen sollte festgestellt werden, ob mit den zur Verfügung stehenden Methoden die

immunmodulierende Wirkung des Shigatoxins 1 auch in der komplexen Umgebung der bovinen Darmschleimhaut nachgewiesen werden kann.

5.4.3.1 Vorversuche mit oral infizierten Kälbern zur Auswahl eines geeigneten Darmabschnittes

Aufgrund anatomischer Widrigkeiten und der Notwendigkeit ausreichend vitale IEL für funktionelle Untersuchungen aus dem Epithel gewinnen zu müssen bot es sich an die Darmschlingen im Bereich des Ileums zu erzeugen. STEC des Serotyps O157:H7 scheinen zwar bei ruminierenden Rindern einen Tropismus für das Epithel über den Lymphfollikeln im terminalen Rektum zu besitzen (228), bei Kälbern kolonisieren *E. coli* O157:H7 aber durchaus auch Ileum, Zäkum und Kolon (23, 44, 47, 48, 91). Non-O157 STEC fehlt sogar der Tropismus für das terminale Rektum (228) und STEC der Serotypen O5 und O111 kolonisieren in grosser Zahl das Kolonepithel (316). Um Unterschiede zwischen IEL aus dem Ileum und dem Kolon besser abschätzen zu können, wurden in einer ersten Versuchsreihe 2 Kälber (20 bzw. 22 Tage alt) oral mit ca. 4×10^{10} Kolonie-bildenden Einheiten des STEC-Stammes PMK5 (STEC-Feldisolat des Serotyps O103:H2, *eae*-Subtyp ε, *stx1*$^{+}$; (201)) bzw. des apathogenen *E. coli* Stammes NADC5738 (Nalidixinsäure-resistente Mutante des porcinen, apathogenen *E. coli* O43:H28 Stammes 123 (47, 215)) inokuliert. Drei Tage später wurden die IEL gewonnen (siehe **6.8**).

Die Zusammensetzung der IEL im Ileum und Kolon der infizierten Tiere war insgesamt sehr ähnlich, obwohl die Präparationen aus dem Ileum höhere Anteile an T-Zellen und Zellen mit MHC-II- und ACT-2-Expression aufwiesen (siehe **6.8**). CD77 war auf der Oberfläche von ca. 50 bzw. 35 % der IEL aus dem Ileum bzw. dem Kolon nachweisbar; ca. 35 % der IEL aus Ileum und Kolon waren in der Lage rStxB1 zu binden. Die Mehrzahl der IEL beider Kälber befand sich *in situ* in der G0/G1-Phase des Zellzyklus´ und auch eine mitogene Stimulation *in vitro* konnte keine Proliferation induzieren. Trotzdem übten die Zellen eine nachweisbare“Natürliche Killerzell-Aktivität” gegenüber einer homologen, nicht MHC-gepaarten Zelllinie aus. Der Nachweis dieser Aktivität gelang nur bei hohen Effektor- zu Zielzell-Verhältnissen. Die Aktivität war in IEL-Präparationen aus dem Kolon geringfügig höher.

5.4.3.2 Einfluss der STEC-Infektion auf die Zusammensetzung der IEL

In der eigentlichen Versuchsreihe wurden bei 5 konventionell gehaltenen Kälbern (9 bis 14 Tage alt) im mittleren Ileum jeweils 4 Darmschlingen abgebunden und entweder mit dem Stamm PMK5, einer isogenen Δ*stx1*-Mutante des PMK5 (313), dem Stamm NADC5738 oder sterilem Bakterienmedium als Kontrolle inokuliert (siehe **6.8**). Zwölf Stunden nach der Inokulation präparierte IEL waren ähnlich zusammengesetzt wie die IEL, die aus dem Ileum oral inokulierter Kälber gewonnen wurden, und bestanden überwiegend aus aktivierten $CD2^+CD3^+CD6^+ACT\text{-}2^+$ T-Zellen. Etwa 40 % aller IEL exprimierten CD77 bzw. banden rStxB1. Im Vergleich zu den Kontrollschlingen führte die Inokulation mit dem STEC1-Stamm PMK5 zu einer signifikanten Verminderung des Anteils $CD8\alpha^+$ T-Zellen um 5.52 ± 3.4 % ($P \leq 0.05$). Diese *in vivo*-Befunde korrelieren mit der *ex vivo* bestimmten differentiellen Expression des Stx-Rezeptors insbesondere durch aktivierte $CD3^+CD6^+CD8\alpha^+$ T-Zellen (siehe **5.3.3**). Die Beobachtung, dass die Inokulation der Darmschlingen die Zahl derjenigen IEL, die rStxB1 binden können, geringgradig reduziert, nicht aber die Zahl der Zellen, die von anti-CD77 erkannt werden, könnte darauf hindeuten, dass bei bovinen Lymphozyten auch *in vivo* zwischen $CD77^+$-Lymphozyten einerseits und Stx-Rezeptor-positiven und dadurch Stx-sensiblen Lymphozyten andererseits unterschieden werden muss.

5.4.3.3 Proliferation der IEL nach STEC-Exposition *in situ*

Ca. 90 % der aus Darmschlingen gewonnenen IEL befand sich in der G0/G1-Phase des Zellzyklus´ (siehe **6.8**). Dennoch sprach ein kleiner Teil der Zellen auf mitogene Stimulation mit einer Transformation zu Blasten an. Allerdings unterschied sich die Stimulierbarkeit der Zellen aus STEC1-inokulierten Darmschlingen nicht von der Stimulierbarkeit der Zellen aus Kontrollschlingen. Es ist nicht auszuschliessen, dass Stx1 zu einer Elimination sensitiver IEL aus der Mukosa STEC1-inokulierter Darmschlingen durch die Induktion von Zelltod geführt hat. Jedoch konnte bislang keine zytoletale Wirkung des Shigatoxins 1 auf bovine Lymphozyten nachgewiesen werden (71, 207). Die nach Inokulation mit dem STEC1-Stamm aufgetretenen Veränderungen in der IEL-Zusammensetzung *in vivo* müssen demnach entweder auf einer Störung der Rekrutierung mukosaler Immunzellen beruhen oder, ähnlich wie *in vitro* (siehe **5.2.1** und **5.3.3**), auf einer Hemmung der Lymphozyten-Proliferation *in situ*. In beiden Fällen könnte sowohl eine Störung des „stromal address code“ der lokalen Gewebsmakrophagen (siehe **5.3.5**) als auch von den IEL selbst gebildete lösliche Faktoren

eine Rolle gespielt haben (9). So induziert vor allem TGF-β die Expression von αEβ7-Integrin und trägt entscheidend zum „homing“ und zur Retention der IEL im Epithel bei (323). In der Tat waren auch in bovinen IEL *ex vivo tgf-β*-Transkripte sowie TGF-β selbst nachweisbar (siehe **5.3.3.5**). Allerdings wurde *in vitro* weder die Zahl der Transkripte noch die Menge des Proteins durch Stx1 messbar verändert. Inwieweit die Induktion der *il-4*-Transkription in IEL durch Stx1 (siehe **5.3.3.5**) in diesem Zusammenhang eine Bedeutung besitzt, muss in weiteren Untersuchungen geklärt werden.

5.4.3.4 Zytokin-mRNA-Gehalt und „Natürliche Killerzell-Aktivität“ von IEL nach STEC-Exposition *in situ*

Mehrere Virulenzfaktoren der STEC, wie Lymphostatin (158, 196) und Stx1 (siehe **5.3.3.5**), können *in vitro* die Synthese von Zytokinen durch mitogen-stimulierte mukosale Lymphozyten beeinflussen. Dennoch unterschied sich die Expression einer Reihe von Zytokinen in *in vitro*-restimulierten IEL aus STEC1-inokulierten Darmschlingen nicht von der der IEL aus Vergleichsschlingen (siehe **6.8**). Der Darmligaturtest hat seine Eignung für Untersuchungen zur immunmodulierenden Wirkung des Shigatoxins 1 prinzipiell unter Beweis gestellt. Für Untersuchungen auf Ebene der Zytokingenexpression bedarf es jedoch der Modifikation der Methoden. So lässt sich vermuten, dass die verwendete semiquantitative Transkriptionsanalyse durch die Vielfalt der Signale zwischen Mukosazellen an die Grenze ihres Auflösungsvermögens stiess. Dies ist insbesondere deshalb anzunehmen, weil unterschiedliche Zellen der bovinen Mukosa unterschiedlich auf Stx1 reagieren.

IEL-Präparationen aus allen Darmschlingen übten eine stärkere “Natürliche Killerzell-Aktivität” aus als IEL der oral inokulierten Kälber in den Vorversuchen (siehe **6.8**). Die Aktivität unterschied sich nicht zwischen IEL aus STEC1-inokulierten Schlingen und Kontrollschlingen. Da Stx1 auch *in vitro* die Fähigkeit boviner IEL zur MHC-unabhängigen Zytotoxizität nicht vermindert (siehe **5.3.3.3**), scheint einer Modulation dieser Funktion bei der dauerhaften Kolonisation der STEC im Darm des Rindes keine Bedeutung zuzukommen.

5.4.4 Immunantwort von Kälbern nach Inokulation mit Stx2-bildenden *E. coli* O157:H7

Die beschriebenen *in vitro*-Befunde führten zur Formulierung der Hypothese, dass Shigatoxine von den *E. coli* vor allem dazu benutzt werden die Entwicklung einer im Entstehen begriffenen adaptiven zellulären Immunantwort zu verzögern (siehe **5.2.1**). Antikörper gegen O157, Stx1 und Stx2 können häufig im Serum und auf den Schleimhäuten (im Kolostrum) von adulten Rindern nachgewiesen werden (137, 255). Der zeitliche Verlauf dieser humoralen Immunantwort nach der STEC-Erstinfektion war jedoch nicht bekannt. Deshalb wurde in Infektionsversuchen die Entwicklung der STEC-spezifischen Immunität bei Kälbern auf humoraler und zellulärer Ebene untersucht.

Dazu wurden 3 Gruppen mit jeweils 5 Kälbern (6 bis 8 Wochen alt) zweimal im Abstand von 3 Wochen mit 10^{10} Kolonie-bildenden Einheiten verschiedener *E. coli*-Stämme oral inokuliert (siehe **6.11**). Eine Gruppe erhielt den Stx2-bildenden *E. coli* O157:H7-Stamm 86-24, die Streptomycin-resistente Mutante eines humanpathogenen EHEC-Isolates (331). Die zweite Gruppe erhielt den *E. coli* O157:H7-Stamm 87-23, eine Nalidixin-resistente *stx*-negative Mutante eines Isolates aus dem gleichen Ausbruch, aus dem Stamm 86-24 isoliert wurde (142, 331), die dritte Gruppe den apathogenen *E. coli* Stamm NADC5738. Drei Wochen nach der zweiten Inokulation wurden die Tiere aller Gruppen mit dem STEC2-Stamm 86-24 belastet.

5.4.4.1 Entwicklung einer STEC-spezischen, zellulären Immunantwort

Eine antigen-spezifische Proliferation peripherer Lymphozyten nach Restimulation mit hitze-inaktiviertem O157:H7-Vollantigen war nur bei Kälbern nachweisbar, die initial mit dem toxin-negativen *E. coli* O157:H7 inokuliert worden waren (siehe **6.11**). Dagegen blieb bei Kälbern, die von Anbeginn den STEC2-Stamm erhielten, die Entwicklung einer zellulären Immunantwort fast vollständig aus. Im Gegensatz zu Schweinen, bei denen nach experimenteller oraler Infektion mit einem Stx1-bildenden *E. coli* O111:NM-Stamm eine generalisierte Immunsuppression auftritt, die auch eine herabgesetzte mitogene Stimulierbarkeit umfasst (36), blieb bei den STEC2-inokulierten Kälbern die mitogene Stimulierbarkeit der PBMC vollständig erhalten. Darüberhinaus entwickelten die Kälber, die mit dem toxin-negativen *E. coli* O157:H7 inokuliert worden waren, eine kontinuierlich ansteigende Reaktivität für das O157:H7-Vollantigen. Vergleichbar mit einer „booster"-Reaktion stieg die Reaktivität sogar noch weiter an, nachdem die Tiere abschliessend mit dem

Stx2-bildenden Stamm belastet worden waren. Bei den verwendeten *E. coli* O157:H7-Stämmen handelt es sich, im Gegensatz zu den in den Darmschlingen-Experimenten verwendeten Stämmen, nicht um isogene Mutanten desselben Stammes. Da Stx2 *in vitro* eine dem Stx1 vergleichbare Wirkung auf bovine Lymphozyten besitzt (C. Menge, E.A. Dean-Nystrom, unveröffentlicht), könnte die Verzögerung der Entwicklung einer STEC-spezifischen Immunantwort nach Inokulation mit einem Stx2-bildenden *E. coli* O157:H7 aber auf das Stx2 selbst zurückzuführen sein. So könnte Stx2 *in vivo* die Entwicklung einer zellulären Immunantwort verhindert haben, wenn es bereits bei den ersten beiden Inokulationen mit dem STEC2-Stamm auf das Immunsystem einwirken konnte. Entwickelten die Tiere aber bereits durch die Inokulationen mit dem *stx*-negativen Stamm eine O157:H7-spezifische Immunantwort, war bei der abschliessenden Belastung das Toxin wirkungslos. Diese Erklärung steht im Einklang mit der Beobachtung *in vitro*, dass bovine Lymphozyten Stx-Rezeptoren nur in einer frühen Phase der Aktivierung exprimieren, später aber gegenüber der Wirkung der Shigatoxine wieder resistent werden (siehe **5.2.1**). Möglicherweise verhinderte das Stx2 jedes Mal nach der Aktivierung STEC-spezifischer Lymphozyten *in vivo* durch Inokulation der Kälber mit dem STEC2-Stamm die weitere Aktivierung und Proliferation der Zellen. In der Folge blieb die Zahl spezifischer Lymphozyten im peripheren Blut unterhalb der Nachweisgrenze des Proliferationstests, während die Zahl der polyklonal durch Mitogene stimulierbaren Zellen unverändert blieb. Bei den zuerst mit dem *stx*-negativen *E. coli* O157:H7 inokulierten Kälbern wurden spezifische Immunzellen dagegen bis zu dem Grad aktiviert, bei dem sie resistent für Stx2 sind. O157:H7-spezifische, aber Stx-resistente Immunzellen waren somit in grosser Zahl vorhanden, wenn die Kälber schliesslich mit dem STEC2-Stamm belastet wurden.

5.4.4.2 Antikörper gegen Stx2 und O157-LPS

Die Kälber aller Gruppen entwickelten im Laufe des Versuches eine humorale Immunantwort gegen *E. coli* O157-LPS. Wie schon in früheren Untersuchungen anderer Arbeitsgruppen (137) waren jedoch Antikörper gegen Stx2 in den Kälbern zu keinem Zeitpunkt nach den Inokulationen nachweisbar (siehe **6.11**). Das Fehlen einer Serokonversion bezüglich Stx2 lässt sich nicht durch eine Schädigung antigen-spezifischer B-Zellen durch Stx2 erklären. Bovine B-Zellen sind zwar *in vitro* ähnlich Stx-sensibel wie $CD8\alpha^+$ T-Zellen (207), jedoch beeinträchtigte Stx2 bei den Kälbern, die von Anbeginn mit dem STEC2-Stamm inokuliert wurden, die Entwicklung der humoralen Antwort gegen O157-LPS *in vivo*

offensichtlich nicht. Eine Diskrepanz zwischen anti-Stx-Titern und anti-O157-LPS-Titern wurde auch schon von Johnson *et al.* beschrieben (137). Das B-Zell-Kompartment des bovinen Immunsystems ist somit möglicherweise *in vivo* weniger sensibel für die Wirkung der Shigatoxine als das T-Zell-Kompartment. Dies könnte vor allem dann der Fall sein, wenn B-Zellen direkt durch ein T-Zell-unabhängiges B-Zell-Antigen wie LPS aktiviert werden.

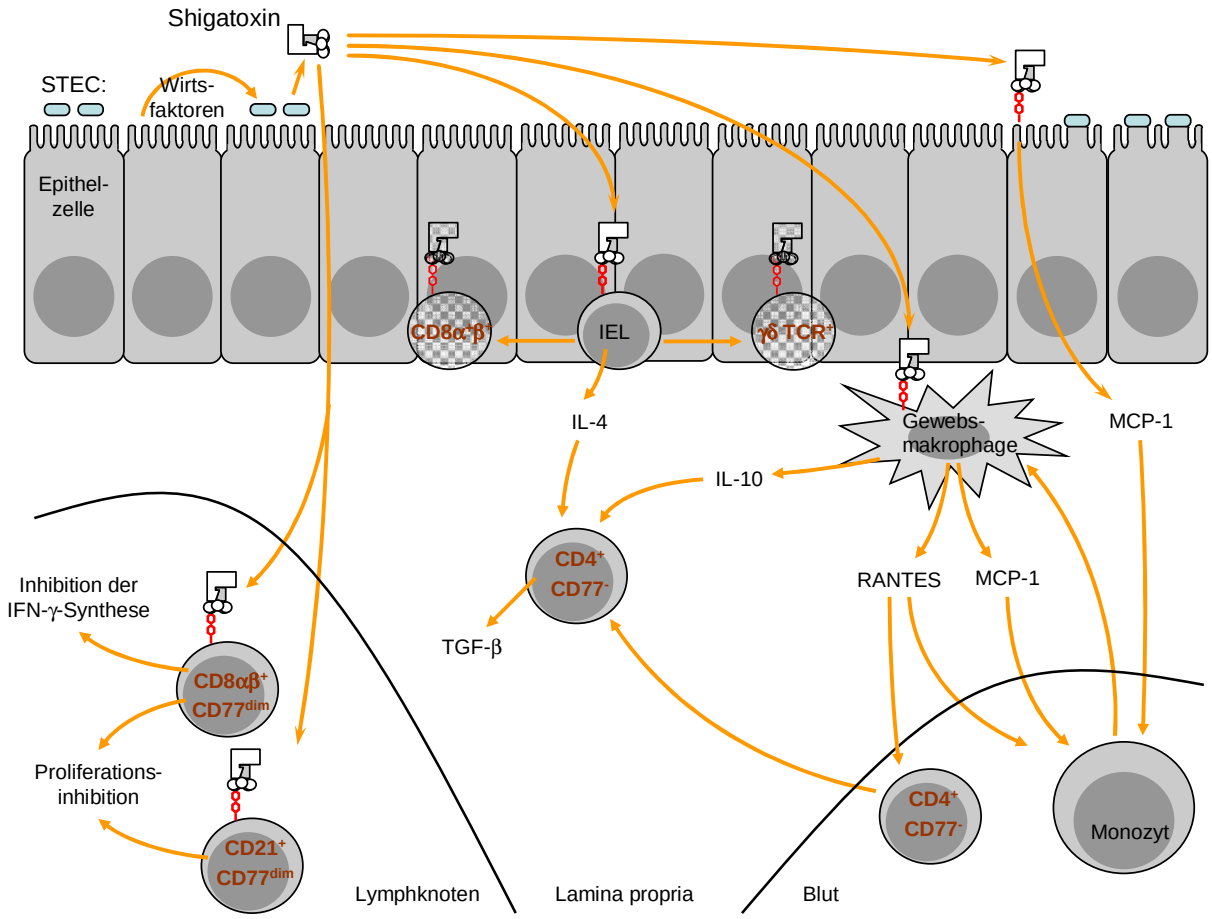

Abb. 5.10: Hypothetisches Modell für die Verzögerung einer antigen-spezifischen Immunantwort durch die Wirkung von Shigatoxinen auf verschiedene Zielzellen im Darm des Rindes

5.5 Schlussfolgerungen

Ein Tiermodell für die durch Shigatoxine ausgelösten, z.T. mit schwerwiegenden Organschädigungen einhergehenden Erkrankungen des Menschen ist nicht bekannt. Die bisherigen Modelle waren lediglich in der Lage Teilaspekte der Pathogenese von HC oder HUS zu reproduzieren. Der wesentliche Grund hierfür dürfte einerseits in den Unterschieden der Verteilung von Stx-Rezeptoren auf Zellen und in Geweben zu suchen sein, aber auch in unterschiedlichen Reaktionen einzelner, den Rezeptor-tragenden Zellarten auf die Toxine. Auch das Rind, das trotz persistenter Kolonisierung durch die STEC keine den Shigatoxinen zuzuschreibenden histopathologisch oder klinisch erkennbaren Krankheitserscheinungen ausbildet, weist eine charakteristische Verteilung von Stx-Rezeptoren auf (**Tabelle 2.2**). Die hier beschriebenen Untersuchungen konnten für alle, zumeist erstmals identifizierten, Stx-Rezeptor exprimierenden Zellen des Rindes nachweisen, dass die Zellen auch in der Lage sind auf Stx zu reagieren. Im Gegensatz zu humanen Zellen war diese Wirkung jedoch nicht zytoletaler, sondern ausschliesslich modulierender Art. Dadurch wird unterstrichen, dass es nicht möglich ist zwischen *per se* Stx-empfindlichen oder Stx-resistenten Spezies zu unterscheiden. Da sich Hinweise für eine Wirkung auch *in vivo* finden, muss die Shigatoxin-Bildung auch bei Infektionen des Rindes als Pathogenitätsmerkmal der STEC angesehen werden. Die spezielle Rezeptorverteilung beim Rind könnte nicht nur erklären, warum die Infektion adulter Rinder klinisch inapparent verläuft, sondern auch bedeuten, dass die Akquisition Stx-konvertierender Phagen durch bestimmte *E. coli*-Klone einen Teil der Adaptation dieser Keime an ihren Reservoirwirt darstellt und die Sekretion der Shigatoxine der Persistenz der Infektion Vorschub leistet.

Die Entdeckung von te Loo *et al.* (332), dass humane Granulozyten die Shigatoxine nicht nur über einen niedrig affinen, von Gb_3/CD77 abweichenden Rezeptor binden, sondern an Gb_3/CD77-exprimierende Endothelzellen auch weitergeben können, könnte erklären, wie die strikt enteralen STEC-Infektionen des Menschen zu extraintestinalen Komplikationen führen. Bovine Granulozyten, denen jeglicher Stx-Rezeptor fehlt, sind dagegen nicht in der Lage die Toxine zu transportieren. Rinder könnten dadurch gegen die extraintestinalen Wirkungen der Shigatoxine gefeit sein. Andererseits ist vorstellbar, dass die CD77-positiven Granulozyten des Schafes Stx mit so hoher Affinität binden, dass sie einmal gebundenes Toxin nicht an sensitive Zellen weitergeben. Vorläufige Ergebnisse unserer Arbeitsgruppe stützen diese Vermutungen (60). Jedoch binden bei Schweinen, die nach STEC-Infektion HUS-ähnliche Nierenveränderungen entwickeln können (257), die Granulozyten Stx ebenfalls über

Gb_3/CD77-artige Rezeptoren (361). Die Bedeutung einer Interaktionen zwischen Stx und Granulozyten für den Verlauf der STEC-Infektion kann deshalb noch nicht abschliessend beurteilt werden. Die gravierenden Unterschiede zwischen Granulozyten von Rindern und Schafen sind jedoch der erste Hinweis für eine mögliche Heterogenität der Stx-Wirkungen im Rahmen von Infektionen bei verschiedenen Spezies, die gleichermaßen als asymptomatische Träger der STEC angesehen werden.

Wie bei den entsprechenden Endothelzellen des Menschen lässt sich auf bovinen Endothelzellen aus Nabelschnurvenen CD77 nachweisen (I. Stamm, C. Menge, unveröffentlicht) und bovine Aorta-Endothelzellen sind Stx-sensitiv (17). Jedoch fehlen im Gegensatz zum Menschen beim Rind Stx-Rezeptoren auf den Endothelzellen in der Mikrovaskulatur des Darmes (260). Hierdurch könnte sich das Fehlen mikrovaskulärer Veränderungen in diesem Bereich des Gefässsystems des Rindes erklären.

Das wichtigste Zielorgan für Shigatoxine ist beim Rind offensichtlich der adaptive Teil des mukosalen Immunsystems, dessen Effektormechanismen durch die Shigatoxine inhibiert werden (**Abb. 5.10**). Mit mukosalen Gewebsmakrophagen wurde im Darm des Rindes eine wichtige Zielzelle für Stx identifiziert, die als Multiplikator der Stx-Wirkung fungieren könnte. Diese Zellen kommen im Kryptenbereich vor, könnten über die Induktion von MCP-1 in Epithelzellen aber auch unterhalb der Kolonisationsstelle der STEC an der Kryptenbasis angelockt werden. Die Expression von CD77 auf diesen Zellen und auf bovinen Monozyten könnte sogar bedeuten, dass Stx die Entwicklung einer Immunantwort bereits auf der Ebene der antigen-spezifischen Aktivierung von T-Zellen stört. Diese Aktivierung würde weiterhin durch eine gesteigerte IL-10-Expression in Gewebsmakrophagen behindert werden. Auch eine vermehrte Sekretion von IL-4 durch IEL würde die Differenzierung von $CD4^+$ T-Zellen der *Lamina propria* zu TGF-β-sezernierenden T_H3-Zellen verstärken und einer durch die STEC-Infektion induzierten intestinalen Entzündung entgegenwirken (121, 209). Auch wenn die Schleimhaut über den Lymphfollikeln im terminalen Rektum der wichtigste Kolonisierungsort für STEC des Serotyps O157:H7 im Rind sein soll (228, 229), können *E. coli* O157:H7, vor allem in den ersten Tagen nach der Infektion, auch die Schleimhaut von Ileum, Zäkum und Kolon kolonisieren (91). Darüberhinaus besitzen non-O157 STEC offensichtlich keinen Tropismus für das terminale Rektum (228, 315). Die starke Ähnlichkeit in der Expression von Oberflächenmarkern einschliesslich CD77 zwischen IEL aus dem Ileum, Kolon und Zäkum (C. Menge, unveröffentlicht) deutet darauf hin, dass IEL im gesamten Magen-Darm-Trakt des Rindes für Shigatoxine empfindlich sind.

Schliesslich werden auch die peripheren Lymphozyten bereits in der Frühphase der Aktivierung, in der sie noch wenige Stx-Rezeptoren ausbilden, von Stx in der weiteren Aktivierung und Proliferation, aber auch in der Synthese von IFN-γ (C. Menge, unveröffentlicht) behindert. Dadurch kann Stx zwar langfristig nicht die Entstehung einer spezifischen Immunität verhindern, aber diese mittelfristig verzögern. Dies wäre aus Sicht der Erreger sehr effizient, da zumindest bei der Entwicklung einer immunologischen Primärantwort geringe Toxinmengen ausreichen würden, um die dann noch wenigen spezifischen Immunzellen zu inhibieren. Auch wenn bislang der Beweis für einen direkten Zusammenhang zwischen einer Immunsuppression und der verlängerten Kolonisierung der Darmschleimhaut des Rindes durch STEC fehlt, wurde die um mehrere Wochen verzögerte Entwicklung einer STEC-spezifischen zellulären Immunantwort bei 9 bis 11 Wochen alten Kälbern (siehe **5.4.4**) von einer signifikant längeren Auscheidung des STEC2-Stammes begleitet. Da z.B.CD45RO$^+$-IEL zu einem hohen Prozentsatz CD77 koexprimieren (C. Menge, unveröffentlicht), könnte sich die Wirkung der Shigatoxine auch auf die (Re)Aktivierung einer immunologischen Gedächtnisreaktion erstrecken. Unlängst wurde über die reduzierte Ausscheidung von STEC des Serotyps O157:H7 durch Rinder nach Vakzinierung mit Proteinen des *lee* berichtet (258). Eine verzögerte Reaktivierung der Impfreaktion nach STEC-Infektion würde die Dauer der Protektivität solcher Vakzinen jedoch auf den Zeitraum beschränken, in dem sich die Effektormechanismen nach der Vakzinierung noch im Zustand hoher Aktivierung befinden.

Die Prävalenz von EHEC-Serovaren (z.B. O157:H7, O26:H11) ist zwar bei Rindern deutlich niedriger als die Prävalenz anderer STEC-Serovare (38). Da die Stx-Gene als Bestandteil von Prophagengenomen einer häufigen Rekombination unterliegen (292), wird aber vermutet, dass sich im Intestinaltrakt der Rinder häufig neue STEC-Stämme bilden, die für den Menschen gefährlich werden können. Deshalb muss die Entwicklung einer erfolgreichen Strategie zur Prävention von EHEC-Infektionen des Menschen auf die Bekämpfung aller STEC-Stämme im Rind abzielen. Die *stx*-Gene sind dabei nicht nur das einzige allen STEC-Stämmen gemeinsame Merkmal, sondern die hier beschriebenen Untersuchungen haben deutlich gemacht, dass Shigatoxine als Immunmodulatoren auch im Rind eine Bedeutung als Pathogenitätsmerkmal besitzen. Aus diesen Gründen bietet sich die Verwendung der Shigatoxine selbst als Zielstruktur für die Entwicklung einer Bekämpfungsstrategie gegen STEC beim Rind an.

6. VORGELEGTE VERÖFFENTLICHUNGEN

* korrespondierender Autor

6.1 “Compensation of preliminary blood phagocyte immaturity in the newborn calf.”

Menge*, C., B. Neufeld, W. Hirt, N. Schmeer, R. Bauerfeind, G. Baljer, and L.H. Wieler

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- Auswertung der Experimente wesentlich
- Erstellung der Publikation weitestgehend eigenständig

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Compensation of preliminary blood phagocyte immaturity in the newborn calf

Ch. Menge [a,*], B. Neufeld [a], W. Hirt [b], N. Schmeer [c], R. Bauerfeind [a], G. Baljer [a], L.H. Wieler [a]

[a] *Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurter Str. 89, D-35392 Giessen, Germany*
[b] *ORPEGEN Pharma, Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion, Czerny-Ring 23, D-69115 Heidelberg, Germany*
[c] *Institut für Infektionskrankheiten, Bayer AG, Gebäude 6210 / Monheim, D-51368 Leverkusen, Germany*

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Abstract

To estimate the functional maturity of the phagocytic defence in neonatal calves, we analyzed the characteristics of blood phagocytes from calves ($n = 10$) 1 h post partum (p.p.) and 4 h p.p. At 1 h p.p., all calves were colostrum-deprived, while 5 calves had received colostrum before the 4 h p.p. sampling. The results were compared to those obtained from 3–9-week-old calves ($n = 10$). Phagocytic and oxidative burst activity of polymorphonuclear leukocytes (PMNL) and monocytes were determined in whole blood and separately analyzed by flow cytometry. In neonates prior to colostrum ingestion (1 h p.p.), phagocytic activity of PMNL against non-preopsonized *E. coli* was lower when compared to PMNL of 3–9-week-old calves. Opsonization of bacteria with pooled plasma from adult animals only partially restituted this lower PMNL phagocytic activity, indicating that humoral as well as cellular aspects of PMNL phagocytosis are altered in neonatal calves. In contrast to PMNL, monocytes of neonates exhibited an enhanced phagocytic activity. The oxidative burst activity of PMNL, as well as of monocytes was higher in newborn calves. During the first 4 h of life, the activities of blood phagocytes changed. Colostrum ingestion was accompanied by an increase in the percentage of phagocytizing PMNL and monocytes. This

Abbreviations: DHR 123, Dihydrorhodamine 123; EDTA, Ethylene diamine tetraacetate; G-CSF, Granulocyte-colony stimulating factor; IL, Interleukin; MNL, Peripheral blood mononuclear leukocyte; PBS, Phosphate-buffered saline; PMA, Phorbol myristate acetate; PMNL, Polymorphonuclear leukocytes; p.p., Post partum; ROS, Reactive oxygen species; RT, Room temperature; TNF-α, Tumor necrosis factor alpha

* Corresponding author. Tel.: +49 641 9938314; fax: +49 641 9938309; e-mail: christian.menge@vetmed.uni-giessen.de

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increase was absent in colostrum-deprived calves. In contrast, the oxidative burst activity of phagocytes decreased with age. In monocytes, the decrease of oxidative burst activity was only observed in colostrum-fed calves. In conclusion, some blood phagocyte functions in calves were found to be immature at birth, but these functions are presumably compensated by high absolute PMNL numbers and by other the more active mechanisms. © 1998 Elsevier Science B.V.

Keywords: Monocytes; Granulocytes; Phagocytosis; Oxidative burst; Newborn calf

1. Introduction

Phagocytosis by granulocytes [polymorphonuclear leukocytes (PMNL)] is the first and major defence mechanism against bacterial and fungal pathogens. However, neonatal calves, like newborns of other species, are deficient in several of the distinct steps of phagocytosis by PMNL (Zwahlen et al., 1992). Sera of neonates opsonize bacteria only poorly (Lombardo et al., 1979). This is partially due to the low immunoglobulin concentration (Adams et al., 1992; Steinhardt et al., 1993). Furthermore, young calves have a low activity in both the classic and the alternative pathway of complement activation (Mueller et al., 1983). In addition to this humoral impairment, the neonatal PMNL only poorly recognize opsonized particles, as concluded from the reduced expression of Fc receptors on these cells, compared to cells from adult animals (Zwahlen et al., 1992). Coincidently, several investigations confirmed that neonatal PMNL possess a low phagocytic activity against bacteria (LaMotte and Eberhart, 1976; Riedel-Caspari and Schmidt, 1991). In addition to the deficiency of PMNL to ingest bacteria, both the generation of reactive oxygen species (ROS) and the myeloperoxidase activity are reduced in PMNL of newborn calves (Doré et al., 1991; Zwahlen et al., 1992). With regard to such impairments, it is assumed that PMNL are functionally immature at birth (LaMotte and Eberhart, 1976).

After birth, phagocytic efficiency increases rapidly, although the time course of this increase is not known exactly. LaMotte and Eberhart (1976) and Lombardo et al. (1979) showed that phagocytosis of *E. coli* increased within 6 h after birth. However, Riedel-Caspari and Schmidt (1991) described that the number of streptococci ingested by 100 leukocytes did not increase until day 5 p.p. This increase of PMNL phagocytic activity after birth was supported by the ingestion of colostrum.

Monocytes represent precursors of antigen-processing and -presenting macrophages, providing the initial signal in the development of antigen-specific immunity. Additionally, monocytes modulate PMNL functions by secreting a variety of cytokines (Clark and Kamen, 1987). Although monocytes are strikingly different from PMNL in their biology and functions, to date they have not been included or separately analyzed in studies on blood phagocyte maturity in neonatal calves.

To better estimate the impact of immature PMNL functions in neonatal calves on the defence against bacterial infections, the level and duration of phagocyte maturity needs to be further elucidated. In the present study, the phagocytic defence mechanism of neonatal calves was characterized distinguishing between PMNL and monocyte functions. Additionally, the impact of serum opsonic activity and colostrum ingestion on the phagocytic functions were examined.

2. Materials and methods

2.1. Animals

Twenty male and female calves (Holstein × German Black Pied) were included in this investigation. Ten calves were delivered in the Clinic for Veterinary Gynaecology and Obstetrics, Justus-Liebig-University, Giessen with minimal or no manual assistance. All calves were separated from their dams before suckling and assigned to two groups. Five calves received colostrum within the second or third hour of life (colostrum-fed group), whereas the others did not receive colostrum until the 4-h blood sample had been taken (colostrum-deprived group). Ten colostrum-fed calves aged 3 to 9 weeks served as control group. All calves appeared clinically healthy throughout the investigation.

2.2. Sampling, sample preparation and differential leukocyte counts

Blood was collected from calves by jugular venipuncture using vials containing heparin or EDTA (Sarstedt, Nümbrecht, Germany). Calves of the control group were bled once, whereas newborn calves were bled twice, 1 h and 4 h post partum (p.p.). Blood samples were stored at room temperature for less than 5 h prior to investigation. Homologous plasma was obtained from heparinized blood samples of 10 cows, pooled, and stored at −20°C.

EDTA blood samples were used for determination of total leukocyte counts in a SYSMEX micro cell counter F800 (Sysmex, Norderstedt, Germany). For the determination of differential leukocyte counts, 200 μl of heparinized blood were incubated with 5 ml of erythrocyte lysing solution (8.26 g NH_4Cl, 1.09 g $NaHCO_3$, 0.037 g Na_2EDTA ad 1000 ml A. dest.) for 5 min (RT). Then the leukocytes were pelleted by centrifugation (200 × g, 5 min, RT) and resuspended in 1.2 ml of PBS (10 g NaCl, 0.25 g KCl, 0.25 g KH_2PO_4, 1.8 g $Na_2HPO_4 * 2H_2O$ ad 1000 ml A. dest.). Analysis was performed on an EPICS ELITE® analyzer (Coulter, Krefeld, Germany). Electronic gates were set according to the light scatter characteristics of lymphocytes, monocytes, and granulocytes, and the proportion of the respective cell type was read.

At the beginning of the functional assays, a volume of heparinized blood containing 3.33×10^5 PMNL calculated from the total and differential leukocyte counts was placed at the bottom of a V-shaped plastic tube (110 × 16 mm, Nunc, Wiesbaden, Germany) in icewater. The volume was brought to 100 μl by adding autologous plasma prepared from an additional heparinized blood sample.

2.3. Phagocytic assay

In vitro phagocytic activity was determined using the Phagotest® kit (ORPEGEN Pharma, Heidelberg, Germany). After addition of fluorescein labeled *Escherichia coli* (ATCC 33572) to whole blood, bacteria are ingested by phagocytes generating a green fluorescence signal that can be quantified by flow cytometry. Phagotest® contained

bacterial suspension and several buffers given in parenthesis throughout this paper. The test was performed according to the instructions of the provider with minor modifications. Briefly, 20 μl of autologous or homologous plasma were added to the precooled blood samples prepared as described above. Twenty or 2 μl of bacterial suspension (1×10^9 bacteria/ml) were added to the blood, generating a bacteria/PMNL ratio of 60:1 or 6:1. *E. coli* bacteria had been cultured overnight in LB medium (Merck, Darmstadt, Germany), washed in PBS, heat-inactivated and labeled with FITC as described by Gelfand et al., 1976. Both non-opsonized *E. coli* and *E. coli* preopsonized with human serum as provided by the vendor (referred to as xenogeneic opsonization) were used as phagocytic particles. When nonopsonized bacteria were applied, the respective blood sample had been supplemented with either autologous or homologous plasma, while bacteria opsonized with xenogeneic serum were just added to blood samples supplemented with autologous plasma. After vortexing all tubes, test tubes were incubated at 39°C in a shaking water bath for 2 or 15 min. A control tube remained on ice. Phagocytosis was stopped by putting the tubes into icewater. To eliminate the fluorescence of non-phagocytized bacteria, 100 μl of 'quenching solution' were added. The cells were washed twice with 4 ml of 'washing-solution' and pelleted by centrifugation ($241 \times g$, 5 min, 4°C). Cells were resuspended and incubated in 2 ml of 'lysing solution' for lysis of erythrocytes and fixation of the leukocytes (20 min, RT). After centrifugation plastic adherent monocytes were recovered by resuspending and incubating the cell pellet in PBS-EDTA (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.42 g $Na_2HPO_4 * 2H_2O$, 2 g Na_2EDTA ad 1000 ml A. dest.) for 5 min in icewater. After a final centrifugation step, the cells of each sample were resuspended in 100 μl of 'DNA-staining solution', and analyzed by flow cytometry as described below.

2.4. Oxidative burst assay

In vitro phagocytic activity was quantified using Bursttest (Phagoburst®) kit (ORPEGEN Pharma, Heidelberg, Germany). In this assay, formation of reactive oxygen species (ROS) by the membrane-bound NADPH oxidase is measured intracellularly by the oxidation of the fluorogenic dihydrorhodamine (DHR) 123 to the membrane adherent fluorescent rhodamin 123. The Bursttest assay was performed according to the instructions of the manufacturer with some modifications. Briefly, 20 μl of autologous or homologous plasma were added to the precooled blood samples. After that, several stimuli were used to induce oxidative burst: fixed but unlabeled *E. coli* (ATCC 33572) were used as stimuli under three opsonizing conditions as described for the phagocytosis assay, whereas PMA (833.33 ng/ml) served as a 'high control'. Twenty microliters of bacterial suspension (1×10^9 bacteria/ml) or 20 μl of PMA solution (5 μg/ml in 'washing solution') were pipetted into the blood. The test tubes were vortexed and placed in a 39°C shaking water bath. A sample without stimulus served as a negative background control. After 10 min of incubation the tubes were cooled in icewater. The samples were supplemented with 20 μl of 'substrate solution' vortexed and incubated for 10 min at 39°C. Four ml of 'washing-solution' were added, and the cells were washed, fixed and stained as described for the phagocytosis assay.

2.5. Flow cytometric analysis of phagocytic and oxidative burst assays

Analysis was performed on an EPICS ELITE® analyzer (Coulter, Krefeld, Germany) equipped with a 488-nm argon laser and standard filter configuration (525 nm bandpass, 630 nm longpass). Data were analyzed with ELITE 3.1 software provided by the vendor. During data aquisition of phagocytic and oxidative burst assays, a live gate was set in the 630 nm (dark red) fluorescence histogram for those events having at least the same DNA-content as diploid cells in order to exclude cell debris and bacterial aggregates. For each sample, the optical features of 10 000 leukocytes were acquired. Monocytes and PMNL were analyzed separately for their 525 nm (green) fluorescence intensity. Percent fluorescence positive events and mean channel of fluorescence, the latter being correlated to the mean number of bacteria ingested by single cells, or the mean oxidative burst activity of single cells, were recorded. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive.

2.6. Statistical analysis

Data were analyzed statistically by use of the BMDP/Dynamic software (Statistical Software, Dixon, 1993). Results were evaluated as follows: $p \leq 0.001$ highly significant ($***$), $p \leq 0.01$ significant ($**$), $p \leq 0.05$ weakly significant ($*$), and $p > 0.05$ not significant (n.s.). Multiple factor analysis was carried out by 3-way or 2-way ANOVA, according to the number of factors that varied throughout the experiment. In the case of significant interactions between the analyzed factors, statistical analysis proceeded in the 2-way ANOVA and Student's *t*-test.

3. Results

3.1. Numbers and differential counts of peripheral blood leukocytes in newborn and older calves

One hour after birth, i.e., prior to colostrum ingestion, calves had more leukocytes per ml blood than 3–9-week-old calves ($13.19 \pm 4.69 \times 10^6$ cells/ml vs. $7.66 \pm 1.01 \times 10^6$ cells/ml; $p \leq 0.01$). The differential leukocyte counts revealed that the portion of granulocytes, referred to as polymorphonuclear leukocytes (PMNL) was higher in peripheral blood of neonates ($76.86 \pm 9.1\%$ vs. $32.04 \pm 14.1\%$; $p \leq 0.001$). In contrast, the percentage of monocytes and lymphocytes, referred to as peripheral blood mononuclear leukocytes (MNL), was lower in newborn calves ($23.14 \pm 9.1\%$ vs. $67.96 \pm 14.1\%$; $p \leq 0.001$). When absolute leukocyte numbers were calculated, it turned out that peripheral blood of calves 1 h p.p. contained about 60% of the MNL numbers of 3–9-week-old calves ($2.91 \pm 1.29 \times 10^6$ cells/ml vs. $5.14 \pm 1.04 \times 10^6$ cells/ml). In contrast, PMNL numbers in the blood of newborns were about 4-fold higher ($10.28 \pm 4.24 \times 10^6$ cells/ml vs. $2.52 \pm 1.35 \times 10^6$ cells/ml). Peripheral blood leukocyte counts, as well as differential counts, did not change significantly within the first 4 h of life ($p > 0.05$), irrespective of colostrum ingestion (data not shown).

3.2. Phagocytic activity in newborn and older calves

In this part of the study, only bacteria opsonized with autologous plasma were admitted to the assay. Irrespective of the animal group or the type of phagocyte studied, the percentage of phagocytizing cells and the mean fluorescence intensity were clearly associated with the test design ($p \leq 0.001$ in each case; Fig. 1). The lowest phagocytic activity was observed under the most limiting conditions, i.e. bacteria/PMNL ratio of 6:1 in the 2-min assay. On the other hand, highest phagocytic activity was measured at a bacteria/PMNL ratio of 60:1 in the 15-min assay. This was referred to as maximum phagocytosis, since prolonging the incubation period did not further enhance the percentage of phagocytizing cells or the mean fluorescence intensity (data not shown).

When the phagocytes of neonates 1 h p.p. and 3–9-week-old calves were compared, monocytes and PMNL exhibited different reaction patterns (Fig. 1). Under limiting conditions, the percentage of phagocytizing PMNL, as well as the average number of ingested bacteria per cell, were lower in newborn calves than in older calves. Nevertheless, maximum phagocytosis by PMNL was identical in both age groups. Monocytes of neonates 1 h p.p. exhibited just a slightly reduced phagocytic activity against bacteria opsonized with autologous plasma as compared to monocytes of older calves in the

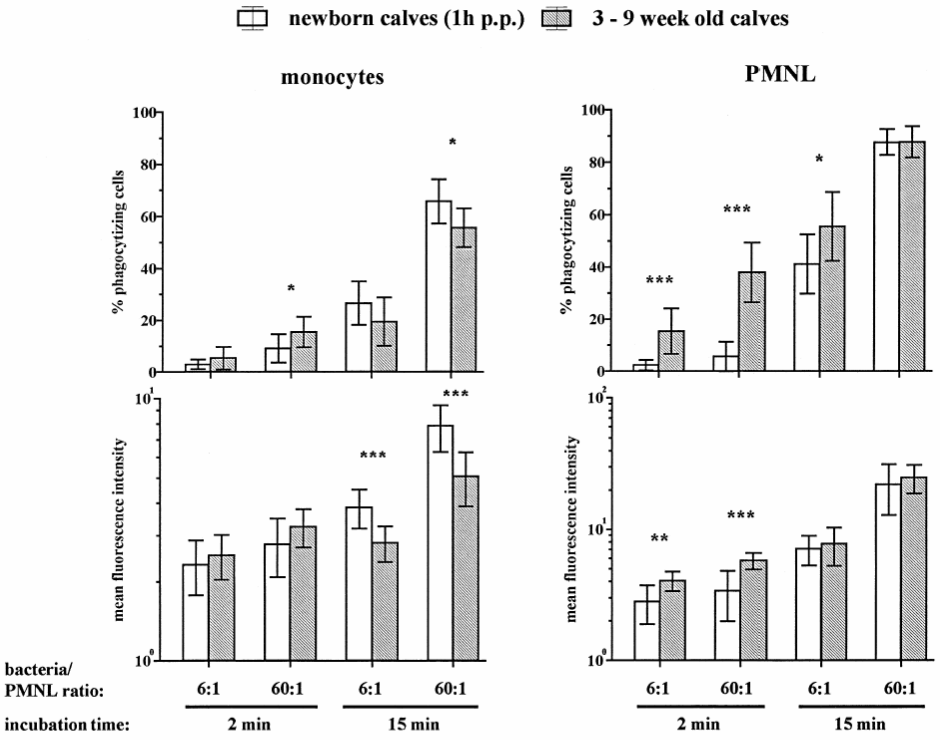

Fig. 1. Percentages of phagocytizing cells (above) among monocytes and polymorphonuclear leukocytes (PMNL) and the mean fluorescence intensity of the phagocytizing cells (below) in peripheral blood of newborn calves 1 h p.p. ($n = 10$) and calves 3–9 weeks of age ($n = 10$). Blood samples were incubated with *E. coli* prelabeled with fluorescein and opsonized with autologous serum. Bars represent mean values ± S.D. ($* p \leq 0.05$, $** p \leq 0.01$, $*** p \leq 0.001$, newborn vs. older calves).

2-min assay. In contrast to PMNL, the phagocytic activity (percentage of phagocytizing cells, mean fluorescence activity) of monocytes was markedly higher in newborn calves than in calves at the age of 3–9 weeks in the 15-min assay.

3.3. Role of opsonization for the phagocytic activity in newborn and older calves

To distinguish humoral from cellular aspects of phagocytosis, blood samples taken from newborn calves 1 h p.p. were incubated with bacteria that were opsonized with autologous or homologous plasma or xenogeneic serum. Irrespective of the opsonin used, the percentage of phagocytizing monocytes and PMNL in the 2-min assay was lower in newborn calves 1 h p.p. than in calves aged 3–9 weeks (Fig. 2, $p \leq 0.05$ for monocytes, $p \leq 0.001$ for PMNL). As it was seen with bacteria opsonized with autologous plasma in the 15-min assay, the percentage of phagocytizing PMNL was identical in both age groups, while the percentage of phagocytizing monocytes was higher in the newborn group ($p \leq 0.05$).

Nevertheless, opsonization had some effects on phagocytosis. These effects were most prominent for PMNL in the 2-min assay ($p \leq 0.001$). In the newborn group the percentage of PMNL ingesting bacteria opsonized with homologous plasma was markedly higher than the percentage of PMNL ingesting bacteria that were opsonized with autologous plasma or xenogeneic serum. In the blood of 3–9-week-old calves bacteria were phagocytized by an equal percentage of PMNL irrespective of the opsonin. Opsonization exhibited similar but less striking effects on the average number of ingested bacteria per cell (data not shown).

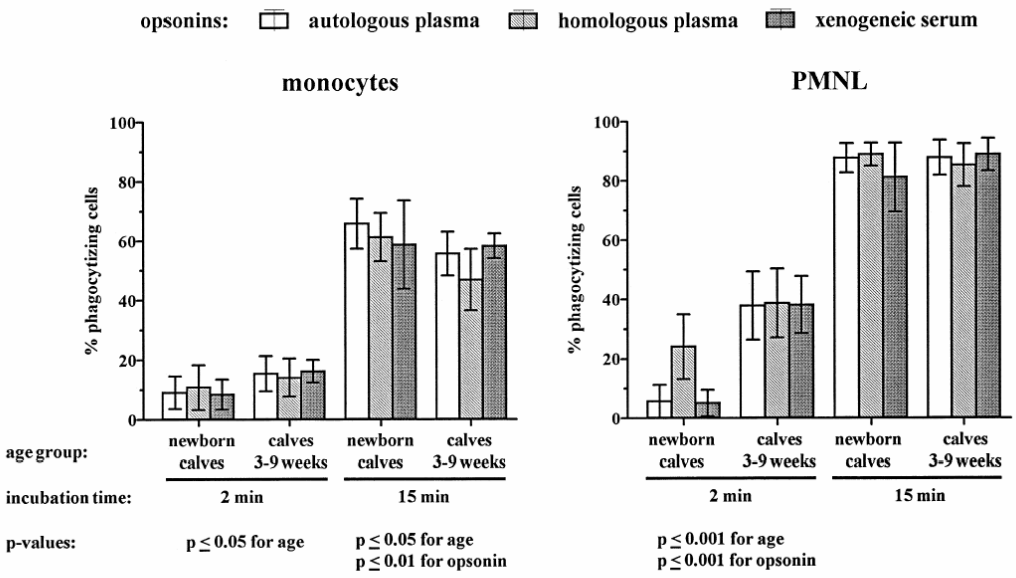

Fig. 2. Effects of three different opsonins on the percentages of phagocytizing monocytes and polymorphonuclear leukocytes (PMNL) in peripheral blood of newborn calves 1 h p.p. ($n = 10$) and calves 3–9 weeks of age ($n = 10$). Cells were incubated with fluorescein-labeled *E. coli* for 2 and 15 min at a bacteria/PMNL ratio of 60:1. Statistical analysis by 2–way ANOVA.

3.4. Effect of aging and colostrum uptake on phagocytic activity in newborn calves

As it is outlined in Fig. 3 (right panel) the percentage of phagocytizing PMNL changed within the first 4 h after birth ($p \leq 0.001$ for the 2-min assay; $p \leq 0.05$ for the 15-min assay). Furthermore, colostrum ingestion had a highly significant effect on the change in the percentage of phagocytizing PMNL from 1 h to 4 h p.p. ($p \leq 0.001$ for the 2-min and 15-min assays each). The percentage of phagocytizing PMNL increased only in those calves that were fed colostrum, but not in colostrum-deprived calves. This increase mainly appeared with bacteria that were opsonized with autologous plasma or xenogeneic serum. However, even the high phagocytic activity against bacteria opsonized with homologous serum was enhanced in colostrum-fed calves. An increase in the percentage of phagocytizing cells in the colostrum-fed, but not in the colostrum-deprived group, was also seen for monocytes (2 min of incubation; Fig. 3, left panel). The average number of bacteria ingested per cell was also influenced by age. In the 15-min assay, the mean fluorescence intensity of PMNL generally declined from 1 h to 4 h p.p. ($p \leq 0.05$), irrespective of colostrum ingestion (data not shown).

3.5. Oxidative burst activity of blood phagocytes in newborn and older calves

Both inactivated bacteria and phorbol myristate acetate (PMA) induced an oxidative burst in monocytes and PMNL (Fig. 4). Comparing newborn calves 1 h p.p. with older

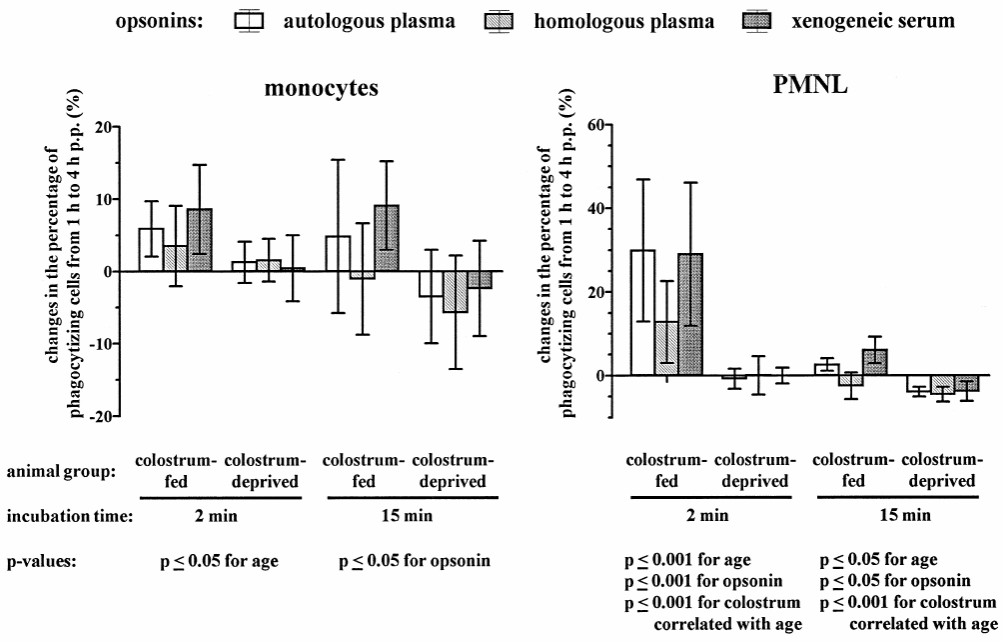

Fig. 3. Changes in the percentages of phagocytizing monocytes and polymorphonuclear leukocytes (PMNL) in peripheral blood of newborn calves between 1 h and 4 h p.p. (bacteria/PMNL ratio: 60:1). Bars represent the calculated mean difference ± S.D. between the 4 h and 1 h p.p. blood sample results of the respective group ($n = 5$ in either group). Three different opsonic stimuli were used as described in Section 2. Statistical analysis by 3-way ANOVA.

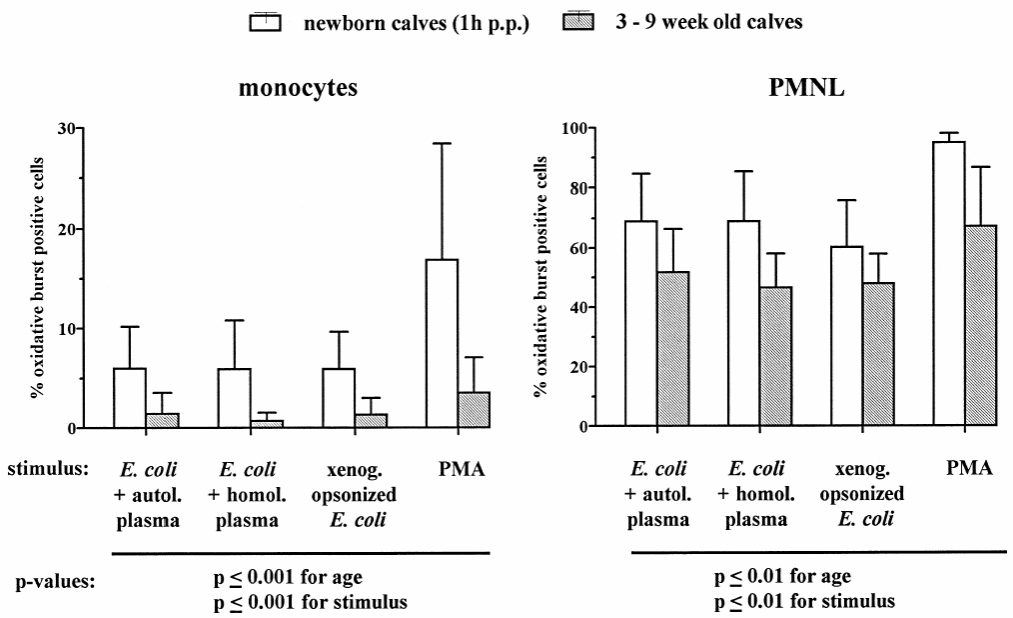

Fig. 4. Percentages of oxidative burst positive monocytes and polymorphonuclear leukocytes (PMNL) in peripheral blood of newborn calves 1 h p.p. ($n = 10$) and calves 3–9 weeks of age ($n = 10$) after incubation with different stimuli (bacteria/PMNL ratio: 60:1; 833.33 ng/ml PMA). Statistical analysis by 2-way ANOVA.

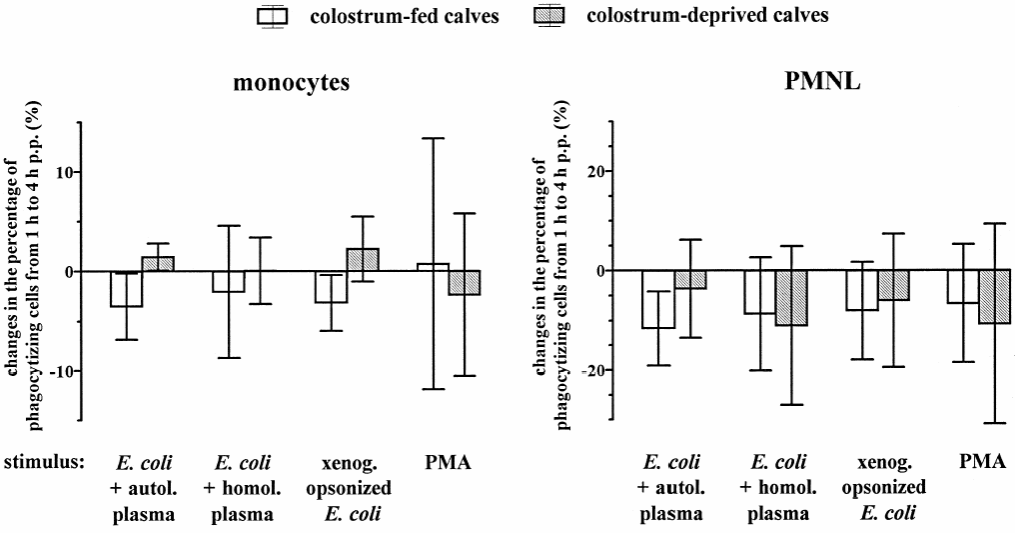

Fig. 5. Changes in the percentages of oxidative burst positive monocytes and polymorphonuclear leukocytes (PMNL) in peripheral blood of newborn calves between 1 h and 4 h p.p. Bars represent the calculated mean difference ± S.D. between the 4 h and 1 h p.p. blood sample results of the respective group ($n = 5$ in the colostrum-fed and $n = 4$ in the colostrum-deprived group). Four different stimuli were used to induce oxidative burst (bacteria/PMNL ratio: 60:1; 833.33 ng/ml PMA).

calves, PMNL as well as monocytes, showed a higher percentage of oxidative burst positive cells in newborn calves irrespective of the stimulus applied ($p \leq 0.001$ for monocytes, $p \leq 0.01$ for PMNL). Additionally, neonatal PMNL exhibited a higher mean fluorescence intensity ($p \leq 0.05$; data not shown).

Within the first 4 h after birth, the percentage of oxidative burst positive PMNL declined, regardless of colostrum ingestion or stimulus applied (Fig. 5). In contrast, the percentage of oxidative burst positive monocytes 4 h p.p. depended on colostrum ingestion ($p \leq 0.05$). Using differentially opsonized *E. coli* as stimuli, the percentage of oxidative burst positive monocytes was reduced in 4 h p.p. blood samples of colostrum-fed calves, whereas in samples of colostrum-deprived calves, the percentage was equal or slightly elevated compared to the values 1 h p.p.

4. Discussion

To determine the level of functional maturity in neonatal calves, blood phagocytes were examined 1 h p.p. and prior to colostrum ingestion. By distinguishing between the phagocytic activity of PMNL and of monocytes, it turned out that only particular activities of the phagocytic defence are impaired in neonatal calves. Under limiting experimental conditions, the phagocytosis of PMNL was impaired compared to PMNL of 3–9-week-old calves. Considering that we and others (LaMotte and Eberhart, 1976; Riedel-Caspari and Schmidt, 1991) found very high leukocyte numbers and a high percentage of PMNL in neonatal calves, the total capacity of neonatal calves for microbial elimination by blood PMNL must be several fold higher than in older calves. In contrast to PMNL, monocytes of neonatal calves showed a higher bacterial uptake than monocytes of 3–9-week-old calves under optimal experimental conditions, probably also contributing to the compensation of low PMNL activities in the newborn animal. Thus, our results contradict the hypothesis of generally impaired phagocytosis in neonatal calves established from previous reports examining only single parameters of blood phagocyte functions (LaMotte and Eberhart, 1976; Lombardo et al., 1979; Riedel-Caspari and Schmidt, 1991).

The lower phagocytic uptake by newborn PMNL was reflected by a lower percentage of phagocytizing cells, as well as a lower average number of bacteria ingested by individual cells in the 2-min assay. Therefore, we assume that under in vivo conditions, PMNL from newborn calves need more time to eliminate bacteria than PMNL of older calves. However, PMNL of calves from both age groups exhibited a similar phagocytosis pattern when blood samples were incubated at a high bacteria/PMNL ratio for a long incubation period. In contrast to our data, LaMotte and Eberhart (1976) and Riedel-Caspari and Schmidt (1991) described a general reduction in the phagocytosis of *E. coli* and streptococci in neonatal calves. These discrepancies may be explained by the fact that various bacterial strains have different opsonic requirements (Moiola et al., 1994), or by the different experimental designs and procedures applied. LaMotte and Eberhart (1976) and Riedel-Caspari and Schmidt (1991) prepared smears after incubation of blood samples with bacteria for 10 min calculating the phagocytic activity of a sample from the number of PMNL that ingested more than 6 *E. coli* and the number of

streptococci ingested by 100 PMNL. In our study, phagocytosis was evaluated by flow cytometry on a single cell level after various incubation times, allowing a more objective evaluation of phagocytic activity.

We assume that the slower phagocytic uptake by PMNL from calves 1 h p.p. is caused by humoral, as well as cellular factors. The involvement of humoral factors was indicated by the result that the slower phagocytosis by PMNL in neonatal calves could be almost accelerated to levels of older calves when the bacteria were opsonized with plasma from adult cows. The poor opsonic activity of neonatal serum was found to be a result of a deficiency in immunoglobulins as well as in serum complement (Adams et al., 1992; Mueller et al., 1983). However, our data show that currently unknown cellular factors must also be involved in the phagocytic impairment of neonatal calves. Homologous opsonization of bacteria only partially restored the reduced phagocytosis, activating an additional portion of blood PMNL to phagocytize bacteria. Obviously, a decreased number of fully competent phagocytes after birth contributed to the impaired phagocytosis. The mechanism of the functional immaturity of neonatal PMNL is not clear, but may involve a reduced expression of Fc receptors and an insufficient capping of ConA binding sites on neutrophils as it has been reported previously (Zwahlen et al., 1992).

Blood PMNL, as well as monocytes in neonatal calves 1 h p.p. generated higher amounts of reactive oxygen species (ROS) than phagocytes from older calves, independent of the type of stimulus applied. Interestingly, the percentage of burst positive phagocytes in neonates was even higher when the identical *E. coli* strain was used as for phagocytosis studies, although PMNL of neonates had difficulties to ingest this strain. Keeping in mind that only those cells are triggered for oxidative burst that actually have ingested bacteria, the portion of phagocytes both able to ingest and efficiently kill bacteria seems to be much higher in newborn than in older calves. Our results are supported by reports, stating a shorter lag time for O_2^- generation in neonatal bovine PMNL (Doré et al., 1991). However, in contrast to our findings, a reduced oxidative burst activity by neonatal neutrophils stimulated with PMA and *E. coli* has been described (Lombardo et al., 1979). This phenomenon was attributed to an altered protein kinase C activity in those cells (Doré et al., 1992). However, it has to be taken into account, that stimulation of PMNL not only leads to the production of intracellular ROS, but also to production of ROS released to the extracellular milieu (Karlsson et al., 1995). We determined oxidative burst activity on a single cell level, hence intracellularly, while other authors measured intracellular, as well as extracellular ROS. Despite these methological differences, we assume that the greater portion of phagocytes producing intracellular ROS represents another line of compensation for the somewhat lower phagocytic activity of those cells in the neonatal calf.

Another goal of this study was to estimate the duration of phagocyte immaturity. Within the first 4 h of life, the phagocytic activity of blood phagocytes was more or less unchanged in colostrum-deprived calves. However, after colostrum ingestion, maximum phagocytosis was unaltered, while the portion of PMNL able to phagocytize bacteria within 2 min was enhanced. Hence, the effect of colostrum on PMNL phagocytosis is mainly characterized by a decrease of the average time needed by the cells to recognize and ingest bacteria, rather than altering the phagocytic capacity of blood PMNL. Colostrum ingestion enhanced phagocytosis against autologous and xenogeneic op-

sonized bacteria more efficiently than against homologous opsonized bacteria. Consequently, the effect of colostrum on phagocytosis in this early period of life is mostly due to promoting plasma opsonic activity. Similar data were already reported for yeasts in lambs (Bernadina et al., 1991) and *E. coli* in calves (Lombardo et al., 1979). However, phagocytosis of bacteria opsonized with homologous plasma was also enhanced by colostrum, although these bacteria were phagocytized very well 1 h p.p. Obviously, colostrum does not only increase the serum opsonic activity but additionally seems to stimulate the cellular activity of blood phagocytes by another yet unknown mechanism. A similiar phenomenon has already been described for phagocytosis of *E. coli* (Lombardo et al., 1979) and *Streptococcus agalactiae* (Riedel-Caspari and Schmidt, 1991) within the first hours or days of calves p.p.. However, we conclude from our data, that PMNL already benefit from colostrum as early as 4 h p.p.. Several authors postulate phagocytosis promoting substances in colostrum, but the nature of these substances is still unknown. While human colostrum contains amounts of TNF-α that affect the defence system of human infants (Straussberg et al., 1995), phagocytosis of *Staphylococcus aureus* by bovine PMNL is not altered by TNF-α (Chiang et al., 1991).

A more suitable model to explain the benefit of colostrum for cellular activity in newborns is given by a possible role of colostral macrophages, vital leukocytes that represent the major portion of colostral cells. On the one hand, macrophage–lymphocyte interactions in milk result in secretion of cytokines, e.g., macrophage migration inhibitory factor and leukaemia inhibitory factor (Riedel-Caspari and Schmidt, 1990). Thus, factors released from colostral cells and absorbed through the gut may have provoked a release of monocyte precursors from the bone marrow of the neonates. Additionally, it has been suggested that macrophages may migrate from the gut lumen to the lamina propria as it has already been shown for lymphocytes (Tuboly et al., 1995). Our data confirm that in addition to its effect on PMNL phagocytosis, colostrum ingestion reduced the average time necessary for monocytes to ingest bacteria, and enhanced the portion of phagocytizing monocytes. Therefore, we speculate that monocytes found in calf's blood after colostrum ingestion are, in part, colostrum-derived. This is supported by the fact that 4 h p.p. colostrum-fed calves show higher percentages of monocytes in the peripheral blood than did colostrum-deprived calves (Menge et al., submitted).

In conclusion, the present study indicates that the phagocytic system in neonatal calves is not immature in the sense of a reduced overall competence to eliminate microorganisms. On the contrary, several impaired functions of phagocytes seem to be compensated by other enhanced phagocyte activities or by high absolute numbers of circulating PMNL, presumably leading to a sufficient antimicrobial protection.

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Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Giessen, Germany

Phenotypical Characterization of Peripheral Blood Leucocytes in the Newborn Calf

CH. MENGE[1,4], B. NEUFELD[1], W. HIRT[2], R. BAUERFEIND[1], G. BALJER[1] and L. H. WIELER[1,3]

Addresses of authors: [1]Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, Frankfurter Str. 89, D-35392 Giessen; [2]ORPEGEN Pharma, Gesellschaft für biotechnologische Forschung, Entwicklung und Produktion GmbH, Czerny-Ring 23, D-69115 Heidelberg, Germany; [3]Institut für Mikrobiologie und Tierseuchen, Freie Universität Berlin, Philippstr. 13, 10115 Berlin, Germany; [4]Corresponding author

With 2 tables

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Summary

The present study was undertaken to establish reference values for the composition of blood leucocyte populations in neonatal calves by differential leucocyte counts and immunophenotyping. Neonatal calves 1 h post partum (p.p.) were found to have a very high absolute number of granulocytes while the number of peripheral blood mononuclear cells was lower than in calves aged 3–9 weeks. The relative numbers of T cell subpopulations were similar in newborn and older calves, but newborn calves had lower percentages of B cells and MHC class II positive cells. Within the first 4 h of life the relative numbers of $CD2^+$, $CD6^+$, and $CD8^+$ T cells declined in colostrum-fed as well as in colostrum-deprived calves. In contrast, the percentage of MHC class II positive cells and monocytes increased from 1 h to 4 h p.p. particularly in colostrum-fed calves. Although there is some evidence for immaturity of lymphocytes in neonatal calves, the immune system of these animals seems to be fully present at birth.

Introduction

Neonatal animals, including newborn calves, are highly susceptible to illness or death caused by micro-organisms which are less pathogenic for the adult animal. The defence against such infections relies on the immune system in the neonate. It is well accepted that the immune system of mammals is functionally immature at birth and subsequently undergoes a process of sequential development which is both programmed genetically and stimulated externally by antigen exposure. Due to the unique structure of the ruminantal placenta, calves are born agammaglobulinaemic (RIEDEL-CASPARI and SCHMIDT, 1991). Thus, the health of neonatal calves heavily depends on a rapid and sufficient uptake of colostral immunoglobulins and an early development of protective immune mechanisms. While the immunoglobulin content of neonatal calf's blood at birth and in the period thereafter has been extensively studied, little is known about the maturity and development of the cellular immunity, such as an increased spontaneous proliferation of blood mononuclear cells in neonatal calves (RIEDEL-CASPARI and SCHMIDT, 1991). Because assays to examine cellular functions are influenced by the relative numbers of cell subsets in the cell preparation tested, an evaluation of the cellular composition is crucial for the interpretation of the results obtained from those studies.

To date, data of blood leucocyte composition in the newborn calf are barely available. High absolute numbers of granulocytes and slightly reduced absolute lymphocyte counts have been described (LAMOTTE and EBERHART, 1976; MENGE et al., 1998). WILSON et al. (1996) reported marked differences in T cell subset distribution between fetal, maturing and older

calves, but cells other than T cells were not investigated. The animals were examined immediately after birth and 48 h later. Because peripheral blood leucocyte composition may be influenced by natal stress immediately after birth and by colostrum ingestion in the days thereafter (LAMOTTE and EBERHART, 1976; PITTARD et al., 1989) the values reported probably do not precisely reflect the leucocyte composition of neonatal calves during the time interval after recreation and before the first uptake of colostrum. Therefore, the aim of the present study was to establish reference values for the leucocyte composition in newborn calves during their first hours of life by differential leucocyte counts and immunophenotyping using flow cytometry.

Materials and Methods

Animals

Ten calves (holstein × german black pied) were delivered in the Clinic for Veterinary Gynaecology and Obstetrics, University of Giessen with minimal or no manual assistance. All calves were separated from their dams before suckling. Thereafter, five calves received colostrum within the second or third hour of life (colostrum-fed group) whereas the others did not until the 4 h blood sample had been taken (colostrum-deprived group). Another 10 colostrum-fed calves (holstein × german black pied) aged 3–9 weeks and maintained on straw served as the control group. All calves appeared clinically healthy throughout the investigation.

Sample preparation and flow cytometric analysis

Blood samples were collected from calves by jugular venipuncture using vials containing heparin or ethylene diamine tetra acetic acid (EDTA) (Sarstedt, Nümbrecht, Germany). The EDTA blood samples were used for total leucocyte counts in a SYSMEX micro cell counter F800 (Sysmex, Norderstedt, Germany). For immunophenotyping, 200 µl of heparinized blood was incubated with 5 ml of erythrocyte lysing solution (8.26 g NH_4Cl, 1.09 g $NaHCO_3$, 0.037 g Na_2EDTA per 1000 ml A. dest.) for 5 min (room temperature (RT)), pelleted by centrifugation (200 ***g***, 5 min), resuspended in 1.2 ml of phosphate-buffered saline (PBS; 10 g NaCl, 0.25 g KCl, 0.25 g KH_2PO_4, 1.8 g Na_2HPO_4*$2H_2O$ per 1000 ml A. dest.) and applied to a V-shaped microtitre plate at 100 µl per well. After centrifugation (200 ***g***, 10 min, 4°C) the pellets were resuspended in the supernatant of one of the following hybridoma cell lines: IL-A11 (detecting BoCD4), IL-A15 (BoCD11b), IL-A16 (BoCD11c), IL-A24 (macrophage differentiation antigen), IL-A29 (BoWC1), IL-A43 (BoCD2), IL-A57 (BoCD6), IL-A65 (BoCD21), IL-A99 (BoCD11a), IL-A105 (BoCD8) and J11 (BoMHC class II) (BALDWIN et al., 1988; ELLIS et al., 1988; HOWARD and MORRISON, 1991; NAESSENS et al., 1993, 1998). Leucocytes were incubated for 20 min on ice, washed once with PBS, resuspended with 50 µl of antimouse polyvalent FITC-conjugate fluorescein isothiocyanate (Sigma, Deisenhofen, Germany) diluted 1:100 in PBS and incubated for another 20 min on ice. The cells were washed twice and analysed on an EPICS ELITE® analyser (Coulter, Krefeld, Germany) using the ELITE 3.1 software. Electronic gates were set according to the negative controls (cells without primary antibodies) included in each test series defining less than 2 % of these cells as positive.

Statistical analysis

Data were analysed by two-way ANOVA and Student's t-test using BMDP/Dynamic (Statistical Software Inc.; DIXON, 1993). The results were evaluated as follows: $P \leqslant 0.001$ highly significant, $P \leqslant 0.01$ significant, $P \leqslant 0.05$ weakly significant, and $P > 0.05$ not significant (n.s.).

Results

Blood leucocyte composition in neonatal calves and calves aged 3–9 weeks

At 1 h post partum (p.p.) calves had more leucocytes per ml blood than calves aged 3–9 weeks ($13.19 \pm 4.69 \times 10^6$ cells/ml vs. $7.66 \pm 1.01 \times 10^6$ cells/ml; $P \leqslant 0.01$). The differential leucocyte counts revealed that the proportion of granulocytes was higher in the neonates (76.86 ± 9.1 % vs. 32.04 ± 14.1 %; $P \leqslant 0.001$). In contrast, the percentage of monocytes and lymphocytes (MNL) was lower in the newborn calves (23.14 ± 9.1 % vs. 67.96 ± 14.1 %; $P \leqslant 0.001$).

Differences between age groups were less striking regarding the proportions of granulocyte

or MNL subsets (Table 1). Among MNL, the percentages of $\alpha\beta$TCR T cells ($CD2^+$) and their subpopulations ($CD4^+$ and $CD8^+$ T cells) as well as $WC1^+$ cells (representing the majority of $\gamma\delta$TCR T cells) were similar in both ages. Differences were observed in the percentage of B cells ($CD21^+$) and MHC class II positive cells. Newborn calves at 1 h p.p. had lower mean percentages of B cells ($P \leqslant 0.01$) and MHC class II positive cells ($P \leqslant 0.05$) than 3–9-week-old calves. On the other hand, the percentage of monocytes among MNL was slightly higher in newborn calves ($P > 0.05$). MNL of newborn calves also differed from those of older calves in their expression of adhesion molecules. While CD11a and CD11c were present on an equal proportion of MNL in both groups, CD11b was present on more MNL and granulocytes in newborn calves 1 h p.p than in 3–9-week-old calves ($P \leqslant 0.01$ for MNL).

Nearly all granulocytes of both animal groups expressed CD11a and the macrophage differentiation antigen recognized by the monoclonal antibody IL-A24. In contrast, CD11b and CD11c were expressed by a higher percentage of granulocytes in newborn than in older calves, although these differences were not significant.

The absolute numbers of the examined leucocyte subsets per ml of blood were calculated from leucocyte counts and immunophenotyping. The MNL numbers in blood of newborn animals were only 60 % of the MNL numbers in 3–9-week-old calves ($2.91 \pm 1.29 \times 10^6$ cells/ml vs. $5.14 \pm 1.04 \times 10^6$ cells/ml). In contrast, granulocyte numbers in newborns were approximately four-fold higher ($10.28 \pm 4.24 \times 10^6$ cells/ml vs. $2.52 \pm 1.35 \times 10^6$ cells/ml).

The absolute numbers of most of the different MNL subsets in newborn calves were again significantly lower and $\approx$60 % of the numbers found in 3–9-week-old calves (Table 1). However, the number of $CD8^+$ T cells and monocytes (IL-A24 positive) did not differ significantly between both groups, indicating that the absolute numbers of these MNL subsets are not as drastically reduced as those of other MNL subsets in neonatal calves.

The four-fold higher number of granulocytes expressing CD11a and the macrophage activation antigen in newborn calves corresponded to the higher total granulocyte numbers

Table 1. Relative and absolute numbers of leucocyte subpopulations in peripheral blood of newborn calves and of calves aged 3–9 weeks

Cell population and surface marker	Mean percentage of positive cells (±SD)		P value of age groups[a]	Mean numbers (cells × 10^6/ml) ± SD		P value of age groups[a]
	1 h	3–9 weeks		1 h	3–9 weeks	
MNL						
CD2	45.4 ± 10.2	42.8 ± 5.9	n.s.	1.39 ± 0.80	2.21 ± 0.60	⩽0.05
CD4	19.4 ± 6.4	20.2 ± 4.0	n.s.	0.59 ± 0.34	1.04 ± 0.28	⩽0.01
CD6	42.6 ± 10.1	39.0 ± 7.0	n.s.	1.31 ± 0.75	2.04 ± 0.67	⩽0.05
CD8	22.5 ± 7.3	20.8 ± 3.8	n.s.	0.71 ± 0.46	1.10 ± 0.40	n.s.
WC1	15.8 ± 7.6	20.2 ± 7.6	n.s.	0.46 ± 0.26	1.05 ± 0.46	⩽0.01
CD21	4.9 ± 1.3	10.0 ± 3.9	⩽0.01	0.15 ± 0.09	0.54 ± 0.28	⩽0.01
MHC class II	26.8 ± 8.0	33.6 ± 5.5	⩽0.05	0.74 ± 0.30	1.70 ± 0.33	⩽0.001
Mø-ag	25.7 ± 10.9	18.9 ± 6.8	n.s.	0.69 ± 0.27	0.94 ± 0.27	n.s.
CD11a	94.5 ± 4.0	97.0 ± 1.8	n.s.	2.77 ± 1.26	4.99 ± 1.00	⩽0.001
CD11b	81.1 ± 6.0	69.9 ± 6.8	⩽0.001	2.41 ± 1.19	3.63 ± 0.93	⩽0.05
CD11c	25.5 ± 8.8	23.4 ± 8.9	n.s.	0.68 ± 0.25	1.22 ± 0.34	⩽0.01
Granulocytes						
Mø-ag	99.1 ± 2.1	98.9 ± 1.0	n.s.	10.17 ± 4.21	2.50 ± 1.36	⩽0.001
CD11a	99.7 ± 0.2	98.1 ± 1.9	n.s.	10.25 ± 4.24	2.48 ± 1.36	⩽0.001
CD11b	52.9 ± 36.3	38.3 ± 24.9	n.s.	5.64 ± 4.44	0.92 ± 0.73	⩽0.01
CD11c	72.3 ± 17.6	52.6 ± 24.1	n.s.	7.29 ± 4.13	1.20 ± 0.73	⩽0.01

MNL, granulocytes: cell populations defined by flow cytometry; Mø-ag: macrophage activation antigen; n.s., not significant; SD, standard deviation.,[a]Calculated by Student's t-test.

observed. However, the number of CD11b- and CD11c-expressing granulocytes was approximately six-fold increased in newborn animals compared with 3–9-week-old calves.

Changes in blood leucocyte composition in colostrum-deprived and colostrum-fed calves between 1 h and 4 h p.p.

Irrespective of colostrum ingestion, peripheral blood leucocyte counts did not differ between 1 h and 4 h p.p. The peripheral blood of colostrum-deprived calves contained $12.62 \pm 4.33 \times 10^6$ and $13.06 \pm 2.61 \times 10^6$ leucocytes/ml, while the peripheral blood of colostrum-fed calves contained $13.76 \pm 5.48 \times 10^6$ and $13.82 \pm 2.64 \times 10^6$ leucocytes/ml 1 h and 4 h p.p., respectively. Differential leucocyte counts revealed slight changes in leucocyte composition from 1 h to 4 h p.p. The percentage of granulocytes increased from 72.83 ± 10.93 % to 79.12 ± 7.98 % in the colostrum-deprived group and from 80.89 ± 5.12 % to 86.38 ± 1.23 % in the colostrum-fed group, thereby improving the high granulocyte count observed in newborn calves 1 h p.p. However, these changes were not significant and independent of colostrum ingestion as calculated by two-way ANOVA.

Immunophenotyping revealed a relative decline of $\alpha\beta$TCR T cells ($CD2^+$; $P \leqslant 0.05$) and mature $\alpha\beta$TCR T cells ($CD6^+$; $P \leqslant 0.05$) during the first hours of life (Table 2). This effect was consistent with a decline in the proportion of $CD8^+$ T cells ($P \leqslant 0.05$). In contrast, the percentages of MHC class II positive cells and monocytes (IL-A24 positive) among MNL increased from 1 h to 4 h p.p. ($P \leqslant 0.05$). As demonstrated for total and differential leucocyte counts, colostrum ingestion had no statistically significant influence on the leucocyte subset distribution in newborn calves in this early period of life. Nevertheless, the proportion of monocytes and MHC class II positive cells was slightly higher in the colostrum-fed group ($P > 0.05$). Changes in the absolute numbers of the leucocyte subsets in neonatal calves from 1 h to 4 h p.p. were not observed in either colostrum-deprived or colostrum-fed calves.

Table 2. Cell surface markers on MNL and granulocytes of newborn calves 1 h and 4 h post partum (p.p.) with and without colostrum ingestion (mean percentage of positive cells ± SD)

Cell population and surface marker	Colostrum-fed		Colostrum-deprived	
	1 h p.p.	4 h p.p.	1 h p.p.	4 h p.p.
MNL				
CD2[a]	45.7 ± 4.1	34.3 ± 8.2	45.1 ± 14.7	35.4 ± 10.9
CD4	18.9 ± 6.2	16.4 ± 6.7	20.0 ± 7.3	19.1 ± 4.9
CD6[a]	42.3 ± 6.6	33.2 ± 8.7	42.8 ± 13.6	37.4 ± 8.8
CD8[a]	24.0 ± 4.2	15.6 ± 1.5	21.0 ± 9.8	15.8 ± 6.7
WC1	14.7 ± 7.2	12.7 ± 5.8	16.9 ± 8.7	17.7 ± 8.1
CD21	5.2 ± 1.1	5.1 ± 1.4	4.6 ± 1.5	4.5 ± 1.9
MHC class II[a]	28.3 ± 9.1	40.4 ± 9.2	25.3 ± 7.5	33.5 ± 10.0
Mø-ag[a]	25.9 ± 8.3	41.3 ± 12.1	25.6 ± 14.0	33.1 ± 9.9
CD11a	95.7 ± 1.5	94.5 ± 2.6	93.2 ± 5.6	92.6 ± 4.8
CD11b	81.1 ± 5.3	84.3 ± 3.9	81.1 ± 7.4	79.5 ± 4.0
CD11c	28.1 ± 9.0	40.1 ± 11.6	21.1 ± 8.2	30.0 ± 8.1
Granulocytes				
Mø-ag	99.7 ± 0.0	99.6 ± 0.2	98.4 ± 3.0	98.6 ± 2.6
CD11a	99.7 ± 0.2	99.7 ± 0.2	99.7 ± 0.2	99.7 ± 0.1
CD11b	52.4 ± 33.1	55.6 ± 26.4	53.5 ± 43.3	54.0 ± 45.8
CD11c	66.1 ± 18.3	59.8 ± 19.4	82.7 ± 12.7	75.3 ± 24.9

MNL, granulocytes: cell populations defined by flow cytometry; Mø-ag, macrophage activation antigen; SD, standard deviation. [a]Two-way ANOVA of this parameter revealed a significance for the time of blood sampling ($P \leqslant 0.05$) but not for the animal group.

Discussion

In the present study similar percentages of the major lymphocyte subsets were found in the blood of neonatal and 3–9-week-old calves. As far as can be concluded from blood values, these data imply that the bovine immune system is fully present at birth. Similar proportions of $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$ cells in cord blood and adult venous blood have also been reported for human neonates (BERRY et al., 1992; ERKELLER-YUKSEL et al., 1992). However, blood lymphocytes of human neonates markedly differed from the respective cells in adults with regard to the expression of differentiation and activation antigens. CD38 and CD45RO, markers for immature or naive cells, were expressed on drastically more cord blood cells than on adult blood cells (BECK and LAM-PO-TANG, 1994). In our study, the CD6 to CD2 ratio did not differ significantly between newborn and 3–9-week-old calves. The bovine CD6 antigen is expressed on most but not all peripheral blood $CD2^+$ cells and indicates mature T cells (HOWARD and MORRISON, 1994). Thus, our results indicate a similar degree of T cell maturity in neonatal and older calves. However, there is also evidence for immaturity of these cells in neonates, as MHC class II values differed between calves of both ages. MHC II has been described as an activation marker on mature memory, but not on resting T cells in the blood of men, sheep and horses (LUNN et al., 1993; MALINOWSKI and RAPAPORT, 1995) and some bovine T cells express MHC II when activated *in vitro* (MORRISON et al., 1988). In 3–9-week-old calves, we found that the proportion of MHC II positive cells was higher than the sum of the proportions of B cells and IL-A24 positive cells. Thus, we speculate that there is a population of mature MHC II positive T cells in the blood of older calves. These cells seem to be absent in newborn calves, as in these animals the proportion of MHC II positive cells is lower than the sum of the proportions of B cells and monocytes.

In addition, we found differences in the absolute numbers of B cells in neonatal calves and 3–9-week-old calves. Neonates had $\approx$70 % less cells than older calves. Because serum antibodies inhibit B cell proliferation and differentiation, BERRY et al. (1992) suggested that in human fetuses the relative decline in B cells during late fetal development may be due to a downregulation of fetal B cell proliferation by maternal immunoglobulins. However, in bovine neonates which are born without any maternal immunoglobulins, it appears more likely that the absence of antigenic stimulation is responsible for the relatively and absolutely low numbers of B cells in peripheral blood.

When reference values for blood cell composition in neonates are established, several factors have to be considered thoroughly. A very critical factor is the preparation procedure. In our study, whole blood samples treated with ammonium chloride for lysis of erythrocytes were used for immunophenotyping. In the study of WILSON et al. (1996), reporting higher CD2, CD4, and WC1 but lower CD8 values, density gradient separated mononuclear cells were characterized. Because it is known that selective cell loss occurs through the use of density gradients, this may be the reason for the differences observed. Another factor with an impact on blood leucocyte values has been extensively studied in humans. A higher percentage of $CD3^+/CD4^+$ cells in infants delivered without preceeding labour has been described (PITTARD et al., 1989). All calves included in the present study were born vaginally without or with minimal manual assistance. However, one calf was examined after delivery by Caesarean section (data not shown). In this animal the percentage of $CD8^+$ cells and $WC1^+$ cells was clearly higher and the percentage of monocytes clearly lower than in every other animal examined. Further work is necessary to evaluate the influence of labour on the immune system of offspring in cattle.

It was stated by RIEDEL-CASPARI and SCHMIDT (1991) that colostrum ingestion and particularly colostral leucocytes have an impact on leucocyte and differential cell counts in maturing calves. Furthermore, maternal lymphocytes are translocated from the gut lumen to the tissues of the porcine and ovine neonate (TUBOLY et al., 1988, 1995). Bovine colostrum predominantly contains $CD8^+$ cells (TAYLOR et al., 1994). When we examined calves 4 h p.p. $CD8^+$ cells had significantly dropped, while the $CD4^+$ cell values remained unaltered, irrespective of colostrum ingestion. Although 1–2 h after colostrum feeding may be too early to detect an effect of colostrum, our results support conclusions drawn by WILSON et al. (1996), that an uptake of maternal T cells does not seem to take place in newborn calves.

In conclusion, absolute lymphocyte numbers are reduced in neonatal calves and these

cells seem to be slightly immature, but the cellular immune system of these animals, not unlike neonates of other species, seems to be fully present at birth. These findings are in accordance with previous data regarding the maturity of blood phagocytes in neonatal calves (MENGE et al., 1998). We found that several impaired functions of phagocytes are compensated by other enhanced phagocytic activities. Thus, the immune system of neonatal calves, although different from that of older animals, seems to provide a sufficient protection against invading pathogens.

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6.3 "Globotriaosylceramide (Gb_3/CD77) is synthesized and surface-expressed by bovine lymphocytes upon activation *in vitro*."

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Globotriaosylceramide (Gb_3/CD77) is synthesized and surface expressed by bovine lymphocytes upon activation in vitro

Christian Menge[a,*], Ivonne Stamm[a], Manfred Wuhrer[b], Rudolf Geyer[b], Lothar H. Wieler[a,c], Georg Baljer[a]

[a]*Faculty of Veterinary Medicine, Institute for Hygiene and Infectious Diseases of Animals, Justus-Liebig-University, Frankfurter Str. 89-91, D-35392 Giessen, Germany*
[b]*Institute for Biochemistry, Academic Hospital Centre, Justus-Liebig-University, Giessen, Germany*
[c]*Institute for Microbiology and Animal Epidemics, Free University of Berlin, Berlin, Germany*

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Abstract

Neutral glycosphingolipids (GSLs) are considered activation markers on human lymphocytes, which are fundamental for studying the immune system. For cattle, only a limited number of activation markers has yet been identified. We recently showed that Shiga toxin 1, known to use globotriaosylceramide (Gb_3 syn. CD77) as a cellular receptor, depresses proliferation of activated bovine lymphocytes [Infect. Immunol. 67 (1999b) 2209]. In order to confirm the expression of Gb_3/CD77 on bovine lymphocytes, we flowcytometrically examined a bovine B-lymphoma cell line (BL-3) and bovine peripheral blood mononuclear cells (PBMC) before and after mitogenic stimulation and biochemically characterized neutral GSLs extracted from PBMC. CD77 was detected on the surface of BL-3 cells and cultured PBMC essentially after mitogenic stimulation. Although expressed by all PBMC subpopulations identified, the portion of $CD77^+$ cells was highest for $BoCD8^+$ cells, followed by B-cells and $BoCD4^+$ cells at day 4 of cultivation. Ceramide trihexoside of stimulated PBMC was structurally determined as Gal(α1-4)Gal(1-4)Glc(1-1)ceramide (Gb_3). Biochemically, Gb_3 was also detected within unstimulated PBMC which contained ceramide monohexoside (CMH) and Gb_3 in a ratio of about 4:1. However, stimulation induced an increase of CMH and Gb_3 by a factor of 2.5 and 10, respectively, implicating that bovine lymphocytes regulate surface expression of Gb_3/CD77 predominantly by quantitative changes in the

Abbreviations: C16:0, hexadecanoic acid; C24:1, tetracosenoic acid; Gb_3, globotriaosylceramide; CDH, ceramide dihexoside; CMH, ceramide monohexoside; CTetH, ceramide tetrahexoside; CTH, ceramide trihexoside; GC/MS, gas chromatography/mass spectrometry; GSLs, glycosphingolipids; HPTLC, high-performance thin-layer chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Stx1, Shiga toxin 1

*Corresponding author. Tel.: +49-641-99-38314; fax: +49-641-99-38309.
E-mail address: christian.menge@vetmed.uni-giessen.de (C. Menge).

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Gb_3 metabolism. This report presents Gb_3/CD77 as the first GSL identified on bovine immune cells and highly recommends this activation dependent antigen as a useful tool to investigate lymphocyte activation within the bovine immune system.

Keywords: Globotriaosylceramide; CD77; Bovine lymphocytes; Shiga toxin 1; Activation marker

1. Introduction

Following activation of lymphocytes, expression of additional membrane molecules is modulated and leads to their upregulation or loss from the cellular surface. Quantification of lymphocytes' activation marker expression was thus widely introduced in measuring cellular immune responses in vitro and in vivo (Menge et al., 1999a; Quade and Roth, 1999; Whist et al., 2000). Functional analysis of activation molecules' interaction with their physiological ligands (e.g. cytokines) clarified the mechanisms of lymphocyte regulation. Moreover, interactions between microbial ligands and lymphocyte surface molecules are found to be important steps in the pathogenesis of infectious diseases (Rietschel and Wagner, 1996; Haig and Fleming, 1999; Jeyaseelan et al., 2000). Unfortunately, the definition of activation molecules in ruminants has so far been limited. The bovine homologues of IL-2R (BoCD25) (Naessens et al., 1992) and transferrin receptor (BoCD71) (Naessens et al., 1996) have been described and MHC-II molecules were found to represent activation markers when expressed by bovine T-cells (Morrison et al., 1988). Activation molecules preliminary designated as BoWC8 (Nthale and Naessens, 1993), ACT1, ACT2, and ACT3 (Davis et al., 1996), mainly expressed by T-cells, were also identified. Since the bovine homologue of CD40 is also expressed by T-cells (Hirano et al., 1997), the only B-cell lineage specific antigen identified in cattle so far is the BoCD21 antigen (Naessens et al., 1990).

Neutral glycosphingolipid (GSLs) are lipid constituents of the cell surface membrane of all mammalian cells and the sugar moieties of many GSLs are exposed on the cell surface (Schwarting, 1980). Nevertheless, GSLs have a unique composition in different functional cell types. In human lymphocytes, monohexosylceramide [Glc(β1-1)Cer], lactosylceramide [Gal(β1-4)Glc(β1-1)Cer], globotriaosylceramide [Gb_3; Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer], and globotetraosylceramide [Gb_4; GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer] were the only neutral glycosphingolipids found by biochemical analysis (Lee et al., 1981). Expression of Gb_3 and Gb_4 on the cellular surface is almost restricted to B-cells and these molecules constitute stage-specific antigens defining a subset of terminally differentiated B-cells located in germinal centers (Taga et al., 1995). Triggering of Gb_3, also designated CD77, induces cell death by apoptosis and is thus the first example of a glycolipid antigen able to transduce a signal leading to apoptosis (Mangeney et al., 1993).

For several human cell types the Gb_3/CD77 molecule has been identified as the eukaryotic cell surface receptor specifically binding the B-subunit of Shiga toxin 1 (Stx1), produced by enterohemorrhagic *Escherichia coli* (EHEC) (Pudymaitis and Lingwood, 1992). It mediates endocytosis of its ligand, gaining Stx1 access to the cytosol where the Stx1A-subunit elaborates its enzymatic activity (O'Brien and Holmes, 1987). To our knowledge, expression of glycolipids by bovine lymphocytes has not yet been described, but our group recently

reported that Stx1 is able to block activation and proliferation of bovine lymphocyte subpopulations in vitro (Menge et al., 1999b). This prompted us to speculate, that Gb_3/CD77 is a good candidate for an activation marker to study the B-cell compartment of the bovine immune system. Therefore, we examined bovine lymphocyte subpopulations for the expression of the CD77 antigen by flow cytometry and defined the conditions under which CD77 expression takes place. The antigen was structurally shown to be Gb_3, and the lipid moiety was characterized as to its sphingoid base and fatty acid composition.

2. Material and methods

2.1. Cell cultures

BL-3 cells (ECACC 86062401), a bovine B-lymphoma cell line originally isolated from a case of spontaneous leukosis, but secondary infected with bovine leukemia virus (Romano et al., 1989) and bovine virus diarrhea virus were maintained in a 1:1 mixture of RPMI 1640 and Leibovitz L15 medium supplemented with 20% fetal calf serum, 2 mM glutamine, 100 U penicillin, and 100 μg streptomycin per ml. Bovine peripheral blood mononuclear cells (PBMC) were separated from bovine blood samples taken from the herd of the teaching and research farm "Oberer Hardthof" of the Justus-Liebig-University by centrifugation on Ficoll-Paque® gradients as described (Menge et al., 1999b). Cells were resuspended at 1.66×10^6 cells/ml in modified cell culture medium (RPMI 1640 supplemented with 0.532 g/l *N*-acetyl-L-alanyl-L-glutamine, 10% fetal calf serum and 3 μm 2-mercaptoethanol). In stimulation assays, medium was additionally supplemented with ConA (5 μg/ml), PHA-P (5 μg/ml), PWM (10 μg/ml), or LPS from *E. coli* O111:B4 (25 μg/ml; Sigma, Deisenhofen, Germany). Microtiterplates or cell culture flasks, respectively, were incubated at 37°C with 5% CO_2 for 1–8 days. Lymphocytes from colonic lymphnodes of a 11-week-old calf were prepared immediately after euthanasia by thoroughly resuspending minced pieces of lymphnodes in cell culture medium. Tissue fragment were allowed to settle, the supernatant was washed twice and the cells were stored at −80°C until flow cytometry analysis.

2.2. Immunophenotyping and flow cytometry analysis

At the end of the cultivation period, cells were submitted to immunolabeling as previously described (Menge et al., 1999b). For some experiments cells were fixed and permeabilized to gain antibodies access to intracellular compartments. Cells were resuspended in 50 μl PBS supplemented with 2% formalin and 50 μl 0.1% Triton X-100/0.1% Na-citrate solution and incubated for 30 and 2 min, respectively. Upon washing with PBS ($200 \times g$, 10 min, 4°C) pellets were resuspended in 50 μl PBS as a negative control, with PBS containing rat IgM (2 μg/ml; Camon, Wiesbaden, Germany) as an isotype control or with anti-CD77 mAb clone 38.13 (diluted 1:10; Coulter-Immunotech Diagnostics, Krefeld, Germany). After incubation (20 min, 4°C), cells were washed once, and resuspended with anti-rat IgM FITC- or R-PE-conjugate (Dianova, Hamburg, Germany) diluted 1:100 in PBS containing 2 μg/ml propidium iodide. Following another 20 min on ice, the cells

were washed twice and analyzed with an EPICS ELITE® analyzer (Beckman-Coulter, Krefeld, Germany) and 5000 events were acquired from each sample. Data analysis was performed using the ELITE 3.1 software provided by the manufacturer. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive.

In double staining experiments, cells were first resuspended in 50 μl of cell culture medium as a negative control or with supernatant of hybridoma cell lines (IL-A11 for BoCD4, IL-A57 for BoCD6, IL-A105 for BoCD8, IL-A16 for BoCD11c, IL-A65 for BoCD21, IL-A29 for BoWC1, J11 for BoMHC-II, and IL-A24 for a bovine differentiation activation antigen; Naessens et al., 1997). After 20 min on ice, cells were washed once with PBS and submitted to the staining protocol as described above. Though, before incubating with anti-rat IgM conjugate cells were additionally incubated with anti-mouse Ig R-PE conjugate (1:100; Sigma).

2.3. Isolation and purification of neutral glycolipids

PBMC of days 0 and 4 (500 million cells each) were washed several times with PBS and lyophilized yielding dry-weights of 21 (day 0) and 41 mg (day 4). Glycolipids were isolated by consecutive extractions, saponification, desalting on a reverse-phase-cartridge and fractionation on a DEAE-Sephadex-A25 column (Dennis et al., 1998). The acidic fraction was desalted on a reverse-phase-cartridge. Neutral fraction glycolipids were further purified by Florisil chromatography (Dennis et al., 1995), and neutral as well as acidic glycolipid fractions were analyzed by HPTLC and orcinol/H_2SO_4-staining as described elsewhere (Dennis et al., 1998). Aliquots of the neutral glycolipids were fractionated on a silica-gel column (40 mg of silica-gel 60; Merck, Darmstadt) to yield CMH and CTH (Dennis et al., 1995).

2.4. Structural analysis

MALDI-TOF-MS and on-target enzymatic cleavage were performed as described previously (Geyer et al., 1999). For on-target enzymatic cleavage, α-galactosidase from green coffee beans (1.5 mU, 6 h; Roche Diagnostics, Mannheim, Germany) was used. For carbohydrate composition analysis, glycolipids were hydrolyzed (4 M trifluoroacetic acid, 4 h, 100°C) and analyzed as their anthranilic acid derivatives by high-performance liquid chromatography (Anumula, 1994). For methylation analysis, glycolipids were permethylated and hydrolyzed as described above. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by GC/MS, using the instrumentation and microtechniques described elsewhere (Geyer and Geyer, 1994). CMH and CTH from days 0 and 4 was treated with 500 μl 1 M HCl and 10 M H_2O in methanol for 16 h at 100°C according to Gaver and Sweeley (1965). The fatty acids, released as their methyl esters, were separated from the remaining sphingoid bases and saccharide residues with a three-fold phase-partition using *n*-hexane. The pooled *n*-hexane partitions were dried down under N_2 and analyzed by GC/MS. For sphingoid base analysis, 2 ml of water and 500 μl of 25% NH_3 were added to the lower methanol phase, and the sphingoid bases purified by a three-fold phase-partition using 2 ml aliquots of chloroform. The chloroform

fractions were dried down under N_2. As a modification of the derivatization method described by Zanetta et al. (1999), after addition of 200 μl acetonitrile and 25 μl pentafluoropropionic anhydride, the sample was heated for 30 min at 150°C, dried down under N_2, taken up in acetonitrile and analyzed by GC/MS. For identification, C18-sphingosine, C18-sphinganine and C18-phytosphingosine standards were used.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as described previously (Wuhrer et al., 1999). Anti-CD77 rat IgM mAb 38.13 (diluted 1:15; Coulter-Immunotech) and rabbit, horseradish peroxidase-conjugated, anti-rat Ig (diluted 1:500; Dako Diagnostics, Hamburg, Germany) were used as primary and secondary antibodies, respectively.

3. Results

3.1. CD77 expression by BL-3 cells

Using flow cytometry to determine the expression of the CD77 antigen by BL-3 cells, viable cells taken from maintenance cultures bound the anti-CD77 mAb only to a very low extent (Fig. 1B). Furthermore, when the cells were analyzed separately according to their light scatter characteristics (Fig. 1A) enabling the discrimination of viable and subvital cells (cells which exhibited light scatter characteristics similar to dead cells, but retained their membrane integrity as concluded from the lack of propidium iodide uptake), it became obvious that CD77 expression is almost restricted to cells of the subvital population (Fig. 1C and D). While immunolabeling of viable cells only detected surface antigen expression, analysis of permeabilized cells revealed that nearly all BL-3 cells bound the anti-CD77 mAb indicating the presence of the CD77 antigen inside the majority of the cells (Fig. 1B). As BL-3 cells are resistant to the apoptosis inducing effect of Stx1 unless they are stimulated by B-cell mitogens (Menge et al., 1999b), we reinvestigated the cells after incubation in the presence of PWM (10 μg/ml) or LPS (25 μg/ml) for 4 days. Mitogenic stimulation led to an enhanced CD77 expression on the cellular surface with LPS being a stronger inductor of CD77 expression than PWM (Fig. 1C and D). While stimulation enhanced the percentage of subvital cells expressing CD77 on the surface it also induced a slight increase in the mean fluorescence intensity of all viable cells coincident with a low CD77 surface expression by the majority of the cells. In addition, mitogenic stimulation also affected expression of other leukocyte markers (data not shown). Incubation in the presence of PWM as well as LPS newly induced the surface expression of BoCD11c and increased the number of BoMHC-II molecules expressed per cell. In contrast, stimulation led to a slight decrease in BoCD4 and BoCD6 expression by BL-3 cells.

3.2. Kinetics of CD77 expression by bovine PBMC

Bovine PBMC freshly prepared from blood samples (day 0 PBMC) did not bind anti-CD77 mAb. When PBMC were cultivated, however, binding of anti-CD77 was detectable

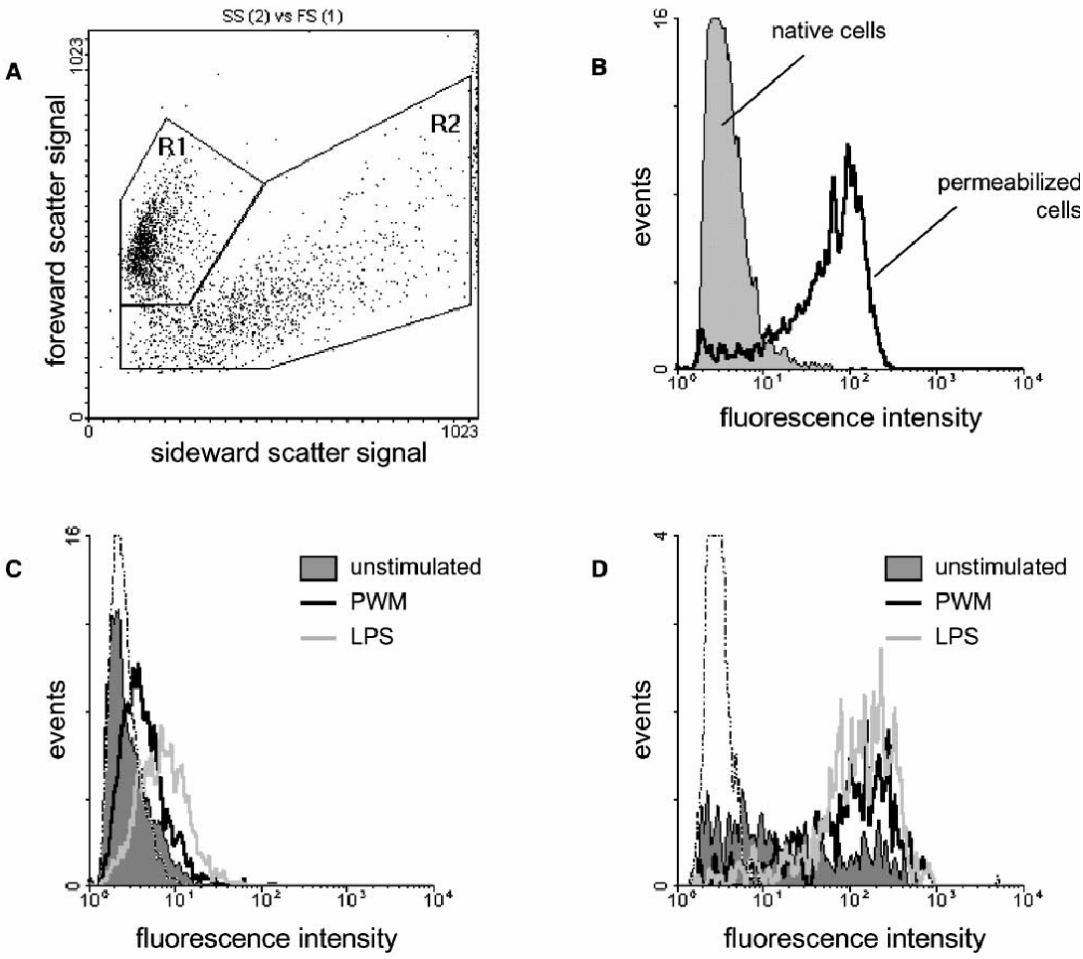

Fig. 1. CD77 expression by the bovine B-lymphoma cell line BL-3. Flow cytometry analysis was used to detect binding of anti-CD77 mAb and FITC-labeled secondary antibody to unstimulated BL-3 cells (B) either in a native state or after permeabelizing treatment. In a separate set of experiments (C and D), BL-3 cells were incubated without mitogens or in the presence of PWM (10 μg/ml) and LPS (25 μg/ml), respectively, for 4 days before immunolabeling was carried out (dashed lines represent isotype controls). Due to their light scatter characteristics (A) viable cells (marked as R1) could be distinguished from subvital cells (marked as R2) and separately analyzed (C viable cells, D subvital cells).

as early as 1 day after initiation of the culture (Fig. 2). Although the cells expressed CD77 even in the absence of mitogenic stimulation, addition of mitogens (ConA, PHA-P, PWM, and LPS, respectively) strongly increased the percentage of positive cells with PHA-P being the most potent stimulus (see Fig. 2 for PHA-P and LPS; ConA and PWM data not shown). The percentage of $CD77^+$ cells constantly rose during the cultivation period, but CD77 expression of the cells differed according to their light scatter characteristics. The populations of lymphoblasts, vital non-blasts and subvital cells were thus monitored separately (Fig. 3A). In the absence as well as in the presence of all mitogens used, the highest percentage of $CD77^+$ cells was found within the blast cell population whereas an intermediate percentage of subvital cells expressed CD77 (Fig. 2). The percentage of

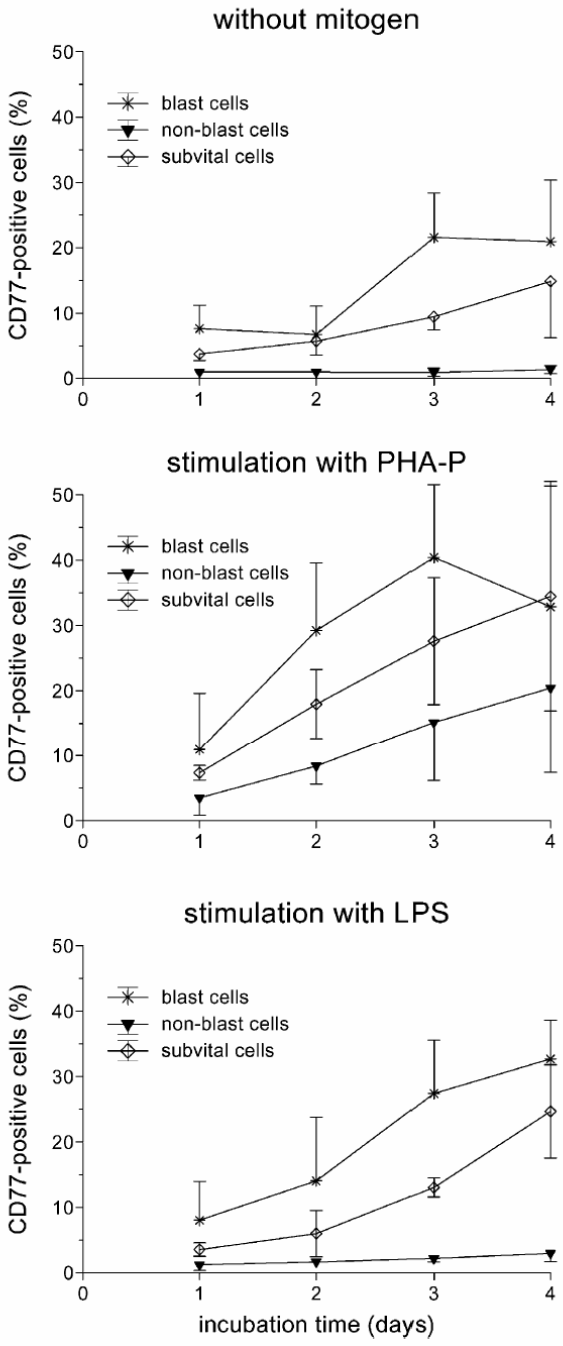

Fig. 2. Time course of CD77 expression by bovine PBMC in vitro and influence of mitogenic stimulation. Bovine PBMC were incubated without mitogen or in the presence of PHA-P (5 μg/ml) and LPS (25 μg/ml) as indicated. Daily, an aliquot of native cells was stained with anti-CD77 mAb and FITC-labeled secondary antibody. The percentage of positive cells was determined separately for blast cells, non-blast cells and subvital cells using a flow cytometer. Data are mean ± S.D. from independent PBMC preparations ($n = 4$). Missing error bars are within symbols.

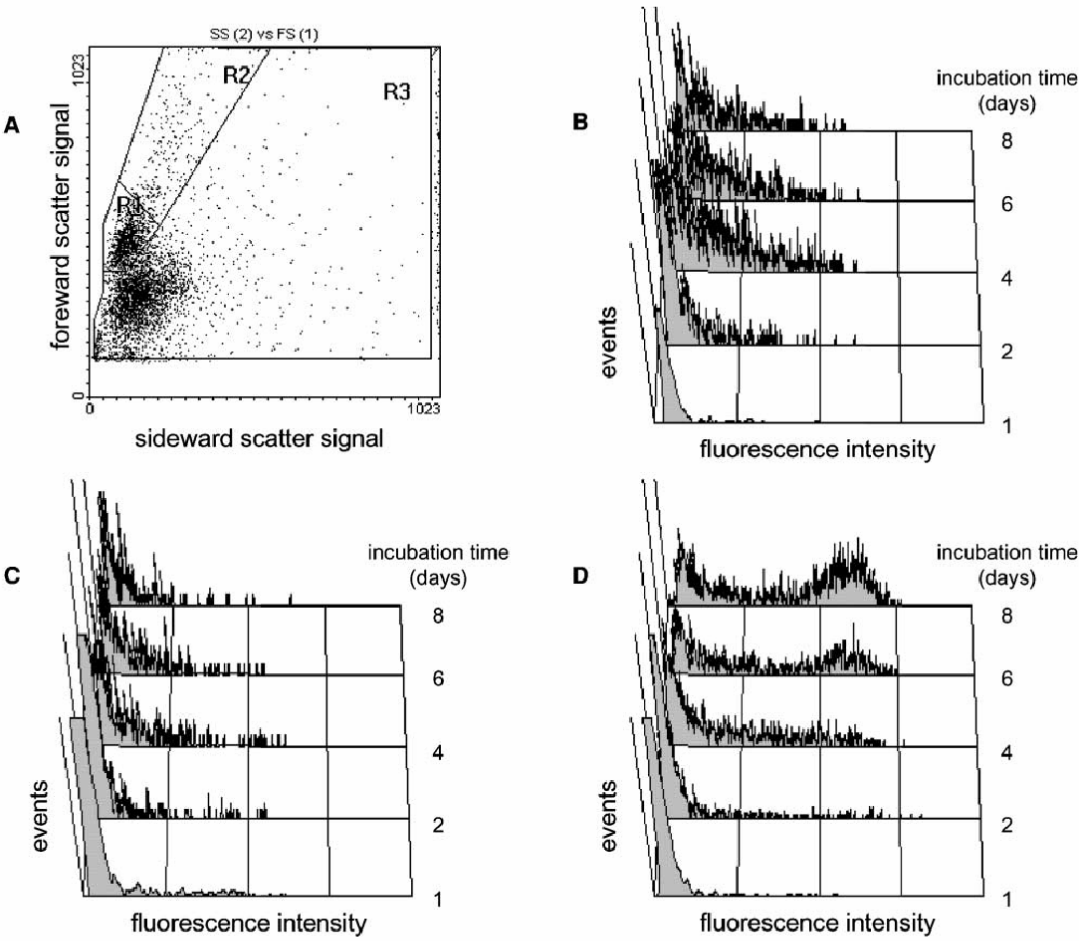

Fig. 3. Representative flow cytometer histograms illustrating CD77 expression pattern by bovine PBMC. PBMC of a 3-year-old cow were incubated in the presence of PHA-P (5 μg/ml) and aliquots of native cells were stained with anti-CD77 mAb and FITC-labeled secondary antibody at the time points indicated. Due to their light scatter characteristics (A PBMC stimulated for 24 h are shown for example, i.e. before significant enlargement of the cells takes place) viable non-blast cells (marked as R1) could be distinguished from viable blast cells (R2) and subvital cells (R3) and separately analyzed for their fluorescence intensity (B viable blast cells, C viable non-blast cells, D subvital cells). The *x*-axes in B, C, and D are scaled logarithmically. Histograms are representative of six-fold determinations performed with cells from one animal.

$CD77^+$ subvital cells constantly rose over the entire cultivation period but the percentage of $CD77^+$ blast cells reached its maximum at days 3–4. Non-blast cells expressed CD77 only in PHA-P (Fig. 2), ConA or PWM (data not shown) stimulated cultures, but neither in the absence of mitogenic stimulation nor in the presence of LPS.

The populations of blast cells, non-blast cells, and subvital cells also differed in the number of CD77 molecules presented on the cellular surface (Fig. 3). Blast cells exhibited a wide range of fluorescence intensities for CD77 detection over the entire cultivation period. Similar expression patterns were found for non-blast cells, although the fluorescence intensities were lower. Contrarily, subvital cells exhibited very high fluorescence intensities from the beginning and $CD77^{high}$ subvital cells accumulated towards the end of

the cultivation period. During analysis of the data presented in this publication dead cells characterized by their ability to take up propidium iodide were excluded to avoid the detection of unspecific binding of anti-CD77 mAb and secondary antibody. Nevertheless, isotype controls confirmed that specific binding of anti-CD77 mAb was also detectable on propidium iodide positive cells. Total anti-CD77 mAb binding was almost equal to propidium iodide positive and negative cells indicating the lack of correlation between mAb binding and cellular death.

3.3. CD77 expression by bovine PBMC subpopulations in vitro and in vivo

As we described recently, Stx1 affects activation and proliferation of different bovine lymphocyte subpopulations to various extents (Menge et al., 1999b). To examine whether these differences in subpopulations' susceptibility are reflected by differences in CD77 expression we performed double labeling experiments. At day 4 after initiation of PHA-P stimulated cultures the CD77 antigen was detected on the surface of all subpopulations identified: TCR1 cells (as defined by the BoWC1 antigen; data not shown), BoCD4$^+$ cells, BoCD8$^+$ cells, B-cells, and even monocytes (Fig. 4). However, the pattern of CD77 expression differed. While the ratio of CD77$^+$ to CD77$^-$ cells was 7.7 for BoCD8$^+$ cells — mainly affected by Stx1 in PHA-P stimulated cultures — it was 4.1 and 3.3 for BoCD21$^+$

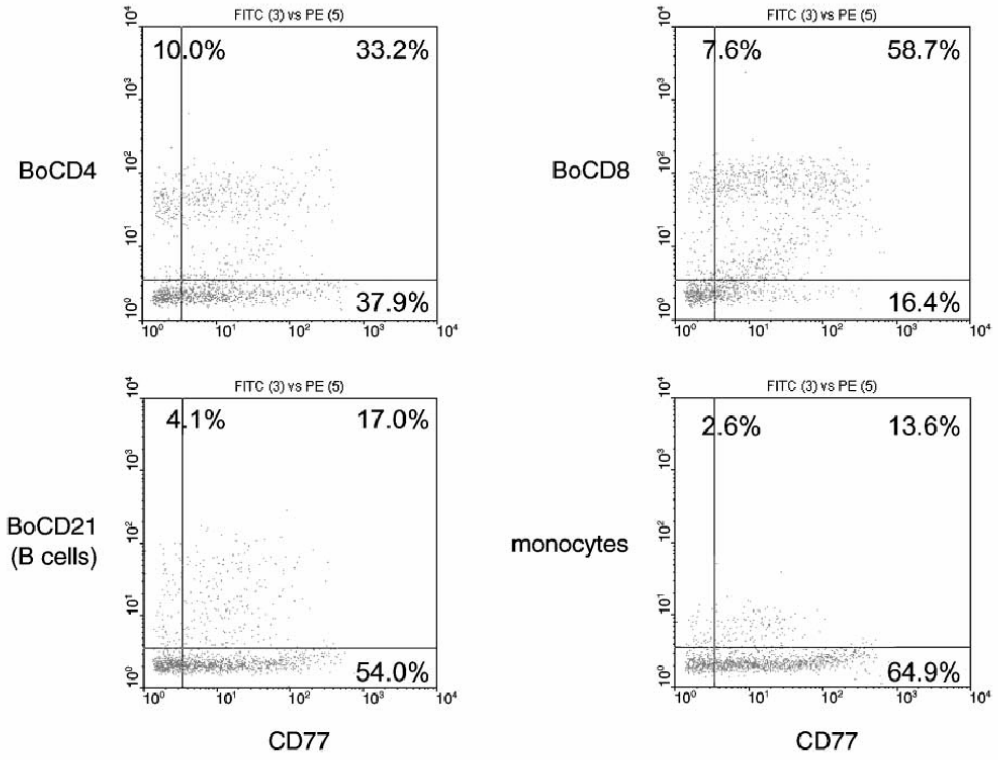

Fig. 4. Flow cytometer dot plots illustrating CD77 expression by bovine PBMC subpopulations in vitro. PBMC of a 3-year-old cow were incubated in the presence of PHA-P (5 μg/ml). On day 4 of the incubation period, native cells were double-labeled with anti-CD77 and subpopulation identifying mAbs. Dot plots depict cells of the blast cell population from a single experiment.

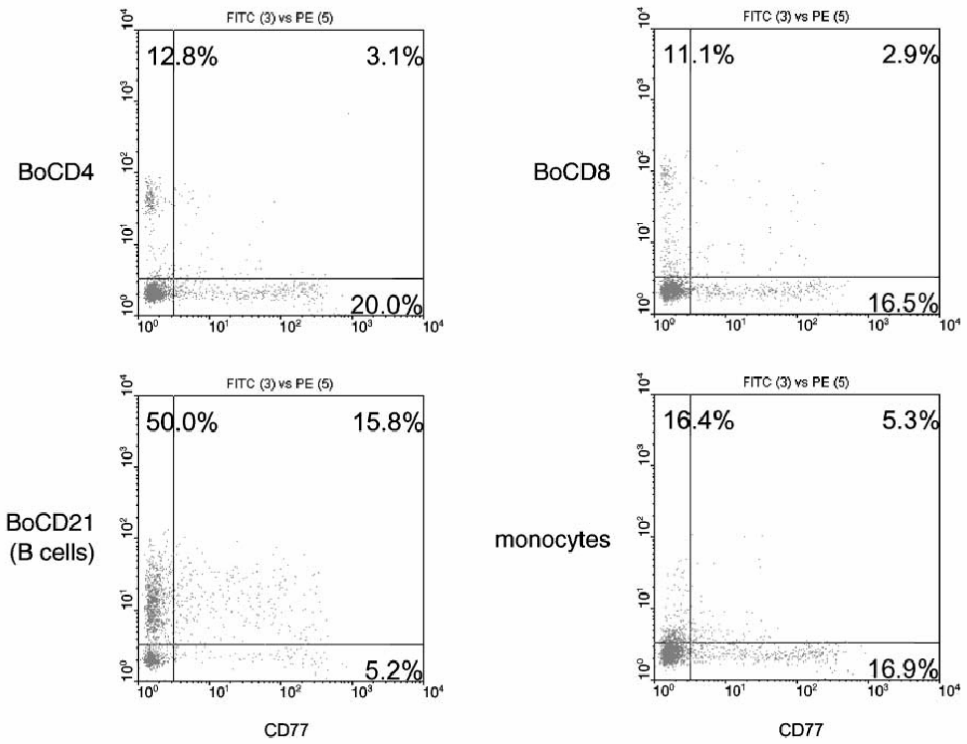

Fig. 5. Flow cytometer dot plots illustrating CD77 expression by bovine lymphocyte subpopulations in vivo. Lymphocytes freshly obtained from *Lnn. colici* of a calf were double-stained with anti-CD77 and subpopulation identifying mAbs. Dot plots depict viable cells from a single experiment.

cells and BoCD4$^+$ cells, respectively. Likewise, the fluorescence intensity for the CD77 detection was highest for BoCD8$^+$ cells. Similar results were seen with cells incubated without mitogen or in the presence of ConA, PWM, and LPS (data not shown). To address the question whether CD77 expression is restricted to in vitro conditions or also takes place in vivo, we phenotyped lymphocytes freshly derived from the colonic lymphnodes of a calf. As it is shown in Fig. 5 about one quarter of B-cells highly expressed the CD77 antigen. Although present in lower numbers as within PBMC, CD77 expression could also be detected on about one-fifth of BoCD4$^+$ and BoCD8$^+$ cells.

3.4. Biochemical analysis of glycolipids from unstimulated and stimulated bovine PBMC

Neutral and acidic glycolipids were isolated from 500 million cells of freshly prepared PBMC (day 0) and PBMC stimulated with PHA-P (5 μg/ml) at day 4 after initiation of the culture, respectively. Glycolipids were visualized in HPTLC by orcinol/H_2SO_4-chemical staining (Fig. 6). PBMC of day 0 had much less neutral glycolipids than those of day 4. The most striking difference was in the amount of ceramide trihexoside (CTH), which had strongly increased from days 0 to 4. To a lesser extent, this was also the case for ceramide monohexoside (CMH). The acidic fractions of days 0 and 4 exhibited related band patterns and were not further analyzed.

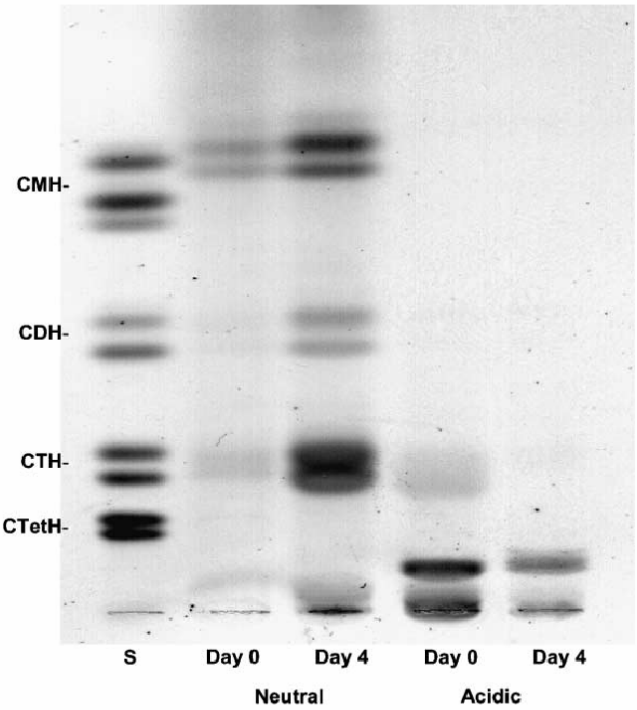

Fig. 6. HPTLC of neutral and acidic fraction glycolipids obtained from bovine PBMC at days 0 and 4 of the incubation period. The amount of PBMC glycolipids per lane were obtained from 2 mg PBMC dry-weight each, which were calculated to correspond to approximately 50 million cells for day 0 and 25 million cells for day 4, respectively. Glycolipids were resolved using the running solvent chloroform:methanol:water, 65:25:4 (by volume) and visualized by orcinol/H_2SO_4-staining. The standard (S) of CMH–CTetH corresponded to globo-series ceramide mono-, di-, tri- and tetrahexosides, respectively.

MALDI-TOF-MS of day 0 neutral fraction glycolipids revealed mainly CMH (*m/z* 722.9, 806.9, 834.7; Fig. 7A), with small amounts of ceramide dihexoside (CDH; *m/z* 884.5) and CTH (*m/z* 1046.7). Day 4 glycolipids revealed a similarly intense signal for CMH (*m/z* 722.8, 806.8, 834.7; Fig. 7B) and CTH (*m/z* 1046.6, 1130.6, 1158.6) and a weaker signal for CDH (*m/z* 884.7), confirming the relative changes observed by HPTLC. By fractionation on a silica-gel column, CMH and CTH were purified for both days 0 and 4 glycolipids and analyzed by MALDI-TOF-MS (Fig. 7C and D) and carbohydrate composition analysis (data not shown). The total carbohydrate amounts derived from 500 million cells each were found to be approximately 650, 450, 1700 and 5000 ng for day 0 CMH, day 0 CTH, day 4 CMH and day 4 CTH, respectively. Calculated from these data, the molar ratios of CMH to CTH were 4:1 at day 0 and 1:1 at day 4. While CMH contained only Glc, CTH contained Gal and Glc in a ratio of approximately 2:1. On-target α-galactosidase treatment of day 4 CTH followed by MALDI-TOF-MS revealed a shift of 162 Da for the CTH signals, indicating the loss of one Gal (Fig. 7E). Methylation analysis of day 4 CTH yielded terminal Gal (2,3,4,6-GalOH), 4-substituted Gal (2,3,6-GalOH) and 4-substituted Glc (2,3,6-GlcOH) in a ratio of 0.8:1.2:1 (Fig. 7F). Together, these data indicated day 4 CTH to be Gal(α1-4)Gal(1-4)Glc(1-1)Cer (globotriaosylceramide; Gb_3).

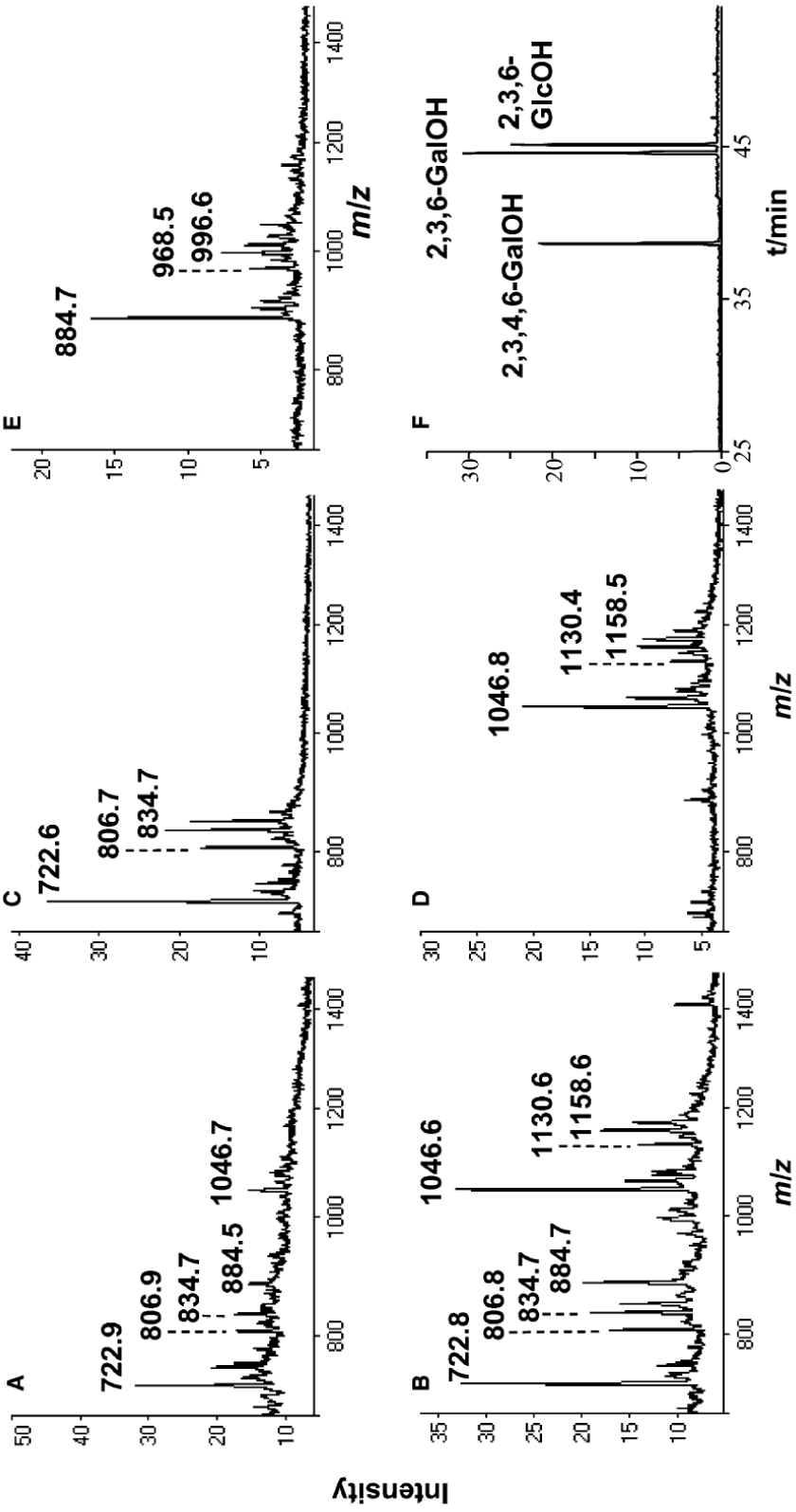

Fig. 7. MALDI-TOF-MS and linkage analysis of PBMC neutral glycolipids. Neutral glycolipid fraction of day 0 (A) and day 4 (B), purified CMH of day 4 (C) as well as CTH of day 4 before (D) and after (E) on target α-galactosidase treatment were subjected to MALDI-TOF-MS in the positive-ion reflection mode. Major pseudomolecular masses $[M + Na]^+$ are given by numbers within the respective graph. (F) Linkage analysis of day 4 CTH. Partially methylated alditol acetates were detected by GC/MS after chemical ionization using ammonia; 2,3,4,6-GalOH or 2,3,4,6-tetra-*O*-methylgalactitol as alditol acetate corresponded to terminal Gal; 2,3,6-GalOH (or GlcOH) or 2,3,6-tri-*O*-methylgalactitol (or glucitol) as alditol acetate corresponded to 4-substituted Gal (or Glc).

Table 1
Fatty acid analysis of PBMC CMH and CTH[a]

Fatty acid methyl esters	Relative amount (%)			
	CMH (day 0)	CTH (day 0)	CMH (day 4)	CTH (day 4)
C16:0	**59**	**79**	**72**	**43**
C17:0	2	2	2	3
C18*	*	*	*	*
C20:0	5	3	2	3
C22:0	**15**	**7**	**8**	**19**
C23:0	–	–	–	1
C24:1	3	–	4	4
C24:0	**16**	**9**	**12**	**27**

[a] Fatty acid methyl esters were analyzed by capillary GC/MS after electron-impact ionization. Relative amounts are based on peak ratios of individual fatty acid derivatives normalized to 100%. Bold type has been used to mark major components. C16:0, saturated fatty acid with 16 carbon atoms, C24:1, monounsaturated fatty acid with 24 carbon atoms, etc. and (*) indicates C18:0, C18:1 and C18:2 contaminations.

Days 0 and 4 CTH were probed in ELISA with the anti-CD77 mAb (data not shown). For both time points, the glycolipid amounts applied per well corresponded to 700,000 cells. Both CTH fractions were recognized by the mAb, but the signal for day 4 CTH was stronger than that for day 0 CTH, which was in accordance with the small amounts of CTH found in day 0 PBMC, which further confirms the biochemical identity of the CD77 antigen detected on bovine lymphocytes' surfaces with Gb_3.

Sphingoid base analysis of pentafluoropropionic acid derivatised species by GC/MS in the positive-ion mode following electron-impact ionization allowed C18-sphingosine to be detected as the only sphingoid base for day 4 CMH, day 4 CTH and day 0 CMH (data not shown). A characteristic ion at *m/z* 409 was observed, which corresponded to a C18-sphingosine with only one pentafluoropropionic acid moiety remaining. The fragmentation pattern was analogous to that observed for a C18-sphingosine standard and similar to that described by Zanetta et al. (1999) for sphingosines derivatized with heptafluorobutyric acid.

Fatty acids were analyzed by GC/MS using electron-impact ionization. C16:0 was found to be the dominant species for all glycosphingolipids (GSLs) analyzed (Table 1), which corresponds to the MALDI-TOF-MS signals at 722 and 1046 Da for CMH and CTH, respectively (Fig. 7A–D). In addition, C22:0 and C24:0 were observed as major species and could be correlated with MALDI-TOF-MS signals at *m/z* 806 and 834 Da for CMH and at *m/z* 1130 and 1158 Da for CTH, respectively. Despite these similarities, day 4 CTH molecular species were unique in a way that they comprised increased amounts of fatty acids with more than 20 carbon atoms.

4. Discussion

So far only some bovine differentiation marker homologues could be identified testing monoclonal antibodies (mAbs) against human leukocyte antigens for crossreactivity (Sopp

and Howard, 1997). However, a comparison of mammalian proteins has demonstrated highest divergence among the ligands and receptors involved in host defense mechanisms. Consequently, only a relatively low proportion of mAbs to human protein antigens presumably recognizes homologous ruminant molecules (Naessens et al., 1997). In contrast, GSLs are much less species-specific, but are sufficiently restricted in their cellular expression to be considered as differentiation markers (Schwarting, 1980). Showing binding of the commercially available mAb raised against the human B-cell antigen CD77 (Wiels et al., 1981) to bovine cells and confirming the biochemical identity of the respective antigen to Gb_3, to the best of our knowledge this has been the first GSL antigen introduced in bovine immunology. Apart from the improvement that the use of this antigen will contribute to our understanding of immune responses in cattle, it can be assumed that the preferential screening of mAbs directed against GSLs for crossreactivity will easily enlarge the number of activation markers available to study the immune system of so far ill defined species.

It is interesting to note, that the CD77 antigen is not restricted to B-cells but has a very broad cellular distribution among bovine lymphocyte subsets. In humans CD77 expression was originally associated with oncogenic transformation in Burkitt lymphomas (Wiels et al., 1981), and subsequently found to be restricted to 10–15% of tonsillar B-lymphocytes in healthy individuals and thus defines a subpopulation of germinal center cells (Mangeney et al., 1991). $CD77^+$ cells express sIgM but not sIgD and are positive for CD10, CD38, LFA-1, LFA-3, and CD44 and for a series of B-cell markers such as CD19, CD20, CD21, CD22, and CD40 (Mangeney et al., 1991, 1993). A number of pre-B-, T-lymphoid and myeloid cell lines were found to be $Gb_3/CD77^-$ (Cohen et al., 1990; Mangeney et al., 1993), but Ramegowda and Tesh (1996) confirmed results of Kniep et al. (1985), who chemically detected Gb_3 within myeloid cell lines at late stages of differentiation, and showed that these cells are able to bind Stx1 on their surface. CD77 expression by T-cells thus appears to be a unique feature of bovine lymphocytes but due to the broad cellular distribution the benefit offered by the use of anti-CD77 mAbs in the evaluation of the bovine immune system is predominantly given by the opportunity to define certain differentiation stages rather than particular lymphocyte lineages.

The data presented here clearly show that $Gb_3/CD77$ is synthesized and surface expressed by bovine lymphocytes in vitro particularly after stimulation. Thereby, the portion of $CD77^+$ cells as well as the average number of CD77 molecules per cell correlated with the activation state. These observations led us to hypothesize that quiescent lymphocytes forming the vital non-blast population are $CD77^-$ or $CD77^{low}$ at the beginning of the cultivation period. Upon activation and transformation to blast cells CD77 expression increased resulting in low to moderate expression pattern by up to 60% of the blast cells 3–4 days after initiation. In 6–8-day-old cultures, $CD77^{moderate}$ cells disappeared from the blast cell population with a subsequent cumulation of $CD77^{high}$ cells within the subvital population. Such a correlation of $CD77^{high}$ expression and subvital cell morphology was also seen with BL-3 cells. These observations coincide with a CD77 expression paralleling the activation of bovine lymphocytes up to a certain stage characterized by moderate CD77 expression. At this point the cells either survive and probably down-regulate CD77 again. Alternatively, they die from apoptosis — coming along with decreased cell size and increased granularity moving the cells to the subvital cytometer

gate — and express high levels of CD77. These patterns of CD77 expression thereby markedly resemble findings for human lymphocytes. Human $CD77^+$ B-cells have a phenotype similar to activated cells but are negative for the classical activation antigens CD23, CD25, and CD71 (Mangeney et al., 1991). Moreover, $CD77^+$ cells exhibit DNA fragmentation and morphological features of apoptosis when cultured in vitro (Mangeney et al., 1991). Although bovine $CD77^+$ lymphocytes partially express CD25 (data not shown) and the correlation of CD77 to other markers of apoptosis needs to be confirmed, this model perfectly corresponds to our findings with bovine lymphocytes stimulated in vitro as well as lymphnode cells that have experienced their stimulation in vivo.

Further studies on the functional relevance of our findings are facilitated by the availability of a well defined Gb_3/CD77 ligand — the receptor binding B subunit of Stx1. Our present knowledge of the Gb_3/CD77 functions was driven by studies on the impact of Stx1 on the human immune response, which found Gb_3/CD77 to interact with two other membrane molecules: (1) Gb_3/CD77 associates with the interferon type I receptor which is a prerequisite for high affinity interferon binding (Ghislain et al., 1994); and (2) it associates with CD19 thus facilitating appropriate CD19 folding (Maloney and Lingwood, 1994). In addition, an unexpected but probably most interesting feature of Gb_3/CD77 was discovered when it was found to transduce extracellular signals: binding of the Stx1B subunit rapidly induces apoptosis in $Gb_3/CD77^+$ Burkitt lymphoma cells (Mangeney et al., 1993). Accordingly, we recently described induction of apoptosis by Stx1 in the bovine B-lymphoma cell line BL-3 (Menge et al., 1999b), pointing to a Gb_3/CD77 linked signal transduction pathway in bovine cells. However, when bovine PBMC cultures were treated with Stx1, the toxin induced a change in the cellular composition — mainly within the blast cell population — without a detectable increase in apoptotic or necrotic cell numbers. Thus, we assume that Gb_3/CD77 is not linked to apoptotic signaling pathways in normal bovine lymphocytes' membranes. An alternate signaling pathway was described very recently by Foster et al. (2000) showing that differentiated human monocytic cells are sensitive to Stx1-mediated TNF-α production via activation of proteinkinase C. Perturbation of cytokine networks as seen with other bacterial products (Wilson et al., 1998) could thus provide a feasible explanation for the inhibition of bovine lymphocyte proliferation. Further studies on this topic also shedding light upon the signal transduction pathways linked to Gb_3/CD77 in the bovine immune system are currently in progress in our laboratory.

Induction of Gb_3/CD77 expression on the surface of bovine lymphocytes obviously depends on several events. Biochemical detection of Gb_3 in lymphocytes at day 0 of the culture as well as the immunological detection of CD77 within fixed and permeabilized BL-3 cells can be explained by a special feature of GSLs named crypticity. Associated with changes in cellular functions, Pudymaitis and Lingwood (1992) reported that, although Gb_3 is chemically present at significant levels, it may not be available at the cell surface for ligand binding. This may be due to the presence of Gb_3/CD77 solely in intracellular membraneous compartments, but glycolipid head groups in general are relatively small and in close proximity to the surface of the cell and may be obscured by intrinsic membrane proteins. Apart from possible changes in the intracellular distribution and the availability of Gb_3/CD77 on the cell surface, stimulation of bovine lymphocytes several fold increased

the amount of CMH and Gb_3. Comparative fatty acid analysis of Gb_3 obtained from days 0 to 4 PBMC revealed that stimulation also altered the relative amounts of the predominant fatty acid derivatives from C16 to fatty acids with more than 20 carbon atoms. These findings implicate that induction of Gb_3/CD77 surface expression on bovine lymphocytes is mainly regulated by sequential activations of the corresponding glycosyltransferases as it has been described for the sequential shifts observed during human B-cell differentiation (Taga et al., 1995).

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6.4 "Protocols to study effects of Shiga toxin on mononuklear leukocytes."

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22

Protocols to Study Effects of Shiga Toxin on Mononuclear Leukocytes

Christian Menge

1. Introduction

Endothelial cells are regarded as the main targets of the Shiga toxins (Stxs) during infections caused by Stx-producing *Escherichia coli* (STEC). However, several investigations also confirmed an effect of these toxins on immune cell functions in species naturally infected with STEC. Human B-cell lines ***(1)*** and tonsillar B-cells ***(2)*** are highly susceptible to the cytotoxic activity of Stx1, which also hampers activation and proliferation of bovine B- and T- cell subpopulations in vitro ***(3)***. Although Stxs appear to be immunosuppressive, they do not prevent the development of a specific antibody response in STEC-infected individuals ***(4–6)***. Thus, the question of an immunosuppressive effect of Stx in the pathogenesis of STEC-mediated diseases needs to be addressed. STEC infections lead to an immunocompromised condition in gnotobiotic pigs and calves ***(7,8)***, which is assumed to contribute to the observed persistency of infection (e.g., in calves and humans) ***(9,10)***.

The investigation of immunomodulation through products of the enteric flora is usually biased by the fact that a variety of those molecules is known to positively or negatively regulate inflammatory responses. Apart from the well-known biological effects of lipopolysaccharide, two factors first decribed for enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium*, but also present in STEC, have been shown to modulate the mucosal immune system. First, lymphostatin, a novel large toxin from EPEC, has been shown to specifically inhibit lymphocyte proliferation and interleukin-2 (IL-2), IL-4, and γ interferon production by murine mucosal lymphocytes ***(11)***. Genes-encoding proteins that are homologous to lymphostatin are present on the large STEC

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plasmid ***(12,13)*** and recent data implicate this STEC protein also in bacterial binding to target cells ***(14)***. Second, the surface protein intimin of EPEC and *C. rodentium* induces a massive T-helper-cell type 1 immune response in the colonic mucosa of mice ***(15)***. Hence, when experiments to study effects of Stx on mononuclear leukocytes are designed, it must be taken into account that STEC lysates most likely contain additional bacterial factors able to interfere with the immune system of the infected host. Thus, these studies greatly rely on the availability of pure toxin preparations. (For a detailed protocol describing the isolation of Stx, the reader is referred to Chapter 15). Only those effects that can be blocked by specific, neutralizing antibodies should be ascribed to Stx. Studies aimed at the detection of Stx receptors on the surface of cells can be performed without purified toxin using CD77-specific antibodies (*see* below).

Mononuclear cells (monocytes and lymphocytes) isolated from peripheral blood (PBMCs) can be obtained easily and repeatedly even from humans without ethical reservations. However, circulating lymphocytes represent just 1–2% of all body lymphocytes and differ significantly from tissue lymphocytes because they lack the interaction with neighboring tissue cells and should thus be regarded as quiescent. A variety of mitogenic stimuli can be included in the experimental design to simulate effects of Stx on activated lymphocytes in the tissue. When PBMCs are incubated in tissue culture plasticware, monocytes tend to adhere tightly within some hours, whereas lymphocytes remain in the medium and can thus easily be submitted to flow cytometry analysis. This analytical approach relies on expensive laboratory equipment, but offers the opportunity to quantitatively determine several parameters in parallel for a large number of single cells within a short time. Thus, most of the protocols presented in this chapter contain a flow cytometry step. However, a protocol for the determination of cellular metabolic activity avoiding flow cytometry is also included. The MTT reduction assay described is a reliable system to detect any decrease in cellular metabolic activity whether it is the result of inhibition of cells or cytotoxicity ***(16)***.

To further discriminate between inhibitory and cytotoxic effects of Stx, loss of cellular membrane integrity can be measured after propidium iodide staining. However, cells dying from apoptosis lose their membrane integrity at a very late stage of the cell death process, and quantification of dead cells by propidium iodide uptake may be insufficient. To monitor apoptotic effects of Stx on lymphocytes, cellular DNA fragmentation can be determined on a daily base using a commercially available kit. Whether or not Stx cause apoptosis in lymphoid cells is a matter of discussion. Human and bovine B-cell lines have been reported to be highly sensitive to the apoptotic effect of Stx1, whereas the toxin inhibits activation and proliferation of primary cultures of bovine lymphocytes without inducing cellular death ***(1,3)***. Analysis of blast cell transfor-

mation ratio and blast cell composition by the protocols described herein are highly sensitive methods for demonstrating effects of Stx on immune cells independently of the underlying mechanism, cytotoxicity, or inhibition.

Most of the diverse biological effects of Stx reported so far are mediated via its binding to the specific cell surface receptors globotriaosylceramide (Gb_3/CD77) and globotetraosylceramide (Gb_4) ***(17,18)***. Most of the Stx variants, except Stx2e, preferentially bind to and act via Gb_3/CD77. Because a monoclonal CD77-specific antibody ***(19)*** is commercially available, immunological detection of this antigen on cell surfaces is a feasible way to identify presumably Stx-sensitive cell populations. However, biochemically diverse Gb_3/CD77 isoforms with varying affinities for Stx have been reported and solely binding studies with Stx holotoxin or the receptor binding B-subunit will confirm whether detected CD77 antigens truly serve as Stx receptors.

2. Materials

1. Disposable plastics: V-shaped centrifugation tubes, 50 mL (Greiner); flat-bottom (Nunc) and V-shaped (Greiner) 96-well microtiter plates; reaction vials, 75 × 12 mm, round bottom (Renner).
2. Ficoll–Paque (Amersham Pharmacia Biotech).
3. Mitogens (e.g., concanavalin A [ConA], phytohemagglutinin P [PHA-P], pokeweed mitogen [PWM], or lipopolysaccharide [LPS]; Sigma).
4. In Situ Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals).
5. Monoclonal antibody against CD77 (clone 38.13, rat IgM; Coulter Immunotech Diagnostics) and leukocyte antigen-specific antibodies suitable for the species of interest and matching anti-immunoglobulin fluorescein–isothiocyanate (FITC) or R-Phycoerythrin (R-PE) conjugates.
6. Purified Stx tested for the absence of endotoxin and neutralizing monoclonal antibody against the respective type of Stx; Stx B-subunit and a monoclonal B-subunit-specific antibody (e.g., mouse monoclonal anti-StxB1 13C4, ATCC cat. no. CRL 1794)
7. Na–citrate solution (3.8% w/v), pH 7.0.
8. Phosphate-buffered saline (PBS): 10.0 g NaCl, 0.25 g KCl, 0.25 g KH_2PO_4, and 1.8 g $Na_2HPO_4 \cdot 2\ H_2O$ per liter of distilled water, pH 7.4.
9. PBS supplemented with EDTA (PBS-EDTA): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.42 g $Na_2HPO_4 \cdot 2\ H_2O$, and 2.0 g Na-EDTA per liter of distilled water, pH 7.4.
10. PBS supplemented with 1% bovine serum albumin fraction V (PBS-BSA; Serva).
11. Lysis buffer: 8.26 g NH_4Cl, 1.09 g $NaHCO_3$, 0.037 g Na-EDTA per liter of distilled water.
12. Modified cell culture medium: RPMI 1640 (Biochrom) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and 3 µ*M* of 2-mercaptoethanol (Sigma).
13. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide, Sigma) stock solution (5 mg/mL in PBS): Freshly dissolved and filter sterilized through

0.2-µm-pore filter. The stock solution should be immediately aliquoted and frozen (–20°C) and is stable for months.
14. Sodium dodecyl sulfate (SDS)-solution: 10% (w/v) SDS, 0.01 *N* HCl in distilled water.
15. Propidium iodide (PI, Sigma) stock solution (100 µg/mL in PBS): keep at 4°C in the dark, stable for years.
16. Formaldehyde solution (4% [w/v] in PBS, pH 7.4); prepare freshly for each assay (*see* **Note 1**).
17. Permeabilizing solution: 0.1% (w/v) Triton X-100 in 0.1% (w/v) Na–citrate; keep at 4°C, stable for days.
18. Multichannel pipets (10–100 µL).
19. Standard cell culture laboratory equipment including laminar-flow bench, CO_2 incubator, and inverse microscope; centrifuge suitable for 50-mL tubes and microtiter plates, with a cooling system and disengageable brake.
20. Incubator set at 37°C equipped with a rocking table adjustable to about 40 rpm.
21. Enzyme-linked immunosorbent assay (ELISA) reader equipped with a filter set to read the optical density at 540 nm and 680 nm.
22. Flow cytometer equipped with an argon laser and standard filter configuration for FITC, R-PE, and PI (525, 575, and 630 nm, respectively).

3. Methods

3.1. Analyzing the Effect of Stx on Lymphocyte Viability

3.1.1. Preparation of Mononuclear Cells

1. Dilute 20 mL of citrated blood (4 mL Na–citrate solution plus 16 mL venous blood) with 17 mL PBS-EDTA (*see* **Note 2**) and layer it carefully onto 12 mL Ficoll–Paque® in a separate centrifugation tube (*see* **Fig. 1**). Avoid pertubation of the gradient.
2. Centrifuge (800*g*, 40 min, 20°C) without break.
3. Carefully recover the pale gray cell layer containing monocytes and lymphocytes, referred to as peripheral blood mononuclear cells (PBMCs) from the Ficoll–buffer interface. Avoid aspirating the erythrocyte sediment. Dilute the suspension thoroughly with 40 mL PBS-EDTA (*see* **Note 3**).
4. Centrifuge: 250*g*, 7 min, 4°C.
5. Discard the supernatant until the soft pellet leaks out and dilute the remaining suspension with 3 vol of lysis buffer to lyse contaminating erythrocytes; incubate for 5 min at room temperature.
6. Wash cells once with PBS-EDTA and then with PBS and adjust to 5×10^6 cells/mL in modified cell culture medium. In stimulation assays, supplement medium with ConA (at a final concentration of 5 µg/mL), PHA-P (5 µg/mL), PWM (10 µg/mL), or LPS (25 µg/mL) (*see* **Note 4**).
7. Transfer 50 µL of the cell suspension to 96-well flat-bottomed microtiter plates prepared as described in **Subheading 3.1.2.**

3.1.2. Preparation of Shiga Toxin Dilution Series on Microtiter Plates

1. To prepare 50 µL of 10-fold dilution series of toxin preparations in microtiter plates dispense 90 µL of 0.15 *M* NaCl per well, leaving the first and the last columns empty.

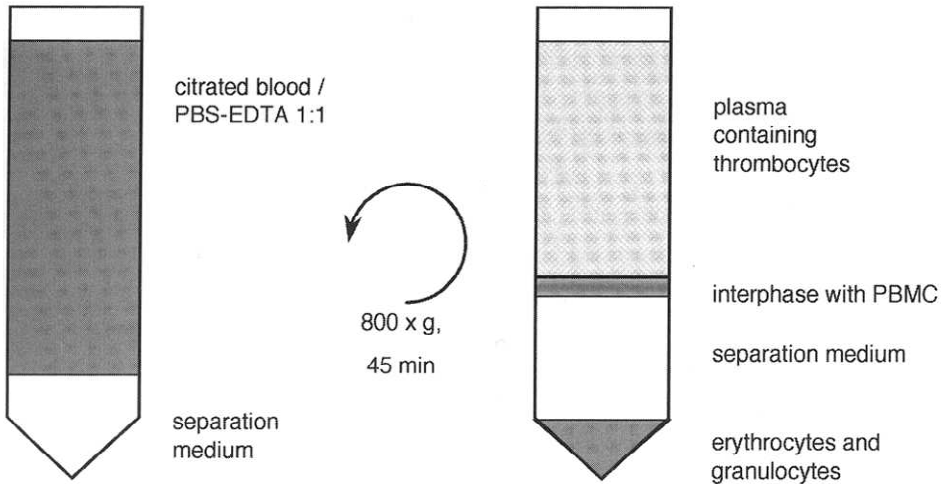

Fig. 1. Schematic drawing of the preparation of bovine PBMCs by density gradient centrifugation.

2. Add 60 µL of the toxin preparation to each well of the first column (*see* **Note 5**).
3. Transfer 10 µL from the first column to the second column and mix. Proceed in the same manner up to the second last column of the microtiter plate. Carefully blow out the pipet tips after each step to prevent excess carryover.
4. Remove 40 µL from each well-except the first and the last columns starting with the lowest dilution.
5. Add 50 µL of 0.15 *M* NaCl and 50 µL of 1% (w/v) SDS in 0.15 *M* NaCl as negative and positive controls, respectively, into separate wells of the last column.
6. Add 50 µL of modified cell culture medium to all wells. In neutralization studies, this medium component can be supplemented with neutralizing Stx-specific antibodies (*see* **Note 5**). In this case, the plates should be incubated for 30–60 min at room temperature before proceeding to **step 7**.
7. Apply 50 µL of cell suspension (5×10^6 cells/mL of cell culture medium) to each well; incubate at 37°C in 5% CO_2.

3.1.3. Measurement of Cellular Metabolic Activity

1. After 2–6 d, add 25 µL of MTT stock solution to each well of the microtiter plates. Place plates on a shaker and move gently for 4 h at 37°C.
2. Stop the reaction and dissolve dye crystals by adding 100 µL SDS solution to each well (*see* **Note 6**).
3. After overnight incubation, read optical density (OD) with an ELISA reader using a test wavelength of 540 nm and a reference wavelength of 690 nm.
4. Calculate percent cellular metabolic activity by the formula: [OD (sample) – OD (positive control)]/[OD (negative control) – OD (positive control)] × 100 (*see* **Fig. 2**).

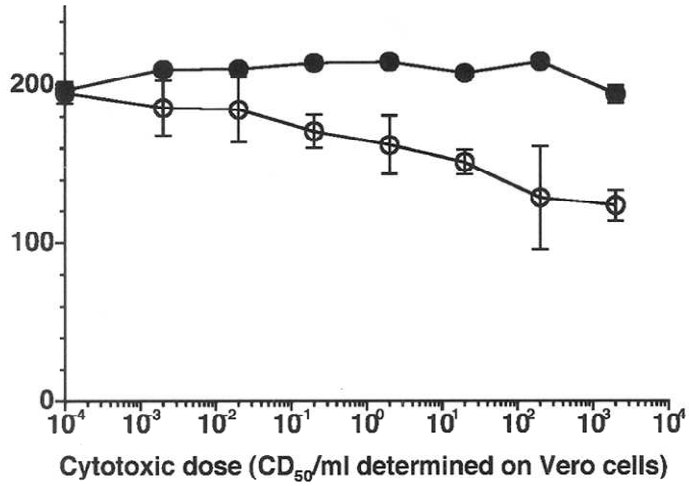

Fig. 2. Effect of purified Stx1 on the cellular metabolic activity of bovine PBMCs. Cells were incubated with 10-fold dilutions of purified Stx1 (0.002 to 2.000 CD_{50}/mL; quantified on Vero cells) for 96 h at 37°C. Culture medium was supplemented with 5 µg/mL PHA-P. Observed effects were assigned to Stx1 by comparison of the results obtained in the absence (open circles) or presence (filled circles) of 1.5 µg/mL monoclonal anti-StxB1 13C4 antibody. Cellular metabolic activity was determined by MTT reduction assay. Cells incubated with medium containing PHA-P alone were used as negative control, whereas cells treated with 1% (w/v) SDS served as a positive control to calculate percent activity. Data are means ± standard deviations of triplicate determinations. (Reprinted from **ref. *3*** with permission.)

3.1.4. Determination of Propidium Iodide Uptake

1. Prepare and incubate the cells as described in **Subheadings 3.1.1.** and **3.1.2.**, but without SDS-treated controls. Daily monitor the cultures for the appearance of dead cells by quantifying the portion of cells able to take up PI.
2. Resuspend cells in the wells of a microtiter plate thoroughly (*see* **Note 7**), transfer them into reaction tubes suitable for the flow cytometer, and add 200 µL of PBS containing 2 µg/mL of PI. Include a blank control in each test series that is resuspended in PBS without PI (*see* **Note 8**).
3. Submit the samples to flow cytometry. Acquire 5000–10,000 events that are presumably leukocytes because of their light-scatter characteristics (*see* **Subheading 3.2.1.**). Plot the events in a one-parameter histogram depicting the PI fluorescence (630-nm filter) vs cell counts. Set an electronic threshold that defines less than 2% of the cells in the blank control to be PI positive.
4. Compare values of samples that received Stx with values of control samples (*see* **Note 9**).

3.1.5. Quantification of DNA Strand Breaks (TUNEL Method)

1. Prepare and incubate the cells as described in **Subheadings 3.1.1.** and **3.1.2.** and monitor them at different time-points for the appearence of DNA strand breaks (*see* **Note 10**). Resuspend cells thoroughly and transfer them to a V-shaped microtiter plate (*see* **Note 7**). To compensate for the loss of cells during the following manipulations, pool two identical samples for each determination.
2. Centrifuge plates (300*g*, 10 min, 4°C) and remove supernatants by inverted flicking of the plate (*see* **Note 11**).
3. Resuspend pellets in 150 µL of PBS-BSA (all volumes throughout this protocol are meant per well), centrifuge, remove supernatants by inverted flicking of the plate, and repeat this washing step once.
4. Add 50 µL of PBS-BSA and 50 µL of formaldehyde solution and resuspend the cells.
5. Following 30 min at room temperature, wash the cells once (*see* **step 3**).
6. Resuspend cells in 50 µL of permeabilizing solution (*see* **Note 11**).
7. Incubate for 2 min at 4°C and wash cells twice (*see* **step 3**).
8. Add 20 µL of the reaction mixture from the In Situ Cell Death Detection Kit, Fluorescein (containing FITC-labeled dUTP and terminal desoxynucleotidyl transferase, prepared according to the instructions of the provider) and mix well.
9. Add 20 µL of the nucleotide solution to a separate sample included as blank control and resuspend.
10. Incubate for 1 h at 37°C in 5% CO_2.
11. Centrifuge and wash cells twice (*see* **step 3**).
12. Optional: Proceed to **Subheading 3.2.2.** or **3.3.1.** for immunostaining (*see* **Note 12**).
13. Transfer the cells into reaction tubes suitable for the flow cytometer and add 200 µL of PBS.
14. Perform flow cytometry analysis and acquire 5000–10,000 events that are presumably leukocytes because of their light-scatter characteristics (*see* **Subheading 3.2.1.** and **Note 13**). Plot the events in a one-parameter histogram depicting the FITC fluorescence (525-nm filter) vs cell counts. Set an electronic threshold that defines less than 2% of the cells in the blank control to be FITC positive (*see* **Fig. 3**).
15. Compare values of samples that received Stx with values of control samples (*see* **Note 14**).

3.2. Analyzing the Effect of Stx on Lymphocyte Transformation and Proliferation

3.2.1. Analysis of Cell Morphology

1. Cells prepared as described in **Subheading 3.1.4.** can additionally be analyzed by flow cytometry recording detailed light-scatter characteristics of the cells.
2. To determine appropriate gates for the differentation of morphologically different PBMCs, analyze freshly isolated cells by flow cytometry (5000–10,000 events). In the forward versus sideward scatter histogram (scattergram), viable lymphocytes should appear as a population of medium size and little granularity. Define a gate surrounding this population and name it (e.g., "viable nonblast cells") (*see* **Fig. 4**).

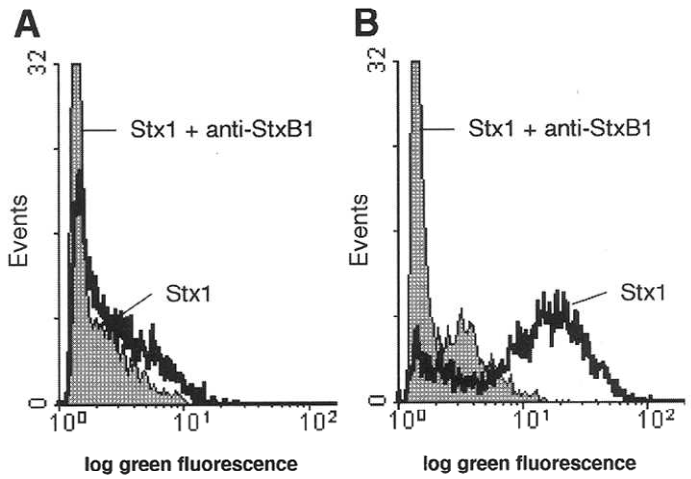

Fig. 3. Flow cytometric histograms illustrating the induction of DNA strand breaks in BL-3 cells (a bovine B lymphoma cell line) by Stx1. Cells were treated with 200 CD_{50}/mL (quantified on Vero cells) for 96 h at 37°C. Culture medium was free of mitogens (**A**) or supplemented with 25 µg/mL LPS (**B**). After incubation, DNA strand breaks were labeled by the TUNEL method. Observed effects were assigned to Stx1 by comparison of the results obtained in the absence or presence of 1.5 µg/mL monoclonal anti-StxB1 13C4 antibody as indicated. (Reprinted from **ref. *3*** with permission.)

3. In the same way, analyze PBMCs incubated for more than 2 d in the absence of mitogens and Stx. As a result of necrosis and apoptosis in primary cultures of PBMCs, a second population of cells should appear in the scattergram characterized by a smaller size and a somewhat increased granularity. Define a gate surrounding this population and name it (e.g., "subvital cells").
4. Analyze PBMCs incubated for more than 2 d in the presence of a potent mitogen (e.g., PHA-P). Because of the mitogen-induced transformation of quiescent lymphocytes to enlarged and polygonal blast cells, a third population of cells should appear in the scattergram, characterized by a prominent increase in cell size and a small increase in granularity. Define a gate surrounding this population and name it (e.g., "viable blast cells") (*see* **Note 15**).
5. Incubate PBMCs in the presence or absence of Stx and analyze daily according to the protocol described above. Perform flow cytometry analysis acquiring 5000–10,000 events from each sample. Exclude cells from further analyis that are PI positive. Plot a scattergram for the viable cells only. Calculate the ratio of blast cell transformation by dividing the number of viable blast cells by the number of viable nonblast cells.
6. Compare values obtained for cells that received Stx and those of control samples (*see* **Note 9**).

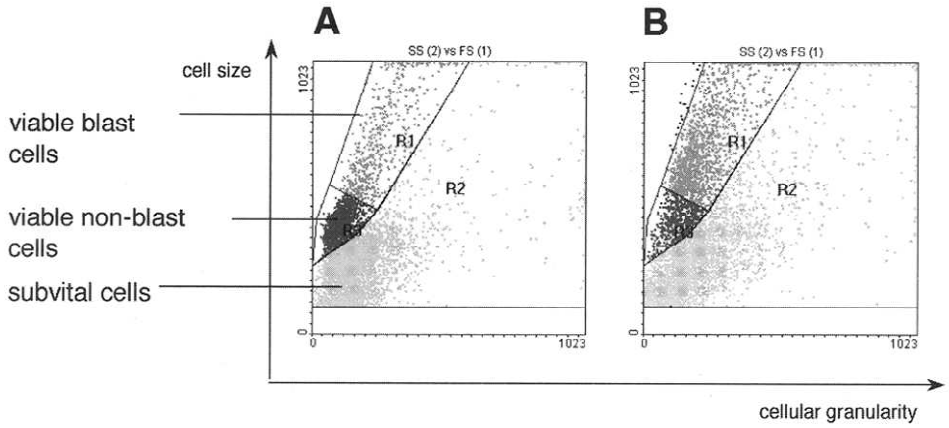

Fig. 4. Cellular morphology of cultured bovine PBMCs as assessed by flow cytometry. PBMCs of a 3-yr-old cow were incubated (96 h, 37°C) in the presence (**B**) or absence (**A**) of phytohemagglutinin P (PHA-P, 5 µg/mL).

3.2.2. Analysis of Blast Cell Composition

1. In order to improve the method described above, examine the effect of Stx on different leukocyte subtypes daily. Resuspend cells thoroughly and transfer them to a V-shaped microtiter plate (*see* **Note 7**). Keep the plate on ice and use pre-cooled (4°C) solutions throughout the entire protocol (*see* **Note 16**).
2. Centrifuge: 150*g*, 10 min, 4°C.
3. Remove supernatants by inverted flicking of the plate.
4. Resuspend pellets in 50 µL of buffer as a blank control or with buffer containing leukocyte subtype-specific antibody (all volumes throughout this protocol are meant per well). If these primary antibodies are already fluorochrome labeled, proceed to **step 8**.
5. Incubate the cells for 20 min on ice, centrifuge, and discard the supernatant.
6. Resuspend the cells in 150 µL of PBS and centrifuge again.
7. Resuspend the cells with 50 µL of a buffer containing a FITC-conjugated antibody recognizing the leukocyte-specific primary antibodies.
8. After 20 min on ice, wash cells twice (*see* **step 6**).
9. Transfer the cells into reaction tubes suitable for the flow cytometer and add 200 µL of PBS containing 2 µg/mL PI (*see* **Note 17**).
10. Perform flow cytometry analysis acquiring 5000–10,000 events from each sample. Define cells of the blast cell population by gating as described in **Subheading 3.2.1.** Exclude cells from further analyis that are PI positive. Create a histogram depicting the FITC fluorescence (525-nm filter) vs cell counts of the viable blast cells only. Set electronic gates according to the blank control included in each test series defining less than 2% of the control cells as positive.

11. Calculate the percentage of viable blast cells that are positive for a certain leukocyte marker among all cells in culture by dividing the absolute number of cells fulfilling all of these three criteria (viable + blast cells + marker positive) by the number of cells acquired in total.
12. Compare values obtained for cells that received Stx and those of control samples (*see* **Note 9**).

3.3. Detection of Stx Receptor Surface Expression

3.3.1. Detection of Gb_3/CD77

1. To examine the surface expression of Gb_3/CD77, freshly isolated PBMCs can be used as well as cells that have been stimulated as described in **Subheadings 3.1.1.** and **3.1.2.**, with the exception that Stx was not included. Using an anti-CD77 antibody and a matching secondary antibody conjugate, the procedure is essentially the same as described in **Subheading 3.2.** To determine the CD77 surface expression by different leukocyte subsets, the procedure described in **Subheading 3.2.2.** can be extended to detect CD77 and certain leukocyte markers simultaneously (*see* **Note 18**).
2. Transfer cells to a V-shaped microtiter plate (*see* **Note 7**). Keep the plate on ice and use precooled (4°C) solutions throughout the entire protocol. Incubate the cells with a leukocyte subtype-specific antibody as described. Spin the cells down once, discard the supernatant to remove the first antibody, and resuspend the pellet in 50 µL of a buffer as a blank control or with buffer containing the CD77-specific monoclonal antibody (all volumes throughout this protocol are meant per well).
3. Incubate on ice for 20 min.
4. Centrifuge (150*g*, 10 min, 4°C), resuspend the cells in 150 µL of PBS, and centrifuge again.
5. Resuspend the cells in 50 µL of a buffer containing a R-PE-labeled antibody recognizing the leukocyte-specific antibody; incubate for 20 min on ice. **Steps 5** and **6** are not necessary if R-PE-labeled leukocyte-specific antibodies are used.
6. Remove the conjugate by centrifugation and discard the supernatant.
7. Resuspend the cells in 50 µL of a buffer containing a rat IgM-specific antibody linked to FITC.
8. Incubate 20 min on ice; wash twice.
9. Transfer cells into reaction tubes suitable for the flow cytometer and add 200 µL of PBS containing 2 µg/mL PI (*see* **Note 17**).
10. Perform flow cytometry analysis acquiring 5000–10,000 events from each sample. Exclude PI-positive cells from further analysis. Create a two-parameter histogram for the viable cells only depicting the FITC (525 nm) vs the R-PE fluorescence (575 nm). Set electronic gates according to the blank controls included in each test series defining less than 2% of the control cells as positive for one or both colors (*see* **Note 19**).

3.3.2. Detection of Surface Toxin Binding

1. As with the detection of Gb_3/CD77 by a CD77-specific antibody, surface Stx-binding experiments can also be performed with both freshly isolated as well as cultivated PBMCs. At the end of the cultivation period, resuspend cells thoroughly (*see* **Note 7**) and transfer them to a V-shaped microtiter plate on ice, centrifuge (150*g*, 10 min, 4°C), and remove supernatants by inverted flicking of the plate. Keep the plate on ice unless otherwise indicated and use precooled (4°C) solutions throughout the entire protocol.
2. Resuspend pellets in 50 µL of buffer as a blank control or with buffer containing Stx or the Stx B-subunit, respectively (*see* **Note 20**).
3. Incubate the cells for 30 min and centrifuge.
4. Resuspend the cells in 150 µL of PBS and centrifuge again.
5. As an option, resuspend the cells in 100 µL PBS and incubate for 1 h at 37°C to induce internalization of the bound toxin. Afterwards centrifuge and discard the supernatant.
6. Resuspend with 50 µL of a buffer containing Stx- or Stx B-subunit-specific antibody, respectively.
7. Repeat **steps 3** and **4**.
8. Resuspend with 50 µL of a buffer containing a FITC-conjugated antibody recognizing the Stx-specific antibody.
9. Following another 30 min, wash the cells twice (*see* **step 4**).
10. Transfer the cells into reaction tubes suitable for the flow cytometer and add 200 µL of PBS containing 2 µg/mL PI (*see* **Note 17**).
11. Perform flow cytometry analysis acquiring 5000–10,000 events from each sample. Exclude PI-positive cells from further analysis. Create histograms containing only viable cells and depicting the FITC fluorescence (525 nm) vs cell counts. Set electronic gates according to the blank control included in each test series defining less than 2% of the control cells as positive.
12. To quantify toxin internalization, compare results of samples warmed up at **step 5** with those that were kept on ice throughout.

4. Notes

1. Formaldehyde is easier to dissolve than paraformaldehyde, which can be used instead, but much less stable; fresh preparation of the solution is thus recommended.
2. Supplementation of PBS with EDTA used as diluent and washing buffer throughout the preparation of bovine PBMCs efficiently prevents clumping of the leukocytes during gradient centrifugation and subsequent washing steps. However, this may not be necessary with leukocytes from other species. Carryover of EDTA into the cell culture medium should be avoided in any case. Thus, at least the last washing step (*see* **Subheading 3.1.1., step 6**) should be carried out with PBS devoid of EDTA.
3. Density of Ficoll–Paque is designed for the isolation of human PBMCs. These cells accumulate on top of the Ficoll layer precisely. In contrast, bovine PBMCs

tend to enter the Ficoll layer upon centrifugation. Therefore, it is convenient to aspirate the pale gray interphase as well as the underlying cloudy Ficoll layer to achieve a good cell recovery.

4. In contrast to several lymphoma cell lines reported to be highly sensitive to the cytotoxic effect of Stx, primary cultures of lymphocytes are affected more gradually. Particularly with bovine PBMCs, an effect of Stx is best seen when the cells are stimulated by mitogens. Because mitogens stimulate PBMCs of different species to different extents, the stimulation protocol should be optimized for cells of the species of interest before including Stx in the experiments.
5. Before examining the effects of Stx on PBMCs in detail, a suitable concentration for both Stx and the neutralizing antibody should be determined by titrating both reagents. With regard to the limited stability of Stx, inclusion of a Vero cell assay in each set of experiments assuring the activity of the used toxin preparation is recommended.
6. Various methods exist to resolve the formazan crystals. In our hands, the method of Tada et al. ***(16)*** adding 10% (w/v) SDS/0.01 *N* HCl is the most reliable technique. The detergent solution is added to the wells without the need to thoroughly remove the medium, which interferes with the dye solubilization in most of the other methods. The addition of HCl changes the color of Phenol Red included in most culture media from red to yellow. A spectral overlap with the purple color of the formazan can thus be avoided. Nevertheless, the plates must be shaken very gently overnight to avoid foaming of the SDS-solution, which then would cause cross-contamination between wells.
7. Transfer of PBMCs cultured in flat-bottom microtiter plates to V-shaped plates and reaction tubes is a prerequisite for immunostaining that requires washing of the cells by centrifugation. Upon cultivation at 37°C, monocytes stick tightly to the surfaces of the flat-bottom wells, whereas lymphocytes do so much less. However, great care must be taken to thoroughly resuspend the cells before transfering them to the V-shaped plates because lymphocytes tend to be trapped in clusters including monocytes, particularly in the presence of mitogens. Microscopic evaluation of the successful transfer is strongly recommended to ensure that almost all lymphocytes are submitted to flow cytometry analysis.
8. In the protocol presented, the nucleic acids staining dye propidium iodide is used to detect cells that lost their membrane integrity, enabling the dye to enter the nucleus. No conclusion can be drawn from this type of experiment about whether stained cells died from necrosis or apoptosis. However, PI can also be used to specifically detect apoptotic cells using a modified protocol that stains the total DNA content of all cells ***(20)***. Cells which have lost apoptotic DNA fragments can then be identified flow cytometrically by their reduced DNA content.
9. Controls should include cells incubated in the absence of Stx as well as cells incubated in the presence of Stx and a defined amount of neutralizing antibody.
10. Usually, apoptosis is a process that takes several hours. However, the best time-point to detect Stx induced apoptosis greatly relies on the time-point the cells experienced the lethal stimulus. This time-point is not necessarily when the cells come into contact with the toxin (i.e., the start of the cultivation period), but

when the cells are able to bind and internalize the toxin. This, in turn, may depend on the induction of the receptor expression. Because of host species variations, the appropriate time-point for analysis may be days after establishment of the culture.

11. This protocol will lead to higher losses of cells during the procedure as compared to the other procedures carried out in microtiter plates (*see* **Subheadings 3.2.** and **3.3.**). To reduce these losses, higher *g*-forces upon centrifugation of the plates (300*g* instead of 150*g*) and supplementation of PBS with 1% (w/v) BSA are strongly recommended. Additionally, keep the incubation time of 2 min when permeabilizing the cells with permeabilizing solution.
12. Performing the TUNEL method in a flow-cytometry-compatible format offers the opportunity to analyze DNA strand breaks in different lymphocyte subsets individually. However, prolonging the protocol by immunostaining will augment the above-mentioned cell losses. Furthermore, binding characteristics of antibodies to fixed and permeabilized cells may be drastically different from those of native cells.
13. In most of the cases, fixation alters the morphological features of cells recorded by flow cytometry. Establishment of cytometer settings suitable for fixed cells before measuring cells subjected to the TUNEL method is thus strongly recommended.
14. Control samples for the establishment of appropriate cytometer settings to quantify DNA strand breaks should include (1) a blank control with PBMCs incubated with labeled nucleotides but without terminal transferase, (2) a positive control with PBMCs incubated with an apoptosis inducing reagent (e.g., 0.15 µ*M* of camptothecin, 1 µ*M* dexamethason, or 0.5 µ*M* of ionomycin), and, optional, (3) a control with cells of a cell line reported to be sensitive to the apoptosis inducing effect of Stx (e.g., Daudi) incubated in the presence of the toxin. When appropriate cytometer settings are found, it will be sufficient to include a blank control in each experiment. The effect of Stx can then be calculated from the comparison of samples as explained in **Note 9**.
15. Freshly isolated PBMCs consist of lymphocytes and monocytes. Because monocytes are larger in size than lymphocytes, these cells exhibit morphological features similar to viable blast cells that appear solely after mitogenic stimulation of PBMCs. On the other hand, monocytes adhere to the plastic surface of the microtiter plates upon cultivation and are barely recovered when the cells are resuspended and transfered to reaction tubes. PBMCs submitted to flow cytometry after incubation in plasticware thus mainly represent lymphocytes. Therefore, cells appearing larger than lymphocytes in the scattergram should be referred to as blast cells if cells had been cultivated previously. In contrast, cells with similar features in the scattergram of freshly isolated PBMCs should be referred to as monocytes.
16. Keeping cells at approx 4°C throughout the preparation process is crucial to avoid capping of surface molecules after binding of a ligand (antibody). Capping will reduce the signal for the detection of this particular antigen.
17. Dead cells tend to bind antibodies and fluorochromes unspecifically. Exclusion of dead cells from further analysis improves the distinction between antigen positive and negative cells.

18. As outlined in the protocol, double staining of the cells requires two primary antibodies and two Ig-specific conjugates, if labeled primary antibodies are not available. To avoid crossreactions between the Ig-specific conjugates, the use of primary antibodies of different isotypes and heavy-chain-specific conjugates is strongly recommended.
19. Because of a marked spectral overlap of FITC and R-PE, one has to put special emphasis on fluorescence compensation when setting up a protocol for the cytometer to simultaneously detect FITC and R-PE signals.
20. In functional assays, Stx exhibits biological activities in the nanogramm range. In contrast, the concentrations of Stx and Stx B-subunit needed to detect surface binding of the proteins to lymphocytes depend on the detection system used and may be in the microgram range.

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6.5 "Bovine lymphocytes express functional receptors for *Escherichia coli* Shiga toxin 1."

Stamm, Ivonne, M. Wuhrer, R. Geyer, G. Baljer and **C. Menge***

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MICROBIAL PATHOGENESIS

Bovine lymphocytes express functional receptors for *Escherichia coli* Shiga toxin 1

Ivonne Stamm[a], M. Wuhrer[b], R. Geyer[b], G. Baljer[a] & Ch. Menge[a]*

[a]*Institut für Hygiene und Infektionskrankheiten der Tiere der Justus-Liebig-Universität, Giessen and* [b]*Biochemisches Institut am Klinikum der Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany*

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Interactions of Shiga toxins (Stxs) and immune cells contribute to the pathogenesis of diseases due to Stx-producing *Escherichia coli* (STEC) infections in humans and facilitate the persistence of infection in asymptomatically infected cattle. Our recent findings that bovine B and T lymphocytes express Gb_3/CD77, the human Stx-receptor, prompted us to determine whether the bovine homologue also mediates binding and internalization of Stx1. In fact, Stx1 holotoxin and recombinant B subunit (rStxB1) bound to stimulated bovine peripheral blood mononuclear cells, especially to those subpopulations (B cells, $BoCD8^+$ T cells) that are highly sensitive to Stx1. Competition and HPTLC-binding studies confirmed that Stx1 binds to bovine Gb_3, but different receptor isoforms with varying affinities for rStxB1 were expressed during the course of lymphocyte activation. At least one of these isoforms mediated toxin uptake. An anti-StxB1 mouse monoclonal antibody, used as a model for bovine serum antibodies specific for Stx1, modulated rather than generally prevented rStxB1 binding to and internalization by the receptors. The presence of functional Stx1-receptors on bovine lymphocytes explains the immunomodulatory effect of Stx1 observed in cattle at a molecular level. Furthermore, expression of such receptors by bovine but not human T cells enlightens the background for the differential outcome of STEC infections in cattle and man, i.e., persistent infection and development of disease, respectively.

Keywords: EHEC, STEC, Shiga toxin 1, receptor, bovine lymphocytes.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC), a subtype of the Shiga toxin-producing *E. coli* (STEC), are the cause of epidemics and sporadic cases of hemorrhagic colitis in humans that can progress to life-threatening diseases as the hemolytic uremic syndrome (HUS) [1]. The main virulence factor of EHEC are the Shiga toxins (Stxs), potent cytotoxins of the ribosome-inactivating type [2]. Upon absorption from the intestine [3] Stxs damage renal endothelial cells resulting in a thrombotic microangiopathy, the histological hallmark of HUS [4]. However, in recent years compelling evidences arose that cells other than endothelial cells i.e., monocytes and granulocytes [5, 6] must be involved in the initiation of this syndrome [7]. To explain the inconsistencies in our current model of HUS pathogenesis, Heyderman *et al.* [8] further

* Author for correspondence: E-mail: christian.menge@vetmed.uni-giessen.de

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speculated that Stxs may modulate intestinal immune cell/epithelial cell interactions resulting in activated T cells, which then target the kidney.

In contrast to man, high percentages of ruminants shed STEC without any clinical symptoms [9–11]. Cattle, sheep, and goats are therefore regarded as 'tolerant' carriers of these bacteria [12], albeit the remarkable persistence of STEC infections in these animals correlates with the ability of the bacteria to synthesize Stx [13]. Owing to findings that Stxs affect bovine B and T lymphocyte functions *in vitro* and *ex vivo* [14–16] we concluded that the persistence of bovine STEC infections has its roots in a Stx-induced immunomodulation, which may prove a suitable target for measures to reduce the STEC prevalence in cattle. If the hypothesis of Heyderman *et al.* [8] holds true, that T cell activation is essential for the pathogenesis of HUS in man, an impact of Stxs on T cell functions would diametrically contribute to the course of bovine and human STEC infections, i.e., asymptomatic persistence and development of disease, respectively.

A reasonable explanation may be the expression of Stx-receptors by bovine but not human T cells. The eukaryotic cell surface receptor for the B subunit of Stxs is globotriaosylceramide (Gb_3, syn. CD77) [17]. Gb_3/CD77 mediates endocytosis of its ligand gaining Stxs access to the cytosol where the A-subunit is enzymatically active [18]. In the human immune system Gb_3/CD77 expression is limited to B cells while T cells lack Stx-receptors [19]. In contrast, we recently showed that Gb_3/CD77 is equally synthesized and surface expressed by bovine B and T cells [16]. However, functional studies revealed that bovine lymphocytes' sensitivity to Stx1 is not strictly correlated to the level of Gb_3/CD77 surface expression: the cells are only transiently sensitive in an early phase of activation characterized by a low to moderate Gb_3/CD77 expression [20]. The broad cellular distribution of Gb_3/CD77 also includes $BoCD4^+$ T cells, the proliferation of which is only marginally affected by Stx1 [15]. It has to be considered that high affinity binding of Stxs to cells depends on several features of Gb_3/CD77 such as length and saturation of the fatty acid incorporated in different isotypes of the molecule [21]. Binding of Stx to cell surface protein of Gb_3/CD77-deficient Vero cell clones has also been described [22]. In order to understand an effect of Stx1 unique for the bovine immune system at a molecular level it became considerably important to prove whether Gb_3/CD77 detected on bovine lymphocytes in fact represents a functional Stx1 receptor. This brought us to perform binding, competition, and internalization studies with stimulated bovine peripheral blood mononuclear cells (PBMC) *in vitro*.

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Results

Stx1 and rStxB1 binding to bovine lymphocytes

Freshly isolated PBMC were unable to bind detectable amounts of Stx1 holotoxin or rStxB1 subunit (data not shown). However, stimulation of PBMC with Phytohemagglutinin P (PHA-P; respective cells are referred to as stimulated PBMC below) rendered a prominent proportion of cells able to bind Stx1 and rStxB1 [Fig. 1(a)]. All lymphocyte subpopulations investigated could bind rStxB1, but the binding pattern reflected the subpopulations' susceptibility to the proliferation inhibitory effect of Stx1: about half of the $BoCD8^+$ cells and two thirds of the $BoCD21^+$ cells, the main targets of Stx1 *in vitro* [15], bound rStxB1 in large amounts, whereas only about one third of $BoCD4^+$ cells and one quarter of $WC1^+$ cells (i.e., the majority of γδT cells in bovine peripheral blood), respectively, did so [Fig. 1(b)].

When neutral and acidic glycosphingolipids (GSL) were separated from PBMC before (day 0) and after (day 4) stimulation and analysed by HPTLC-overlay, rStxB1 bound specifically to the ceramide tri-hexoside bands of the standard and to two distinctive bands of the day 4 neutral GSL [Fig. 2(b)], which we previously structurally determined as Gal(α1-4)Gal(1-4)Glc(1-1)ceramide (Gb_3) [16]. No binding could be observed to day 0 neutral GSL – due to the low amount of Gb_3 detected at this time point [16] – or to acidic GSL.

Double staining experiments yielded rStxB1-Gb_3/CD77 double positive cells finally proving that rStxB1 is able to bind bovine Gb_3/CD77 even in its physiological environment within a membrane (Fig. 3). However, binding of rStxB1 was not closely linked to binding of the anti-CD77 mab 38.13. A prominent population

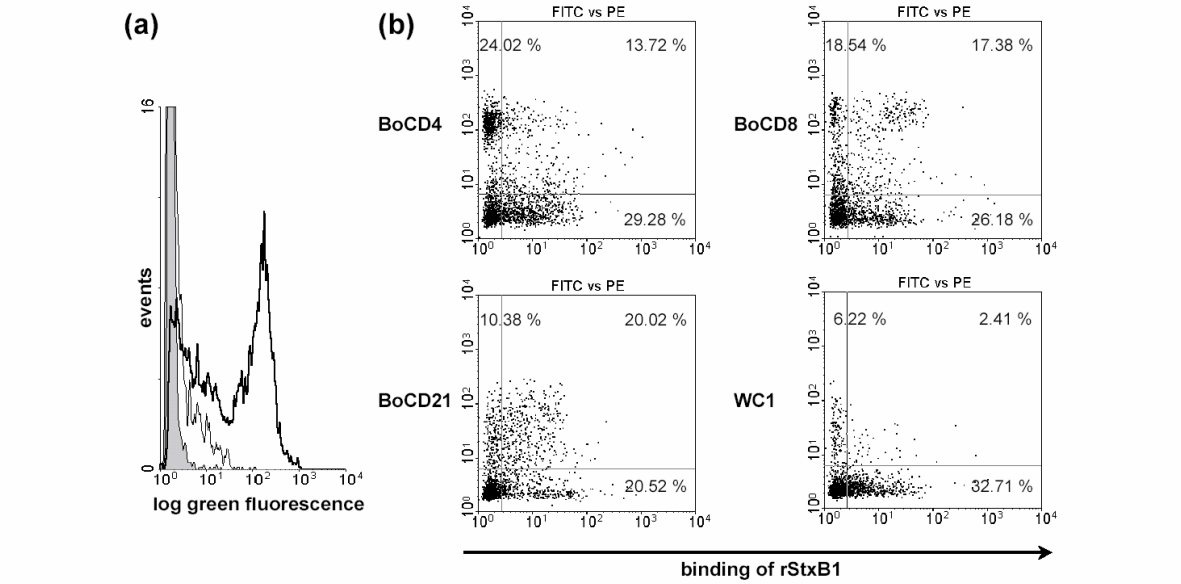

Figure 1. Binding of Stx1 and rStxB1 by bovine PBMC after cultivation for 4 days in the presence of PHA-P (5 μg/ml). (a) Representative flow cytometric histograms illustrating the binding of Stx1 (thin solid line; 50,000 CD_{50}/ml, quantified on Vero cells) and rStxB1 (thick solid line; 30 μg/ml) to the blast cell population of bovine PBMC. Grey shaded histogram denotes the corresponding antibody control. Histograms are from one representative of twelve PBMC preparations. (b) PBMC subpopulation binding of FITC-labelled rStxB1 (10 μg/ml). Representative two-colour dot plots are shown from one out of four PBMC preparations. Percentage of cells from the blast cell-gated population are indicated in the quadrants.

of anti-CD77 negative cells that bound rStxB1 could be detected at the beginning of the incubation period on day 2. In turn, a population of anti-CD77 positive cells unable to bind rStxB1 appeared later on and its percentage constantly increased during the following days of incubation (Fig. 3).

Binding of rStxB1 to Gb_3/CD77 isoforms expressed by bovine lymphocytes

In subsequent competition studies by using the anti-CD77 mab 38.13 and rStxB1 as competitors and stimulated PBMC (day 4) as targets, concentrations of rStxB1 above 3.75 μg/ml were able to prevent subsequent binding of anti-CD77 as concluded from the reduction in the percentage of anti-CD77 positive cells [Fig. 4(b)]. It is remarkable that the percentage of anti-CD77 positive cells was almost identical when the cells were incubated first with either mab 38.13 or with rStxB1. Obviously, at this time a major portion of bovine PBMC expressed an isoform of Gb_3 with an affinity for rStxB1 that was much higher than that for mab 38.13. On the other hand, even at the highest concentration of rStxB1 tested, anti-CD77 positive cells could still be detected, pointing to a second population of cells expressing Gb_3 molecules with very high affinity for mab 38.13. As shown in Figure 4(a), the percentage of cells positive for binding of rStxB1 decreased at concentrations of rStxB1 lower than 7.5 μg/ml. Interestingly, in the range from 1.875 to 3.75 μg/ml the percentage of cells positive for rStxB1-binding was influenced by the order the ligands were given to the cells. Within this range, preincubation of the cells with mab 38.13 prevented binding of rStxB1 indicating a third population of cells expressing Gb_3 isoforms that bound mab 38.13 and rStxB1 with almost similar affinity.

Effect of rStxB1 on bovine lymphocyte transformation and proliferation

When PBMC were incubated in the presence of rStxB1 (10 μg/ml) for 4 days, the B subunit was able to exert a suppressive effect on lymphocyte proliferation on its own (Fig. 5) comparable to

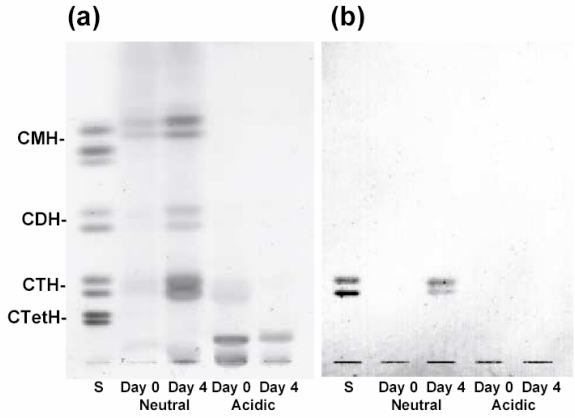

Figure 2. rStxB1 binding to bovine PBMC glycosphingolipids (GSL). The total neutral and acidic GSL fractions of bovine PBMC freshly after preparation (day 0) and after stimulation with PHA-P (5 μg/ml; day 4) were spotted onto HPTLC plates. The amount of PBMC GSL per lane corresponded to 2 mg (a) and 0.5 mg (b) PBMC dry weight, respectively. GSL were resolved by using the running solvent chloroform:methanol:water, 65:25:4 (by volume) and visualized chemically with orcinol/H_2SO_4-staining (a) or by HPTLC-overlay with rStxB1 (b). The standard (S) of CMH-CTetH corresponded to globo-series ceramide mono-, di-, tri- and tetrahexosides, respectively.

that of the holotoxin in the nanogram range [15, 20]. Addition of rStxB1 to stimulated PBMC from the beginning of the cultivation reduced the percentage of viable $BoCD8^+$ and $BoCD21^+$ blast cells remaining on day 4 and also significantly reduced the percentage of $Gb_3/CD77^+$ blast cells. The latter effect could even be detected at rStxB1 concentrations as low as 20 ng/ml [Fig. 6(a)]. To exclude that this effect was simply due to capping of Gb_3/CD77 molecules the concentration threshold for the detection of rStxB1-binding to stimulated PBMC (day 4) was determined. In fact, rStxB1-binding was undetectable below 937.5 ng/ml [Fig. 6(b)] clearly arguing against a capping phenomenon at 20 ng/ml.

Internalization of Stx1 and rStxB1 by bovine lymphocytes

Internalization studies proved that a minority of the Gb_3/CD77 isoforms of bovine PBMC was even able to mediate uptake of the toxin. When stimulated PBMC (day 4) were loaded with toxin at 4°C and incubated for further 30 min at 37°C surface fluorescence was decreased compared to cells that were kept at 4°C throughout.

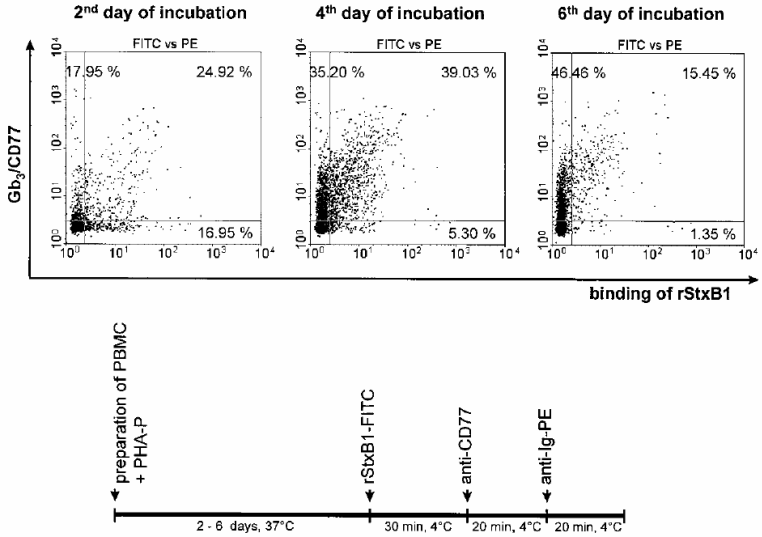

Figure 3. Gb_3/CD77 expression and rStxB1 binding by bovine PBMC. Flow cytometry analysis of PBMC stimulated with PHA-P (5 μg/ml) for 2–6 days. At the end of the incubation period cells were labelled with anti-CD77 and FITC-labelled rStxB1 (10 μg/ml) as depicted in the lower part of the figure. Percentage of cells from the blast cell-gated population are indicated in the quadrants. Representative two-colour flow cytometry results are shown from one out of four PBMC preparations.

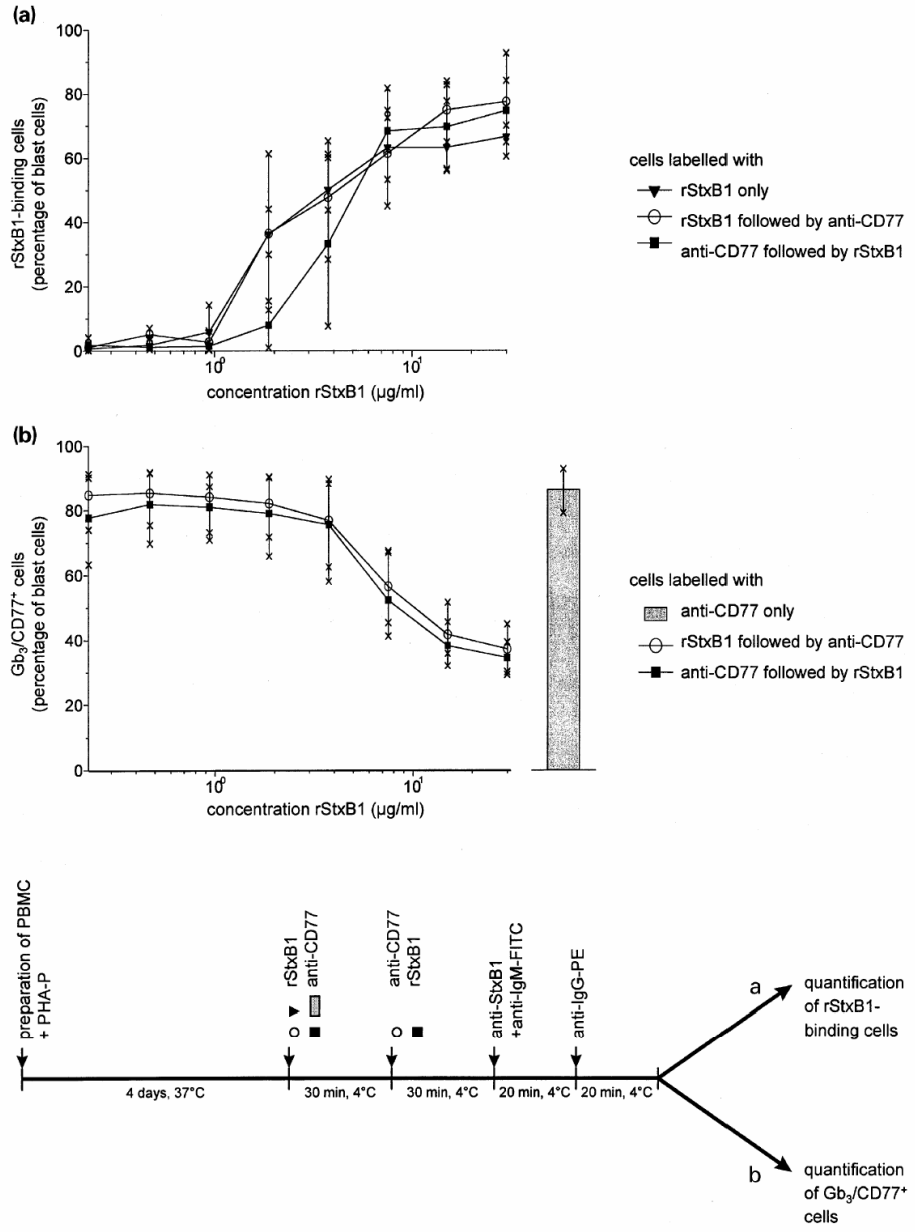

Figure 4. Competition of rStxB1 and anti-CD77 for binding to bovine PBMC. Graphs represent data obtained by flow cytometry analysis of PBMC stimulated with PHA-P (5 µg/ml) for 4 days. At the end of the incubation period cells were labelled with anti-CD77 (15 µg/ml) and rStxB1 as depicted in the lower part of the figure. Graphs represent mean, max and min of the data obtained from blast cells of four PBMC preparations.

In this course mean fluorescence intensities were 0.732 ± 0.12 and 0.719 ± 0.0817 ($P > 0.05$) for cells labelled with Stx1 holotoxin and 3.605 ± 0.62 and 2.465 ± 0.571 ($P \leq 0.01$) for cells labelled with rStxB1 after incubation at 4°C and 37°C, respectively (mean $\pm$ SD of 4 PBMC preparations). Incubation at 37°C also slightly reduced the percentage of Stx1 and rStxB1-binding cells, but differences missed statistical significance (Fig. 7, bar A, B; data not shown for Stx1).

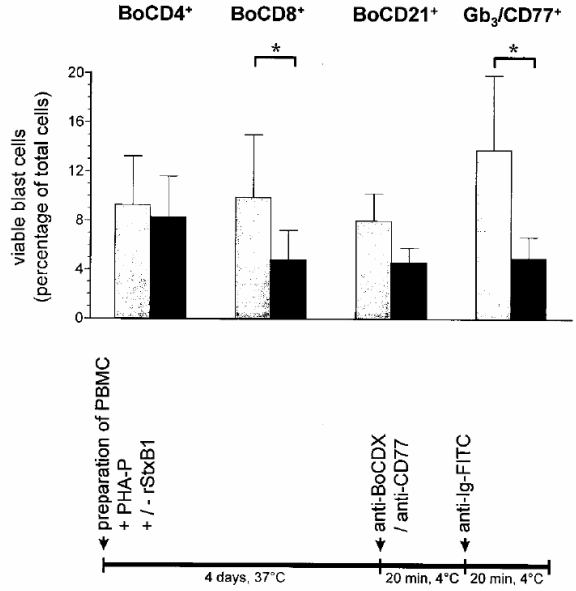

Figure 5. Effect of rStxB1 on transformation and proliferation of bovine PBMC. PBMC were stimulated with PHA-P (5 μg/ml) for 4 days in the presence (black bars) or absence (grey bars) of rStxB1 (10 μg/ml) and analysed by flow cytometry at the end of the incubation period as depicted in the lower part of the figure. Lymphocyte subpopulations were identified by immunophenotyping. Data analysis was performed by calculating the percentage of viable blast cells. Bars represent the mean ± SD of the data obtained from four PBMC preparations. Comparison of cells incubated with and without rStxB1 was performed by the paired *t*-test. Horizontal brackets with centered asterisks enclose bars that are significantly different ($P < 0.05$).

Effect of anti-Stx on binding and internalization of rStxB1

High proportions of cattle harbour Stx1-neutralizing antibodies in their sera [23]. Indeed, 19 out of 20 plasma samples from blood donors included in the present and previous studies [20] contained Stx1-neutralizing antibodies with at least 1 neutralizing unit/ml (data not shown). Non-parametric correlation analysis revealed that there is no significant correlation between antibody titers and the level of the suppressive effect of Stx1 on the number of $BoCD8^+$, $BoCD21^+$, and $Gb_3/CD77^+$ blast cells in PBMC cultures obtained from these animals (Spearman rank correlation coefficients were 0.145, 0.073, and −0.049, respectively; critical value = 0.447 at a two-sided significance level of 5%). Because PBMC are separated from other blood components including immunoglobulins, these findings stress that the sensitivity of lymphocytes towards the effect of Stx1 is an inherent feature of cattle independent of a certain animals' immune status.

However, possible implications of the host's immune response became apparent by the dichotomous effect of anti-StxB1 mab 13C4 [24] used as a model to study the role of Stx1-specific serum immunoglobulins. Preincubation of up to 50,000 CD_{50}/ml Stx1 holotoxin with anti-StxB1 (45 μg/ml) – shown to neutralize the effect of Stx1 on bovine lymphocytes [15, 20] – abrogated binding of the holotoxin (data not shown). In contrast, preincubation of rStxB1 with anti-StxB1 (1.5 μg/ml) only partially neutralized the effect of rStxB1 on the percentage of $Gb_3/CD77^+$ cells remaining in PBMC cultures after 4 days [Fig. 6(a)]. Moreover, anti-StxB1 even significantly ($P \leq 0.01$) enhanced the percentage of stimulated PBMC (day 4) able to bind rStxB1 and rStxB1/anti-StxB1-complexes at least at intermediate concentration ratios [Fig. 6(b)]. The idea that rStxB1/anti-StxB1-complexes retain their binding capability to cells was finally confirmed, because such complexes could still be detected on a prominent number of PBMC after preincubation of 30 μg/ml rStxB1 with 45 μg/ml of anti-StxB1 (Fig. 7, bar C). Moreover, these complexes could even be internalized at 37°C (Fig. 7, bar D). Controls included in every set of experiments, i.e. cells that were only incubated with anti-StxB1 and anti-mouse conjugate, always gave negative results proving that even the rStxB1/anti-StxB1-complexes bound via $Gb_3/CD77$ and not via Fc receptors. Anti-StxB1 completely blocked internalization in this model only when given to the cells after they had already bound rStxB1 (Fig. 7, bar E) or Stx1 holotoxin (data not shown).

Discussion

Most STEC infections in ruminants are characterized by the lack of clinical symptoms, but the persistence of enteral infection with prolonged fecal shedding [13] is of critical importance for human food safety. We are currently investigating the hypothesis that a Stx1-induced immunomodulation fundamentally contributes to the persistence of bovine STEC infection. Confirming our previous assumption that Stx1 directly acts on bovine lymphocytes [20] we show here

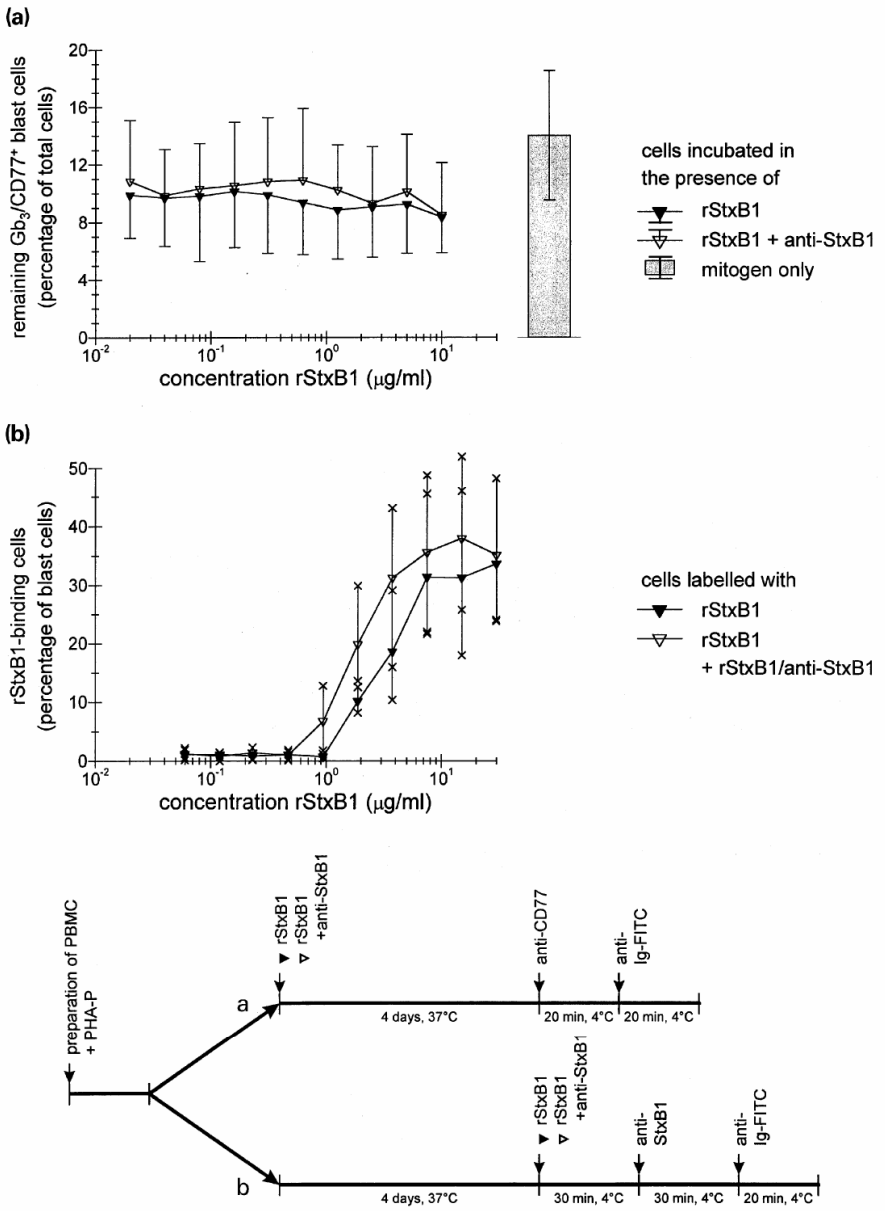

Figure 6. Effect of rStxB1 on Gb_3/CD77 expression by bovine PBMC (a) and determination of rStxB1-binding as a function of rStxB1 concentration (b). Flow cytometry analysis of PBMC stimulated with PHA-P (5 μg/ml) for 4 days. PBMC were incubated in the presence of rStxB1 (10 μg/ml) and the presence and absence of anti-StxB1 (1.5 μg/ml) as depicted in the lower part of the figure. Data analysis was performed by calculating the percentage of viable blast cells. Graphs represent the mean ± SD (a) and mean, maximum and minimum (b) of the data obtained from four PBMC preparations each. Two-way ANOVA revealed significances for the curves presented in graph B ($P \leq 0.01$ for anti-StxB1 and $P \leq 0.001$ for the concentration of rStxB1).

that these cells are in fact capable of binding Stx1 holotoxin as well as recombinant B subunit (rStxB1). Competition and HPTLC-binding studies demonstrated that the binding site for Stx1 is a glycosphingolipid which we recently biochemically characterized as Gb_3, thus representing the bovine homologue of the human Stx-receptor CD77 [16]. Thereby, cattle

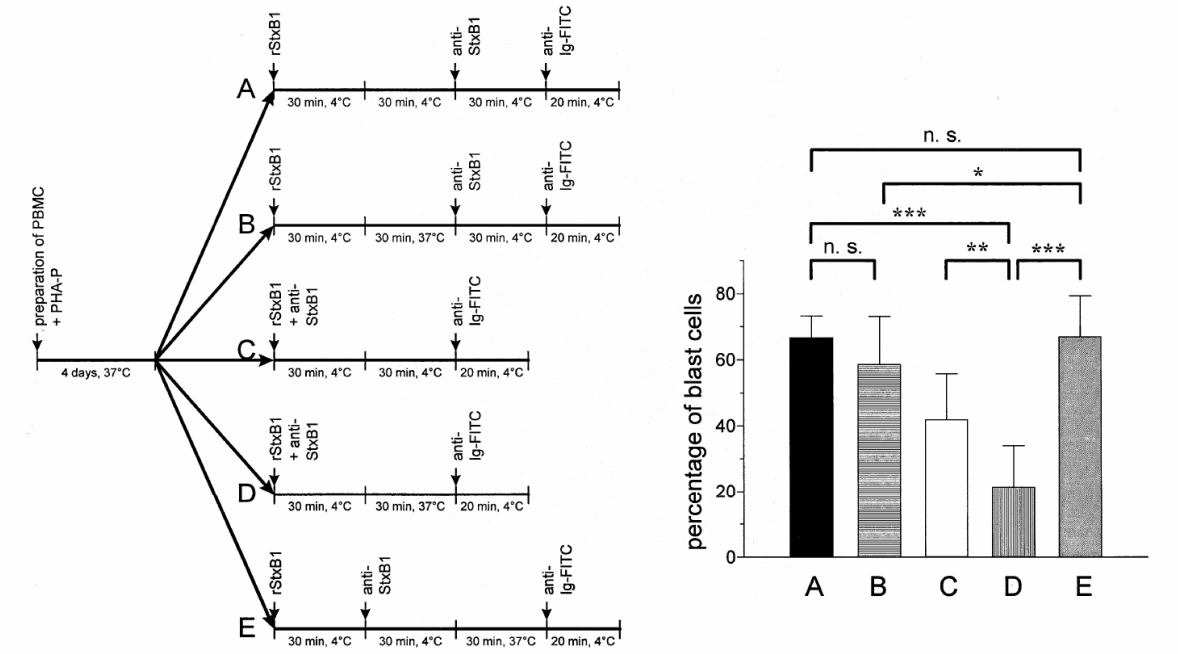

Figure 7. Binding and internalization of rStxB1 and rStxB1/anti-StxB1-complexes by bovine PBMC. Flow cytometry analysis of PBMC stimulated with PHA-P (5 μg/ml) for 4 days. At the end of the incubation period cells were labelled with rStxB1 (30 μg/ml) after preincubation with (C, D) or without (A, B) anti-StxB1 (45 μg/ml), and kept either on ice throughout or warmed to 37°C for 30 min as depicted in the left-handed part of the figure. In addition, cells were labelled with rStxB1 followed by incubation with anti-StxB1 prior to warming to 37°C (E). Bars represent the mean ± SD of four PBMC preparations. Data were analysed by the paired *t*-test. Horizontal brackets with centered asterisks enclose bars that are significantly different [$P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***); n.s. = not significantly different ($P > 0.05$)].

is the first species reported to date that expresses functional Stx-receptors on the surface of T cells. Because bovine intraepithelial lymphocytes, the first immune cells which gain contact to the toxin, predominantly are T cells and equally express Gb_3/CD77 *in vivo* [25], the direct targeting of T cells by Stx1 must be of particular importance for the colonization of the bovine intestine by STEC.

Bovine lymphocytes' CD77-expression was not congruent to rStxB1-binding by the cells, although rStxB1 and anti-CD77 both specifically recognize the terminal galabiose in Gb_3/CD77 [17, 26]. Competition studies revealed three subtypes of cells expressing receptors with varying affinities for the two ligands. The fact that rStxB1-binding cells mainly appeared at the beginning of the incubation period suggests that only at an early time point the cells exhibit a reasonable sensitivity against Stx1. This idea is supported by our previous findings that Gb_3/CD77 expression by bovine lymphocytes parallels the activation of the cells [16]. Nevertheless, Stx1 only affects cells between the $Gb_3/CD77^{low}$ and the $Gb_3/CD77^{moderate}$ state of activation while cells that reached the $Gb_3/CD77^{high}$ state before experiencing Stx1 become refractory again [20]. Comparative fatty acid analysis of Gb_3/CD77 obtained from bovine PBMC prior to (day 0) and after (day 4) stimulation had shown that stimulation altered the fatty acid pattern with hexadecanoic acid dominating on day 0 and fatty acids with more than 20 carbon atoms prevailing on day 4 [16]. Pellizzari *et al.* [21] found Gb_3/CD77 molecules in human kidneys which contained hexadecanoic acid as the major component having a consistently higher affinity for Stx1 than other ceramide trihexosides. Given that bovine lymphocytes express high affinity Stx1-receptors only during early states of activation, STEC by secreting Stx1 prevent the onset of an immune response rather than down regulate an established one. This may be of particular benefit for the bacteria because low amounts of Stx are sufficient to target small numbers of sensitive cells.

The biochemical target of the Stx1 holotoxin is the ribosome [2], and internalization from coated pits after clustering of randomly distributed Stx binding sites [18] is a prerequisite for the toxin to exert its toxicity [27]. To prove the ability of bovine lymphocytes to internalize Stx we applied a method previously described for Stx1 [18, 19] and quantified the amount of immunologically detectable Stx1 and rStxB1 on the cellular surface after incubation of the cells at 37°C by flow cytometry. Our findings that cells labelled by anti-StxB1 mab after warming to 37°C exhibited a lower fluorescence intensity compared to cells kept at 4°C strongly suggest the existence of a toxin translocation mechanism linked to surface glycolipids. However, only a minor portion of surface-bound Stx was internalized by bovine PBMC. Moreover, rStxB1 can be detected on the cellular surface throughout when the cells are incubated at 37°C in the presence of rStxB1 for days (data not shown). A suitable explanation for that is the existence of two populations of receptors as predicted by Lindberg *et al*. [17] and meanwhile confirmed by studies on the aglycone modulation of human Gb_3/CD77 receptor function [28]: one isoform of bovine Gb_3/CD77 binds toxin only and another isoform, possibly present in smaller numbers, mediates toxin uptake.

Interestingly, the isolated B subunit on its own exhibited an effect similar to that of Stx1 in bovine PBMC cultures. Although the B subunit has no inhibitory effect on protein synthesis itself, a series of recent studies indicates that it interferes with cell signalling [29–32]. Nevertheless, the fact that anti-StxB1 mab in some instances is able to facilitate rStxB1-binding to bovine lymphocytes but fails to equally enhance the biological effect of rStxB1 and Stx1 stands against the hypothesis of a signal solely derived from binding of the protein. Because even the Stx1 B subunit can exert cytotoxicity when expressed inside transfected cells [33] it may be that internalization into bovine lymphocytes is additionally required. With respect to the course of events during bovine STEC infections it must be stressed, however, that a 50,000-fold higher concentration of the B subunit was required compared to incubation with the holotoxin (10 µg/ml *vs.* 200 pg/ml of Stx1 holotoxin) [15], and toxin concentrations in the microgram range are unlikely to occur *in situ*. In other *in vitro* systems inhibition of protein synthesis by Stx1 holotoxin sensitizes cells for a separate cell surface-derived signal induced by minute concentrations of the B subunit that then leads to apoptosis [32, 34]. Apoptosis does not occur in Stx1-treated bovine PBMC cultures [15], but before inhibiting protein synthesis Stx1 first damages 28S rRNA and induces a 'ribotoxic stress response' resulting in an induction of mRNAs of primary response genes [35]. We thus assume that signals originating from the surface after Gb_3/CD77-binding of the B subunit *in vivo* (or the B pentamer of the holotoxin) act in concert with the induction of primary response genes after internalization of the Stx1 holotoxin and thereby contribute to the suppressive effect Stx1 exerts on bovine immune cells (Fig. 8).

Active or passive immunization against Stxs, prior to experimental inoculation with STEC, prevents the systemic complications of STEC infection in animal models [36–38] and Stx-specific antibodies prevent humans from developing HUS [39, 40]. However, it is still a matter of discussion in literature whether the protective effect of these antibodies is based on the capability to prevent binding or to prevent

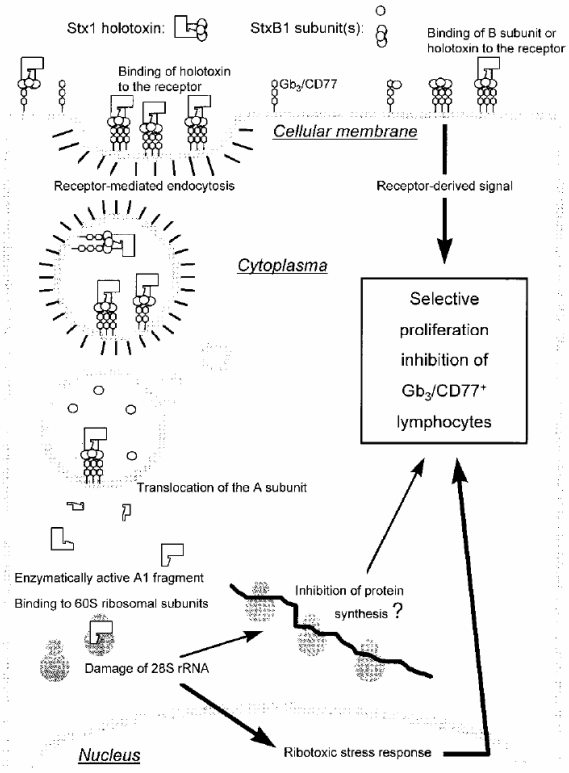

Figure 8. Proposed model for the pathways involved in the suppressive effect of Stx1 on bovine lymphocytes. For details see text.

internalization of the toxins. Nakao *et al*. [41] showed that a neutralizing monoclonal antibody to Stx2 blocks receptor binding and Lindberg *et al*. [17] as well as Eiklid and Olsnes [42] observed that cells cannot be rescued by addition of antiserum after toxin binding. In contrast, Sandvig *et al*. [18] were able to partially protect cells by addition of antiserum even 15 min after toxin binding. A similar instance was reported for human microvascular endothelial cells [43]. In our study an anti-StxB1 mab, used as a model for bovine serum antibodies specific for Stx1 [23], prevented rStxB1 binding at high antibody-antigen ratios (data not shown) and also effectively blocked internalization of cell-bound rStxB1 under certain conditions (see Fig. 7, bar E). High titers of Stx-specific antibodies as reported for cattle should therefore be highly protective [23, 44, 45]. There is some circumstantial evidence, however, that these antibodies' effects are not limited to neutralization but also may influence the way the toxin binds to cells. While at least a prominent portion of rStxB1/anti-StxB1-complexes formed in solution during preincubation retained their ability to bind to and translocate into bovine lymphocytes (see Fig. 7, bars C and D), rStxB1 molecules that were recognized by anti-StxB1 after cell surface binding did not internalize during 30 min at 37°C (see Fig. 7, bar E). Because Gb_3/CD77 exists in different isoforms and a single Stx1 B subunit harbours three independent Gb_3/CD77 binding sites [46] these dichotomous effects of anti-StxB1 may result from the usage of an alternative binding site of rStxB1 or the usage of a different receptor isoform or both. Anti-StxB1 induced alterations in Stx1/receptor interactions may consequently modify the cellular effects of the toxin because the three receptor binding sites of Stx1 are linked to different biological activities [47]. Furthermore, the ability of anti-StxB1 at subinhibitory concentrations to promote rStxB1 binding to bovine lymphocytes increases masking of surface Gb_3/CD77 molecules. This will profoundly influence cellular interactions between immune cells as well as interactions of immune cells with other tissue cells *in situ* [48]. As a consequence – although specific antibodies are able to neutralize Stx1 in principle – their activity fundamentally depends on the antibody-antigen ratios within the tissues and the time point (before or after receptor binding) the antibodies bind to the toxin. Hence, the detection of Stx-specific antibodies in sera of cows must not necessarily result in an efficient protection against immunomodulatory effects of Stx1. In fact, serological responses in cattle do not correlate with elimination of STEC infection [45] or protection against reinfection [14, 45].

Bovine intestinal epithelial cells express receptors for Stx1 [49; Menge, unpublished data] but the same holds true for some human intestinal cell lines [50] which are able to translocate significant amounts of Stx1 [3]. Because bovine endothelial cells also resemble their human counterpart in their susceptibility to Stx1 [51], expression of functional Stx1-receptors by bovine but not human T cells is the only fundamental species difference discovered to date and thus probably represents the missing link to explain the differential outcome of STEC infections – persistent infection or disease – in these species. Detailed investigations of T cell/epithelial cell interactions in the presence and absence of Stx1 are currently under way in our laboratory and will help to understand the underlying mechanisms.

Materials and Methods

Toxin purification

Stx1 was produced from the bovine STEC1 strain 2403 (rough, H^-) [10] and purified by a procedure that was described previously [15]. At the end of the purification process, toxin preparations were passed through Detoxi-Gel™ columns (Pierce, Old-Beijerland, Holland) to reduce contaminations with endotoxin. The Stx1 preparation contained 50,000 CD_{50} of Stx1 (see below) and 0.85 ng of Endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay.

Purification of B subunit protein

Recombinant StxB1 (rStxB1) was purified from *E. coli* DH5α [pSU108] [52] by the method of Niebuhr [53] with slight modifications. Bacteria were grown at 30°C overnight in Luria broth supplemented with ampicillin (50 μg/ml), diluted to an optical density at 560 nm (OD_{560}) of 0.4 and incubated further until the OD_{560} reached 0.8. The culture was induced at 42°C for 4 h, and the cells were harvested by centrifugation (8200 × *g* for 15 min). The pellet was washed

twice with 10 mM Tris–HCl (pH 8.0), resuspended in a solution containing 25% sucrose (wt/vol), 1 mM Na_2EDTA, and 10 mM Tris–HCl (pH 8.0), and gently shaken at 30°C for 10 min. The cells were harvested and immediately resuspended in ice-cold distilled water (osmotic shock treatment) and shaken gently at 4°C for 10 min. After centrifugation, the supernatant fraction (periplasmic fraction) was collected and diluted in the same volume of 20 mM Tris-HCl (pH 7.6), followed by another centrifugation step (100,000 × *g*, 1.5 h) immediately before further purification.

Purification of B subunit was performed by use of a fast protein liquid chromatography system (FPLC®; Amersham Pharmacia, Freiburg, Germany). The periplasmic fraction was applied to a MonoQ HR 5/5 column (Amersham Pharmacia) equilibrated with 20 mM Tris–HCl (pH 7.6). Elution was carried out with a gradient from 0 to 1 M NaCl in the same buffer. The B subunit was eluted at a salt concentration of 50 to 90 mM. Afterwards, the preparation was supplemented with aprotinin (50 KIE/ml) (Trasysol®, Bayer, Leverkusen, Germany) and 5% glycerol (v/v), and passed through a Detoxi-Gel™ column (Pierce) to reduce endotoxin contaminants. Further analysis of the B subunit preparation was carried out by subsequent SDS-PAGE and Western blot techniques. Protein concentration was determined with BCA protein assay® (Pierce, Old Beijerland, The Netherlands) according to the instructions of the manufacturer. The rStxB1 preparation used in the present study contained 330 µg/ml of rStxB1 and 0.92 ng Endotoxin per ml.

Cytotoxicity assay

The cytotoxic activities of toxin preparations were determined on Vero cells (ATCC CRL 1587) by the method of Gentry and Dalrymple [54] with minor modifications [15]. Cellular metabolic activity was assessed by MTT reduction assay as described previously [15, 55]. CD_{50} was calculated from dose response curves geometrically as the reciprocal of the toxin dilution causing 50% reduction in cellular metabolic activity. In studies determining the neutralizing activity of plasma samples obtained from the blood donors, medium was supplemented with plasma that had been collected from the upper phase of the Ficoll preparation and heated (56°C, 30 min). Neutralizing activity of plasma samples was calculated as: × neutralizing units/ml = reduction of CD_{50}/ml by 10^x [23].

Cell preparation and stimulation

Blood samples were taken from healthy cows (Holstein × german black pied) from the dairy herd of the farm of the Justus-Liebig-University. Samples were diluted 1:1 with Ca^{2+}-Mg^{2+}-free PBS and layered onto Ficoll-Paque® (Amersham Pharmacia) as described by Bøyum [56]. After centrifugation (800 × *g*, 20°C, 45 min), cells were recovered from the Ficoll buffer interface. Contaminating erythrocytes were removed by incubating the cell suspension with lysis buffer (8.26 g NH_4Cl, 1.09 g $NaHCO_3$, 0.037 g Na_3EDTA ad 1000 ml A. dest.) at RT for 5 min. The cells were washed twice with PBS and resuspended at 5×10^6 cells/ml in modified cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 3 µM 2-mercaptoethanol, and Phytohemagglutinin P (PHA-P; Sigma, Taufkirchen, Germany) at a final concentration of 5 µg/ml). The cell suspension was subsequently added to 96-well flat-bottomed microtiter plates (Nunc, Wiesbaden, Germany) with 50 µl per well. The plates had been prepared with 50 µl per well of dilution series of toxin preparations generated with 0.15 M NaCl in triplicate plus 50 µl of cell culture medium. In neutralization studies, medium was additionally supplemented with purified anti-StxB1 (mAb 13C4) [24]. Plates were incubated at 37°C under 5% CO_2 environment.

Immunophenotyping and flow cytometry analysis

At the end of the cultivation period, cells were thoroughly resuspended and transferred to V-shaped microtiter plates (Greiner, Frickenhausen, Germany) for immunolabelling as described previously [55, 57]. In short, the cells were centrifuged (150 × *g*, 4°C, 7 min) and resuspended in 50 µl of cell culture medium as a negative control or with supernatant of hybridoma cell lines (IL-A11 for BoCD4, IL-A105 for BoCD8, IL-A65 for BoCD21 and IL-A29 for WC1). Alternatively, the cells were resuspended with 25 µl of rat IgM (1 mg/ml, 1:50 in PBS;

Camon, Wiesbaden, Germany) as a negative control or with anti-human CD77 antibody (1:10 in PBS; Beckman-Coulter, Krefeld, Germany). Upon incubation (20 min, 4°C), the cells were washed once and resuspended with either 50 µl of anti-mouse IgG PE-conjugate (Sigma) diluted 1:100 in PBS or anti-rat IgM FITC- or PE-conjugate (Dianova, Hamburg, Germany) 1:200 diluted in PBS containing 2 µg/ml propidium iodide (Sigma). Following another 20 min on ice, the cells were washed twice and analysed with an EPICS ELITE® Analyser (Beckman–Coulter). Five thousand events were aquired from each sample. Data analysis was performed by using the ELITE 4.01 software provided by the manufacturer. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive. Populations of enlarged lymphoblast cells were defined according to its light scatter characteristics as described [16, 55] and analysed separately.

Binding and internalization studies

Cells were transferred to V-shaped microtiter plates and incubated with 50 µl of Stx1 (50,000 CD_{50}/ml) or rStxB1 (either 30 µg/ml or varying concentrations as indicated) for 30 min on ice [55]. In the case of neutralization studies, Stx1 and rStxB1, respectively, had been preincubated with anti-StxB1 for 1 h. After incubation cells were washed once. [In some studies FITC-conjugated rStxB1 (kindly provided by C. A. Lingwood, The Hospital for Sick Children, Department of Microbiology, Toronto, Canada) was used (10 µg/ml). The following steps were omitted in this case.] For internalization studies the cells were next incubated at 37°C for 30 min to allow uptake of the bound proteins. Afterwards the cells were washed once and resuspended in 50 µl of anti-StxB1 (45 µg/ml), followed by another incubation on ice for 30 min. Cells were washed again and resuspended in 50 µl of anti-mouse IgG FITC-conjugate (Dianova) diluted 1:400 in PBS and containing 2 µg/ml propidium iodide (Sigma). Following another 30 min on ice, the cells were washed twice and analysed by flow cytometry analysis as described above. In some instances cells were additionally immunolabelled as described above prior to the last incubation step.

Isolation and purification of neutral glycolipids

PBMC of day 0 (the day of the preparation of the cells) and day 4 (500 million cells each) were washed several times with PBS and lyophilized yielding dry-weights of 21 (day 0) and 41 mg (day 4). Acidic fraction and neutral fraction glycolipids were isolated and analysed by HPTLC and orcinol/H_2SO_4-staining as described elsewhere [16, 58].

Binding of rStxB1 to glycolipids on HPTLC plate

After glycolipid separation by HPTLC, the air-dried plates were dipped three times in a hexane solution of 0.5% Plexigum (Aldrich, Steinheim, Germany) for 60 s and then dried under warm air. The plates were blocked by incubation with 2% bovine serum albumin (w/v) in PBS supplemented with 0.5% Tween 20® (v/v) for 1 h at RT. After washing, the plates were overlayed for a further 2 h with rStxB1 (5 µg/ml) diluted in the same buffer. The plates were washed six times and incubated with anti-StxB1 (3 µg/ml) for 1 h. After washing six times the plates were overlayed with anti-mouse Ig conjugated to alkaline phosphatase (Dako, Hamburg, Germany) diluted 1:200 in buffer for 1 h. The plates were washed again three times, and bound antibody was visualized with the 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazoliumchloride substrate system (Biomol, Hamburg, Germany).

Statistical analysis

Data were analysed statistically by the paired *t*-test, the Wilcoxon signed rank test, two-way analysis of variance (ANOVA) and the Spearman rank order correlation by using SigmaStat 2.0 software (1992; SPSS Inc., Chicago, IL, USA). Significant differences were separated at $P \leq 0.001$ (***), $P \leq 0.01$ (**), and $P \leq 0.05$ (*).

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6.6 "Verotoxin 1 from *Escherichia coli* affects $Gb_3/CD77^+$ bovine lymphocytes independent of Interleukin 2, Tumor-Necrosis-Factor α, and Interferon α."

Menge*, C., Ivonne Stamm, Maike Blessenohl, L.H. Wieler, and G. Baljer

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Eigener Anteil an der Publikation:

- Initiative — weitestgehend eigenständig
- Projektplanung — weitestgehend eigenständig
- Durchführung der Versuche — unterstützend
- Auswertung der Experimente — unterstützend
- Erstellung der Publikation — wesentlich

SUPPLEMENT

Verotoxin 1 from *Escherichia coli* Affects Gb_3/CD77$^+$ Bovine Lymphocytes Independent of Interleukin-2, Tumor Necrosis Factor-α, and Interferon-α

CHRISTIAN MENGE,*[,1] IVONNE STAMM,* MAIKE BLESSENOHL,* LOTHAR H. WIELER,*† AND GEORG BALJER*

*Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität, D-35392 Giessen; and †Institut für Mikrobiologie und Tierseuchen, Freie Universität Berlin, D-10115 Berlin, Germany

Verotoxin (VT)-induced immunomodulation has been implicated in the ability of VT-producing *Escherichia coli* (VTEC) to cause persistent infections in cattle. VT1, also referred to as Shiga toxin 1, is a potent cytotoxin that modulates cytokine secretions and functions. This prompted the current investigation to examine whether the inhibiting effect of VT1 on bovine lymphocytes correlates with the expression of the cellular VT1 receptor Gb_3/CD77 or is mediated instead via perturbation of cytokine secretion. Using blood mononuclear cells stimulated by mitogens as a model, VT1 significantly blocked lymphoblast transformation and proliferation in the BoCD8$^+$ T cell and BoCD21$^+$ B cell population. In contrast, VT1 dramatically reduced the number of viable Gb_3/CD77$^+$ blast cells within all subpopulations identified (BoCD2$^+$, BoCD4$^+$, BoCD8$^+$, WC1$^+$ [i.e., γδ T cells] BoCD21$^+$, and BoCD25$^+$). Similar effects of VT1 were observed when the culture medium was supplemented with selected cytokines: tumor necrosis factor-α-sensitizing endothelial cells against VT1, interferon-α (IFN-α) as bovine IFN-α receptors are partially homologous to the B-subunit of VT1, and interleukin-2 that is critical for lymphocyte proliferation *in vitro*. The addition of these cytokines was neither able to mimic nor to overcome the effects of VT1. Therefore, it is concluded that VT1 directly acts on bovine lymphocytes rather than inducing a cytokine-mediated effect. VT1 considerably affects all main bovine lymphocyte subpopulations, implicating that the immune system is a predominant target for VT1 in cattle. Exp Biol Med 228:377–386, 2003

Key words: verotoxins; immunity; cattle; Shiga toxin; *Escherichia coli*; food safety

Killing the host immune cells by exotoxins (1) represents a mechanism of pathogenic bacteria to survive inside their host. However, evasive strategies also include those that counteract cytokine action by either blocking production of particular cytokines, mimicking cytokines and/or cytokine receptors, and inhibiting cytokine release or action (2). Analogous to the established classes of virulence factors such as adhesins, invasins, aggressins, and impedins, Henderson *et al.* (3) suggested the term "modulin" to describe this class of molecules. Although classified as aggressins, many bacterial exotoxins, originally defined by cytopathic effects, may possess additional modulating activities. The capacity of exotoxins (3) to elicit synthesis and secretion of pro- and anti-inflammatory cytokines may be as important as their direct toxic effects in pathogenesis. Verotoxins (VT; also referred to as Shiga toxins) are commonly known as potent cytotoxins that efficiently truncate protein synthesis and cause subsequent death of susceptible cells (4). VT-producing *Escherichia coli* (VTEC) are foodborne pathogens causing dramatic illnesses such as the hemolytic uremic syndrome (HUS) in humans (5). During pathogenesis of HUS, kidney failure predominantly results from VT cytotoxic effects on endothelial cells (6). VTs also significantly interfere with cytokine secretion and function

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[1] To whom request for reprints should be addressed at Institute für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig Universität, Frankfurter Strasse 85-89, D-35392 Giessen, Germany. E-mail: christian.menge@vetmed.uni-giessen.de

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within tissues (7). VT-induced liberation of tumor necrosis factor-α (TNF-α) and interleukin-1 from macrophages (8) renders endothelial cells susceptible to VT by upregulating the VT-receptor Gb_3/CD77 (9). In addition, the receptor-binding B-subunit of VT1 and the Type 1 interferon receptor (IFNAR) share a Gb_3/CD77-binding domain and one of the physiological functions of Gb_3/CD77 is to facilitate the binding of α2 interferon to the IFNAR (10). VT1 and IFNAR efficiently compete for Gb_3/CD77 binding and VT1 pretreatment of cells resulted in a diminished IFN-α sensitivity (10, 11).

Ruminants show a high prevalence of asymptomatic VTEC infections and represent the biological reservoir of these pathogens (12, 13). The work of Cornick *et al.* (14) suggested that VT are profoundly involved in the ability of VTEC to cause persistent infections in ruminants. This suggestion is underlined by the findings of our group and others that VT are able to block proliferation of bovine lymphocytes *in vitro* (15) and *in vivo* (16). Therefore, a VT-induced immunomodulation represents a highly attractive hypothesis to explain how VTEC facilitate persistence of infection. However, the exact mechanisms are poorly understood. VT1 efficiently induced apoptosis in a bovine lymphoid cell line (15), and only inhibited proliferative responses to mitogens in primary cultures of bovine lymphocytes without inducing cell death (15, 17). The latter effect was putatively due to a blockage of cellular activation particularly in cells of the $BoCD8^+$ T cell and $BoCD21^+$ B cell subpopulations (15). Although bovine lymphocytes are able to express Gb_3/CD77 *in vivo* and *in vitro*, Gb_3/CD77 expression itself relies on activation of the cells (18). It should be noted that Gb_3/CD77 expression is not limited to $BoCD8^+$ and $BoCD21^+$ cells as it has a very broad cellular distribution, including $BoCD4^+$ T cells proliferation of which is marginally affected by VT1. It is not known whether VT1 can act as a leukotoxin to affect $Gb_3/CD77^+$ bovine lymphocytes. VT1 may function as a modulin by interfering with cytokines that subsequently cause negative effects such as inhibition of $BoCD8^+$ and $BoCD21^+$ cell proliferation (15). The objective of this study was to investigate whether the inhibition of bovine lymphocytes by VT1 is linked to the activation-dependent Gb_3/CD77 expression by lymphocytes and whether a selected number of cytokines are involved in the immunomodulation caused by VT1 in cattle.

Materials and Methods

Experimental Animals. Blood samples were collected from 25 healthy, lactating cows (Holstein × German black pied) from the dairy herd of the Teaching and Research Farm of our institution. All cows were healthy throughout the study.

Toxin Purification. VT1 was purified from the bovine VTEC1 strain 2403 H^- (19) by a procedure that was described previously (15). Briefly, bacteria were grown at 37°C for 12 hr in minimal essential medium, harvested, and sonicated. The supernatant was applied to a column containing Cibacron blue 3G-A linked to agarose beads (HiTrap blue, Pharmacia, Freiburg, Germany). The bound material was eluted with a gradient from 0 to 1 *M* NaCl in 10 m*M* sodium phosphate buffer (pH 7.4). Fractions with the highest verotoxicity (determined by using the Vero [African Green Monkey kidney] cell toxicity assay) were pooled. For subsequent immunoaffinity chromatography, protein A/G agarose (Schleicher & Schuell, Dassel, Germany) was loaded with mouse anti-VT1 B-subunit (anti-VTB1) monoclonal antibodies (mAb clone 13C4) that became noncovalently linked to the gel matrix (20). The partially purified and dialyzed toxin was applied to this column and bound material was eluted with a pH gradient from 3.5 to 2.15 in 0.75 *M* NaCl. The fractions with the highest verotoxicity were pooled and dialyzed against 0.15 *M* NaCl overnight. Finally, toxin preparations were passed through Detoxi-Gel columns (Pierce, Old-Beijerland, The Netherlands) to remove endotoxin contaminants.

Cytotoxicity Assay. The cytotoxic activity of VT1 preparation was determined on Vero cells (ATCC CRL 1587) as described by Gentry and Dalrymple (21) with minor modifications. Briefly, 50 μl of 10-fold dilution series of toxin preparations were generated with 0.15 *M* NaCl in microtiter plates (Nunc, Wiesbaden, Germany) in triplicate. Fifty microliters of 0.15 *M* NaCl served as a negative control, whereas 50 μl of 1% SDS in 0.15 *M* NaCl were used as a positive control. A total of 50 μl of cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin per milliliter) then were added to each well. In neutralization studies, medium was additionally supplemented with purified anti-VTB1 (mAb 13C4) leading to a final concentration of 1.5 μg of immunoglobulin per milliliter. Initial experiments in our laboratory revealed that this concentration of antibody is sufficient to completely neutralize the biological activity of at least 200 CD_{50}/ml of VT1 (for definition see below) used in this study. After incubation (at room temperature for 30 min) 50 μl of Vero cell suspension (8×10^5 cells/ml of culture medium) were applied to each well, and the plates were incubated at 37°C for 96 hr under a 5% CO_2 environment. Cellular metabolic activity was assessed by 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Cytotoxic dose 50% (CD_{50}) was calculated from dose-response curves geometrically as the reciprocal of the toxin dilution causing 50% reduction in cellular metabolic activity.

Peripheral Blood Mononuclear Cell (PBMC) Preparation and Stimulation. Bovine PBMC were prepared according to the method of Bøyum (22) by using Ficoll-Paque (Pharmacia) with minor modifications (15). The cells were resuspended at 5×10^6 cells/ml in a modified cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum and 3 μ*M* 2-mercaptoethanol). The cell suspension was subsequently added to 96-well flat-bottomed microtiter plates (50 μl per well). The plates were prepared in a manner similar to that for the cytotoxicity

assay. In the PBMC cultures, the medium also was supplemented with concanavalin A (ConA), phytohemagglutinin P (PHA-P), pokeweed mitogen (PWM), or lipopolysaccharide from *E. coli* O111:B4 (LPS; Sigma, Taufkirchen, Germany) at final concentrations of 5, 5, 10, and 25 μg/ml, respectively. In some studies, the medium was supplemented with VT1 (leading to a final concentration of 200 CD_{50}/ml as determined in the Vero cell assay) in the absence or presence of monoclonal anti-VTB1 (a final concentration of 1.5 μg/ml) and varying concentrations of selected cytokines. Cytokines were rboIL-2 (supplied by R.A. Collins, IAH, Compton, UK), rboTNF-α (supplied by R.F. Steiger, CIBA-GEIGY, Basel, Switzerland), and rhuIFN-α(2b) purchased from TEBU (Frankfurt, Germany). Plates were incubated at 37°C for 1 to 8 days under 5% CO_2.

MTT Reduction Assay. Cellular metabolic activity was assayed by measuring the reduction of MTT (Sigma, Taufkirchen, Germany) as described previously (15). Twenty-five microliters of MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) were added per well. Upon incubation at 37°C for 4 hr, the reaction was stopped by adding 100 μl of 10% sodium dodecyl sulfate in distilled water. After an overnight incubation, the optical density (OD) was read on a Titertek Multiscan MCC/340 ELISA plate reader (Flow, Meckenheim, Germany) by using a test wavelength of 540 nm and a reference wavelength of 690 nm. The percentage of cellular metabolic activity was calculated as: (OD sample − OD positive control)/(OD negative control − OD positive control) × 100.

Immunophenotyping and Flow Cytometry Analysis. After stimulation, PBMC were thoroughly resuspended and transferred to V-shaped microtiter plates (Greiner, Frickenhausen, Germany) at the end of the cultivation period and were pelleted by centrifugation (150*g* at 4°C for 7 min) as described by Menge *et al.* (23). The pellets were resuspended in 50 μl of cell culture medium as a negative control or with supernatant of hybridoma cell lines (IL-A 43 for BoCD2, IL-A11 for BoCD4, IL-A105 for BoCD8, IL-A65 for BoCD21, IL-A111 for BoCD25, and IL-A 29 for WC1). The cells were incubated on ice for 20 min, pelleted, resuspended with 25 μl of rat IgM (1 mg/ml, 1:50 in PBS; Camon, Wiesbaden, Germany) as a negative control or with anti-human CD77 antibody (1:10 in PBS; Beckman-Coulter, Krefeld, Germany) and incubated for an additional 20 min. The cells then were washed once and resuspended with 50 μl of anti-mouse PE-conjugate (Sigma) diluted 1:100 in PBS. After 20 min, the cells were pelleted and resuspended in 50 μl of anti-rat IgM FITC-conjugate (Dianova, Hamburg, Germany) 1:200 diluted in PBS and containing 2 μg/ml propidium iodide (Sigma). After another 20 min on ice, the cells were washed twice and analyzed with an EPICS ELITE Analyser (Beckman-Coulter, Krefeld, Germany). A total of 5000 events were acquired from each sample. Data analysis was performed by using the ELITE 4.01 software provided by the manufacturer. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive. Populations of enlarged lymphoblast cells and untransformed nonblast cells were defined according to its light scatter characteristics as described (18) and were analyzed separately.

Statistical Analysis. Data were analyzed statistically by paired *t* test and the Wilcoxon signed rank test

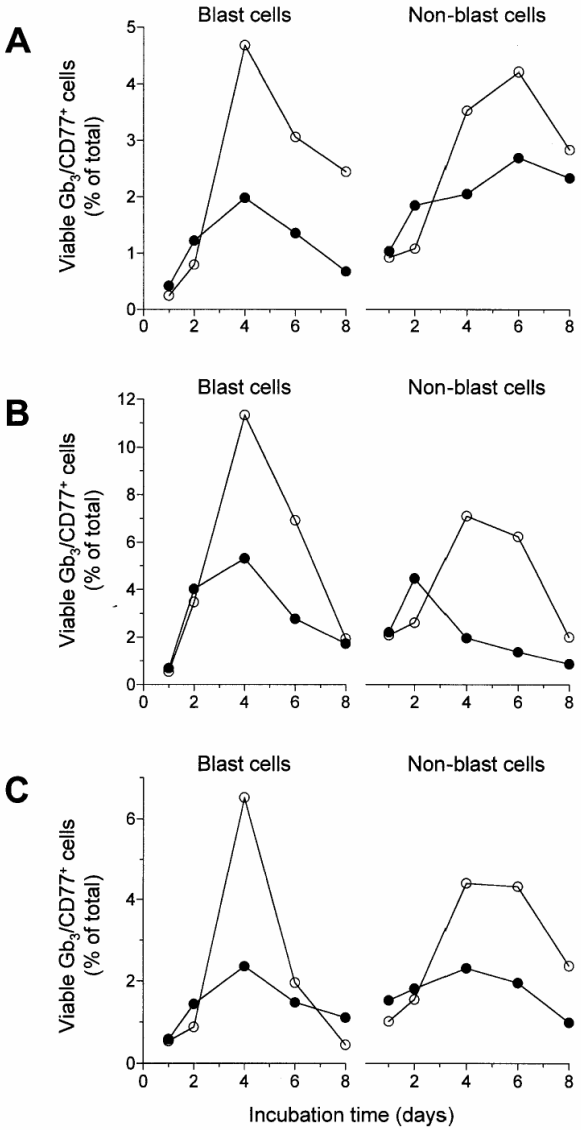

Figure 1. Effect of VT1 on Gb_3/CD77 expression by bovine PBMC. Cells were incubated with VT1 (200 CD_{50}/ml, quantified on Vero [African Green Monkey kidney] cells) at 37°C. Culture medium was free of mitogen (A) or was supplemented with 5 μg/ml PHA-P (B) or 25 μg/ml LPS (C). Observed effects were assigned to VT1 by comparison of the results obtained in the absence (●) or presence of 1.5 μg/ml anti-VTB1 mAb 13C4 (○). Expression of Gb_3/CD77 was quantified by flow cytometry at the indicated time points. Data analysis was performed by calculating the percentage of viable Gb_3/CD77$^+$ cells belonging to the blast cell or nonblast cell population of single determinations of one cow.

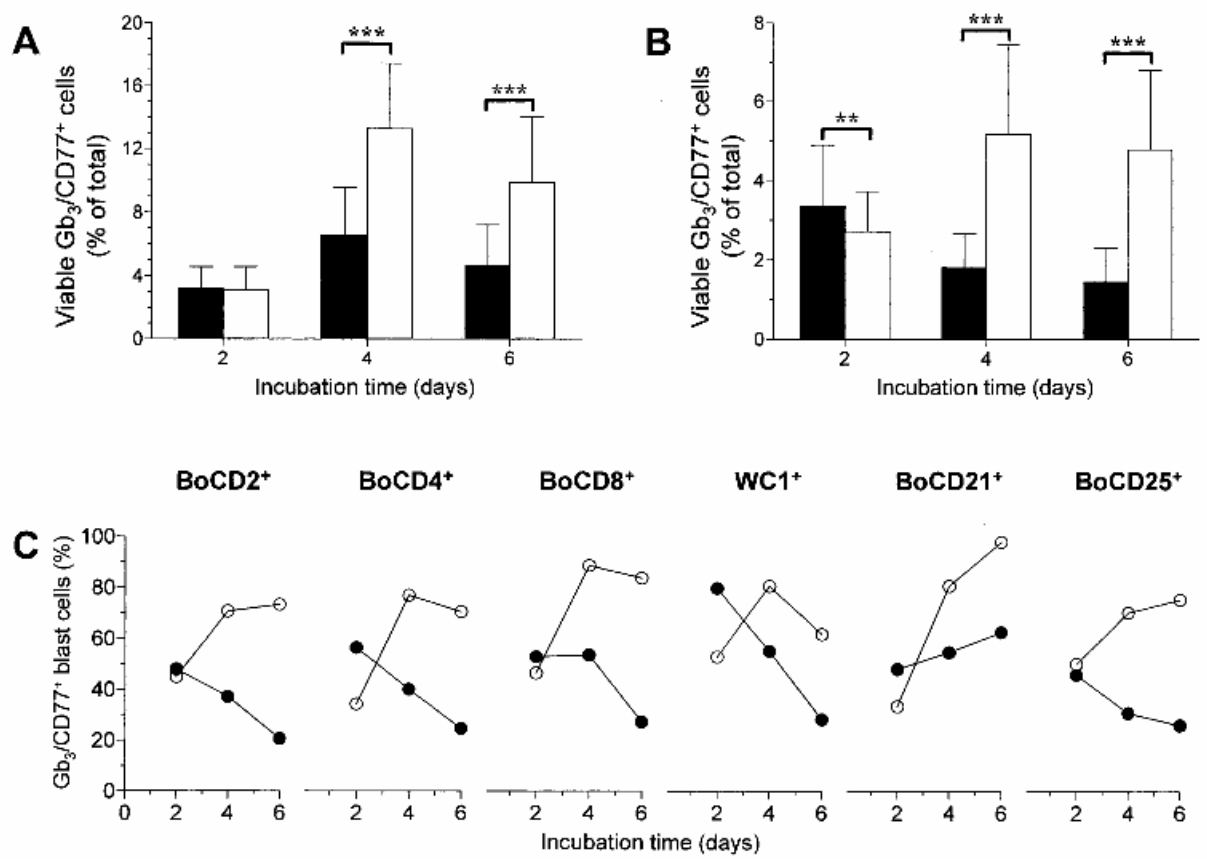

Figure 2. Effect of VT1 on Gb_3/CD77 expression by bovine PBMC subpopulations. Cells were incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Observed effects were assigned to VT1 by comparison of the results obtained in the absence (●) or presence of 1.5 µg/ml anti-VTB1 mAb 13C4 (○). Expression of Gb_3/CD77 was quantified at the time points indicated by flow cytometry. Effect of VT1 on Gb_3/CD77 expression within the blast cell (A) and nonblast cell (B) population is shown by calculating the percentage of viable, Gb_3/CD77$^+$ cells belonging to the blast cell and nonblast cell population, respectively. Bars represent mean ± SD of 20 cows. Horizontal brackets enclose bars that are different ($P \leq 0.01$ [**]; $P \leq 0.001$ [***]). (C) Effect of VT1 on Gb_3/CD77 expression by PBMC subpopulations of one cow was depicted by calculating the percentage of Gb_3/CD77$^+$ blast cells belonging to the indicated subpopulations (single determinations; WC1, antigen solely expressed by bovine γδ T cells).

using SigmaStat 2.0 software (1992; SPSS Inc., Chicago, IL). Significant differences were separated at $P \leq 0.001$ [***], $P \leq 0.01$ [**], and $P \leq 0.05$ [*].

Results

Effect of VT1 on Gb_3/CD77$^+$ Bovine PBMC. Using PBMC cultures as a model, the percentage of Gb_3/CD77$^+$-enlarged lymphoblast cells as well as Gb_3/CD77$^+$ nonblast cells increased constantly within 1 to 2 days after the initiation of culture and peaked at Day 4 in the absence or the presence of the mitogens PHA-P and LPS, respectively (Fig. 1). The presence of nanogram concentrations of VT1 (200 CD_{50}/ml as determined on Vero cells; one verotoxic dose 50% was previously calculated to be equivalent to 0.4–0.8 pg/ml of purified VT) (24) dramatically reduced the number of viable Gb_3/CD77$^+$ cells in the blast cell and nonblast cell population over time (Fig. 1). The effect of VT1 became obvious only after 4 days of incubation, implicating that Gb_3/CD77 expression on Days 1 and 2 is a

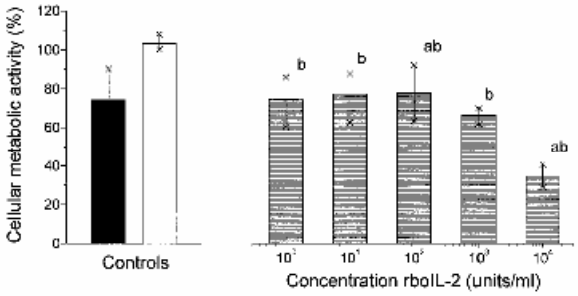

Figure 3. Effect of rboIL-2 and VT1 on the cellular metabolic activity of bovine PBMC. Cells were either incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) with (open bar) or without (black bar) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with indicated concentrations of rboIL-2 (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Cellular metabolic activity was determined by MTT reduction assay after 96 hr of incubation. Data represent mean, minimum, and maximum of nine determinations with cells from three cows. Differences ($P \leq 0.05$) to VT1 (a) and VT1 + anti-VTB1 (b) were detected.

prerequisite for the effect of VT1 to occur. Analysis of PHA-P-stimulated PBMC of a significant number ($n = 20$) of cows (Fig. 2, A and B) indicated that VT1 did not completely eliminate $Gb_3/CD77^+$ cells. Even in the presence of VT1, viable $Gb_3/CD77^+$ cells were detectable within the cultures over the entire cultivation period, although initial experiments had shown that the concentration of VT1 applied was sufficient to induce maximum effects (data not shown). Surprisingly, at an early phase of cultivation the number of viable $Gb_3/CD77^+$ nonblast cells in VT1-treated cultures significantly exceeded the number of positive cells in cultures treated with VT1 plus anti-VTB1 mAb (Fig. 2B). Irrespective of susceptibility of different PBMC subpopulations to the inhibitory effects of VT1, with $BoCD8^+$ T cells and $BoCD21^+$ B cells being the most sensitive cells, a marked reduction of $Gb_3/CD77^+$ blast cells in the presence of VT1 was detectable for all lymphocyte subsets investigated (Fig. 2C).

Interference of Selected Cytokines and the Proliferation Inhibiting Effect of VT1. Checking the hypothesis that VT1 might have impaired a paracrine IL-2 release in the cultures, addition of recombinant bovine interleukin-2 (rboIL-2; 1–10,000 units/ml) did not overcome the reduction of the cells' metabolic activity caused by VT1 (Fig. 3). At the lowest rboIL-2 concentration used (1 unit/ml), the metabolic activity was as much reduced as could be seen with VT1 alone. This reduction was even amplified at increasing rboIL-2 concentrations. Likewise, rboIL-2 neither influenced inhibitory effects of VT1 on $BoCD8^+$ and $BoCD21^+$ cells nor the toxin-induced reduction of $Gb_3/CD77^+$ blast cells at a any significant level (Fig. 4; $P > 0.05$ for VT1 + rboIL-2 versus VT1).

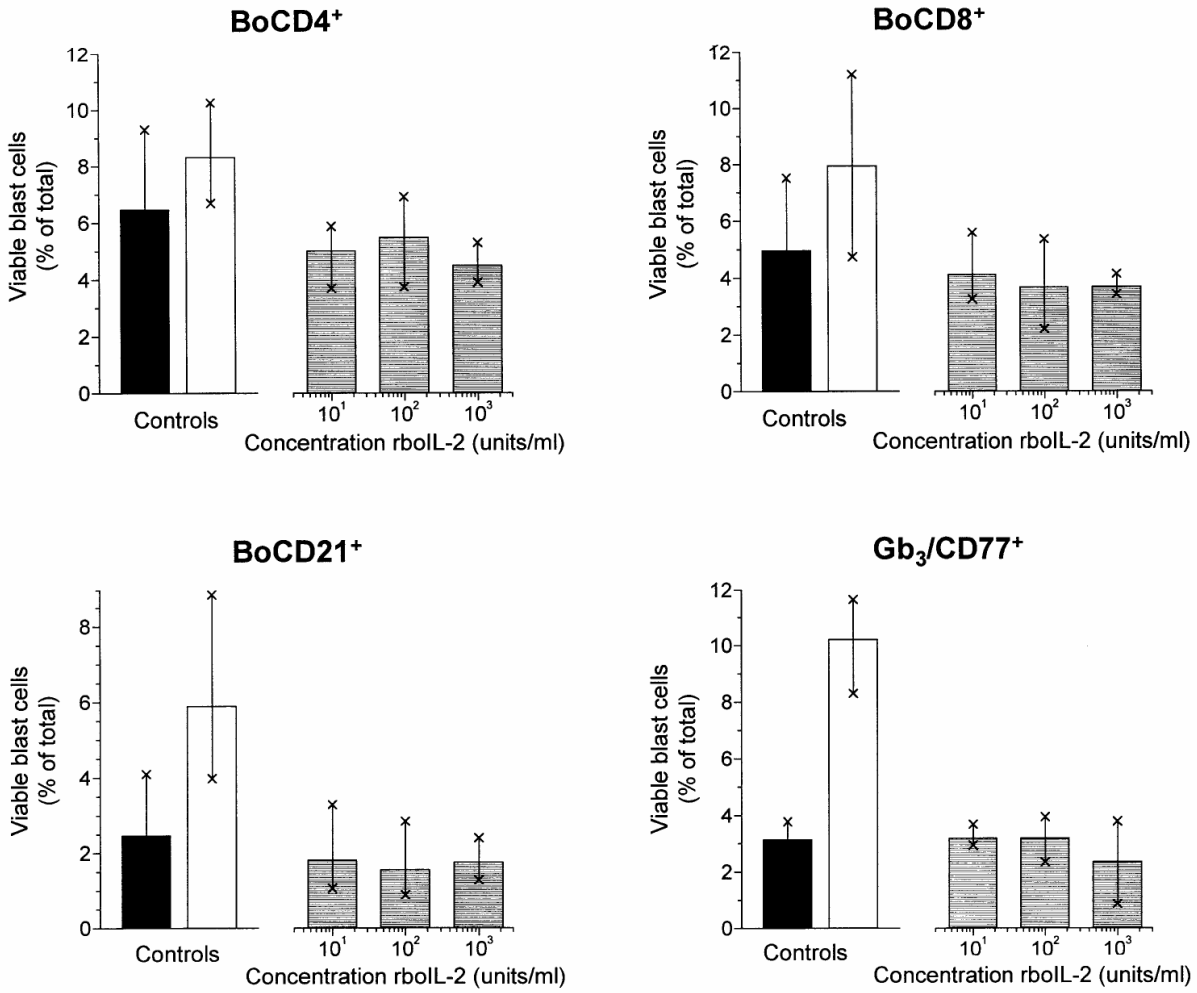

Figure 4. Effect of rboIL-2 and VT1 on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with indicated concentrations of rboIL-2 (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean, minimum, and maximum of determinations from three cows.

The effects of VT1 on bovine lymphocytes also were not due to a VT1-induced release of TNF-α from monocytes because addition of rboTNF-α (1–10,000 ng/ml) alone did not mimic the effect of VT1 and did not reduce the cellular metabolic activity (data not shown). Although rboTNF-α (10–1,000 ng/ml) led to a slight decrease ($P \leq 0.05$ for 100 and 1000 ng/ml rboTNF-α each versus VT1 + anti-VTB1) in the percentage of $BoCD21^+$ blast cells (Fig. 5), the percentages of $BoCD8^+$ and $Gb_3/CD77^+$ blast cells were not different ($P > 0.05$ for rboTNF-α versus VT1 + anti-VTB1) in rboTNF-α-treated and negative control cultures (Fig. 5).

Recombinant human interferon-α (rhuIFN-α; 1.5–1,500 units/ml) alone did not significantly alter the transformation and proliferation of PBMC subpopulations and the number of viable $Gb_3/CD77^+$ blast cells, but slightly depressed ($P \leq 0.05$) the metabolic activity of the cells at high concentrations (15,000 units/ml; data not shown). To test whether IFN-α was able to modulate the effect of VT1, the cells were incubated in the presence of 1.5–150 units/ml rhuIFN-α simultaneously to VT1. In this experiment, the metabolic activity of PBMC was reduced to a level similar to that when incubated with VT1 alone (Fig. 6). Moreover, rhuIFN-α augmented the VT1-induced reduction at higher concentrations. The addition of rhuIFN-α (15–1,500 units/ml) to VT1-treated cultures only marginally affected the transformation and proliferation of PBMC subpopulations (Fig. 7). In cultures with rhuIFN-α and VT1, the percentage of $Gb_3/CD77^+$ blast cells was reduced to a level similar to that in cultures treated with VT1 alone ($P > 0.05$ for VT1 + rhuIFN-α versus VT1).

Effect of VT1 on Prestimulated Bovine PBMC. The results described above suggest that induction of Gb_3/CD77 expression occurred before the inhibitory effect of VT1 when the toxin is present in the culture from the be-

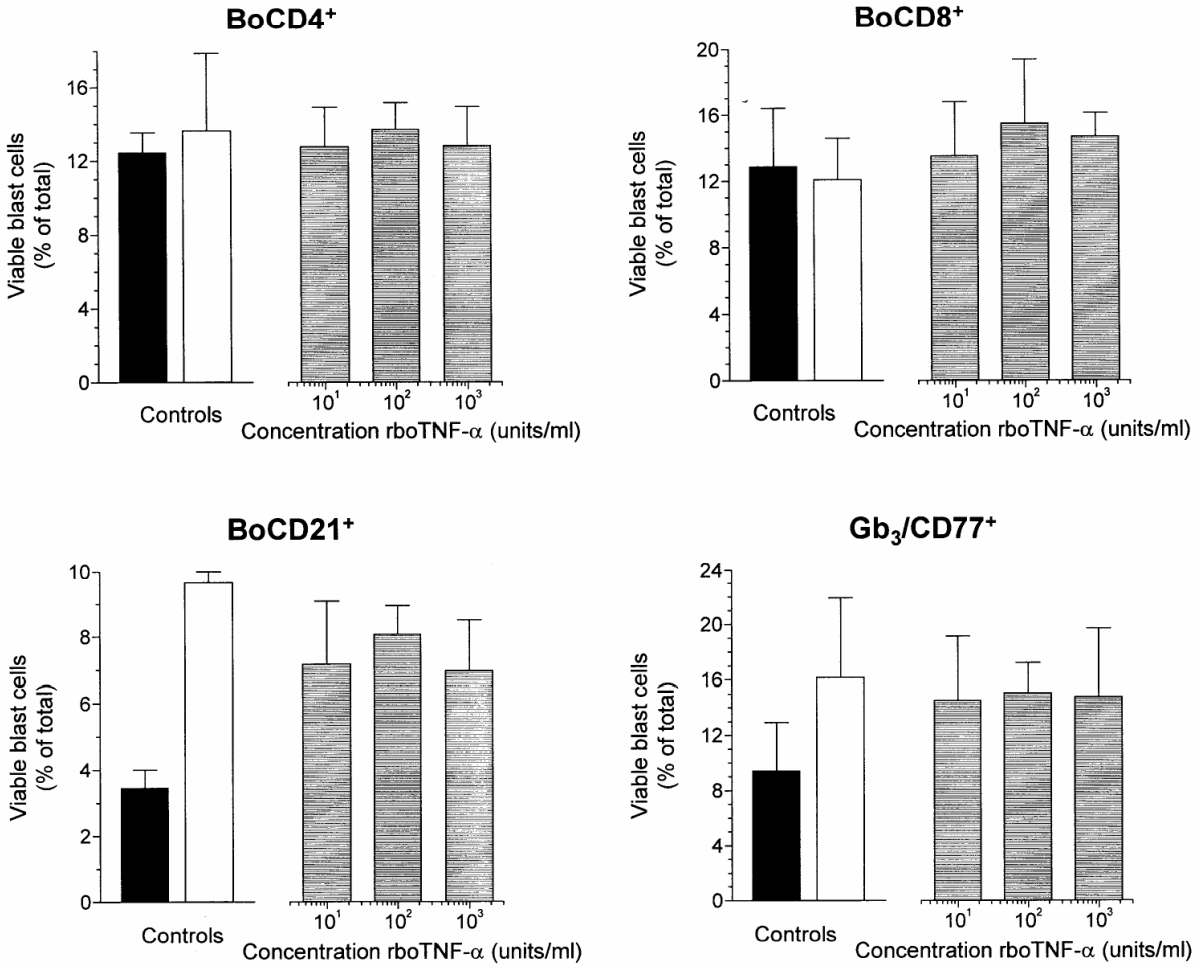

Figure 5. Effect of rboTNF-α on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 μg/ml) or with rboTNF-α at the indicated concentrations (striped bars) at 37°C. Culture medium was supplemented with 5 μg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determinations from four cows.

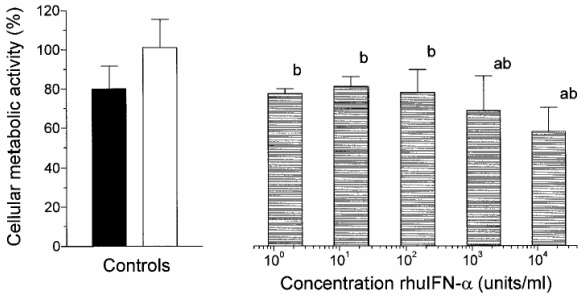

Figure 6. Effect of rhuIFN-α and VT1 on the cellular metabolic activity of bovine PBMC. Cells were either incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) with (open bar) or without (black bar) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with indicated concentrations of rhuIFN-α (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Cellular metabolic activity was determined by MTT reduction assay after 96 hr of incubation. Percentage activity was calculated and data represent mean ± SD of 12 determinations with cells from four cows. Differences ($P < 0.01$) to VT1 (a) and VT1 + anti-VTB1 (b) were detected.

ginning. Consequently, PBMC that were prestimulated to express Gb_3/CD77 at high levels before they experienced VT1 should have been much more sensitive to the toxin. We compared PHA-P-stimulated PBMC treated with VT1 from the beginning of the incubation (Day 0) until Day 4 with cells that received toxin after a 3-day prestimulation period and were characterized on Day 6 (Fig. 8). In fact, VT1 led to a more pronounced decrease in the number of viable $BoCD8^+$ cells and $BoCD21^+$ cells when added to prestimulated PBMC. However, reduction of cell numbers was not notably different from that achievable in cultures treated from Day 0 until Day 4 (Figs. 1–7). Even when VT1 was added to the cells on Day 3, a prominent number of $Gb_3/CD77^+$ cells were still detectable after 3 days of cultivation. Based on our data, susceptibility of bovine PBMC cultures for VT1 did not directly correlate with the percentage of $Gb_3/CD77^+$ cells.

Discussion

Several studies using experimental infections and cultures of lymphoblastoid cell lines suggested that VT can cause immunomodulatory effects in animals infected with VTEC (16, 25–27). Based on our finding that VT1 inhibits bovine lymphocyte proliferation *in vitro* (15), we attempted to elucidate the mechanism underlying the immunomodulation by using primary cultures. Expression of Gb_3/CD77 (recognized as the VT1 receptor in other cell systems) in fact preceded the inhibitory effect of VT1 on bovine lymphocytes. After activation-induced Gb_3/CD77 surface expression, VT1 subsequently reduced the portion of $Gb_3/CD77^+$ cells. Inhibition by VT1 predominantly affects $BoCD8^+$ and $BoCD21^+$ cells (15), but $Gb_3/CD77^+$ cells were eliminated from all subpopulations identified. Therefore, the bovine immune system appears to be more sensitive to VT1 than the human immune system because in the latter, the activity of VT1 is restricted to the B cell compartment (25, 28). The significance of VT1 in bovine VTEC infections has been questionable since Pruimboom-Brees *et al.* (29) published information that cattle lack vascular receptors for VT. However, the present study indicates that in contrast to other species in which endothelial cells are the main targets for VTs, immune system cells are a predominant target for VT1 in cattle.

The impact of immunomodulatory effects caused by bacterial products *in vitro* on the course of bacterial infections can only be appropriately estimated if it is understood whether this effect truly results from a direct action on immune system cells or is rather due to a perturbation of cytokine profiles (30). Thus, we investigated the effects of selected cytokines in the presence and absence of VT1. First, TNF-α is secreted by human monocytes such as after stimulation with VT1 and sensitizes human endothelial cells by inducing Gb_3/CD77 (8). The PBMC preparations used in our studies consisted of up to 15% monocytes (15). Consequently, inhibition of lymphocytes could be secondary to a VT1-induced release of TNF-α from monocytes. However, the addition of recombinant bovine TNF-α (rboTNF-α) alone did neither augment the expression of Gb_3/CD77 nor did it mimic the effect of VT1 within a wide concentration range. Accordingly, we were not able to detect significant amounts of TNF-α in VT1-treated bovine monocyte cultures (C. Menge, unpublished data). Second, IFN-α only binds to its receptor (IFNAR) in its high-affinity conformation complexed to Gb_3/CD77 (10). Due to the partial homology between the bovine IFNAR and the receptor-binding B-subunit of VT1 (10), it appeared that IFN-α might have been able to block the activity of VT1 by stabilization of the Gb_3/CD77-IFNAR complex. However, the fact that rhuIFN-α augmented the inhibitory effect of VT1 in a concentration-dependent manner suggested that both substances use different signal transduction pathways. Third, because VT1 increased and prolonged expression of the bovine IL-2 receptor BoCD25 (15), we speculated that the effect of VT1 could have been due to a blockage of the paracrine boIL-2-release. This phenomenon is induced in human lymphocyte cultures (31) by a factor encoded for by the *lifA* gene of enteropathogenic *E. coli* (32). The fact that exogenous rboIL-2 was unable to overcome the effect of VT1 on bovine lymphocytes in a concentration range from 10 to 1000 units/ml suggests a direct effect of VT1.

An impact of cytokines cannot be ruled out completely because cytokines other than those used in our study may be involved, but VT1 drastically decreased the number of $Gb_3/CD77^+$ cells and predominantly acted as a leukotoxin rather than a modulin. Interestingly, low numbers of positive cells were still detectable during the entire cultivation period, even in the presence of VT1. Because VT1 also lowered the mean number of Gb_3/CD77 molecules on the surface of positive cells as concluded from a reduced mean fluorescence intensity (data not shown), VT1 seemed to induce a cytotoxic mode of action by eliminating $Gb_3/CD77^{high}$ cells.

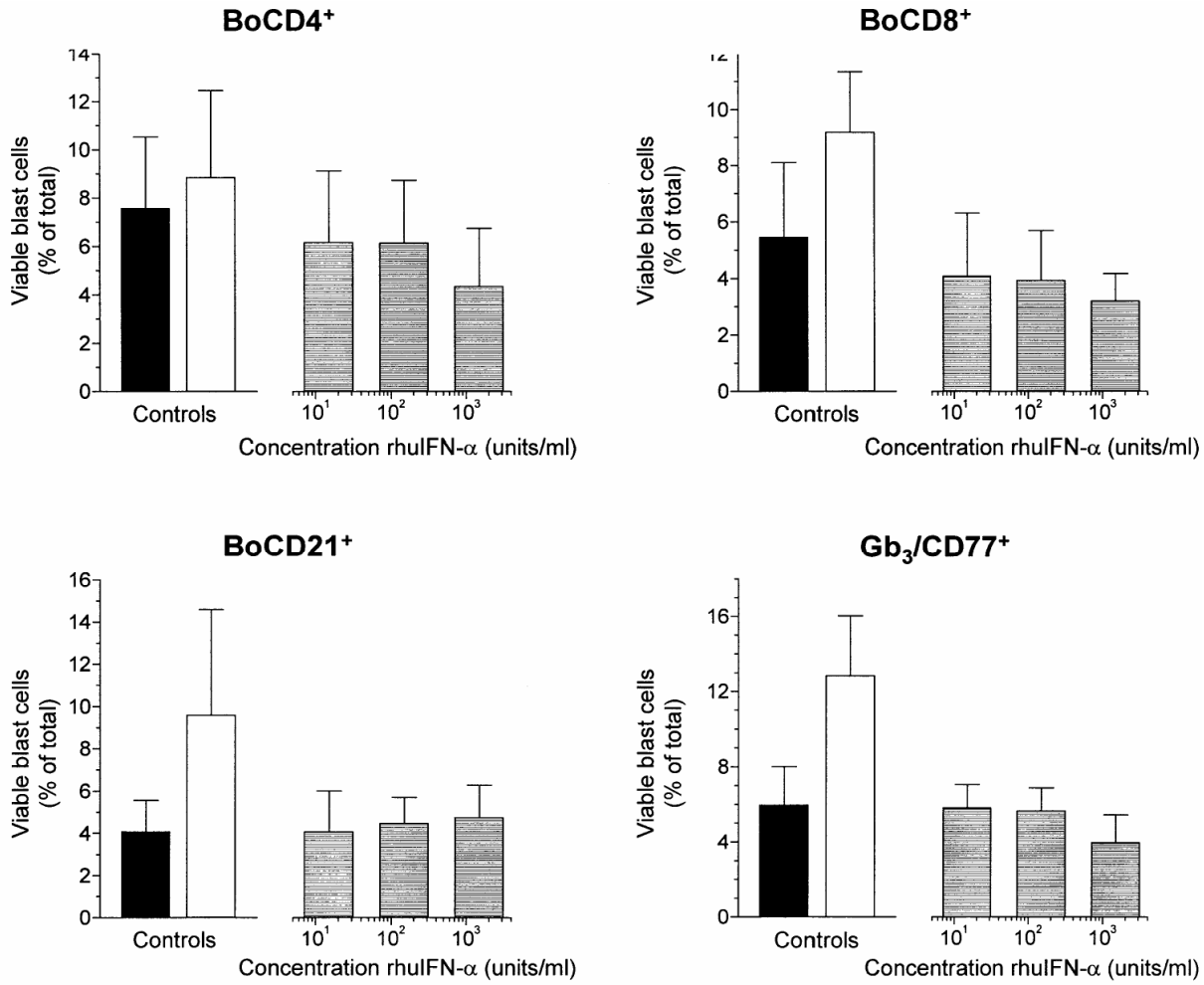

Figure 7. Effect of rhuIFN-α and VT1 on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with various concentrations of rhuIFN-α (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determination from four cows.

Such a correlation between the susceptibility of cells for VT1 and the number of Gb_3/CD77 molecules expressed on the cellular surface has already been described for other cell lines (33). However, there is evidence that the effect of VT1 on bovine cymphocytes is much more sophisticated. First, there is a marked contradiction between the prominent reduction in cellular metabolic activity caused by VT1 and the lack of a detectable increase in the number of apoptotic cells on Day 4 of culturing (15). Second, the percentage of Gb_3/$CD77^+$ cells was drastically decreased by VT1, even in those subpopulations of bovine lymphocytes proliferation of which was only marginally affected (Fig. 2). Third, the reduction of Gb_3/$CD77^+$ cells also was significant within the nonblast population of lymphocytes (Figs. 1 and 2) that expressed Gb_3/CD77 just at low to moderate levels (18). Gb_3/CD77 expression by bovine lymphocytes parallels the activation of the cells up to a certain stage characterized by moderate Gb_3/CD77 expression (18). At this point, the cells either survive and probably downregulate Gb_3/CD77 again or die from apoptosis expressing high levels of Gb_3/CD77. The effect of VT1 thus likely occurred early in cultivation on apparently small numbers of sensitive cells between the Gb_3/$CD77^{low}$ and the Gb_3/$CD77^{moderate}$ state of activation. These cells then end up in apoptosis after transient high-level Gb_3/CD77 expression. This might explain the significantly enhanced percentage of Gb_3/$CD77^+$ nonblast cells in VT1-treated cultures on Day 2 (Figs. 1 and 2). This notion is further supported by the fact that the addition of VT1 on Day 3 of the cultures still induced effects in a magnitude similar to that associated with addition of the toxin at the beginning. The cells must have become refractory again when they reached the Gb_3/$CD77^{high}$ state before experiencing VT1. Because bovine lymphocytes are VT1 sensitive only transiently during the activation process, VTEC

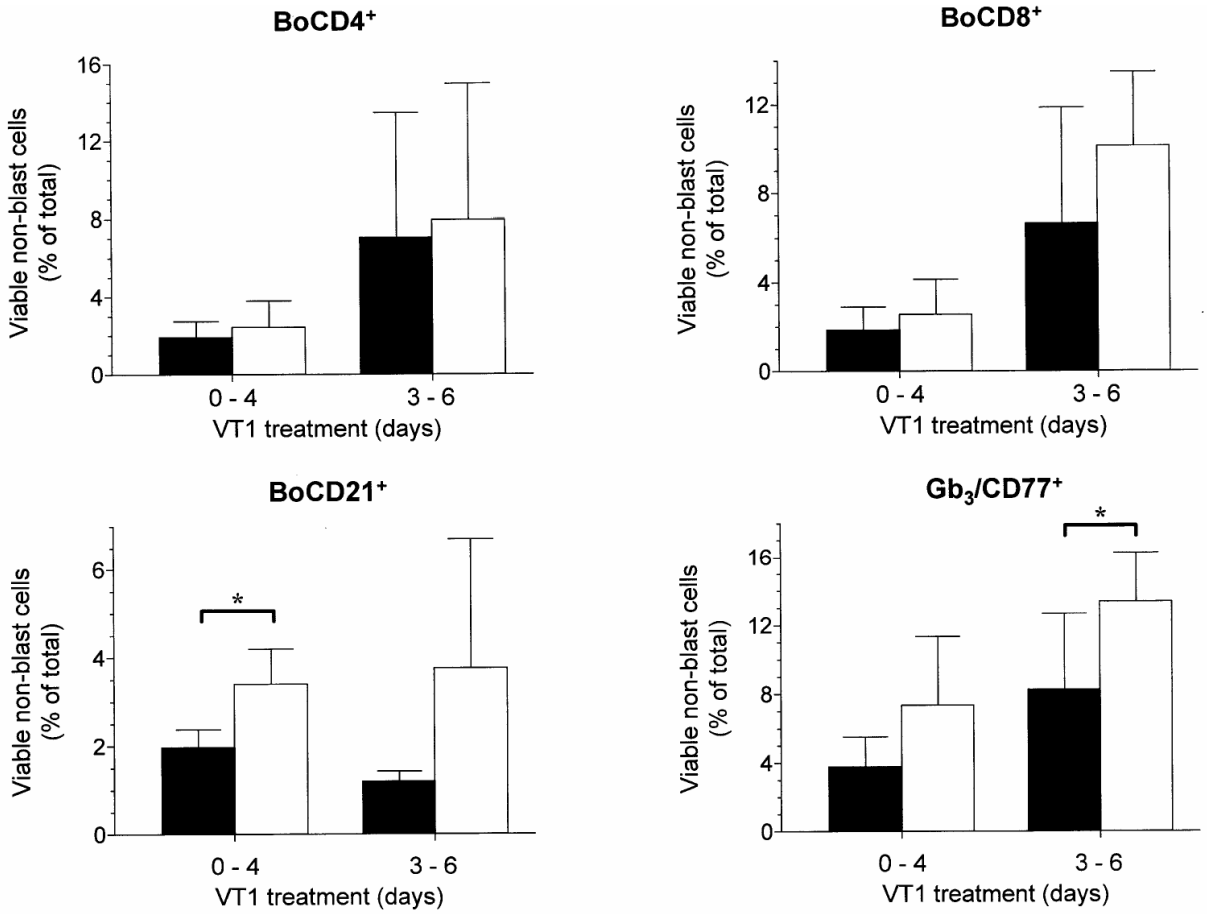

Figure 8. Effect of VT1 on prestimulated PBMC. Cells were incubated with VT1 (200 CD_{50}/ml, quantified on Vero cells) either with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 µg/ml) at 37°C starting either on the day of PBMC preparation (Day 0) or on Day 3 of incubation. Culture medium was supplemented with 5 µg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping on Days 4 and 6 of incubation, respectively, quantified by flow cytometry, and the percentage of viable nonblast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determinations from four cows. Horizontal brackets enclose bars that are different ($P \leq 0.05$ [*]).

secreting VT1 predominantly block the onset of an immune response in their host rather than downregulating an established one. From the bacterial point of view, this strategy is highly efficient as less amounts of toxin are necessary to target smaller numbers of immune cells.

The fact that VT1 directly targets several subsets of lymphocytes at an early phase of activation underlines that the immunomodulation via secretion of VT1 is a very specific feature of VTEC. The impact of these findings on the course of VTEC infections can only be a matter of speculation. Currently, the impact of the interactions between VT and immunocytes on pathogenesis of HUS is under intense review (34–36). In cattle, Hoffman *et al.* (16) showed that experimental VTEC infection of calves led to a reduced mitogenic responsiveness of PBMC *in vivo*, confirming the relevance of our *in vitro* results. However, adult cattle are asymptomatic carriers of VTEC and frequently possess antibody titers against VT (37). Therefore, VT1 presumably modulates the host's immune response of adult cattle on a local level rather than causing a systemic immune deficiency. Because Paton *et al.* (38) showed that LPS-specific antibodies can prevent the adhesion of VTEC to epithelial cells, VT1 may facilitate the colonization of the bovine gut by acting on local B cells, hindering antibody production or secretion. This would explain why VTEC are shed for longer periods compared with the closely related but VT-negative enteropathogenic *E. coli* strains as it has been shown to occur in sheep (14). Accordingly, a prominent portion of bovine B cells separated from mesenteric lymph nodes are $Gb_3/CD77^+$ (18). Interestingly, the bovine intestinal intraepithelial lymphocytes also express $Gb_3/CD77$ *in vivo* and are sensitive to the proliferation inhibiting effect of VT1 *in vitro* (39). Further investigations on VT effects on mucosal immune cells will, therefore, be of considerable importance in increasing our understanding of VTEC's adaptation to their bovine reservoir.

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Bovine Ileal Intraepithelial Lymphocytes Represent Target Cells for Shiga Toxin 1 from *Escherichia coli*

Christian Menge,* Maike Blessenohl, Tobias Eisenberg, Ivonne Stamm, and Georg Baljer

Institut für Hygiene und Infektionskrankheiten der Tiere der Justus-Liebig-Universität Giessen, D-35392 Giessen, Germany

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The discovery that bovine peripheral lymphocytes are sensitive to Stx1 identified a possible mechanism for the persistence of infections with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) in the bovine reservoir host. If intraepithelial lymphocytes (IEL) are also sensitive to Stx1, the idea that Stx1 affects inflammation in the bovine intestine is highly attractive. To prove this hypothesis, ileal IEL (iIEL) were prepared from adult cattle, characterized by flow cytometry, and subjected to functional assays in the presence and absence of purified Stx1. We found that 14.9% of all iIEL expressed Gb_3/CD77, the Stx1 receptor on bovine lymphocytes, and 7.9% were able to bind the recombinant B subunit of Stx1. The majority of Gb_3/CD77$^+$ cells were activated CD3$^+$ CD6$^+$ CD8α^+ T cells, whereas only some CD4$^+$ T cells and B cells expressed Gb_3/CD77. However, Stx1 blocked the mitogen-induced transformation to enlarged blast cells within all subpopulations to a similar extent and significantly reduced the percentage of Gb_3/CD77$^+$ cells. Although Stx1 did not affect the natural killer cell activity of iIEL, the toxin accelerated the synthesis of interleukin-4 (IL-4) mRNA and reduced the amount of IL-8 mRNA in bovine iIEL cultures. Because the intestinal system comprises a rich network of interactions between different types of cells and any dysfunction may influence the course of intestinal infections, this demonstration that Stx1 can target bovine IEL may be highly relevant for our understanding of the interplay between STEC and its reservoir host.

Persistently infected ruminants—especially cattle—are the main natural reservoir of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) (14). STEC can be detected in as many as 60% of bovine fecal samples from several countries all over the world (5). The pathogenicity of STEC for cattle is low and limited to sporadic cases of diarrhea in young calves. For adult animals, no disease condition has been linked to STEC infections to date (35). Nevertheless, STEC represents an emerging group of zoonotic enteric pathogens (39): when transmitted to humans, some STEC strains cause epidemics and sporadic cases of diarrhea, and in most cases intestinal inflammation then progresses to hemorrhagic colitis (HC). Patients with HC may develop life-threatening sequelae such as the hemolytic-uremic syndrome (HUS) (32). Once HUS is clinically established, there is no curative therapy (44). Most human STEC infections for which a source has been identified have been traced to direct or indirect contact with bovine feces (13). Strategies aimed at reducing the incidence of human STEC infections by lowering the prevalence of STEC in cattle must be based on an understanding of how STEC is able to persist in the intestine of the bovine host.

Because there are no overt signs of intestinal inflammation in STEC-infected cattle, it has been hypothesized that STEC has adapted to a purely "commensal" lifestyle in cattle (35). However, even under normal conditions, the intestinal mucosa displays a state of "physiological inflammation," manifested by the presence of abundant leukocytes in the intraepithelial and subepithelial compartments (7). Most STEC strains have an array of virulence determinants, including the induction of a characteristic histopathological feature known as the "attaching and effacing" lesion (2, 6, 10, 46), that allows them to interact with intestinal cells in multiple ways. It is tempting to assume that at least one of these determinants represents a danger signal that generates a response beyond physiological inflammation and terminates STEC colonization, even in the bovine host (34, 40). The frequent detection of anti-Stx antibodies in the sera and colostrum of cows (17, 31) provides additional evidence that the persistence of bovine STEC infections does not result from a general inability of cattle to respond to STEC or its products. STEC must have elaborated a mechanism that actively limits intestinal inflammation, maintains intestinal homeostasis, and finally allows persistent colonization.

The main virulence factors of STEC are the Stxs, potent cytotoxins of the ribosome-inactivating type comprising five B subunits that mediate Stx binding to a eukaryotic cell surface receptor (globotriaosylceramide [Gb_3]/CD77) and one A subunit that contains the enzymatic activity (33). Stxs may also play a role in facilitating intestinal persistence of STEC. Experimentally infected adult sheep shed STEC strains longer than they shed strains lacking the *stx* gene (4). Our hypothesis that Stxs are able to modulate intestinal inflammation in the bovine mucosa was based on observations that activated bovine blood-derived lymphocytes express Gb_3/CD77 molecules, a subset of which represents functional Stx1 receptors (23, 37). Stx1 binds directly to bovine blood lymphocytes and blocks activation and proliferation of these cells in vitro (21, 24). However, Stx1 does not affect all subpopulations equally but mainly blocks proliferation of CD21$^+$ B cells and CD8$^+$ T cells (24).

* Corresponding author. Mailing address: Institut für Hygiene und Infektionskrankheiten der Tiere, Frankfurter Str. 89, D-35392 Giessen, Germany. Phone: 49-641-99-38314. Fax: 49-641-99-38309. E-mail: christian.menge@vetmed.uni-giessen.de.

Approximately 10 to 15% of the cells in the bovine intestinal epithelium are intraepithelial lymphocytes (IEL), and the majority of those belong to the $CD8^+$ subset (C. Menge, unpublished data). IEL are located between the epithelial cells adjacent to the basement membrane and are the only lymphocyte population situated so close to Stxs in the entire body (27). Given that bovine IEL resemble blood-derived lymphocytes with regard to Stx receptor expression and susceptibility to Stx1, we hypothesized that Stx1 modulates intestinal inflammation by acting directly on $Gb_3/CD77^+$ IEL. In a first attempt to prove this hypothesis, we obtained IEL from the intestines of adult cattle and characterized them phenotypically ex vivo and functionally after incubation in the presence and absence of purified Stx1 in vitro.

MATERIALS AND METHODS

Protein purification. Stx1 was produced from bovine STEC1 strain 2403 (rough, H^- [47]) and purified by a procedure described previously (24). At the end of the purification process, toxin preparations were passed through Detoxi-Gel columns (Pierce, Old-Beijerland, The Netherlands) to reduce contamination with endotoxin. Cytotoxic activities of toxin preparations were determined on Vero cells (ATCC CRL 1587) by the method of Gentry and Dalrymple (9) with minor modifications (24). Cellular metabolic activity was assessed by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as described previously (22, 24). The 50% cytotoxic dose (CD_{50}) was calculated from dose-response curves geometrically as the reciprocal of the toxin dilution causing a 50% reduction in cellular metabolic activity. The Stx1 preparation contained 50,000 CD_{50}s of Stx1 and 0.17 ng of endotoxin per ml, as determined by the *Limulus* amoebocyte lysate assay. Recombinant StxB1 (rStxB1) was purified from *E. coli* DH5α(pSU108) (42) by the method of Niebuhr (29) with slight modifications (37). The rStxB1 preparation used in the present study contained 411 μg of rStxB1/ml.

Preparation and stimulation of bovine ileal IEL (iIEL). Gut specimens (distal ileum) were obtained from healthy adult (>24-month-old) cows (German black pied) slaughtered at the local slaughterhouse. Digesta were removed by washing with tap water. After incubation with phosphate-buffered saline (PBS) supplemented with 1 mM dithiothreitol at 37°C and 100 rpm for 25 min, the specimens were cut into strips (width, 0.5 to 1 cm). The strips were then transferred to 50-ml centrifugation tubes filled with 35 ml of PBS-EDTA-AB solution (PBS supplemented with 2 mM EDTA, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 50 μg of gentamicin/ml) and incubated (at 37°C and 100 rpm for 20 min). After vortexing (for 2 min at maximum speed), the liquid contents of the tubes were passed through nylon wool. Cells were collected by centrifugation (at 250 × *g* for 8 min), resuspended in 25 ml of Percoll solution (density adjusted to 1.0500 g/ml with PBS-EDTA [PBS supplemented with 5.4 mM EDTA]; Biochrom, Berlin, Germany), and layered onto 10 ml of Percoll solution (density adjusted to 1.0816 g/ml) in a 50-ml centrifugation tube. The gradient was covered with 15 ml of Leibowitz L15 medium. After centrifugation (at 652 × *g* for 20 min), cells were recovered from the Percoll-Percoll interface, washed twice with PBS-EDTA solution (at 202 × *g* for 7 min), and resuspended at 5×10^6 cells/ml in modified cell culture medium (IEL medium, containing 89% RPMI 1640 [Biochrom], 10% fetal calf serum [Life Technologies GmbH, Karlsruhe, Germany], 100 IU of penicillin/ml–100 μg of streptomycin/ml [Life Technologies GmbH], and 3 μM mercaptoethanol [Fluka, Taufkirchen, Germany]).

For stimulation assays, 2.5×10^5 IEL/150 μl of IEL medium were plated in triplicate onto 96-well flat-bottom microtiter plates (Nunc, Wiesbaden, Germany). The medium was additionally supplemented with recombinant human interleukin-2 (rhuIL-2) (200 IU/ml; kindly provided by H. Jomaa, Institute for Biochemistry, Academic Hospital Centre, Giessen, Germany) with or without purified Stx1 (24) at 200 CD_{50}s/ml, and for neutralization studies the latter was preincubated (for 90 min at room temperature) with purified anti-StxB1 (41) (monoclonal antibody 13C4; 1.5 μg of immunoglobulin per ml). Previous experiments had shown that this concentration of antibody is sufficient to completely neutralize the biological activity of at least 200 CD_{50}s/ml in the Vero cell assay (21, 24). For IEL stimulation, the medium was supplemented with nine different combinations (see Fig. 3) of commonly used mitogens with different modes of action (all purchased from Sigma, Taufkirchen, Germany): concanavalin A (final concentration, 2.5 μg/ml), phytohemagglutinin P (PHA-P; 2.5 μg/ml), pokeweed mitogen (2.5 μg/ml), phorbol-12-myristate-13-acetate (PMA; 5 and 20 ng/ml), and ionomycin (500 ng/ml). The plates were incubated for 72 h at 37°C under 5% CO_2 at 95% humidity. Blast cell transformation of lymphocytes was quantified by flow cytometry according to the cells' light scatter characteristics by acquiring 5,000 events as described previously (22).

Immunophenotyping studies. Immediately after preparation or at the end of the cultivation period, IEL were thoroughly resuspended and transferred to V-shaped microtiter plates (Greiner, Frickenhausen, Germany) for immunolabeling as described previously (22, 25). Briefly, the cells were centrifuged (at 137 × *g* and 4°C for 7 min) and resuspended in 50 μl of cell culture medium as a negative control or with supernatants or diluted ascites fluid of hybridoma cell lines. Antibodies kindly provided by J. Naessens (International Livestock Research Institute [ILRI], Nairobi, Kenya) were as follows: IL-A11 (specific for bovine CD4), IL-A57 (CD6), IL-A105 (CD8α), IL-A65 (CD21), IL-A111 (CD25), IL-A118 (CD44), IL-A77 (CD71), IL-A30 (surface immunoglobulin M [IgM]), J11 (major histocompatibility complex class II), IL-A29 (WC1), and IL-A96 (WC9). Antibodies purchased from VMRD (Pullman, Wash.) were MM1A (specific for CD3), BAT82A (CD8β), CACT61A (TcR1-N12), CACTB6A (TcR1-N6), CACTB81A (TcR1-N7), and CACT26A (ACT-2). Alternatively, the cells were resuspended with 25 μl of rat IgM (1 mg/ml; dilution, 1:50 in PBS; Camon, Wiesbaden, Germany) as a negative control or with an anti-human CD77 antibody (clone 38.13; dilution, 1:10 in PBS; Beckman-Coulter, Krefeld, Germany). Cells were incubated on ice for 20 min, washed once with PBS supplemented with 1% bovine serum albumin and 0.01% sodium azide (BSA-azide), resuspended with either 50 μl of an anti-mouse γ-chain antibody conjugated with fluorescein isothiocyanate (dilution, 1:100 in PBS; Medac, Hamburg, Germany) or an anti-rat μ-chain antibody conjugated with R-phycoerythrin (R-PE) (diluted 1:200 in PBS containing 2 μg of propidium iodide [PI; Sigma]/ml; Beckman-Coulter), and kept on ice for 20 min. In double-staining experiments, cells were incubated with anti-CD77 and an anti-rat μ-chain antibody conjugated with PE (Beckman-Coulter) after the staining procedure described above. Finally, the cells were washed twice and analyzed with an EPICS ELITE Analyser (Beckman-Coulter). A total of 5,000 events were acquired from each sample. Data analysis was performed with the ELITE (version 4.01) software provided by the manufacturer. Electronic gates were set according to the negative control included in each test series, defining less than 2% of the cells as positive. When freshly prepared iIEL were analyzed, a region was set around a dense population of events with indistinguishable light scatter characteristics. Similarly, when cultured iIEL were investigated, two populations of non-blast cells and enlarged blast cells were defined according to their light scatter characteristics as described previously (22, 23) and were analyzed separately.

rStxB1 binding studies. For binding studies, cells were sequentially incubated with 50 μl of rStxB1 (30 μg/ml) for 30 min on ice (37), washed once with BSA-azide, resuspended in 50 μl of anti-StxB1 (45 μg/ml), incubated for 30 min on ice, washed, resuspended in 50 μl of an anti-mouse γ-chain antibody conjugated with fluorescein isothiocyanate (Dianova, Hamburg, Germany), incubated for 30 min on ice, washed twice, and analyzed by flow cytometry as described above. In some instances, cells were additionally immunolabeled as described above, with the primary antibodies from ILRI used in biotinylated form and detection carried out with PE-conjugated streptavidin (Dianova). A supernatant of a hybridoma cell line producing an irrelevant antibody was used between the staining steps to block nonspecific binding.

Quantitation of natural killer cell activity of bovine iIEL. Cells from a homologous B lymphoma cell line (BL-3; ECACC 86962401) were used as target cells. Cells were maintained in a medium (BL-3 medium) consisting of 56% RPMI 1640 (Biochrom), 23% Leibowitz L15 medium, 20% fetal calf serum, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (all purchased from Life Technologies), and 3 μM 2-mercaptoethanol (Fluka). To differentiate between target and effector cells, 10^6 BL-3 cells were stained with 25 μg of 3,3′-dioctadecyloxacarbocyaninperchlorate (DiO; Molecular Probes, Leiden, The Netherlands) in 2 ml of the medium at 37°C (for 4 h at 100 rpm). The cells were washed twice with PBS and incubated in the medium at 37°C under 5% CO_2 and 95% humidity overnight. Prior to addition of the cells to the test mixture, the washing step was repeated twice and cells were resuspended in 2 ml of BL-3 medium.

Freshly prepared effector cells (iIEL) were incubated in the presence or absence of Stx1 (200 CD_{50}s/ml) with or without anti-StxB1 (1.5 μg/ml) at a density of 2.5×10^5/150 μl of IEL medium in 6-well multiwell plates (5 to 10 ml per well) for 24 h at 37°C under 5% CO_2 and 95% humidity. Anti-StxB1 (final concentration, 1.5 μg/ml) was then added to wells supplemented only with Stx1. After an additional incubation period of 2 h, IEL were then harvested and plated onto 96-well flat bottom microtiter plates in triplicate in threefold dilution series starting with 10^6 cells/well in 200 μl of IEL medium. A total of 10^4 BL-3 cells (stained with DiO as mentioned above) were added to each well, resulting in effector/target ratios of 100:1, 33:1, 11:1, 3.6:1, and 1.2:1. Target cell controls

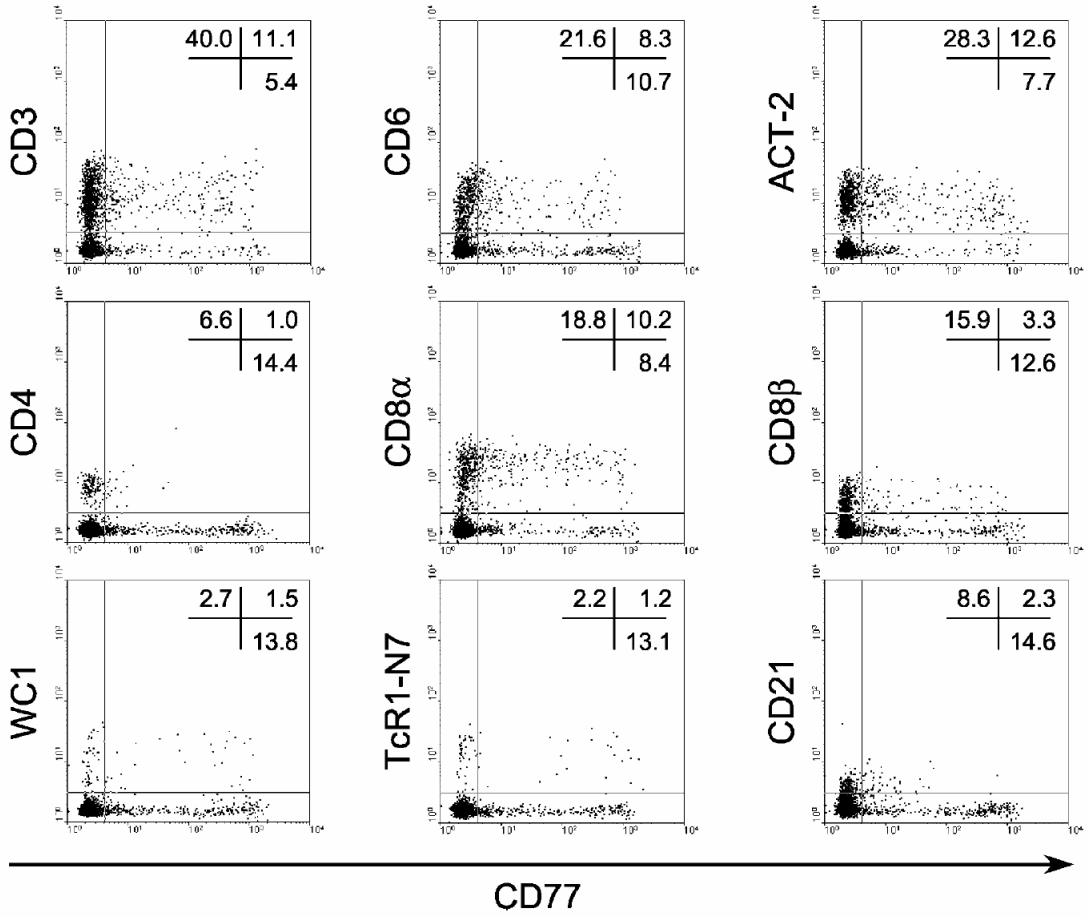

FIG. 1. Flow cytometer dot plots illustrating Gb_3/CD77 expression by bovine iIEL subpopulations ex vivo. IEL were prepared from the ileum of an adult animal at slaughter and were subsequently double labeled with anti-CD77 and subpopulation-identifying monoclonal antibodies. Dot plots depict cells of the lymphocyte region in the forward- versus side-scatter plot from a single representative of six experiments. Percentages of cells positive for one or both of the antigens are given in the upper right corners of the dot plots.

without effector cells were included. After incubation for 18 h (at 37°C under 5% CO_2), cells were analyzed by flow cytometry, as described above. Samples were measured in PBS containing 2 μg of PI/ml. The ratio of dead (PI-positive) to viable (PI-negative) target cells was calculated after gating on 2,500 target cells according to their FL1 (DiO) signal. Natural killer cell activity was determined as the difference between the mean percentages of lysed target cells in test and control samples.

Preparation of mRNAs from bovine iIEL. A total of 3.75×10^7 freshly prepared IEL in 15 ml of IEL medium (with the fetal calf serum concentration raised to 20%) were plated onto 10-cm-diameter petri dishes. The medium was supplemented with 2.5 μg of PHA-P/ml. Cells were incubated for 4.5 h at 37°C under 5% CO_2 in the presence or absence of purified Stx1 (200 CD_{50}s/ml). Cells were then harvested and counted by trypan blue exclusion. A total of 10^7 viable cells from each treatment were washed twice with ice-cold PBS and finally resuspended in 2 ml of RNAzol (Wak-Chemie, Steinbach, Germany). Samples were stored at −80°C for as long as 3 days until isolation of RNA.

After thawing, samples were intensively mixed with 200 μl of chloroform, incubated on ice for 5 min, and centrifuged (at 12,000 × *g* and 4°C for 15 min). The upper clear phase was recovered, mixed with the same volume of isopropanol, and incubated on ice for 15 min. After centrifugation (at 12,000 × *g* and 4°C for 15 min), pellets were washed twice in 75% ethanol, air dried, and resuspended in diethyl pyrocarbonate-treated distilled water. The nucleic acid content was estimated spectrophotometrically. The quality of isolated RNA was checked by gel electrophoresis.

Reverse transcription. cDNA was obtained from RNA by reverse transcription of 1 μg of RNA per sample by using 200 U of Moloney murine leukemia virus reverse transcriptase (H^-) (Promega, Mannheim, Germany) and 0.1 nmol of $oligo(dT)_{16}$ primers (Applied Biosystems, Darmstadt, Germany) in a 40-μl reaction volume according to the manufacturer's instructions. After annealing at 70°C for 5 min, the reaction was performed at 37°C for 60 min, followed by 94°C for 2 min. Negative controls were performed without Moloney murine leukemia virus reverse transcriptase (H^-).

Quantitation of cytokine-specific mRNA from bovine iIEL. Cytokine-specific PCR was performed in a 20-μl reaction volume including 1 U of AmpliTaq polymerase (Applied Biosystems), 1 μM concentrations of each primer, and 1 μl of cDNA template. Previously published cytokine primers (11, 26) were used with minor modifications: for IL-2, 5′-TCT TGC ATT GCA CTA ACT CT-3′ (sense) and 5′-GCT TTG ACA AAA GGT AAT CC-3′ (antisense); for IL-4, 5′-GCC ACT TCG TCC ATG GAC AC-3′ (sense) and 5′-TCC CAA GAG GTC TCT CAG CG-3′ (antisense); for IL-8, 5′-GCA GTT CTG TCA AGA ATG AG-3′ (sense) and 5′-GGA TCT TGC TTC TCA GCT C-3′ (antisense); for IL-10, 5′-TGT TGC CTG GTC TTC CTG-3′ (sense) and 5′-TCT CTT CAC CTG CTC CAC-3′ (antisense); for gamma interferon (IFN-γ), 5′-GCT TTA CTG CTC TGT GTG CT-3′ (sense) and 5′-GAC TTC TCT TCC GCT TTC

TABLE 1. Gb_3/CD77 expression by bovine iIEL subpopulations ex vivo

Surface antigen defining subpopulation	% of cells coexpressing Gb_3/CD77[a]	Fluorescence intensity for Gb_3/CD77 expression[a]
CD44	23.3 (16.5–32.1)	2.52 (1.97–3.24)
CD3	22.3 (14.0–37.5)	2.67 (1.08–6.07)
CD4	20.0 (9.8–28.2)	1.53 (0.61–6.85)
CD8α	38.7 (23.7–52.7)	3.28 (2.33–4.24)
CD8β	18.2 (10.6–31.3)	2.84 (1.53–5.04)
TcR1-N7	47.8 (25.9–91.7)	5.30 (0.81–10.0)
WC1	43.9 (15.4–70.0)	4.58 (0.60–11.9)
WC9	21.0 (11.5–36.7)	2.17 (0.72–6.45)
CD21	30.2 (0.0–50.0)	1.00 (0.70–1.36)
Surface IgM	16.9 (12.5–27.8)	0.89 (0.40–1.27)
MHC-II[b]	28.4 (20.8–48.1)	1.50 (0.93–1.96)
CD25	25.8 (0.0–46.7)	2.40 (0.60–8.87)
ACT-2	29.3 (17.2–47.2)	3.78 (1.64–5.98)

[a] Values are means (ranges) from 4 to 12 determinations with 2 to 6 iIEL preparations. Fluorescence intensity is expressed in arbitrary units.
[b] MHC-II, major histocompatibility complex class II.

TG-3′ (antisense); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ATC ACT GCC ACC CAG-3′ (sense) and 5′-CAT GCC AGT GAG CTT-3′ (antisense). GAPDH was used as control for constitutive gene expression. The amplification reaction was carried out for a total of 35 cycles as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a precycle of 94°C for 15 s and postextension at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.75% agarose gel and stained with ethidium bromide. Densitometry values for cytokine signals were evaluated with EasyWin 32 software (Herolab, Wiesloch, Germany) and normalized to those for GAPDH.

Quantitation of the migratory activity of bovine granulocytes. Neutrophil migration was assayed by the method of Galligan and Coomber (8) with some modifications. Transwell inserts with 3-μm pores (diameter, 12 mm; Corning Costar, Badhoevedorp, The Netherlands) were used in 12-well multiwell plates (Corning Costar), thus producing a two-chambered system. Neutrophils were separated from whole blood by density gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences, Freiburg, Germany). After that, cells were resuspended in 2 ml of IEL medium. The purity of each fraction was assessed by flow cytometry. Only fractions with a purity higher than 90% were pooled. Purified neutrophils were diluted to 5×10^5 cells 0.5 ml^{-1} in IEL medium and were added to the top well. At the same time, 1.5 ml of an agonist was added to the bottom well. Agonists were supernatants of bovine iIEL preparations, which were incubated in either IEL medium alone, IEL medium plus Stx1 (200 CD_{50}s/ml), or IEL medium plus Stx1 (200 CD_{50}s/ml) and anti-StxB1 (1.5 μg/ml). Filter plates were incubated at 37°C under 5% CO_2 and 95% humidity for 2 h. Fluids of top and bottom wells were resuspended. A suspension of counting beads (Fluoresbrite Calibration Grade 3.0 Micron YG Microspheres; Polysciences, Eppelheim, Germany) was added, and neutrophils were then quantitated by flow cytometry.

Statistical analysis. After $\log_{10}$ transformation, data were analyzed statistically by one-way repeated-measures analysis of variance followed by the Student-Newman-Keuls test using SigmaStat (version 2.03) software (SPSS Inc., Chicago, Ill.). Results were evaluated as highly significant ($P \leq 0.001$), significant ($P \leq 0.01$), weakly significant ($P \leq 0.05$), or not significant ($P > 0.05$).

RESULTS

Stx receptor expression by bovine iIEL. When analyzed by flow cytometry after preparation from intestinal specimens, 14.9 ± 1.96% of iIEL (mean ± standard error of the mean [SEM] from 12 determinations with 6 iIEL preparations) stained with anti-CD77, but the intensity of anti-CD77 staining differed among different iIEL subpopulations ex vivo (Fig. 1 and Table 1). The majority of cells expressing high levels of the Gb_3/CD77 antigen (estimated from the mean fluorescence intensity of the cells) were activated mature T cells expressing CD3, CD6, ACT-2, and CD8α. A high level of Gb_3/CD77 expression was also found on $CD8\beta^+$ cells and subpopulations of γδ T cells (WC1, TcR1-N7). Although mean fluorescence intensities for Gb_3/CD77 expression were different for iIEL prepared from different intestinal specimens (i.e., different donor animals), $CD4^+$ T cells and B cells ($CD21^+$; surface IgM^+) consistently expressed only low numbers of Gb_3/CD77 molecules on their surfaces. The notion of a differential Gb_3/CD77 expression was supported by the calculation of the percentage of $Gb_3/CD77^+$ cells within a given subpopulation of iIEL (Table 1). While more than 40% of iIEL belonging to γδ T-cell subpopulations ($TcR1\text{-}N7^+$, $WC1^+$) expressed Gb_3/CD77 ex vivo, only 20.0% of $CD4^+$ iIEL were $Gb_3/CD77^+$. The calculation also revealed further differences within certain subpopulations, since 30.2% of $CD21^+$ B cells coexpressed Gb_3/CD77 ex vivo but only 16.9% of cells positive for surface IgM were Gb_3/CD77 positive. Moreover, 38.7% of $CD8\alpha^+$ T cells coexpressed Gb_3/CD77 as opposed to 18.2% of the $CD8\beta^+$ T cells. Gb_3/CD77 was only partially coexpressed with the activation markers CD25 and ACT-2.

Binding studies with rStxB1 (the subunit responsible for receptor binding of the holotoxin) showed that 7.9% (mean from 10 determinations with 5 iIEL preparations; range, 5.7 to 11.5%) of the iIEL ex vivo bound the protein. In particular, rStxB1 bound to $CD8\alpha^+$ iIEL and to iIEL coexpressing TcR1-N12, a pan-γδ T-cell marker in cattle (Fig. 2). Double-staining experiments with rStxB1 and anti-CD77 at limiting concentrations that avoided complete masking of Gb_3/CD77 by one of the ligands (37) showed that the populations of iIEL that bound these two ligands overlapped but were not identical. Although double-positive cells were detected (Fig. 2, right), a significant number of cells bound only one of the ligands.

Effect of Stx1 on lymphoblast transformation and natural killer cell activity in bovine iIEL cultures. Although iIEL generally respond poorly to mitogenic stimulation in vitro (27), detectable levels of blast cell transformation were induced in bovine iIEL cultures by applying a number of different stimuli alone or in combination. The percentage of blast cells in iIEL cultures incubated with Stx1 for 72 h was reduced (40 to 70% depending on the stimulus) compared to that with the respective mitogen control (Fig. 3). The presence of the anti-StxB1 monoclonal antibody 13C4 partially neutralized the effect of Stx1 on the average proliferative response of five iIEL preparations under seven out of nine conditions tested. Stx1 reduced the percentage of blast cells within all subpopulations identified to similar extents (Fig. 4). Individual differences between the iIEL preparations from different donors occurred, but Stx1 significantly reduced the percentage of Gb_3/CD77-expressing blast cells, and this effect was completely neutralized by preincubation with anti-StxB1. Although Gb_3/CD77 was expressed mainly by cells with an activated morphology, i.e., blast cells, Stx1 also affected the percentage of $Gb_3/CD77^+$ cells within the non-blast cell population. The cellular distribution of Gb_3/CD77 on the iIEL further stimulated in vitro was strikingly different from that observed ex vivo (Table 2). Following incubation, comparable portions of $CD8\alpha^+$ and $CD8\beta^+$ iIEL coexpressed Gb_3/CD77. Furthermore, the highest percentage of cells coexpressing Gb_3/CD77 could now be detected within the $CD4^+$ and $CD21^+$ subpopulation. Pretreatment with Stx1

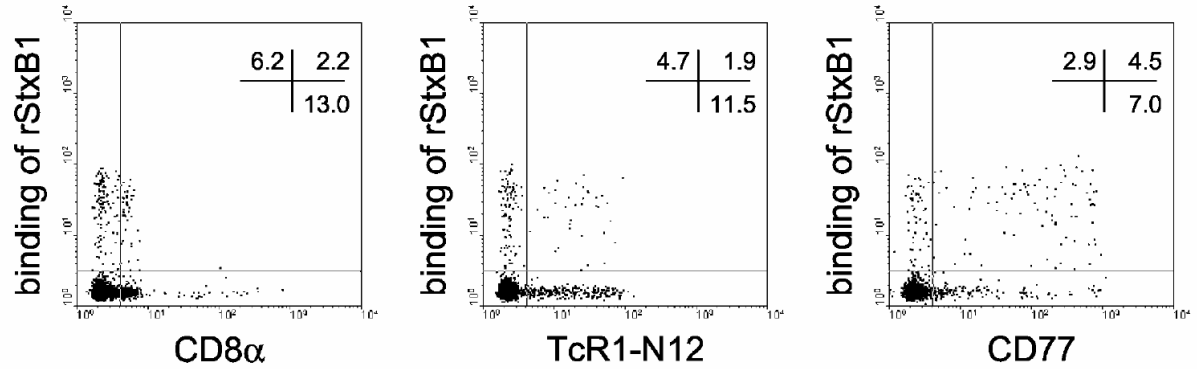

FIG. 2. Abilities of bovine iIEL subpopulations to bind rStxB1 ex vivo. IEL were prepared from the ileum of an adult animal at slaughter and subsequently double labeled with rStxB1 and subpopulation-identifying monoclonal antibodies. Each flow cytometer dot plot shows results for lymphocytes from a single experiment representative of five experiments. Percentages of cells positive for one or both of the antigens are given in the upper right corners of the dot plots.

did not affect the ability of iIEL to exert natural killer cell activity against a homologous lymphoma cell line (Fig. 5).

Effect of Stx1 on cytokine expression in bovine iIEL cultures. iIEL preparations obtained from different donors showed different levels of cytokine gene expression and different effects of Stx1 (Fig. 6). Stx1 reduced the IL-2 and IFN-γ signals in some preparations, but the expression of these TH1-type cytokines was unaffected or even slightly enhanced in other preparations. IL-10-specific mRNA was undetectable in four preparations, and Stx1 did not consistently affect its expression in the remaining two preparations. In contrast, Stx1 enhanced the expression of IL-4 in all of the four iIEL preparations in which IL-4-specific mRNA was detectable (four of six preparations). In addition, treatment with Stx1 for 4.5 h reduced the amount of IL-8-specific mRNA in all of the iIEL preparations investigated. The migration of bovine granulocytes through a filter membrane toward the supernatants obtained from two Stx1-treated iIEL cultures did not differ from that of supernatants of controls treated with Stx1 plus anti-StxB1 (data not shown).

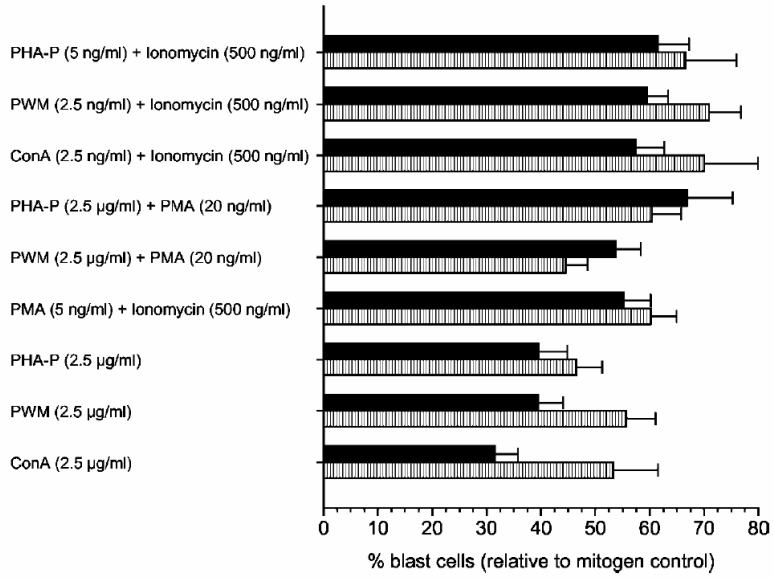

FIG. 3. Effect of purified Stx1 on transformation and proliferation of bovine iIEL subpopulations in vitro. Cells were incubated with purified Stx1 (200 CD_{50}s/ml; quantified on Vero cells as described in Materials and Methods) at 37°C for 72 h either in the absence (solid bars) or in the presence (striped bars) of 1.5 µg of the anti-StxB1 monoclonal antibody 13C4/ml. Culture medium was supplemented with rhuIL-2 (200 U/ml) and different stimuli as indicated. Lymphocytes that were transformed to blast cells were identified by flow cytometry according to their light scatter characteristics and quantified by acquiring 5,000 events in total. Data analysis was performed by calculating the percentage of viable (PI-negative) cells belonging to the blast cell population in relation to a mitogen control (i.e., cells that were incubated in the absence of Stx1 and anti-StxB1). Data are arithmetic means + SEMs from 15 determinations with 5 IEL preparations. PWM, pokeweed mitogen; ConA, concanavalin A.

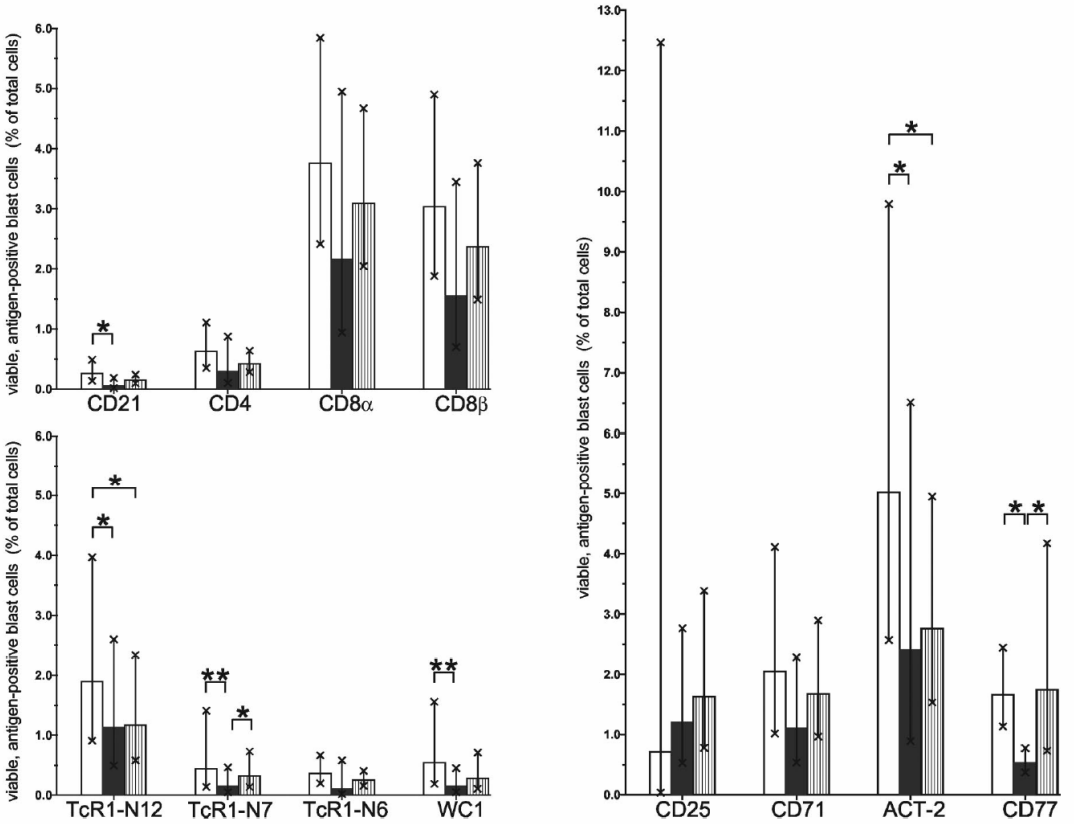

FIG. 4. Effect of purified Stx1 on transformation and proliferation of bovine iIEL subpopulations in vitro. Cells were incubated with purified Stx1 (200 CD_{50}s/ml; quantified on Vero cells as described in Materials and Methods) at 37°C for 72 h either in the absence (solid bars) or in the presence (striped bars) of 1.5 μg of the anti-StxB1 13C4 monoclonal antibody/ml. Cells that were incubated in the absence of Stx1 and anti-StxB1 were included as controls (open bars). The culture medium was supplemented with rhuIL-2 (200 U/ml) and PHA-P (2.5 μg/ml). Lymphocyte subpopulations were identified by immunophenotyping and quantified by flow cytometry acquiring 5,000 events in total. Data analysis was performed by using the software provided with the instrument to calculate the percentages of viable (PI-negative) and antigen-positive blast cells among all cells in culture. Data are geometric means and dispersion factors from 6 to 8 determinations with 4 IEL preparations (and from 56 determinations with 4 IEL preparations in the case of CD77). Statistical significance was obtained after $\log_{10}$ transformation by one-way repeated-measures analysis of variance followed by the Student-Newman-Keuls test. Horizontal brackets indicate bars that are different (*, $P \leq 0.05$; **, $P \leq 0.01$).

DISCUSSION

Some STEC strains are able to induce intestinal inflammation in infected humans. Neutrophils attracted and activated by Stxs may contribute to the pathogenesis of HC (18), and it has been further suggested that intestinal T cells are activated in the course of human STEC infections (15). In contrast, intestinal STEC infections in bovines do not induce inflammatory signs. These differences might be partially explained by the absence of functional Stx receptors on the surfaces of bovine granulocytes (C. Menge, T. Eisenberg, I. Stamm, and G. Baljer, Abstr. 5th Int. Symp. Shiga Toxin [Verocytotoxin]-Producing *Escherichia coli* Infect. 2003, abstr. O-11, p. 22, 2003). Another possible explanation is that Stx receptors are expressed on bovine T cells but not on human T cells (37). The observation that Stx1 suppresses bovine peripheral blood mononuclear cell (PBMC) functions (24) led us to hypothesize that by secreting Stx, STEC is able to limit the activation of intraepithelial T cells in cattle. This could explain both the persistence of bovine STEC infections and the absence of inflammation. Consistent with this hypothesis, the present study showed that bovine iIEL from adult animals express the Stx receptor ex vivo and are sensitive to purified Stx1 in vitro. The terminal rectum had recently been reported as the principal site of STEC O157:H7 colonization in the bovine host (28), but colonization of the ileum, cecum, and colon was shown to occur in calves infected with *E. coli* O157:H7 (5, 12), and non-O157 enterohemorrhagic *E. coli* apparently does not share a tropism for the terminal rectum (28, 39). The high similarity among the patterns of expression of cell surface molecules, including Gb_3/CD77, by IEL obtained from ileal, colonic, or cecal sites (Menge, unpublished) suggests that sensitivity to Stx1 is widely distributed among IEL of the intestinal tract in cattle.

CD77 expression was detected on the surfaces of all iIEL

TABLE 2. In vitro effect of Stx1 on the percentage of bovine iIEL subpopulations coexpressing Gb_3/CD77[a]

Surface antigen defining subpopulation	Morphology of cells[b]	% of cells[c] coexpressing Gb_3/CD77 after incubation (72 h) in the presence of:		
		Mitogen only	Stx1	Stx1 + anti-StxB1
CD4	Blast cells	73.3 ± 10.5	71.1 ± 9.7	85.7 ± 5.7
	Non-blast cells	50.7 ± 10.9	42.1 ± 10.1	68.0 ± 9.6
CD8α	Blast cells	38.8 ± 7.2	34.3 ± 5.0	39.0 ± 5.7
	Non-blast cells	18.2 ± 4.6	10.1 ± 1.4	18.3 ± 3.0
CD8β	Blast cells	38.6 ± 6.3	20.4 ± 3.3	39.4 ± 4.5
	Non-blast cells	16.9 ± 3.9	10.1 ± 1.5	19.1 ± 3.2
CD21	Blast cells	98.2 ± 1.8	87.5 ± 12.5	100.0 ± 0.0
	Non-blast cells	65.0 ± 8.1	74.5 ± 8.4	87.1 ± 8.4
WC1	Blast cells	60.8 ± 9.3	45.0 ± 13.5	52.5 ± 11.2
	Non-blast cells	31.7 ± 6.2	28.8 ± 7.6	68.5 ± 11.9
TcR1-N7	Blast cells	49.6 ± 8.3	24.0 ± 7.3	48.1 ± 9.4
	Non-blast cells	38.4 ± 7.1	24.8 ± 4.8	53.8 ± 9.6
ACT-2	Blast cells	33.3 ± 7.8	9.6 ± 2.4	48.1 ± 4.7
	Non-blast cells	16.9 ± 4.6	4.4 ± 0.8	15.6 ± 3.1

[a] iIEL were incubated for 72 h with PHA-P (2.5 μg/ml) and rhuIL-2 (200 U/ml).
[b] Populations of blast cells and non-blast cells were defined by their light scatter characteristics, with blast cells representing cells with increased size compared to quiescent non-blast cells.
[c] Values are means ± SEMs from eight determinations with IEL preparations from four animals.

subpopulations, including αβ and γδ T cells, that were analyzed directly ex vivo. It was recently confirmed by biochemical analysis that the antigen on bovine lymphocytes recognized by an anti-human CD77 antibody belongs to the Gb_3 family (23). Gb_3 molecules share the carbohydrate moiety that serves as the Stx binding site in human Stx receptors (19). However, double-labeling studies with anti-CD77 and rStxB1 identified three distinct population of cells. One population clearly stained with both of the ligands; the other two populations bound only rStxB1 or anti-CD77. Although corresponding experiments utilizing the Stx1 holotoxin have not been performed yet, bovine iIEL resemble their PBMC counterparts in expressing different isoforms of Gb_3/CD77 with different affinities for the two ligands (37). Resting PBMCs predominantly express Gb_3 isoforms with high affinity for rStxB1, but upon activation these cells synthesize Gb_3 molecules with a preference for anti-CD77. This shift in affinity comes along with an increase in the percentage of Gb_3 molecules that have incorporated long-chain fatty acids (23), a feature known to lower the affinity of Stx receptors for the holotoxin (30). Expression of anti-CD77 binding Gb_3 isoforms by bovine iIEL suggests, therefore, that the cells display an activated state. Waters et al. (45) defined an activated phenotype of bovine IEL by the expression of the null cell and $CD8^+$ T-cell activation marker ACT-2. ACT-2 was expressed by 42.8% (mean from 12 determinations with 6 iIEL preparations; range, 28.4 to 61.2%) of the iIEL in the present study. However, Gb_3/CD77 expression did not correlate closely with the expression of activation markers (Table 1). Gb_3/CD77 was found mainly on the $TcR1\text{-}N7^+$ and $WC1^+$ subsets of γδ T cells as well as on $CD8\alpha^+$ cells. Detailed analysis of the IEL subsets further revealed that CD8α is coexpressed by 31.8% (mean from nine determinations with five iIEL preparations; range, 22.5 to 48.1%) of $TcR1\text{-}N12^+$ cells (pan-γδ T cells), while only 18.3% (range, 7.3 to 38.9%) of $TcR1\text{-}N12^+$ cells coexpress CD8β. Hence, CD8αα homodimer-positive γδ T cells—as identified by Wyatt et al. (48)—and probably other $CD8\alpha^+$ IEL represent the iIEL subpopulation with the highest proportion of $Gb_3/CD77^+$ cells in cattle. In mice and humans, $CD8\alpha\beta^+$ αβ T IEL are consid-

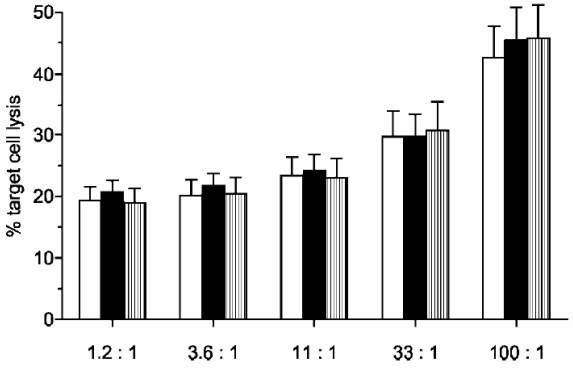

FIG. 5. Effect of purified Stx1 on natural killer cell activity of bovine iIEL in vitro. iIEL were incubated with purified Stx1 (200 CD_{50}s/ml; quantified on Vero cells as described in Materials and Methods) at 37°C either in the absence (solid bars) or in the presence (striped bars) of 1.5 μg of the anti-StxB1 monoclonal antibody 13C4/ml. Cells that were incubated in the absence of Stx1 and anti-StxB1 were included as controls (open bars). The culture medium was supplemented with rhuIL-2 (200 U/ml). Upon 24 h of incubation, homologous dye-labeled lymphoma cells (BL-3) were added to the cultures for an additional 18 h. The percentage of target cell lysis was quantified by flow cytometry. By live-gating, 2,500 events identified as target cells by their FL-1 fluorescence were acquired and analyzed with regard to their membrane integrity (PI uptake). Data are arithmetic means + SEMs from 15 determinations with 5 IEL preparations.

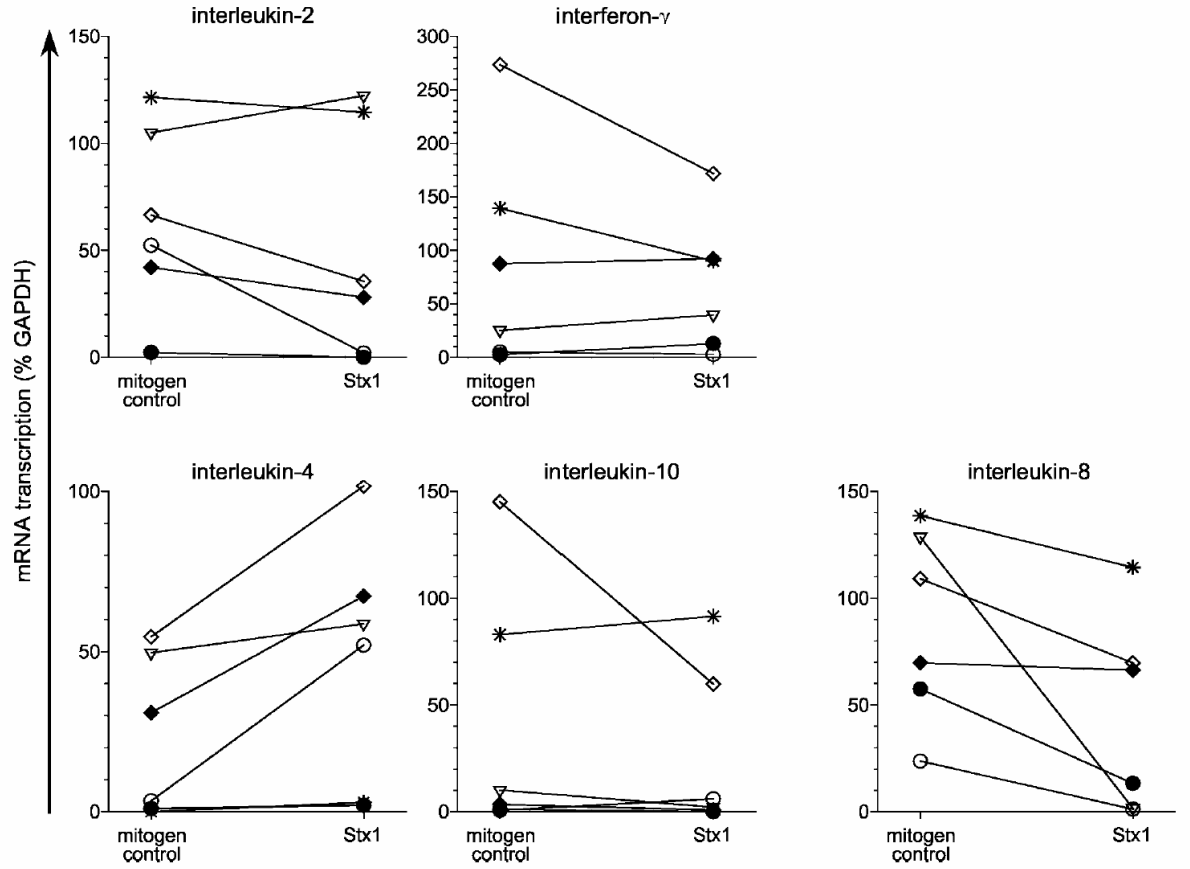

FIG. 6. Effects of purified Stx1 on cytokine gene expression by bovine iIEL in vitro. iIEL were incubated in the absence and presence of purified Stx1 (200 CD_{50}s/ml; quantified on Vero cells as described in Materials and Methods) at 37°C. The culture medium was supplemented with PHA-P (2.5 μg/ml). Upon 4.5 h of incubation, mRNA was harvested from the cells and subjected to semiquantitative reverse transcription-PCR. Each value is the band intensity of the specific PCR product expressed as a percentage of the GAPDH signal obtained from the same sample. Each symbol represents results for a particular IEL preparation.

ered to lyse infected cells, while γδ T IEL (and perhaps $CD8\alpha\alpha^+$ αβ T IEL) may stimulate renewal of the epithelial layer. Although the information available on the function of bovine IEL is very limited, it is tempting to speculate that, by affecting this type of cell, STEC is able to interfere with the local immune response as well as with epithelial-cell turnover. In fact, both aspects of intestinal barrier homeostasis have previously been linked to the persistent character of STEC infections in ruminants (20; M. Hoffman, T. Casey, and B. Bosworth, Abstr. 3rd Int. Symp. Workshop Shiga Toxin (Vero-cytotoxin)-Producing *Escherichia coli* Infect. 1997, abstr. V67/VIII, p. 117, 1997).

Comparable to what is seen with PBMCs (24), the addition of Stx1 to iIEL cultures from several donors reduced the average proliferative response to several mitogenic stimuli. This effect of Stx1 was partially prevented by preincubation of the toxin with anti-StxB1. However, the addition of anti-StxB1 seemed to have had an adverse effect when the cells were stimulated with PHA-P plus PMA or with pokeweed mitogen plus PMA. One possible explanation, based on previous findings (37), is that anti-StxB1 at low antigen-to-antibody ratios ameliorates rather than prevents the binding of rStxB1 to bovine lymphocytes. In contrast to the differential expression pattern of Gb_3/CD77 by iIEL subpopulations ex vivo, Stx1 affected all subpopulations of cultured iIEL similarly and, in particular, reduced the percentage of $Gb_3/CD77^+$ cells. Calculation of the percentages of $Gb_3/CD77^+$ cells within the different subpopulations after 72 h of culture showed that high percentages of $CD4^+$ and $CD21^+$ cells now coexpressed Gb_3/CD77, suggesting that each type of mucosal lymphocyte is capable of expressing functional Stx1 receptors in principle.

When we quantified the mRNAs specific for TH1 or TH2 prototype cytokines in iIEL after 4.5 h of treatment, Stx1 was found to induce a prominent increase in IL-4-specific mRNA levels in four out of six iIEL preparations examined. Previous investigations of peripheral lymphocytes from cattle showed that IL-4 is solely produced by $CD4^+$ cells with a $CD45RO^+$ memory phenotype (36), while activated γδ T cells primarily secrete IFN-γ (1). The proliferation experiments in this study showed that Stx1 is able to affect $CD4^+$ cells at least upon

stimulation in vitro, despite the low level of Gb_3/CD77 expression on the surfaces of these cells ex vivo. Recently, several apoptosis-inducing toxins that, like Stx1, belong to the ribosome-inactivating proteins were found to stimulate intracellular IL-4 expression in human PBMCs (38). Although the proliferation-inhibiting effect of Stx1 on bovine iIEL is not accompanied by a significant increase in the number of apoptotic cells (data not shown), we cannot rule out the possibility that induction of IL-4 in bovine iIEL cultures by Stx1 results from the onset of apoptosis in one or several lymphocyte subpopulations. IL-4 represents a highly attractive target for future investigations, because it is able to retard granulocyte migration into and across human intestinal epithelial cell monolayers (3). IEL-regulated granulocyte migration at mucosal sites is part of the local immune response against colonizing bacteria (43), and Stx1-induced IL-4 from IEL might thus prevent STEC microcolonies from being attacked by granulocytes in situ. Strikingly, Stx1 also acts directly on Gb_3/CD77$^+$ bovine colonic epithelial cells and causes a reduction in the release of chemoattractive signals by the epithelial cells (Menge et al., Abstr. 5th Int. Symp. Shiga Toxin-Producing *E. coli* Infect.). Stx1-treated iIEL examined in the present study also showed reduced levels of IL-8-specific mRNA synthesis. However, IL-8 is only one of numerous chemokines discovered thus far. The observation that supernatants of Stx1-treated iIEL cultures did not consistently contain reduced chemotactic activities led us to hypothesize that Stx1 differentially influences inflammatory signals in the intestinal barrier rather than generally downregulating them.

The intestinal system comprises a rich network of reciprocal and finely orchestrated interactions between immune, epithelial, endothelial, mesenchymal, and nerve cells and the extracellular matrix (7). Dysfunction of any component of this highly integrated mucosal system may lead to a disruption in communication which influences the course of intestinal infections. The proof that bovine IEL represent target cells for Stx1, together with recent reports on the expression of functional Stx receptors on bovine intestinal epithelial cells (16; Menge et al., unpublished data), is thus highly relevant for our understanding of the interplay of STEC with its bovine reservoir host and for the development of strategies aimed to limit these infections.

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6.8 "Phenotypic and functional characterisation of intraepithelial lymphocytes in a bovine ligated intestinal loop model of enterohaemorrhagic *Escherichia coli* infection."

Menge*, C., Ivonne Stamm, Pauline M. van Diemen, P. Sopp, G. Baljer, T. S. Wallis and M. P. Stevens

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Phenotypic and functional characterization of intraepithelial lymphocytes in a bovine ligated intestinal loop model of enterohaemorrhagic *Escherichia coli* infection

Christian Menge,[1] Ivonne Stamm,[1] Pauline M. van Diemen,[2] Paul Sopp,[3] Georg Baljer,[1] Timothy S. Wallis[2] and Mark P. Stevens[2]

[1]Institute for Hygiene and Infectious Diseases of Animals, Justus-Liebig-University, D-35392 Giessen, Germany

[2,3]Division of Microbiology[2] and Division of Immunology & Pathology[3], Institute for Animal Health, Compton Laboratory, Compton, Berkshire RG20 7NN, UK

Correspondence
Christian Menge
christian.menge@vetmed.uni-giessen.de

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Ruminants are a major reservoir of enterohaemorrhagic *Escherichia coli* (EHEC), which cause acute gastroenteritis in humans with potentially life-threatening sequelae. The mechanisms underlying EHEC persistence in ruminant hosts are poorly understood. EHEC produce several cytotoxins that inhibit the proliferation of bovine lymphocytes *in vitro* and influence EHEC persistence in calves, suggesting that bacterial suppression of mucosal inflammation may be important *in vivo*. In order to address this hypothesis, intraepithelial lymphocytes (IEL) obtained from ligated intestinal loops of five 9–14 day old calves were characterized 12 h after inoculation with *E. coli* strains. Loops were inoculated with an EHEC O103 : H2 strain, an isogenic Δ*stx1* mutant incapable of producing Shiga toxin 1 (Stx1) and a porcine non-pathogenic *E. coli* strain. The IEL mainly comprised activated $CD2^+$ $CD3^+$ $CD6^+$ $CD8\alpha^+$ T cells and resembled IEL obtained from the intestinal mucosa of orally challenged calves. Forty per cent of all IEL were potentially sensitive to Stx1 in that they expressed the receptor for Stx1. Nevertheless, analysis of IEL from inoculated loops failed to detect a significant effect of the different *E. coli* strains on proliferative capacity, natural killer cell activity or the cytokine mRNA profile. However, the EHEC wild-type strain reduced the percentage of $CD8\alpha^-$ T cells in the ileal mucosa compared with loops inoculated with the Δ*stx1* mutant. This shift in IEL composition was not associated with inhibition of IEL proliferation *in situ*, since the majority of the IEL from all loops were in the G_0/G_1 phase of the cell cycle. These studies indicate that the ligated ileal loop model will be a useful tool to dissect the mechanisms underlying suppression of mucosal inflammation by EHEC in the reservoir host.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) infections in humans are often acquired by direct or indirect contact with ruminant faeces and can have life-threatening consequences (Paton & Paton, 1998; Roe & Gally, 2000). Strategies to lower the incidence of EHEC in cattle and sheep are expected to reduce the incidence of human infections (Stevens *et al.*, 2002b); however, the mechanisms underlying EHEC persistence in ruminants are poorly understood. Previous studies indicated that the locus of enterocyte effacement (LEE)-encoded type III secretion apparatus mediates intestinal colonization in animal models of attaching and effacing *E. coli* infection (Abe *et al.*, 1998; Mundy *et al.*, 2003), and our laboratory has recently shown that the LEE is required for colonization of the bovine intestine by EHEC serotypes O157 : H7 and O26 : H– (P. M. van Diemen, F. Dziva, M. P. Stevens and T. S. Wallis, unpublished observations). Several of the LEE-encoded effector proteins do not influence adherence per se, indicating that they may affect colonization by subverting or inhibiting the activity of host cells (Elliott *et al.*, 2001; McNamara *et al.*, 2001; Tu *et al.*, 2003). Indeed, EspB or a protein(s) dependent on EspB for secretion was recently reported to suppress activation of the nuclear transcription factor NF-κB and the synthesis of proinflammatory cytokines *in vitro* (Hauf & Chakraborty, 2003).

EHEC also produce several cytotoxins including Shiga toxin(s) (Stx1 and/or Stx2) and lymphostatin, the latter of

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; IEL, intraepithelial lymphocytes; rStxB1, recombinant B-subunit of Shiga toxin 1; Stx1, Shiga toxin 1.

which influences intestinal colonization of calves (Stevens *et al.*, 2002c). Both Stx1 and lymphostatin inhibit the proliferation of bovine peripheral blood lymphocytes *in vitro* (Menge *et al.*, 1999; Ferens & Hovde, 2000; Stevens *et al.*, 2002c). In addition, lymphostatin can block the proliferation of human and murine intestinal lymphocytes *in vitro* (Klapproth *et al.*, 1996; Malstrom & James, 1998). Bovine intestinal intraepithelial lymphocytes (IEL) express functional Stx1 receptors, and Stx1 blocks proliferation and affects the expression of cytokines in these cells (Stamm *et al.*, 2002; Menge *et al.*, 2004). Studies are required to confirm that such immunomodulatory strategies are relevant in the complex environment of the intestine in the target animal species (Smith *et al.*, 2002; Hein & Griebel, 2003).

Intestinal loop models have been used in ruminants to study enteropathogenic responses to bacterial pathogens. EHEC elicit enteropathogenic responses in such loops and adhere to the epithelium, forming attaching and effacing lesions (Sandhu & Gyles, 2002; Stevens *et al.*, 2002a). Recently, Gerdts *et al.* (2001) established that intestinal loops are a valuable model for the analysis of mucosal immune responses. We therefore assessed the phenotype and function of IEL in a bovine ligated intestinal loop model of EHEC infection in order to identify bacterial and host factors that modulate inflammatory responses during EHEC infection of cattle.

METHODS

Bacterial strains, toxin and anti-toxin. The bacterial strains used were PMK5 (wild-type EHEC O103 : H2 *eae* subtype-ε *stx1*$^{+}$; Mariani-Kurkdjian *et al.*, 1993), an isogenic PMK5 Δ*stx1* mutant (Stevens *et al.*, 2002a) and NADC5738 (Dean-Nystrom *et al.*, 1997), which is a nalidixic acid-resistant derivative of the porcine non-pathogenic *E. coli* O43 : H28 strain 123 (Moon *et al.*, 1968). Bacterial strains were cultured in brain heart infusion (BHI) broth for 18 h at 37 °C and the optical density at 600 nm of inocula was adjusted to within 0·1 units. Viable bacteria in adjusted inocula were enumerated by plating serial tenfold dilutions onto MacConkey agar. Recombinant B-subunit of Stx1 (rStxB1) was purified by anion-exchange chromatography as described by Stamm *et al.* (2002). Anti-StxB1 was purified by Protein A/G (Schleicher & Schuell) affinity chromatography from the mouse hybridoma cell line 13C4 (Strockbine *et al.*, 1985).

Animals. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (UK) and were approved by the local Ethical Review Committee. Conventional Friesian bull calves were fed on milk replacer twice daily with free access to water and were screened for excretion of EHEC or *Salmonella* by enrichment on sorbitol MacConkey agar containing tellurite and cefixime or Brilliant green agar, respectively. Calves were observed twice daily for 7 days prior to surgery and animals with diarrhoea or excreting EHEC were excluded from the analysis.

Oral inoculation of calves. Calves aged 20–22 days were challenged orally with $3{\cdot}97 \times 10^{10}$ c.f.u. of strain PMK5 or $4{\cdot}63 \times 10^{10}$ c.f.u. of NADC5738 in antacid as described by Stevens *et al.* (2002c).

Ligated intestinal loop assay. The bovine ligated ileal loop assay has been described previously (Stevens *et al.*, 2002a). Briefly, calves were anaesthetized for the duration of the experiment (approx. 14 h) with pentobarbitone sodium [Sagatal, 0·44 ml (kg body weight)$^{-1}$] and the mid-ileum was flushed with intestinal wash solution (5·61 mg NaCl; 0·11 mg KCl; 1·09 mg KH_2PO_4; 0·16 mg Na_2HPO_4; 7·04 mg trisodium citrate and 5 mg *N*-acetyl cysteine ml^{-1}). Calves were maintained at 38·5–39·5 °C by the use of heated mats. Four mid-ileal loops per animal approx. 40 cm in length with 10 cm spacers were ligated with surgical silk and inoculated with 40 ml bacterial culture (PMK5, $4{\cdot}58 \pm 0{\cdot}70 \times 10^{10}$ c.f.u. per loop; PMK5 Δ*stx* $4{\cdot}02 \pm 0{\cdot}67 \times 10^{10}$ c.f.u. per loop; NADC5738 $5{\cdot}84 \pm 1{\cdot}37 \times 10^{10}$ c.f.u. per loop) or sterile medium (BHI) as a negative control. Each strain was tested once per animal and the experiment was repeated in a total of five calves.

Isolation of IEL. Twelve hours after loop inoculation and immediately after the administration of an overdose of anaesthetic, the infected mucosa was collected into ice-cold PBS. IEL (approx. 2×10^8 cells per loop) were isolated from the recovered mucosa after treatment with 1,4-DTT (1 mM in PBS, 15–25 min at 37 °C with shaking) by incubation with an EDTA solution containing an inhibitory concentration of antibiotics (2 mM EDTA in PBS, 100 U penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, 2·5 µg gentamicin ml^{-1}; 20 min at 37 °C with shaking) and mechanical detachment (vortexing). The cells were resuspended in Percoll at a density of 1·05 g ml^{-1}, layered onto Percoll at a density of 1·0816 g ml^{-1} and separated by centrifugation at 677 *g* for 20 min (Menge *et al.*, 2004).

Immunophenotyping. Freshly isolated IEL were transferred to microtitre plates for the staining of cell differentiation and activation markers for flow cytometry as described (Menge *et al.*, 1999; Stamm *et al.*, 2002). IEL were incubated with antibodies/rStxB1 in the dark for 20 min. Detected antigens and the respective antibodies used were: CD2 (IL-A 43), CD4 (IL-A 11), CD6 (IL-A 57), CD8α (IL-A 105), CD21 (IL-A 65), a macrophage/granulocyte differentiation antigen (IL-A 24), WC1 (γδ T cells, IL-A 29), CD25 (IL-A 111), CD71 (IL-A 77), MHC-II (IL1) (all antibodies provided by J. Naessens, International Livestock Research Institute, Nairobi, Kenya), CD3 (MM1A), CD8β (BAT82A), ACT-2 (CACT26A), TcR1-N7 (CACTB81A), TcR1-N6 (CACTB6A), TcR1-N12 (CACT61A) (all antibodies purchased from VMRD, Pullman, WA, USA) and CD77 (clone 38.13; Beckman Coulter). Binding of rStxB1 (30 µg ml^{-1}) was detected with anti-StxB1 (45 µg ml^{-1}). Visualization was carried out with FITC-labelled secondary antibodies. Cells were analysed with a FACSCalibur flow cytometer acquiring 5000 events for each sample.

Natural killer (NK) cell activity assay. A bovine lymphoma cell line (BL-3, ECACC 86962401) was used as target cells. Target cells (T) prestained with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were added to IEL as effector cells (E) in different ratios (E : T 100 : 1, 33 : 1 and 11 : 1) and incubated for 18 h at 37 °C. Affected target cells were detected by flow cytometry according to their altered morphology (i.e. increase in granularity).

Cell cycle determination/DNA analysis. IEL were fixed with ethanol (70 %, v/v) immediately after isolation. DNA was stained with propidium iodide (PI) after RNase A digestion and proportions of cells in the different cell cycle compartments were assessed by flow cytometry analysis according to their PI signal (Noguchi, 1991).

Lymphocyte stimulation-proliferation assay. To examine proliferative capacity after mitogen stimulation, IEL were cultured for 3 days at 37 °C in medium supplemented with rhuIL2 (200 U ml^{-1}) and either phytohaemagglutinin (PHA-P, 2·5 µg ml^{-1}) or phorbol-12-myristate-13-acetate (PMA, 5 ng ml^{-1}) and ionomycin (500 ng ml^{-1}). The proportion of viable cells transformed to blast cells and non-transformed non-blast cells relative to an unstimulated control was determined by flow cytometry according to the light scatter characteristics of cells with PI exclusion of dead cells.

Cytokine mRNA profile. After incubation of IEL in medium supplemented with 2·5 μg PHA ml^{-1} and 200 U rhuIL2 ml^{-1} for 30 min, RNA was isolated from cells using an RNeasy MiniKit (Qiagen), treated with DNase I and reverse transcribed to cDNA. Cytokine-specific PCRs for *il2*, *il4*, *il8*, *il10* and *ifn-γ* were carried out following standard procedures. Previously published cytokine primers (Gohin *et al.*, 1997; Morsey *et al.*, 1996) were used with minor modifications: *il2* (sense, 5′-TCTTGCATTGCACTAACTCT-3′; antisense, 5′-GCT TTGACAAAAGGTAATCC-3′), *il4* (sense, 5′-GCCACTTCGTCCAT GGACAC-3′; antisense, 5′-TCCCAAGAGGTCTCTCAGCG-3′), *il8* (sense, 5′-GCAGTTCTGTCAAGAATGAG-3′; antisense, 5′-GGATCT TGCTTCTCAGCTC-3′), *il10* (sense, 5′-TGTTGCCTGGTCTTCCTG-3′; antisense, 5′-TCTCTTCACCTGCTCCAC-3′), *ifn-γ* (sense, 5′-GCT TTACTGCTCTGTGTGCT-3′; antisense, 5′-GACTTCTCTTCCGCTT TCTG-3′) and *gapdh* (sense, 5′-ATCACTGCCACCCAG-3′; antisense, 5′-CATGCCAGTGAGCTT-3′). The GAPDH gene was used as a control for constitutive gene expression. The amplification reaction was carried out for a total of 35 cycles as follows: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, with a precycle of 94 °C for 15 s and final extension at 72 °C for 5 min. Cytokine signals were evaluated after gel electrophoresis from densitometry measurements and values were normalized to a GAPDH signal.

Statistical analysis. Data were analysed statistically using BMDP (Statistical Software) and SigmaStat software (SPSS). *P* values were calculated by Student–Newman–Keuls test and one-way repeated measures ANOVA and considered significant when $P \leq 0{\cdot}05$.

RESULTS AND DISCUSSION

Comparative phenotypic and functional characterization of bovine ileal and colonic IEL in orally challenged calves

Anatomical constraints and the need to recover sufficient viable IEL from the epithelial layer for functional studies led us to focus on the small intestine for ligated loop experiments to study the effect of *E. coli* strains on mucosal inflammation. Despite a recent report suggesting that lymphoid follicle dense epithelium in the terminal rectum is the principal site of *E. coli* O157 : H7 colonization in weaned calves and adult cattle (Naylor *et al.*, 2003), colonization of the ileum, caecum and colon has been reported in calves infected with *E. coli* O157 : H7 (Cray & Moon, 1995; Brown *et al.*, 1997; Dean-Nystrom *et al.*, 1997, 1999; Grauke *et al.*, 2002). Non-O157 EHEC apparently do not share a tropism for the terminal rectum (Naylor *et al.*, 2003), and serotypes O5 and O111 have been observed to adhere extensively to the colonic epithelium (Stevens *et al.*, 2002c). In order to determine whether phenotypic or functional differences were detectable between IEL from ileal and colonic sites, conventional calves were challenged orally with either EHEC strain PMK5 or non-pathogenic *E. coli* strain NADC5738, and intestinal mucosa was obtained 3 days after inoculation to prepare IEL. The general composition of IEL subpopulations was very similar in the ileum and colon of orally challenged calves (Fig. 1), with the exception that ileal IEL preparations contained higher percentages of T cells and of cells expressing MHC-II and ACT-2 (a tissue-specific activation marker) as compared with colonic IEL preparations. The Stx receptor Gb_3/CD77 was found on approx. 50 and 35 % of the IEL from ileal and colonic preparations, respectively. We previously observed that bovine lymphocytes express different isoforms of Gb_3/CD77 molecules that have incorporated fatty acids of varying length and display different affinities for anti-CD77 and rStxB1 (Menge *et al.*, 2001, 2004; Stamm *et al.*, 2002). Consistent with that, only approx. 35 % of ileal as well as colonic IEL were capable of binding rStxB1 in the present study.

IEL composition differed little between calves that were inoculated with PMK5 or NADC5738. It is noteworthy, however, that the portion of Gb_3/CD77$^+$ and rStxB1 binding ileal IEL was reduced in the calf that received the EHEC strain. In addition, the portion of ileal and colonic intra epithelial T cells expressing CD8α and CD8β was markedly lower in this animal. In turn, other T cell populations including CD3$^+$, CD4$^+$, CD6$^+$ and N12$^+$ $\gamma\delta$ T cells were enhanced at colonic sites in the PMK5-inoculated animal.

The majority of IEL from both calves were in the G_0/G_1 phase of the cell cycle and mitogen stimulation did not result in proliferative responses in comparison to unstimulated cells (data not shown). IEL are known to respond only poorly to mitogens and nominal antigens *in vitro*, despite their activated appearance and phenotype (Mowat & Viney, 1997). Nevertheless, isolated IEL were still functionally active in that they exerted an NK cell activity towards a homologous cell line, and inoculation with EHEC did not influence this activity (Fig. 2). Detection of this activity required high effector to target cell ratios and was slightly higher in colonic IEL preparations.

Immunophenotype of IEL exposed to EHEC *in situ* in a ligated intestinal loop model of EHEC infection

Mid-ileal loops were constructed in a total of five conventional calves aged 9–14 days and inoculated with either PMK5, PMK5 Δ*stx1*, NADC5738 or sterile medium as a control. Since it is known that Stx1 inhibits the activity of bovine lymphocytes *in vitro* (Stamm *et al.*, 2002; Menge *et al.*, 2003), the *stx1* mutant was included to determine whether mucosal immunomodulatory effects due to Stx1 could be detected *in vivo*. Viable IEL could be isolated after 12 h from all mid-ileal loops inoculated with the different *E. coli* strains. Immunophenotyping of the IEL revealed their composition was very similar to IEL derived from ileal mucosa from orally inoculated calves (Figs 1 and 3). IEL from mid-ileal loops comprised mainly activated CD2$^+$ CD3$^+$ CD6$^+$ ACT-2$^+$ T cells, with approximately 60 % CD8α^+, 50 % CD8β^+, 10 % CD4$^+$ and 25 % $\gamma\delta$ T cells, suggesting that the integrity of the mucosal layer had been maintained (Fig. 3). Forty per cent of all IEL expressed the Stx receptor Gb_3/CD77 and were capable of binding rStxB1. Few changes in the phenotype of the recovered IEL could be detected between loops inoculated with EHEC and control loops filled with NADC5738 or sterile medium (Fig. 3). Student–Newman–Keuls test following one-way repeated measures ANOVA revealed that the wild-type Stx1-producing EHEC strain PMK5 significantly reduced the percentage of CD8α^+ T cells

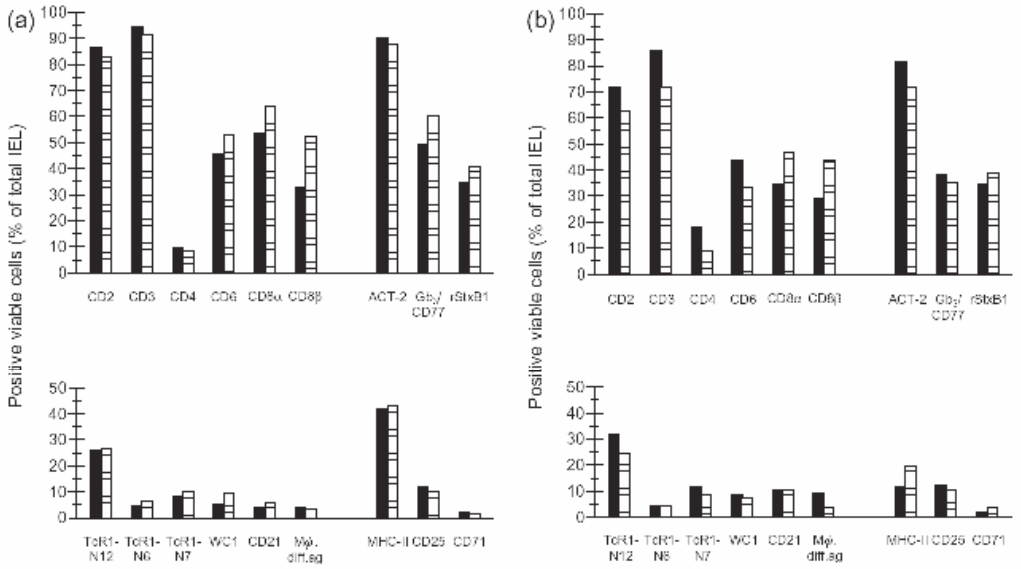

Fig. 1. Phenotype analysis of IEL isolated from the ileum (a) and colon (b) of orally challenged calves 3 days after inoculation with EHEC wild-type strain PMK5 (filled bars) or non-pathogenic *E. coli* strain NADC5738 (striped bars). Analysis was performed by flow cytometry and subsequent calculation of the percentage of viable lymphocytes positive for the respective antigen. Values represent means from duplicate determinations of one animal per strain. Mφ diff. ag., macrophage/granulocyte differentiation antigen.

by 5·52 ± 3·4 % ($P \leq 0{\cdot}05$) compared with loops inoculated with the PMK5 Δ*stx1* mutant or the non-pathogenic *E. coli* strain. A slight decrease in the portion of $CD8\beta^+$, $CD6^+$ and $CD2^+$ IEL in PMK5-inoculated loops could also be detected, although differences reached significance only in the latter case. These findings reflect the differential expression of Stx receptors by several IEL subpopulations: in the ileum of adult cattle, the majority of $Gb_3/CD77^+$ IEL are activated $CD3^+$ $CD6^+$ $CD8\alpha^+$ T cells, whereas $CD4^+$ T cells and B cells express much less $Gb_3/CD77$ (Menge *et al.*, 2004). Inoculation of the loops with PMK5 did not influence the number of $Gb_3/CD77^+$ IEL, but slightly reduced the number of rStxB1-binding cells. *In vitro*, Stx1 affects bovine peripheral lymphocytes early in the activation process (Stamm *et al.*, 2002) when the cells express an isoform of $Gb_3/CD77$ with a high affinity for rStxB1 that is not recognized by anti-CD77 (Menge *et al.*, 2003). Stx1 thus probably eliminated from the loop mucosa only those IEL that were not recognized as $Gb_3/CD77^+$ cells in either loop.

The finding that Stx1 specifically depletes a subset of bovine lymphocytes in the complex environment of the intestine is a significant step forward in our understanding of the modulation of mucosal immune responses by EHEC. To the best of our knowledge, the present study provides the first direct evidence that Stx1 acts as a virulence factor in cattle. Suppression of immune function in the gut through depletion of $CD8\alpha$ and probably $CD8\beta$ T cells may facilitate intestinal colonization; however, there is presently a paucity of published data to support this role (reviewed by Smith *et al.*, 2002). Stx-positive *E. coli* O157 : H7 have been reported to colonize the intestines of weaned calves more effectively than Stx-negative strains (Dean-Nystrom *et al.*, 1998); however, the strains used were not isogenic and the differences could be due to traits other than Stx production.

Cell cycle progression and proliferation of IEL exposed to EHEC *in situ*

Between 88·65 ± 2·11 and 90·14 ± 2·43 % of IEL (mean ± SD of triplicate determinations of five animals) from the loops were in the G_0/G_1 phase of the cell cycle. The response to mitogen stimulation was low and did not differ between IEL preparations obtained from the different loops (data not shown). The effect of Stx1 on IEL composition is therefore not a consequence of inhibition of cell proliferation *in situ*. We recently reported that the ability of Stx1 to block the proliferation of bovine peripheral $CD8\alpha^+$ $Gb_3/CD77^+$ T lymphocytes is due to direct toxic action and is not mediated via perturbation of autocrine cytokine release within the lymphocyte cultures (Menge *et al.*, 2003). Although previous reports failed to detect a cytotoxic activity of Stx1 for bovine lymphocytes (Menge *et al.*, 1999; Ferens & Hovde, 2000), Stx1 was recognized as a potent cytotoxin in other cellular systems (Sandvig, 2001). Therefore, we cannot exclude the possibility that Stx1 eliminated sensitive IEL from the mucosa of PMK5-inoculated loops.

The IEL composition in loops inoculated with a non-pathogenic *E. coli* strain did not significantly differ from

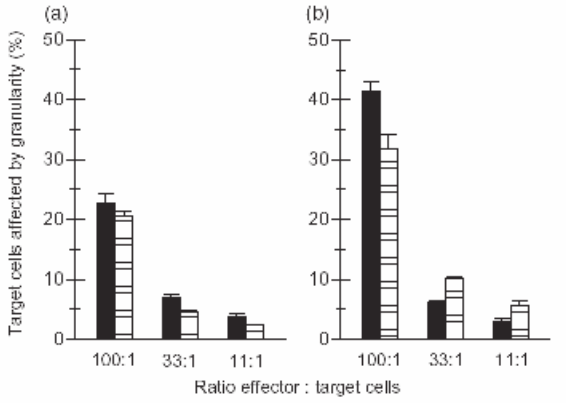

Fig. 2. NK cell activity of IEL isolated from the ileum (a) and colon (b) of orally challenged calves 3 days after inoculation with EHEC wild-type strain PMK5 (filled bars) or non-pathogenic *E. coli* strain NADC5738 (striped bars). After co-incubation with bovine lymphoma cells as target cells for 18 h *in vitro*, NK cell activity of IEL was determined by flow cytometry. Specific target cell lysis was assessed by calculating the difference between the percentage of target cells exhibiting increased granularity in test samples and controls without effector cells. Values represent means and SD from triplicate determinations of one animal per strain.

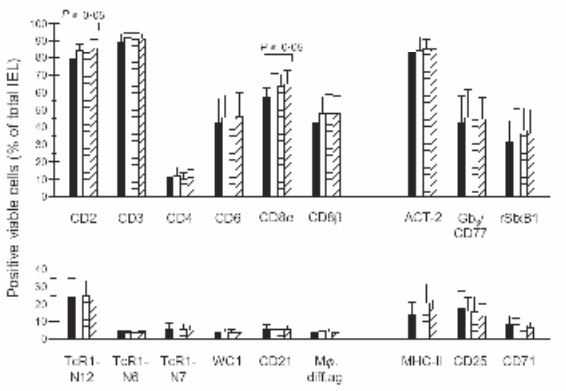

Fig. 3. Phenotype analysis of ileal IEL isolated from ligated loops after 12 h of inoculation with EHEC wild-type strain PMK5 (filled bars), isogenic PMK5 (*stx1* mutant; open bars), non-pathogenic *E. coli* strain NADC5738 (striped bars) or BHI broth (hatched bars). Analysis was performed by flow cytometry and subsequent calculation of the percentage of viable lymphocytes positive for the respective antigen. Values represent means and SD from duplicate determinations of five animals. For statistical analysis, one-way repeated measures ANOVA and Student–Newman–Keuls test were performed (for details see text). Mφ diff. ag., macrophage/granulocyte differentiation antigen.

loops inoculated with PMK5 Δ*stx1*. The latter strain, but not NADC5738, contains the gene for lymphostatin (*lifA*), which influences colonization of the bovine intestine by EHEC serotypes O5 and O111 (Stevens *et al.*, 2002c). Lymphostatin represents another EHEC factor that blocks lymphocyte proliferation *in vitro* (Klapproth *et al.*, 2000; Stevens *et al.*, 2002c); however, no lymphostatin-like effects on IEL phenotype and function were detected in the present study.

Cytokine mRNA synthesis and NK cell activity of IEL exposed to EHEC *in situ*

Several EHEC virulence factors inhibit the synthesis of cytokines by mitogen-activated mucosal lymphocytes *in vitro*, including lymphostatin (Klapproth *et al.*, 1996; Malstrom & James, 1998) and Stx1 (Menge *et al.*, 2004). We therefore assessed the effect of exposure of bovine IEL *in situ* to different *E. coli* strains on the mitogen-activated transcription of *il2* , *il4*, *il8*, *il10* and *ifn-γ*. Upon mitogenic stimulation of recovered IEL for 30 min *in vitro*, suitable amounts of intact RNA could be recovered and subjected to semi-quantitative RT-PCR. IEL preparations obtained from different donor animals varied in their cytokine gene expression (Fig. 4). While inoculation of loops with PMK5 increased the IL2 signal in four out of five animals in comparison with loops inoculated with PMK5 Δ*stx1*, the

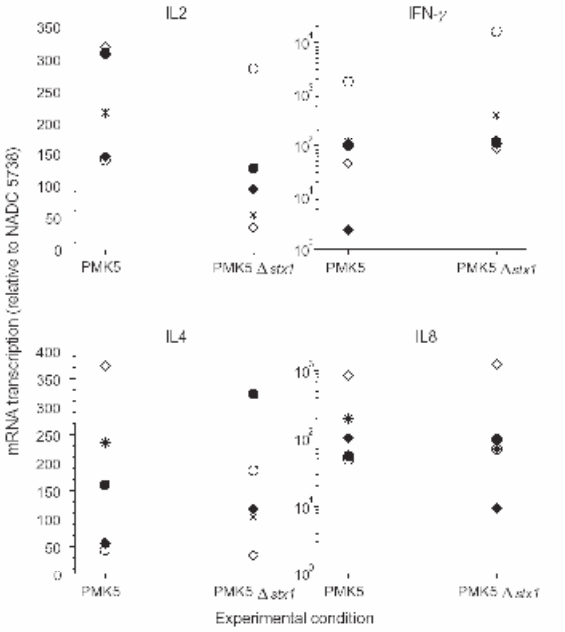

Fig. 4. Cytokine gene expression of ileal IEL isolated from ligated loops after 12 h of inoculation with EHEC wild-type strain PMK5 or isogenic PMK5 (*stx1* mutant). IEL were incubated *in vitro* for 30 min at 37 °C in culture medium supplemented with 2·5 μg PHA-P ml^{-1} and 200 U recombinant huIL2 ml^{-1}. RNA was harvested from cells and subjected to semiquantitative RT-PCR. Values are band intensities of the specific PCR product relative to the GAPDH signal obtained from the same sample and expressed relative to loops inoculated with NADC5738. Symbols represent IEL preparations from different animals.

expression of IFN-γ was reduced to different extents in all five animals. The amounts of IL4-, IL8- and IL10-specific mRNA (Fig. 4; data for IL10 not shown) varied inconsistently between the loops and the animals.

IEL preparations from all the inoculated loops exhibited a higher NK cell activity than ileal IEL obtained from the orally challenged calves, but no significant differences between IEL exposed to the different *E. coli* strains were detectable (Fig. 5).

Gut-associated lymphoid tissues trap antigen at sites of infection and present it to migratory lymphocytes, leading ultimately to the development of antigen-specific mucosal immunity. It may be speculated that EHEC cytotoxins suppress these events once the bacteria are intimately associated with the epithelium in order to prevent clearance. Accordingly, Stx1 had been shown to hinder peripheral lymphocyte functions *in vitro* (Menge *et al.*, 1999), and presumably *in vivo* (Hoffman *et al.*, 1997). However, the fact that humoral and mucosal immune responses against EHEC antigens can be readily detected after experimental and natural infection of cattle (Johnson *et al.*, 1996; Pirro *et al.*, 1995) argues against a general immune suppression. Moreover, IEL differ strikingly functionally from peripheral lymphocytes (Mowat & Viney, 1997). IEL do exhibit cytotoxicity against virus-infected cells (Müller *et al.*, 2000), but, in the first place, these cells form an indispensable part of the mucosal regulatory network that maintains intestinal homeostasis (Fiocchi, 1997). By releasing soluble factors, IEL control the migration and activation of inflammatory cells as well as a number of epithelial cell functions including proliferation (Mowat & Viney, 1997). It is thus tempting to assume that, by modulating IEL functions, EHEC prevent the onset of mucosal inflammatory responses that would otherwise follow bacterial adhesion to the mucosal surface (Zhou *et al.*, 2003). In addition to directly affecting epithelial cell functions (Hoey *et al.*, 2002, 2003), EHEC would be able, by this mechanism, to influence epithelial cell turnover indirectly, which correlates with the duration of EHEC shedding in calves (Magnuson *et al.*, 2000). Using Stx1 as a prototype for EHEC factors with immunomodulatory activity, the bovine ligated intestinal loop model proved suitable to dissect the effect of bacterial factors on different aspects of the intestinal immune response during EHEC O103:H2 infections in this reservoir host.

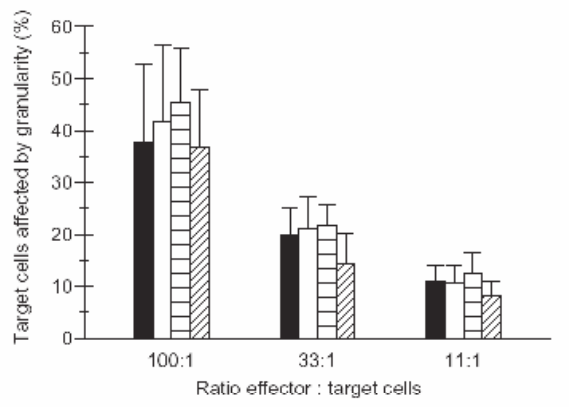

Fig. 5. NK cell activity of ileal IEL isolated from ligated loops after 12 h of inoculation with EHEC wild-type strain PMK5 (filled bars), isogenic PMK5 (stx1 mutant; open bars), non-pathogenic *E. coli* strain NADC5738 (striped bars) or BHI broth (hatched bars). After co-incubation with bovine lymphoma cells as target cells for 18 h *in vitro* NK cell activity of IEL was determined by flow cytometry. Specific target cell lysis was assessed by calculating the difference between the percentage of target cells exhibiting increased granularity in test samples and controls without effector cells. Values represent means and SD from triplicate determinations of five animals. Statistical analysis with one-way repeated measures ANOVA revealed no significant differences between the loops.

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6.9 "Comparison of binding and effects of *Escherichia coli* Shiga toxin 1 on bovine and ovine granulocytes."

Menge*, C., T. Eisenberg, Ivonne Stamm, and G. Baljer

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Comparison of binding and effects of *Escherichia coli* Shiga toxin 1 on bovine and ovine granulocytes

Christian Menge *, Tobias Eisenberg, Ivonne Stamm, Georg Baljer

Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebig-Universität Giessen, Frankfurter Str. 89, D-35392 Giessen, Germany

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Abstract

Granulocytes play a pivotal role in the pathogenesis of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) related diseases in humans. Granulocytes are attracted and activated by Stxs in the enteric mucosa and are believed to thereby contribute to the intestinal inflammation. Mature ruminants, the main reservoir hosts of STEC, do not develop pathological changes that can be attributed to the Stxs. To prove whether the latter phenomenon correlates with the inability of the Stxs to affect granulocytes of ruminants, we investigated the ability of Stx1 to bind to granulocytes of cattle and sheep and analysed the effects of Stx1 on viability, phagocytosis, and oxidative burst activity. Bovine granulocytes from blood and milk did not express Stx1-binding sites even after activation of the cells and also were resistant to Stx1. In contrast to bovine granulocytes, granulocytes of sheep constitutively expressed Stx1-receptors of the Gb_3/CD77 type ex vivo and bound the recombinant B-subunit of Stx1 (rStxB1). Stx1 holotoxin induced apoptosis in ovine granulocytes after prolonged incubation (18 h) but Stx1 only slightly altered the phagocytosis and oxidative burst activities. The rStxB1 had no effect on granulocytes of either species. While arguing in favour of our initial hypothesis, that granulocytes of both, cattle and sheep are not activated by Stxs, the results of our study are the first evidences for differences in the cellular distribution of Stx-receptors in species equally regarded as STEC carriers.

Keywords: Shiga toxin; Granulocyte; Cattle; Sheep; Gb_3/CD77

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) of the pathovar of Shiga toxin-producing *E. coli* (STEC) are emerging foodborne pathogens and the cause of Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) in humans (Karmali, 1989). Shiga toxins (Stxs) are regarded as the main virulence factors of STEC in that they damage endothelial cells, one of the key events in the pathogenesis of HC and HUS (Paton and Paton, 1998). It was only recently recognized that interactions of Stxs with granulocytes also play a central role in pathogenesis (Proulx et al., 2001). Stxs delay the onset of apoptosis in human granulocytes (Liu et al., 1999) and trigger the generation of reactive oxygen intermediates which ought to damage the mucosal tissue (King et al., 1999). In addition, Stxs induce the release of chemotactic factors from epithelial cells (Yamasaki et al., 1999; Thorpe et al., 2001) and thereby enhance the transmigration of granulocytes (Hurley et al., 2001). Both effects contribute to intestinal inflammation and are assumed prerequisites for the resorption of significant amounts of Stxs from the gut lumen (Proulx et al., 2001). Once the toxins have passed the intestinal barrier, there is

* Corresponding author. Tel.: +49 641 99 38314; fax: +49 641 99 38309.
E-mail address: christian.menge@vetmed.uni-giessen.de (C. Menge).

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evidence that granulocytes may even be capable of transporting Stxs in the blood stream to extraintestinal organs such as the kidneys (te Loo et al., 2000). Granulocytes of pigs – animals that develop Stx-related symptoms after natural and experimental STEC infection – were recently also found to bind Stxs (Winter et al., 2004).

In contrast to humans and pigs, mature ruminants do not show any overt clinical signs during intestinal colonization with STEC, even though they sometimes shed 10^5 and 10^6 STEC/g feces (Smith et al., 2002). Ruminants, especially cattle and sheep, have been identified, however, as the major source for human STEC infections and a number of surveys detected STEC in high percentages of bovine and ovine fecal samples from several countries worldwide (Dean-Nystrom et al., 1998; Hancock et al., 1998; Zschöck et al., 2000). The role of Stxs in bovine STEC infections had been unclear and the absence of Stx-receptors on bovine intestinal endothelial cells even led to the speculation that ruminants are ''Stx-tolerant'' STEC carriers (Pruimboom-Brees et al., 2000). However, bovine B and T lymphocytes express globotriaosylceramide (Gb_3/CD77) molecules in vitro that function as Stx-receptors (Menge et al., 2001, 2003; Stamm et al., 2002). Bovine ileal and colonic intraepithelial lymphocytes (IEL) also express Gb_3/CD77 and Stx1 blocks the activation and proliferation of peripheral and mucosal lymphocytes in vitro and in vivo (Menge et al., 1999b, 2003, 2004a,b). A suppression of the local adaptive immune response by Stx1 may therefore contribute to the ability of these bacteria to colonize the intestinal mucosa of their natural host for prolonged periods (Cray and Moon, 1995; Grauke et al., 2002).

Granulocytes principally represent effector cells of the innate immune system that contribute to the limitation of mucosal colonization by bacteria (Svanborg et al., 1999). Granulocytes infiltrate the colonic mucosa of STEC-infected calves (Schoonderwoerd et al., 1988), but their impact on the course of STEC infections in ruminants is not known. The absence of intestinal inflammation in STEC-infected mature ruminants may indicate that their granulocytes are not attracted to the mucosa and activated by Stxs like their human counterparts. Consistent with this hypothesis, inflammatory responses induced by some STEC strains in ligated intestinal loops in calves are independent of Stx1 (Stevens et al., 2002). We hypothesized that granulocytes of ruminants differ from human granulocytes in that the former are resistant to Stx-induced activation. The objectives of the present study, therefore, were to determine whether granulocytes represent target cells for Stx in cattle and sheep. For that purpose, we analysed these cells for the presence of Stx binding sites and the effects of purified Stx1 on viability and the phagocytic and oxidative burst activities.

2. Materials and methods

2.1. Protein purification

Stx1 was produced from bovine STEC strain 2403 (rough, H^-; Wieler et al., 1992) and purified as described before (Menge et al., 1999b). At the end of the purification process, toxin preparations were passed through Detoxi-Gel™ columns (Pierce, Old-Beijerland, The Netherlands) to reduce contamination with endotoxin. Cytotoxic activities of toxin preparations were determined on Vero cells (ATCC CRL 1587) by the method of Gentry and Dalrymple (1980) with minor modifications (Menge et al., 1999b; Menge, 2003). CD_{50} was calculated from dose–response curves geometrically as the reciprocal of the toxin dilution causing 50% reduction in cellular metabolic activity. The Stx1 preparation contained 50,000 CD_{50} of Stx1 and 166 pg of endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay (Kinetic QCL®, Cambrex, Verviers, Belgium). Recombinant StxB1 (rStxB1), previously shown to recognize Gb_3 but not Gb_4 (Niebuhr, 1991; Stamm et al., 2002), was purified from *E. coli* DH5α[pSU108] (Su et al., 1992) as described before (Stamm et al., 2002). The rStxB1 preparation contained 411 μg/ml of rStxB1 and 600 pg/ml of endotoxin.

2.2. Animals and sample collection

Blood samples were taken from adult, lactating cows and 13–18 day old calves (Holstein × German Black Pied) from the dairy herd of the Teaching and Research Farm of the Justus-Liebig-University, and from adult sheep (German Whiteheaded and German Blackheaded Mutton breed) kept at our institute. Blood samples were drawn by venipuncture of *V. jugularis externa* by using 60 ml syringes with 2.500 International Units (IU) heparine (Liquemin®N; Braun, Melsungen, Germany). They were stored at room temperature and transported directly to our laboratory for further processing. Milk samples were taken from the cows described above and from sheep (East Friesian breed) at a local farm.

2.3. Ex vivo phenotyping of leukocytes from peripheral blood

Leukocytes were obtained from whole blood samples by hypotonic lysis of erythrocytes. After

preparation, 2×10^5 cells were thoroughly resuspended in phosphate buffered saline (PBS) and transferred to V-shaped microtiter plates (Greiner, Frickenhausen, Germany) for immunolabeling as described previously (Menge et al., 1999a; Menge, 2003). Cells were centrifuged (4 °C, 137 × *g*, 7 min) and resuspended as described below. All incubations were for 20 min on ice and all washing steps were carried out as described above and with PBS (4 °C) if not otherwise indicated.

To detect Gb_3/CD77, cells were resuspended in 25 μl of rat IgM (dilution 1:50 in PBS; Camon, Wiesbaden, Germany) as a negative control or with anti-human CD77 antibody (rat IgM, clone 38.13; dilution 1:10 in PBS; Beckman-Coulter, Krefeld, Germany). Cells were incubated, washed once, and then resuspended in 50 μl of fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragment goat anti-rat μ-chain (Beckman-Coulter) diluted 1:200 in PBS containing 2 μg/ml propidium iodide (PI; Sigma–Aldrich Chemie, Taufkirchen, Germany), and kept on ice for 20 min.

To detect rStxB1 binding, cells were incubated with 50 μl of cell culture medium (referred to as "medium" throughout) containing 89% (v/v) RPMI 1640 (Biochrom, Berlin, Germany), 10% (v/v) fetal-calf-serum (FCS; Invitrogen, Karlsruhe, Germany), 100 IU/ml penicillin/100 μg/ml streptomycin (Invitrogen) and 3 μM mercapto-ethanol (Fluka, Taufkirchen, Germany) as a negative control or medium supplemented with rStxB1 (30 μg/ml; Stamm et al., 2002), washed once, resuspended in 50 μl of purified mouse anti-StxB1 (IgG1, clone 13C4; 45 μg/ml; Strockbine et al., 1985), incubated, washed, resuspended in 50 μl of anti-mouse IgG (H + L) FITC-conjugate (dilution 1:400 in PBS; Dianova, Hamburg, Germany) containing 2 μg/ml PI, and incubated again.

Finally, all samples were washed twice and analysed with an EPICS ELITE® Analyser (Beckman-Coulter). Five thousand events were aquired per sample and analysed by the ELITE 4.01 software. Granulocytes were defined by their light scatter characteristics. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive.

2.4. Ex vivo phenotyping of leukocytes from milk

Milk leukocytes were prepared following the method of Taylor et al. (1994). Briefly, cream and the aqueous phase of milk samples were removed by pipetting after centrifugation (RT, 249 × *g*, 8 min). The resuspended cells were transferred to a new centrifugation tube and washed twice with PBS. After preparation, 2×10^5 cells were thoroughly resuspended in medium, transferred to V-shaped microtiter plates, centrifuged. In order to triple label the cells with anti-CD172a, anti-CD77, and PI, cells were resuspended in 50 μl of medium as a negative control or with the cell culture supernatant of the mouse hybridoma cell line IL-A24 (producing IgG1 recognizing CD172a on bovine and ovine myeloid cells (Brooke et al., 1998; Ellis et al., 1988; Haig et al., 1991); kindly provided by J. Naessens, International Livestock Research Institute, Nairobi, Kenya). Cells were incubated, centrifuged, and resuspended in 25 μl of rat IgM as a negative control or with anti-CD77. Cells were incubated, washed once, and incubated in 50 μl of goat anti-mouse γ-chain FITC-conjugate (dilution 1:200 in PBS; Medac, Hamburg, Germany). After centrifugation, cells were resuspended in 50 μl of anti-rat μ-chain R-phycoerythrin (R-PE)-conjugate diluted 1:200 in PBS containing 2 μg/ml PI, and kept on ice for 20 min. Finally, the cells were washed twice and analysed by flow cytometry.

2.5. In vitro cultures of granulocytes

Granulocytes were separated from mononuclear cells by density gradient centrifugation with Ficoll-Paque™ Plus (Amersham Pharmacia, Freiburg, Germany; Menge, 2003). Two millilitre aliquots from the pellet were diluted 1:2 with PBS supplemented with 5.4 mM EDTA (PBS-EDTA) and mixed with 27 ml of distilled water. After 50 sec 3 ml of a 10-fold concentrated solution of PBS and 16 ml of RPMI 1640 were added. Remaining granulocytes were washed once in PBS (20 °C, 202 × *g*, 7 min) and resuspended in medium. The purity of each aliquot was assessed by flow cytometry and only aliquots containing more than 90% granulocytes as defined by their light scatter characteristics were pooled. Granulocyte suspensions were adjusted to 2×10^5 cells/150 μl medium and either used for blocking experiments (see next section) or cultured in 96-well U-shaped microtiter plates (Nunc, Wiesbaden, Germany).

For stimulation of the cells prior to labeling with anti-CD77, medium was supplemented with lipopolysaccharide from *E. coli* O111:B4 (LPS; Sigma–Aldrich Chemie) at 1, 10 or 100 ng/ml. Plates were incubated at 37 °C with 5% CO_2 for 0.5–72 h. At the end of the incubation, cells were labeled with anti-CD77 as described for ex vivo phenotyping studies.

For studies aimed at detecting effects of Stx1 on viability, phagocytic and oxidative burst activity, cells were incubated with Stx1 (200 CD_{50}/ml) or rStxB1 (10 μg/ml). For neutralizing conditions, the medium was additionally supplemented with purified anti-StxB1 13C4 (final concentration 1.5 μg immunoglobulin/ml).

LPS (1 ng/ml) was included (in lieu of Stx1) as a control. Plates were incubated at 37 °C with 5% CO_2 for 0.5 or 18 h.

2.6. Blocking of anti-CD77 binding with rStxB1

Purified granulocytes (2×10^5 cells/150 μl medium) were transferred to V-shaped microtiter plates, centrifuged (4 °C, 137 × *g*, 7 min) and incubated (4 °C, 30 min) with 50 μl of a two-fold dilution series of rStxB1 (starting at 30 μg/ml). After centrifugation, the pelleted cells were incubated in 25 μl of anti-CD77 (1:10 in PBS equivalent to 15 μg/ml; 4 °C, 30 min). In separate wells, cells were incubated with the two ligands in reverse order. Finally, binding of anti-CD77 was quantified in all samples by incubation of the cells with 50 μl of anti-rat μ-chain R-PE (4 °C, 20 min), followed by two washing steps (PBS, 4 °C) and flow cytometry analysis.

2.7. Viability test

After cultivation for 0.5 or 18 h, granulocytes were resuspended in medium, transferred to V-shaped microtiter plates and centrifuged (4 °C, 137 × *g*, 7 min). Cells were then resuspended in 100 μl of Annexin-V-binding-buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ of distilled water; pH 7.4) and three fluorescent probes were added: 5 μl Calcein AM (0.01 μM; Molecular Probes, Leiden, The Netherlands), 10 μl Annexin-V (PE-conjugated; 1:10 in PBS; Caltag Laboratories, Hamburg, Germany), and 2 μl PI (100 μg/ml). Labeling of the cells was performed in duplicates per culturing condition (as described in 2.5) in separate wells. Cells were incubated for 15 min in the dark. After washing with Annexin-V-binding buffer, cells were analysed by flow cytometry. By plotting the signals of the cells for Calcein and Annexin-V, viable cells were defined as Calcein positive but Annexin-V negative, while early apoptotic cells were defined as Calcein positive and Annexin-V positive. Late apoptotic/necrotic cells were enumerated in a second plot displaying the Annexin-V signal versus the signal for PI and defined as Annexin-V positive and PI positive granulocytes. Since a small number of cells apparently stained with all three dyes the sum of data depicted in Figs. 4 and 5 may slightly exceed 100%.

2.8. Phagocytic assay and oxidative burst assay

After cultivation for 0.5 or 18 h, granulocytes were thoroughly resuspended, transferred to V-shaped microtiter plates and centrifuged (4 °C, 137 × *g*, 7 min). After removal of the supernatant, the phagocytic and oxidative burst activity of the granulocytes was quantified by applying Phagotest® and Bursttest® kits (ORPEGEN-Pharma, Heidelberg, Germany). All samples were performed in duplicate. The tests were carried out as described before (Menge et al., 1998) with some modifications. For the quantitation of phagocytic activity, 2×10^5 granulocytes were resuspended in 150 μl ice-cold autologous plasma (diluted 1:2 with PBS-EDTA as it was recovered from the upper phase of the Ficoll gradient during the preparation of the cells). Then 10 μl of a suspension of FITC-labeled *E. coli* (ATCC 33572) preopsonized with human serum as provided in the assay kit were added. After 10 min in a shaking water bath at 38 °C, quenching of surface-bound bacteria, fixation and permeabilisation of the granulocytes were carried out as recommended by the manufacturer. For the quantitation of the inducible oxidative burst activity, 2×10^5 granulocytes were resuspended in 150 μl ice-cold PBS and 10 μl phorbol 12-myristate 13-acetate solution (PMA; 8,1 μM; ORPEGEN-Pharma) were added. After 10 min in a shaking water bath at 38 °C, 10 μl of "substrate-solution" containing dihydrorhodamine 123 were added and the cells were incubated for another 10 min. Fixation and permeabilisation was carried out as recommended by the manufacturer. Both the granulocytes prepared for the phagocytic assay and those prepared for the oxidative burst assay were finally incubated in PBS-EDTA (at 4 °C for 5 min) to recover plastic adherent cells and counterstained with "DNA-staining solution" as provided in the assay kits. Cells were subsequently analysed by flow cytometry. During data acquisition, a "live" gate was set in the 630 nm fluorescence histogram for those events having at least the same DNA-content as diploid cells in order to exclude cell debris and bacterial aggregates. Granulocytes were monitored for their 525 nm (FITC) fluorescence. Electronic gates were set according to the negative controls for the phagocytic and the oxidative burst activity (cells incubated on ice throughout and cells incubated without PMA, respectively) included in each test series defining less than 2% of the cells as positive.

2.9. Statistical analysis

Data were analysed statistically by multi-factorial analysis of variance (ANOVA) by using BMDP/Dynamic-software (Statistical Software Inc.; Dixon, 1993) Results were evaluated as follows: $^{***}P \leq 0.001$,

highly significant; $^{**}P \leq 0.01$, significant; $^{*}P \leq 0.05$, weakly significant; $P > 0.05$, not significant. In the case of two-way ANOVA different P-values indicate significance regarding (i) influence of Stx1 and rStxB1, respectively ($P_{additive}$); (ii) influence of adding monoclonal antibody 13C4 ($P_{antibody}$); (iii) interaction between influence of additive and antibody ($P_{interaction}$).

3. Results

3.1. StxB1-receptor expression by granulocytes from cattle and sheep

Neither anti-CD77 nor the recombinant B-subunit of Stx1 (rStxB1) bound to granulocytes obtained from the peripheral blood of five adult cattle by hypotonic lysis (Fig. 1) when the cells were directly submitted to the labeling procedure (''ex vivo''). Similarly, neither anti-CD77 nor rStxB1 bound to any of the granulocytes obtained from four additional cows and three calves (13–18 days of age) which were prepared by density gradient centrifugation and incubated in the absence or presence (1, 10 or 100 ng/ml) of LPS for 0.5, 6, 18, 24, 48, and 72 h (in vitro stimulation) prior to the labeling procedure (data not shown).

In contrast, both anti-CD77 and rStxB1 bound to granulocytes from the blood of adult sheep ($n = 3$) without preceding stimulation ex vivo (Fig. 1). Flow cytometry detection of rStxB1 binding resulted in a population of positive events that displayed a high fluorescence signal and formed a narrow peak clearly separated from the negative population. Identical staining patterns were achieved when purified granulocytes were labeled in vitro with rStxB1 at

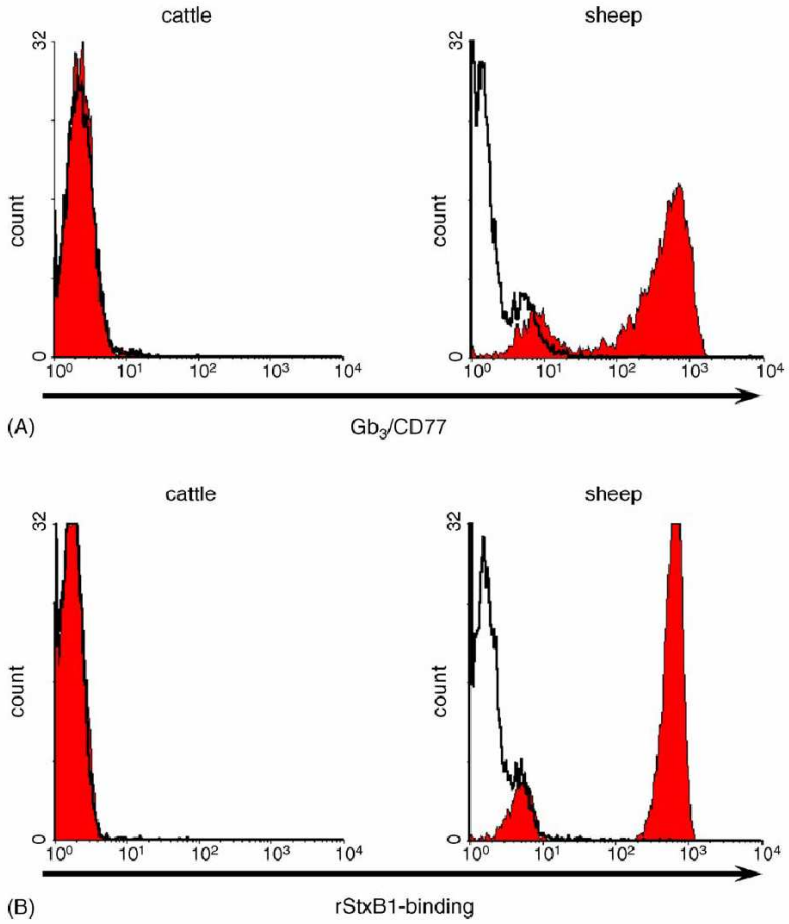

Fig. 1. Expression of Gb_3/CD77 (A) and rStxB1 (B) binding sites by peripheral blood granulocytes of cattle and sheep ex vivo. Granulocytes were obtained from peripheral blood by hypotonic lysis and distinguished from mononuclear cells by their light scatter characteristics. Flow cytometry histograms illustrate specific binding (shaded histograms) of the ligand in comparison to the corresponding antibody control (solid line). Results are representative of repeated experiments with leukocyte preparations from adult cattle ($n = 5$) and sheep ($n = 3$).

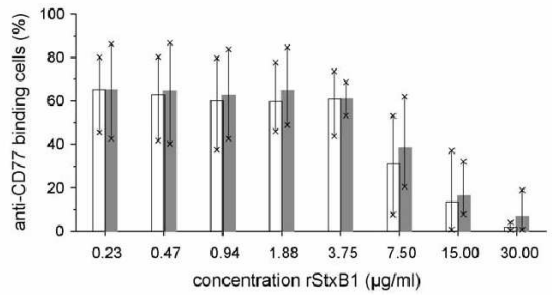

Fig. 2. Blocking of anti-CD77 binding to ovine blood granulocytes in vitro by pre-incubation with rStxB1. Purified granulocytes were incubated with a two-fold dilution series of rStxB1 followed by incubation with anti-CD77 (15 μg/ml; shaded bars) at 4 °C for 30 min each. Alternatively, cells were incubated with anti-CD77 first, followed by a two-fold dilution series of rStxB1 (open bars). In both instances, bound anti-CD77 was subsequently detected by labeling with anti-rat μ-chain R-PE. Bars represent mean, max and min of the results obtained by flow cytometry from six determinations with preparations from three different animals. Statistical analysis by two-way repeated measures ANOVA revealed a significant ($P \leq 0.001$) influence of the rStxB1 concentration on the percentage of anti-CD77 labeled cells. The order of the incubation with the two ligands did not influence the results ($P > 0.05$).

concentrations of ≥7.5 μg/ml (corresponding to ≥1.875 pg/cell; data not shown). Pre-incubation of ovine granulocytes with rStxB1 reduced the subsequent binding of anti-CD77 in vitro in a concentration dependent manner and completely abolished binding at 30 μg/ml (corresponding to 7.5 pg/cell; Fig. 2). Interestingly, pre-incubation with anti-CD77 before the addition of rStxB1 also reduced the number of anti-CD77 binding cells to the same extent. CD77-positive granulocytes (cells which had migrated from blood vessels through an epithelial cell layer into milk) were also detected in ovine milk, but not in bovine milk (Fig. 3).

3.2. Effect of Stx1 on functions of granulocytes

Neither incubation of bovine granulocytes obtained from the blood of five adult cattle with purified Stx1 nor incubation with rStxB1 affected the viability of the cells within 0.5 h (data not shown). After 18 h the percentage of viable, early apoptotic and late apoptotic/necrotic cells slightly varied between the conditions (Fig. 4). However, no effect could be attributed to Stx1 or rStxB1 since the variations were within the range between the medium only and the LPS control and results were similar in the absence and presence of anti-StxB1. Neither Stx1 nor rStxB1 altered the phagocytic or the oxidative burst activity of granulocytes prepared from five adult cattle within 0.5 and 18 h of incubation (data not shown).

Stx1 also did not alter the viability of granulocytes from five adult sheep within 0.5 h of incubation. However, Stx1 significantly increased the percentage of early apoptotic cells after 18 h by nearly two-fold (Fig. 5). Concomitantly, the percentage of viable cells was reduced after incubation with Stx1 although the differences did not reach statistical significance. The numbers of late apoptotic/necrotic cells were unaffected by Stx1. Induction of apoptosis by Stx1 could be blocked by pre-incubation of the toxin with anti-StxB1 and was not detected when the cells were incubated

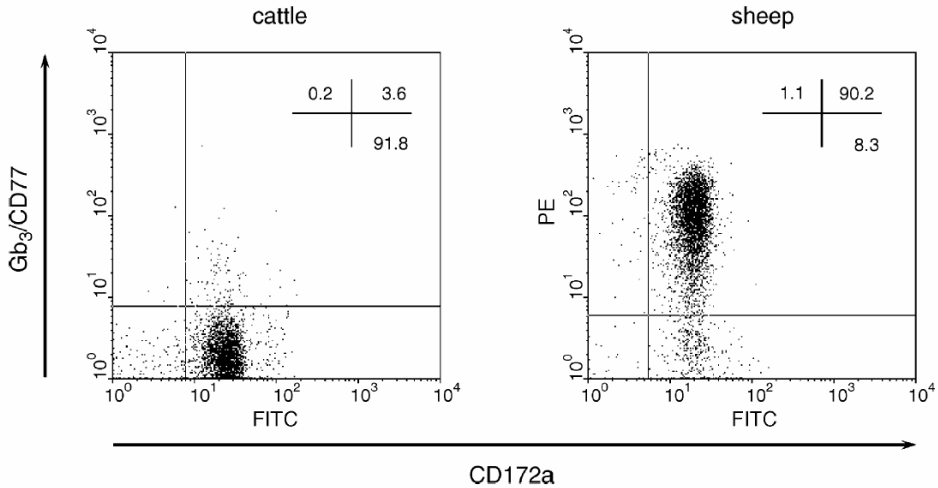

Fig. 3. Expression of Gb_3/CD77 by milk granulocytes from cattle and sheep ex vivo. Representative dot plots of milk leukocytes stained for Gb_3/CD77 and CD172a (to positively identify myeloid cells; Ellis et al., 1988) are shown from one out of three preparations. Percentages of cells positive for one or both of the antigens are indicated in the upper right corner of the dot plots.

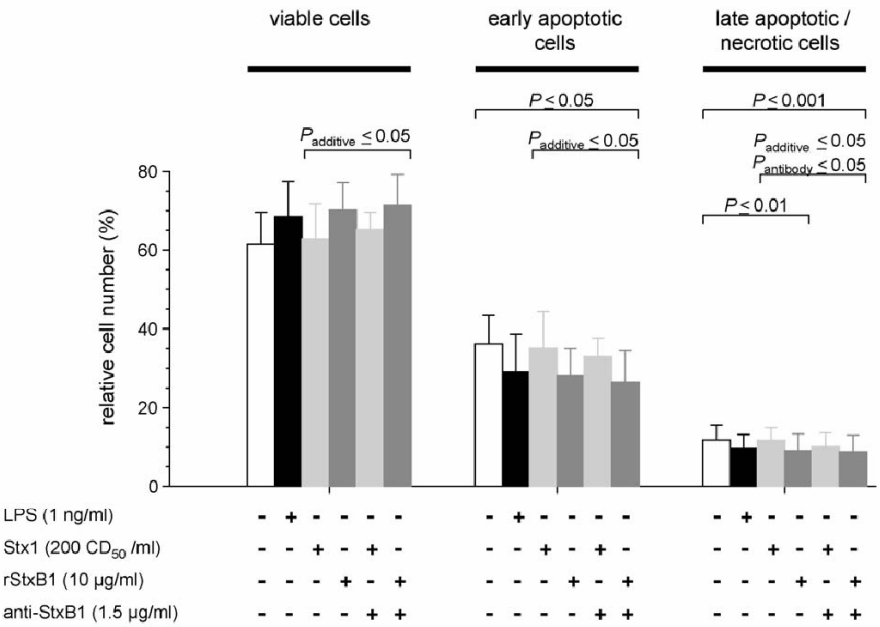

Fig. 4. Effect of Stx1 on the viability of bovine blood granulocytes in vitro. Granulocytes were separated from mononuclear cells and incubated in the presence and absence of Stx1 and rStxB1 at 37 °C for 18 h. After that, the percentages of viable, early apoptotic, and late apoptotic/necrotic cells were determined by flow cytometry. Values represent mean and standard deviations from experiments with five granulocyte preparations from different animals. P values were calculated by one-way and two-way repeated measures ANOVA but omitted from the figure when $P > 0.05$. Brackets embrace groups of bars within which significant differences occured.

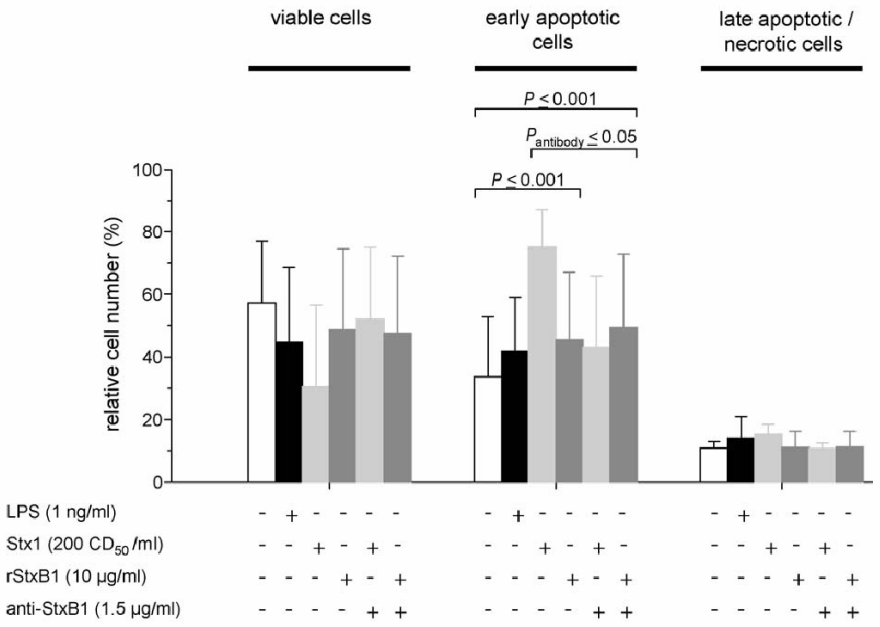

Fig. 5. Effect of Stx1 on the viability of ovine blood granulocytes in vitro. Granulocytes were separated from mononuclear cells and incubated in the presence and absence of Stx1 and rStxB1 at 37 °C for 18 h. After that, the percentages of viable, early apoptotic, and late apoptotic/necrotic cells were determined by flow cytometry. Values represent mean and standard deviations from experiments with five granulocyte preparations from different animals. P values were calculated by one-way and two-way repeated measures ANOVA but omitted from the figure when $P > 0.05$. Brackets embrace groups of bars within which significant differences occured.

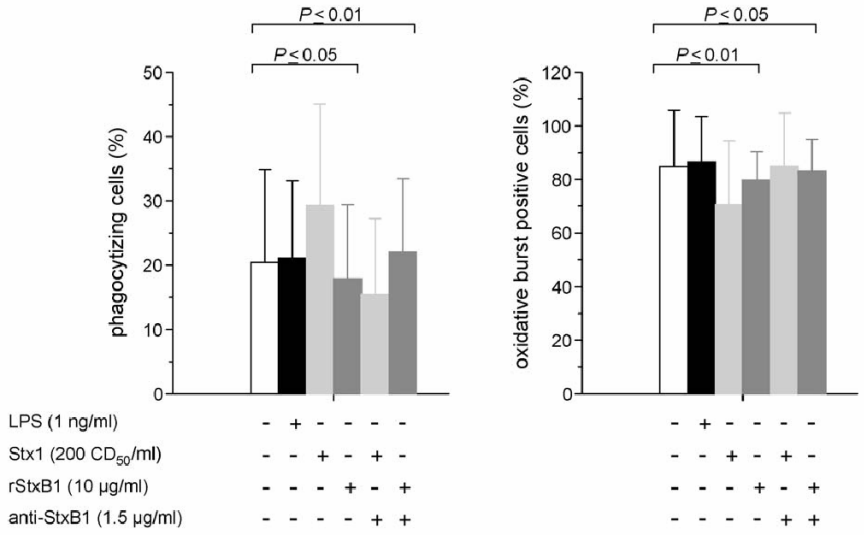

Fig. 6. Effect of Stx1 on the phagocytic activity and induced oxidative burst activity of ovine blood granulocytes in vitro. Granulocytes were separated from mononuclear cells and incubated in the presence and absence of Stx1 and rStxB1 at 37 °C for 18 h. After that, the percentages of cells capable of phagocytizing heat-inactivated *E. coli* (left hand figure) and capable of performing an oxidative burst upon stimulation with PMA (right hand figure) were determined by flow cytometry. Values represent mean and standard deviations from experiments with five granulocyte preparations from different animals. *P* values were calculated by one-way and two-way repeated measures ANOVA but omitted from the figure when $P > 0.05$. Brackets embrace groups of bars within which significant differences occured.

with rStxB1 only which lacks the enzymatic activity of the holotoxin. Stx1 also slightly altered the phagocytic activity of ovine granulocytes and the capability of these cells to perform an oxidative burst (Fig. 6). Although incubation with Stx1 increased the phagocytic activity it depressed the oxidative burst activity when the cells were stimulated with PMA. Again, effects of Stx1 were detectable after 18 h but not after 0.5 h of incubation, could be blocked by pre-incubation of Stx1 with anti-StxB1, and were not induced by rStxB1.

4. Discussion

Some STEC strains cause severe diseases in the human but not in the ruminant host (Smith et al., 2002). It was assumed previously that this is based – at least in part – on the differences in the expression of Stx-receptors (Gb_3/CD77) by endothelial cells, epithelial cells, and lymphocytes in man and cattle, respectively (Pruimboom-Brees et al., 2000; Menge et al., 2001; Hoey et al., 2002; Stamm et al., 2002). The present data confirm our initial hypothesis that Stx-receptor expression by granulocytes and the susceptibility of these cells to Stxs represent another significant difference between ruminants and humans. It thereby becomes increasingly apparent that the different outcomes of STEC infections in different species cannot solely be explained by the presence or absence of Gb_3/CD77 on a single type of cell as assumed before (Pruimboom-Brees et al., 2000). Despite the opposed findings for cattle and sheep with regard to Stx-receptor expression on granulocytes, results from functional studies with these cells provide convincing evidence that a direct effect of Stx1 on these cells of the innate immune system is not implicated in the adaptation of STEC to these reservoir hosts.

Bovine peripheral blood granulocytes lacked Stx-receptors. Owing to previous findings that bovine lymphocytes only express Gb_3/CD77 upon activation in vitro or in vivo (Menge et al., 2001; Stamm et al., 2002), we also analysed granulocytes incubated under conditions shown to directly stimulate the cells to express surface molecules such as CD11b (Diez-Fraile et al., 2000). Bovine granulocytes neither bound anti-CD77 nor rStxB1, even after incubation for up to 72 h in the absence or presence of LPS (1–100 ng/ml). Migration across the blood-milk barrier, which affects different granulocytes' functions (Smits et al., 1999), also did not result in expression of Gb_3/CD77. We ruled out age-related effects as described for rabbit intestinal cells (Mobassaleh et al., 1988) by analyzing granulocytes from calves less than 3 weeks of age. None of these attempts led to a detection of Gb_3/CD77 or rStxB1 binding sites on bovine granulocytes.

In contrast to bovine granulocytes, granulocytes of sheep bound rStxB1 and the monoclonal antibody "38.13" that specifically reacts with the terminal

galabiose of globotriaosylceramide (Gb_3) on human (Nudelman et al., 1983) and bovine cells (Menge et al., 2001; Stamm et al., 2002). High concentrations of rStxB1 completely blocked subsequent binding of anti-CD77, strongly implying that the vast majority of Gb_3/CD77 molecules (e.g. recognized by "38.13") also serves as Stx1-receptors. Interestingly, however, anti-CD77 binding to ovine granulocytes was reduced by rStxB1 to the same extent when the cells were incubated with anti-CD77 first, suggesting that rStxB1 is able to remove bound anti-CD77 from Gb_3/CD77 molecules. In similar experiments with bovine lymphocytes, upon which we biochemically confirmed the existence of various Gb_3 isoforms differing in the fatty acids length of their lipid moiety, we showed that the same concentrations of rStxB1 only partially blocked anti-CD77 binding (Stamm et al., 2002). In accordance to Pellizzari et al. (1992), we concluded that various Gb_3 isoforms on bovine lymphocytes exhibit different affinities for different ligands. This interpretation is particularly supported by recent studies showing that anti-CD77 "38.13" recognizes Gb_3 molecules in human kidneys that may be different from those recognized by Stx1 (Chark et al., 2004). Although the structure of Gb_3 molecules synthesized by ovine granulocytes awaits further biochemical characterization, the present data argue in favour of the notion that, in contrast to lymphocytes in cattle, only a single class of binding sites with a considerably higher affinity for Stx1 than for anti-CD77 is expressed by ovine granulocytes. Owing to the fact that Gb_3 is not behaving as a single species in Stx binding studies but shows dose-dependent apparent cooperative binding kinetics at select Gb_3 concentrations (Binnington et al., 2002), it remains to be determined whether Stx binding sites on ovine granulocytes represent a single class of Gb_3 isoforms or a distinct isoform mixture, density, and spatial organisation of the receptor.

Ovine Gb_3/CD77 receptors appear to mediate the effect of Stx differently than the Stx-receptors identified on human granulocytes. Superoxide production by PMA-stimulated human granulocytes is impaired by addition of Stx1 as well as the enzymatically inactive B-subunit of the toxin (King et al., 1999). Accordingly, the inhibitory effect of Stx2 on the apoptosis of human granulocytes can be overcome by an inhibitor of protein kinases (Liu et al., 1999) indicating the presence of a signal transduction pathway that originates from Stx-receptors on the surface. A similar pathway is linked to Gb_3/CD77 in human B cells (Taga et al., 1997), but seems to be absent in ovine granulocytes, because only Stx1 holotoxin but not rStxB1 was able to induce apoptosis in these cells.

An antiphagocytic phenotype of enteric bacteria may be advantageous in that it allows the bacteria to colonize the intestinal lining and M cells without being transported to the gut-associated lymphoid tissue (Goosney et al., 1999; Ernst, 2000). One may speculate that STEC affect cells of the innate immune response in sheep by secreting Stx1 and thereby contribute to the prolongation of intestinal colonization (Cornick et al., 2000). However, the toxin required 18 h to increase the percentage of early apoptotic cells in ovine granulocyte cultures, still leaving the number of late apoptotic cells almost unchanged. Within this period of time the effect of Stx1 on the phagocytic and the induced oxidative burst activity was comparatively low. Phagocytosis of bacteria by granulocytes requires some minutes (Menge et al., 1998) and enteropathogenic *E. coli* are able to display their antiphagocytic phenotype by products of the *lee*-encoded type III secretion pathway within 120 min after infection of macrophage-like cell lines (Goosney et al., 1999). Provided that the same course of events also takes place upon contact of *lee*-positive STEC with ovine phagocytizing cells, the meaning of such a delayed effect of Stx1 remains elusive.

Stxs can directly act on human granulocytes (King et al., 1999; Liu et al., 1999), but also induce the release of chemokines from intestinal epithelial cells (Yamasaki et al., 1999; Thorpe et al., 2001), which results in enhanced granulocyte transmigration and Stx translocation across intestinal epithelial cells in vitro (Hurley et al., 2001). Stx1 neither affected the cellular viability nor did it interfere with other cellular functions of bovine granulocytes. The obvious resistance of bovine granulocytes to a directly activating activity of Stx1 is another step towards an understanding why mature cattle while harbouring STEC do not develop any clinical signs. However, it remains to be determined whether Stx or other STEC factors modulate the expression of signalling substances (e.g. chemokines) in the bovine intestinal mucosa that alter granulocyte functions indirectly. Intestinal inflammatory responses induced by some STEC strains in ligated intestinal loops in calves are independent of Stx1 (Stevens et al., 2002), but the observation that bovine intestinal epithelial cells express Gb_3/CD77 (Hoey et al., 2002; Hoey et al., 2003; I. Stamm, E. Schröpfer, M. Mohr, G. Baljer, C. Menge, unpublished data) might indicate that these cells are capable of acting as sensors for the toxin and encourages further investigations.

The discovery that human granulocytes express a yet unknown low-affinity Stx-receptor that is distinct

from Gb_3/CD77 and that the cells are able to transfer Stx1 to cocultured endothelial cells expressing Gb_3/CD77 (te Loo et al., 2000) is thought to explain how intestinal STEC infections lead to tissue damage in extraintestinal organs such as the kidneys and the brain. If this hypothesis holds, bovine granulocytes that lack any type of Stx-receptors should be unable to transport Stx1 and thereby protect cattle from the extraintestinal effects of Stx. Gb_3/CD77 positive granulocytes in sheep, on the other hand, probably do not release the toxin again once it is bound. In fact, preliminary data from our laboratory seem to support this notion. The neutralizing capacity of ovine granulocytes may even be further improved by anti-Stx1 antibodies, present in the sera of sheep (Eisenberg, 2003) that ought to mediate Stx1-binding to Fc-receptors. Notably, we also detected Gb_3/CD77 molecules that bound rStxB1 on granulocytes from goats (data not shown), animals that also function as asymptomatic STEC carriers. In conflict with the hypothesis that Gb_3/CD77 positive granulocytes serve as Stxs scavengers, however, it was recently discovered that porcine granulocytes also bind Stx1 and Stx2 via Gb_3/CD77 (Winter et al., 2004) though the functional consequences have not been investigated yet. Since Shiga-toxigenic *E. coli*-inoculated neonatal piglets develop kidney lesions that are similar to those in humans with HUS (Pohlenz et al., 2005), further comparative studies on the meaning of Stx/granulocyte-interactions for the course of STEC infections in different species are strongly encouraged.

In anticipation of such studies, the results presented here reveal for the first time differences between species equally regarded as STEC carriers (Heuvelink et al., 1998). The development of strategies for the reduction of STEC prevalence in animals is mainly hampered by the limited understanding of the mechanisms by which the bacteria interact with their host. Studies performed to address this problem have made use of the sheep model based on the presumption that the STEC require similar colonization factors in cattle and sheep (Naylor et al., 2005). In fact, experimental infections resulted in similar STEC shedding pattern and intestinal recovery to those reported for cattle (Cornick et al., 2000). However, this is, to the best of our knowledge, the first investigation that addresses the cellular distribution of Stx-receptors in sheep and has instantly revealed significant differences to cattle. The assumption that sheep are essentially identical to cattle may be a major obstacle for an appropriate interpretation of results obtained with ovine models unless more extensive comparative studies have been performed.

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6.10 "*Escherichia coli* Shiga toxin 1 enhances *il-4* transcripts in bovine ileal intraepithelial lymphocytes."

Moussay, E., Ivonne Stamm, Anja Taubert, G. Baljer and **C. Menge***

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Escherichia coli Shiga toxin 1 enhances *il-4* transcripts in bovine ileal intraepithelial lymphocytes

Etienne Moussay [a], Ivonne Stamm [a,1], Anja Taubert [b], Georg Baljer [a], Christian Menge [a,*]

[a] *Institute for Hygiene and Infectious Diseases of Animals, Frankfurter Strasse 85-89, Justus-Liebig-University, D-35392 Giessen, Germany*
[b] *Institute for Parasitology, Justus-Liebig-University, D-35392 Giessen, Germany*

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Abstract

Shiga toxin 1 (Stx1) blocks the activation of bovine peripheral and intraepithelial lymphocytes (IEL), implying that the toxin has the potential to retard the host's immune response during intestinal colonization of cattle with human pathogenic Stx-producing *Escherichia coli* (STEC). Since Stx1 does not eliminate affected lymphocytes by causing cellular death, we assumed that Stx1 disturbs the integrity of the immune regulatory network. We therefore assessed the impact of Stx1 on the expression of selected chemokine and cytokine genes *in vitro* by real-time RT-PCR and by quantitation of intracellular cytokine proteins. While Stx1 did not alter the amount of mRNA specific for interleukin (IL)-2, IL-10, gamma interferon (IFN-γ), transforming growth factor beta (TGF-β), IL-8, 10 kDa interferon inducible protein (IP-10), and monocyte chemoattractant protein 1 (MCP-1) in cultured ileal IEL (iIEL), minute concentrations of Stx1 led to an up to 40-fold increase of *il-4* transcripts within 6–8 h of incubation. Comparative experiments with peripheral lymphocytes revealed that the effect was specific for iIEL. The enhancement of *il-4* transcripts in iIEL was not accompanied by apoptosis but required the enzymatic activity of the holotoxin. Nevertheless, iIEL retained their ability to synthesize proteins in the presence of Stx1: 40% of iIEL could be stimulated to synthesize IFN-γ while less than 10% expressed IL-4 or TGF-β. Furthermore, iIEL were found to produce granulocyte chemoattractants, but the release of these substances was not different in iIEL cultures incubated with or without Stx1. Although Stx1 did not affect the numbers of iIEL producing either cytokine, these findings point to an altered responsiveness of IEL during bovine STEC infections and shed light on the initial effects Stx1 exerts on the local adaptive immune system.

Keywords: Shiga toxin 1; Intraepithelial lymphocytes; *il-4*; Cattle

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) infections are a major cause of bloody diarrhoea, hemorrhagic colitis and haemolytic uremic syndrome in humans (Bennish, 1991). Following initial major outbreaks (Riley et al., 1983; Wells et al., 1983), STEC are considered as human pathogens of significant public health concern. *E. coli* Shiga toxins (Stx, consisting of two major groups, Stx1 and Stx2) are heteromeric

* Corresponding author. Tel.: +49 641 99 38314; fax: +49 641 99 38309.
E-mail address: christian.menge@vetmed.uni-giessen.de (C. Menge).
[1] Present address: Vet Med Labor GmbH, Mörikestrasse 28/3, D-71636 Ludwigsburg, Germany.

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toxins (1A:5B) and were identified as main STEC virulence factors (O'Brien et al., 1982) that principally target endothelial cells (Louise and Obrig, 1991), leading to vascular damages in human kidneys, brain, pancreas and intestine. After binding of the B-subunit(s) to the glycosphingolipid Gb_3/CD77 (Fuchs et al., 1986), Stx is retrogradly transported (Sandvig et al., 1992) and the A_1-subunit translocates to the cytosol, where it exerts its cytotoxicity and rapidly kills the affected cells (Sandvig and van Deurs, 1996). Stx are ribosome-inactivating toxins that inhibit the protein synthesis by removing the adenine residue A-4324 in the 28S rRNA of the 60S ribosomal subunit (Endo et al., 1987, 1988) and eventually block the eEF-1 and eEF-2 dependent elongation process (Obrig et al., 1987). Additionally, cross-linking of Gb_3/CD77 by Stx1 on the cell surface activates the BCR-signaling cascade and induces apoptosis of human B cells (Mangeney et al., 1993; Mori et al., 2000). An increasing body of evidence suggests, however, that Stx1 also modulates the expression of certain cytokines (IL-1, TNF-α, IL-6) and chemokines (IL-8 and MCP-1) in epithelial cells (Thorpe et al., 1999, 2001), mesangial cells (Simon et al., 1998) and monocytes and macrophages (Harrison et al., 2005, 2004; Tesh et al., 1994; van Setten et al., 1996) in mice and man. Remarkably, peritoneal murine macrophages resist the cytolethal effects of Stx1 but produce large amounts of cytokines in response to the toxin (Tesh et al., 1994).

In cattle, representing the main source of human infections (Hancock et al., 1997), intestinal STEC-infections are mostly asymptomatic (Smith et al., 2002) but result in a high percentage of animals shedding STEC for prolonged periods (Dean-Nystrom et al., 1998; Dunn et al., 2004). We recently hypothesized (Menge et al., 2004a) that STEC have evolved strategies to limit intestinal inflammation and the mucosal immune defense in cattle, thus permitting a commensal-like lifestyle as suggested by Smith and coworkers (Smith et al., 2002). Indeed, Stx1 suppresses bovine lymphocyte functions (Menge et al., 2003b, 1999) and presumably represents a STEC virulence factor even in cattle (Menge et al., 2004b). In contrast to human lymphocytes, bovine B and T cell subsets both express functional Stx-receptors and are affected by the toxin (Stamm et al., 2002). Stx1 blocks the activation and the proliferation of these cells *in vitro* (Ferens and Hovde, 2000; Menge et al., 1999, 2003b) and *in vivo* (Hoffmann et al., 1997). Intestinal intraepithelial lymphocytes (IEL), the first immune cells that gain contact to the toxin, likely represent the main targets for Stx1 in the bovine gut (Menge et al., 2004a). Stx1 inhibits the activation of certain subsets of Gb_3/CD77-expressing IEL *in vitro* and depletes the ileal mucosa of $CD8\alpha^+$ T IEL in an ileal loop model of STEC infections (Menge et al., 2004a,b). However, since Stx1 neither induces cellular death nor affects the natural killer (NK) activity of IEL *in vitro* (Menge et al., 2004a) and *in vivo* (Menge et al., 2004b), the consequences of this effect of Stx1 for mucosal immune responses remained to be elucidated.

IEL represent effector cells against bacterial (Nencioni et al., 1983) and viral infections (Godson et al., 1992; Muller et al., 2000). While TCR αβ IEL participate in immune reactions to lumenal antigens, TCR γδ IEL mainly secrete cytokines (e.g. TGF-β and IL-4) involved in the surveillance and regulation of the epithelial homeostasis (Barrett et al., 1992; Fujihashi et al., 1993a,b; Inagaki-Ohara et al., 2004). IEL are also a potent source of chemokines (e.g. IL-8, MIP-1α and -1β) (Boismenu et al., 1996; Lundqvist et al., 1996). Based on our hypothesis that Stx1 modulates the local immune response during STEC infections in cattle, the objectives of this study were to investigate whether Stx1 binding to or internalization by bovine ileal IEL (iIEL) changes the cellular expression of selected cytokine and chemokine genes on the transcriptional and translational level.

2. Materials and methods

2.1. Preparation of bovine ileal intraepithelial lymphocytes (iIEL) and peripheral blood mononuclear cells (PBMC)

Ileal IEL were isolated following the procedure previously described (Menge et al., 2004a) with slight modifications. Briefly, gut specimen (distal ileum) were obtained from freshly slaughtered cattle older than 24 months from the local slaughterhouse and washed. The ileum was incubated 25 min with phosphate-buffered saline (PBS) supplemented with 1 mM 1,4-dithiotreitol at 37 °C under constant shaking (100 rpm). Specimen were cut into strips (0.5–1 cm), transferred to PBS-EDTA-AB (PBS supplemented with 2 mM EDTA, 100 IU/ml penicillin and 100 μg/ml streptomycin [PAA Laboratories GmbH, Pasching, Austria] and 50 μg/ml gentamicin [Biochrom AG, Berlin, Germany]), and incubated 20 min at 37 °C under constant shaking (100 rpm). The tissues were vortexed 2 min at maximum speed and supernatants were passed through nylon wool (Biotest AG, Dreieich, Germany). Cells were collected by centrifugation (250 × *g*, 8 min, RT), resuspended in 25 ml Percoll solution (Sigma–Aldrich, Steinheim, Germany), density adjusted to $\delta = 1.0500$ g/ml with

PBS-EDTA [PBS supplemented with 5.4 mM EDTA], and layered on 10 ml Percoll solution (density adjusted to $\delta = 1.0816$ g/ml). After centrifugation (652 × *g*, 20 min, 20 °C), iIEL were carefully recovered from the Percoll–Percoll interface, washed with PBS-EDTA (202 × *g*, 7 min, 20 °C) and then with PBS. Ileal IEL (2×10^7) were directly lysed for RNA isolation in 600 μl of RLT buffer (RNeasy® Mini Kit, QIAGEN) supplemented with 1% of β-mercaptoethanol (Fluka, Taufkirchen, Germany) or were resuspended at the density of 2×10^7 iIEL/ml in IEL-medium (RPMI 1640 with 2 mM stabilized L-glutamin and 2.0 g/l $NaHCO_3$ [PAN™ BIOTECH GmbH, Aidenbach, Germany] supplemented with 20% fetal calf serum [FCS, Biowest, Essen, Germany], 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/l amphotericin B [PAA Laboratories GmbH] and 2.5 μg/ml gentamicin [Biochrom AG]).

An immunophenotyping protocol was used to monitor the quality of the ileal IEL preparations by flow cytometry (Menge et al., 2004a). Briefly, iIEL (4×10^5) were stained for surface-expressed antigens in the presence of 1 μg/ml of 7-amino actinomycin D (7-AAD) to exclude dead cells from further analysis. Antibodies used were IL-A11 (specific for bovine CD4), IL-A105 (CD8α), IL-A65 (CD21), and IL-A111 (CD25) (hybridoma cells kindly provided by J. Naessens, International Livestock Research Institute, Nairobi, Kenya). Antibodies purchased from VMRD (Pullman, USA) were BAT82A (CD8β) and CACT61A (TcR-N12). Anti-human CD77 (clone 38.13) was purchased from Beckman-Coulter (Krefeld, Germany). Binding of the primary antibodies to the cells was detected by goat anti-mouse IgG (H + L) conjugated with FITC (Dianova, Hamburg, Germany) or by an anti-rat IgM conjugated with PE (Beckman-Coulter). Five thousand events were acquired by a FACSCalibur™ flow cytometer (Becton-Dickinson, Heidelberg, Germany).

Bovine PBMC were isolated as previously described (Menge et al., 1999) by density centrifugation using Ficoll-Paque® Plus (Amersham Biosciences Europe GmbH, Freiburg, Germany). Blood was obtained from healthy cows of the dairy herd of the Teaching and Research Farm of the University. Cells were resuspended in PBMC-medium containing RPMI 1640 (PAN™ BIOTECH GmbH), 10% FCS (Biowest) and 3 μM β-mercaptoethanol (Fluka).

2.2. Cultivation of bovine lymphocytes for RNA isolation

Freshly prepared iIEL were seeded in dishes in IEL medium (2×10^7 in 9 ml). For stimulation, the medium was supplemented with 2.5 μg/ml phytohemaglutinin-P (PHA-P, Sigma–Aldrich), with or without purified Stx1 (7, 22, 66, or 200 CD_{50}/ml), as determined on Vero cells (Menge et al., 1999) with an equivalence of about 1 CD_{50}/ml to 1 pg/ml (Olsnes et al., 1981). The methods for Stx1 preparation and purification were previously published (Menge et al., 1999). The Stx1 preparation contained 65,000 CD_{50} of Stx1 and 0.17 ng of endotoxin per ml as determined by the *Limulus* amoebocyte lysate assay. Ileal IEL were incubated 4, 6, 8, 18, or 24 h at 37 °C in 5% CO_2 and 95% humidity, and then resuspended in the wells, transferred to 50 ml-centrifugation tubes, washed with PBS (202 × *g*, 7 min, 20 °C) and lysed in 600 μl of RLT buffer (RNeasy® Mini Kit, Qiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol and stored at −70 °C. Supernatants were recovered 8 and 18 h after initiation of the cultures and immediately frozen at −20 °C. These supernatants were then used for granulocyte migration assays.

PBMC were seeded in PBMC medium (2×10^7 in 9 ml) supplemented with 5 μg/ml PHA-P, incubated 6 h at 37 °C with or without purified Stx1 (33 CD_{50}/ml), then resuspended, washed, and lysed as described for iIEL in 600 μl RLT buffer and stored at −70 °C.

2.3. RNA isolation procedure

Samples of all conditions (iIEL without incubation, iIEL incubated and mitogen-stimulated and PBMC incubated and mitogen-stimulated) were handled in the same way. The samples were thawed 5 min at 37 °C and homogenized by passing through a 20 Gauge-needle fitted to a 5 ml-syringe. For RNA extraction, the RNeasy® Mini Kit (Qiagen) was used following the instructions of the manufacturer with slight modifications. A first DNA digestion step was made on the column with 27 units of DNase I (RNase-free-DNase set, Qiagen) during 20 min at room temperature. After a wash in RW1 buffer (Qiagen), and elution of RNA in DEPC-treated water (Fermentas GmbH, St Leon-Rot, Germany), a second DNA digestion step was performed with 30 units of DNase I (Amersham Biosciences Europe GmbH) for 20 min at 37 °C. RNA was protected by 80 units of Ribonuclease Inhibitor (RNasin, Fermentas GmbH). The DNase was then inactivated by chemical (10 μl of 3 M sodium acetate, pH 4.6) and physical (vortexing) denaturations. RNA was precipitated with 99% ethanol for 2 h at −70 °C. After centrifugation (12.000 × *g*, 60 min, 4 °C), RNA was washed, air-dried and resuspended in DEPC-treated water supplemented with 40 units of RNasin.

The absence of genomic DNA in the preparation was checked by PCR for a housekeeping gene encoding the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplified with 1 unit of AmpliTaq® DNA polymerase, 25 mM $MgCl_2$, 10× PCR buffer II (Applied Biosystems, Darmstadt, Germany) and 5 μM primers (Table 1, MWG Biotech AG, Ebersberg, Germany). After a denaturation step of 2 min at 94 °C, the amplification was performed in 40 sequential cycles (15 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C) followed by post-elongation of 2 min at 72 °C. PCR products were analysed on a 2% agarose gel.

2.4. Reverse transcription and cytokine and chemokine-specific real-time PCR

One microgram of total RNA was reverse transcribed by using an oligo $d(T)_{16}$ primer (Applied Biosystems). After annealing for 5 min at 70 °C with 0.5 μg oligo $d(T)_{16}$, the reverse transcription (RT) reaction was carried out in a volume of 40 μl for 60 min at 40 °C with 200 units of M-MLV reverse transcriptase H(−) (Promega, Mannheim, Germany) using the recommendations of the manufacturer in the presence of 40 units RNasin. A cytokine and chemokine mRNA profile was established from freshly isolated iIEL in the absence of Stx1. cDNA from iIEL were subjected to semi-quantitative PCR using the reagents and PCR conditions described above for the GAPDH control PCR (for primers see Table 1).

For mitogen-stimulated iIEL and PBMC a real-time PCR was performed; primers (purchased from MWG Biotech AG) and probes (purchased from Eurogentec, Liège, Belgium) are shown in Table 1. Probes were labelled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). PCR amplification was performed on an automated fluorometer (ABI PRISM™ 5700 Sequence Detection System, Applied Biosystems) using 96-well optical plates. Each sample was analysed in duplicates. For PCR, 1.5 μl cDNA (corresponding to 37 ng of total cDNA) were used in a 25 μl PCR reaction mixture containing 12.5 μl qPCR™ MasterMix (Eurogentec), 300 nM of each primer and 200 nM probe. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s.

Table 1
Sequences of primers and probes used in this study

Specificity	Primers (5′–3′) and probes (5′-6-FAM-TAMRA-3′)	Reference
IL-2 Forw	GGA TTT ACA GTT GCT TTT GGA GAA A	Leutenegger et al. (2000)
IL-2 Rev	GCA CTT CCT CTA GAA GTT TGA GTT CTT	
TaqMan® probe	CGT GCC CAA GGT TAA CGC TAC AGA ATT GAA	
IL-4 Forw	CAT GCA TGG AGC TGC CTG TA	Waldvogel et al. (2000)
IL-4 Rev	AAT TCC AAC CCT GCA GAA GGT	
TaqMan® probe	TGC TGC CCC AAA GAA CAC AAC TGA GAA G	
IL-8 Forw	CAC TGT GAA AAA TTC AGA AAT CAT TGT TA	Leutenegger et al. (2000)
IL-8 Rev	CTT CAC CAA ATA CCT GCA CAA CCT TC	
TaqMan® probe	AAT GGA AAC GAG GTC TGC TTA AAC CCC AAG	
IL-10 Forw	CCA AGC CTT GTC GGA AAT GA	Present study
IL-10 Rev	GTT CAC GTG CTC CTT GAT GTC A	
TaqMan® probe	AGC CTG TGG CAT CAC CTC TTC CAG GTA A	
IFN-γ Forw	CAG CTC TGA GAA ACT GGA GGA CTT	Waldvogel et al. (2000)
IFN-γ Rev	TGG CTT TGC GCT GGA TCT	
TaqMan® probe	AGC TGA TTC AAA TTC CGG TGG ATG ATC T	
TGF-β Forw	GGC CCT GCC CTT ACA TCT G	Present study
TGF-β Rev	CGG GTT GTG CTG GTT GTA CA	
TaqMan® probe	CCT GGA TAC ACA GTA CAG CAA GGT CCT GG	
IP-10 Forw	AAG TCA TTC CTG CAA GTC AAT CCT	Taubert et al. (2006)
IP-10 Rev	TTG ATG GTC TTA GAT TCT GGA TTC AG	
TaqMan® probe	CCA CGT GTC GAG ATT ATT GCC ACA ATG A	
MCP-1 Forw	CGC TCA GCC AGA TGC AAT TA	Taubert et al. (2006)
MCP-1 Rev	GCC TCT GCA TGG AGA TCT TCT T	
TaqMan® probe	CCC AAG TCG CCT GCT GCT ATA CAT TCA A	
GAPDH Forw	GCG ATA CTC ACT CTT CTA CCT TCG A	Taubert et al. (2006)
GAPDH Rev	TCG TAC CAG GAA ATG AGC TTG AC	
TaqMan® probe	CTG GCA TTG CCC TCA ACG ACC ACT T	

Semiquantitative analyses used the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the manufacturer of the 5700 sequence detector and reported as differences in comparison to the "medium + PHA-P" control (which was set as 100%) after normalizing the samples referring to the housekeeping gene GAPDH. The analysis of real-time PCR C_T values revealed that the expression of GAPDH was conserved between iIEL cultured in medium supplemented with PHA-P and iIEL cultured with PHA-P + Stx1 (25.9 ± 3.8 versus 25.5 ± 2.1, respectively, after 4 h of culture; 24.8 ± 2.5 versus 24.8 ± 1.7 after 6 h; 25.7 ± 2.1 versus 25.5 ± 0.9 after 8 h; 22.4 ± 0.7 versus 22.8 ± 0.8 after 24 h; mean C_T ± S.D. of five to nine experiments).

2.5. Bovine granulocyte migration assay

Derived from Galligan and Coomber (2000), an original method using dyed-cells was established to count migrating granulocytes by flow cytometry. Granulocytes were separated from PBMC by density gradient centrifugation (Menge, 2003a) with Ficoll-Paque® Plus (Amersham Biosciences Europe GmbH). Two millilitres from the pellet were diluted 1:1 with PBS-EDTA and erythrocytes were lysed with 27 ml of distilled water. After 50 s, 3 ml of 10-fold concentrated PBS-EDTA and 16 ml of RPMI 1640 were added to restore the osmolarity. Granulocytes were washed with PBS and resuspended in medium containing RPMI 1640, 10% FCS, and 3 μM β-mercaptoethanol. The purity was checked by flow cytometry. Preparations with a purity greater than 90% granulocytes were pooled. After culturing PHA-P-stimulated iIEL for 8 or 18 h in the absence or presence of Stx1, supernatants (agonists) were obtained by centrifugation at 10,000 × *g* for 10 min and transferred into 12-well plates (i.e. lower compartment of the migration chamber). Culture supernatant of PBMC stimulated with 5 μg/ml concanavalin A and 200 IU/ml of rhuIL-2 was used as a positive control for induced migration. The upper compartments of all migration chambers (12 mm-Transwell® clear culture inserts, 3 μm-pores, Corning Costar, Germany) were placed in the wells and each filled with medium containing 5×10^5 granulocytes. After a 2 h-incubation at 37 °C, granulocytes of both compartments were harvested.

BL-3 cells (ECACC 86062401), a bovine B lymphoma cell line, were incubated with 1.5 μg/ml of 3-3′-dioctadecyloxacarbocyanine perchlorate (DiO, Molecular Probes, Leiden, The Netherlands) for 1 h at 37 °C in the dark under constant agitation (100 rpm). DiO-BL-3 cells were centrifuged (202 × *g*, 7 min at RT), washed twice in PBS and resuspended at the density of 1×10^6 ml^{-1}. The cells were fixed by diluting 1:1 in paraformaldehyde 4% (PFA in PBS, 2% f.c.), and incubated 30 min in the dark at 20 °C. Until the end of the procedure, BL-3 cells were protected from light and stored on ice. Finally, DiO-BL3 cells were centrifuged (202 × *g*, 7 min at 4 °C), washed and resuspended in PBS. The morphology and the fluorescence were checked by flow cytometry.

Granulocytes were counted with DiO-BL-3 cells as counting particles. DiO-BL-3 cells (1.5×10^4) were added to each of the 500 μl volumes derived from the different compartments of the migration chambers. A document was created with the acquisition-software Cell Quest Pro (Becton-Dickinson) to discriminate the different populations of cells. Acquisition was stopped after 1,000 DiO-BL-3 cells were counted. The spontaneous migration of granulocytes, evaluated as the migration of granulocytes in chambers in which the lower compartment was filled with non-conditioned medium (freshly prepared IEL-medium not having been in contact with cells), was used as a reference (100%).

2.6. Quantitation of intracellular cytokines by flow cytometry

Ileal IEL were resuspended in 2.5 ml of IEL-medium at the density of 2×10^6 ml^{-1}. For stimulation, IEL-medium was supplemented with 50 ng/ml of phorbol-12-myristate-13-acetate (PMA, Phagoburst® Kit, ORPEGEN Pharma, Heidelberg, Germany) plus 1 μg/ml of ionomycin (Sigma–Aldrich). Ileal IEL were then incubated with or without purified Stx1 (200 CD_{50}/ml) for 6 or 24 h at 37 °C in 5% CO_2 and 95% humidity. Brefeldin A (10 μg/ml, Sigma–Aldrich) was added 1 or 8 h, respectively, after the incubation began.

At the end of the incubation period, iIEL were resuspended, centrifuged (202 × *g*, 7 min, and 20 °C), washed once with PBS-EDTA, and once with PBS. The staining procedure was performed in 96-well plates (Nunc, Wiesbaden, Germany) with 4×10^5 cells per well. Briefly, cells were centrifuged (150 × *g*, 7 min, 4 °C), fixed in PFA 1% (Merck, Darmstadt, Germany) for 10 min in the dark on ice, washed once and permeabilized 5 min on ice in PBS supplemented with 0.1% saponin (Merck) and 0.1% sodium azide (Merck). The detection of cytokines was performed by incubation for 20 min in the dark on ice with antibodies against bovine IFN-γ (clone CC302), bovine IL-4 (CC303), and human TGF-β (TB21; all antibodies purchased from Serotec, Germany, diluted in PBS supplemented with 1% bovine serum

albumin, 0.1% saponin, and 0.1% sodium azide) or with isotype controls (irrelevant antibodies directed against *C. perfringens* phospholipase C and β2-toxin). The homology between the mature forms of human and bovine TGF-β proteins (Alevizopoulos and Mermod, 1997; Van Obberghen-Schilling et al., 1987) allowed us to use an anti-human TGF-β antibody. A goat anti-mouse IgG (H + L) antibody conjugated with FITC (3.4 μg/ml, Dianova) was used as a secondary antibody. Cells were analysed with a FACSCalibur™ flow cytometer. Five thousand events were acquired for each sample. Gates were defined according to the negative control (PBS) and the respective isotype controls defining less than 2% of the cells as positive.

2.7. Experiments addressing the impact of the enzymatic activity of Stx1 and the induction of apoptosis

For stimulation, iIEL (2.5×10^7) were cultivated in 9 ml of IEL-medium supplemented with 2.5 μg/ml of PHA-P for 6 or 20 h at 37 °C and 5% CO_2 in the presence of either (a) actinomycin D (2 μg/ml, Sigma–Aldrich), (b) purified Stx1 (200 CD_{50}/ml), (c) purified rStxB1 subunit (10 μg/ml), (d) anti-CD77 antibody (clone 38.13, 50 μg/ml; for the functional assays kindly provided by J. Wiels [Institut Gustave Roussy, Villejuif, France]) pre-incubated 1 h at 37 °C with a goat anti-rat IgM (10 μg/ml, Dianova), (e) LPS (1 ng/ml, Sigma–Aldrich), or (f) 10 μg/ml of Brefeldin A and 200 CD_{50}/ml of Stx1. Ileal IEL were also incubated with the corresponding controls (Table 2). The recombinant rStxB1 subunit was prepared from the *E. coli* DH5α [pSU108] strain as already described (Stamm et al., 2002). Ramos cells were used as a positive control for Stx1- and rStxB1-induced apoptosis. Ramos cells, a kind gift of M. Oppermann (Georg-August University, Göttingen, Germany), were cultured in 2.5 ml of medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS) at the density of 6×10^5 ml^{-1} in the conditions mentioned in Table 2.

After the incubation, iIEL and Ramos cells were centrifuged for 5 min at 350 × *g* at 20 °C, and washed once in PBS. RNA was isolated only from iIEL (2×10^7 cells) using the QIAGEN RNeasy® Mini Kit. Reverse transcription and real-time-RT-PCR were performed as described above to investigate the IL-4 mRNA production by iIEL.

In order to evaluate phosphatidylserine surface expression and membrane integrity, iIEL or Ramos

Table 2
Percentages of apoptotic cells in cultures of Ramos cells and bovine iIEL

Cell type and condition of incubation	6 h incubation		20 h incubation	
	Early apoptotic	Late apoptotic/necrotic	Early apoptotic	Late apoptotic/necrotic
Ramos cells				
Medium	n.t.	n.t.	17.9 ± 8.7	3.3 ± 1.4
Actinomycin D (2 μg/ml)	n.t.	n.t.	77.2 ± 2.5	15.8 ± 2.0
Stx1 (200 CD_{50}/ml)	n.t.	n.t.	78.8 ± 4.1	14.5 ± 4.0
Stx1 + anti-StxB1 Mab (1.5 μg/ml)	n.t.	n.t.	21.2 ± 9.1	3.5 ± 1.2
rStxB1 (10 μg/ml)	n.t.	n.t.	52.7 ± 7.2	8.2 ± 4.3
Anti-StxB1 Mab (1.5 μg/ml)	n.t.	n.t.	20.2 ± 8.6	4.0 ± 2.0
Gb_3/CD77 cross-linking[a]	n.t.	n.t.	17.6 ± 9.2	5.2 ± 3.1
Brefeldin A (10 μg/ml)	n.t.	n.t.	34.0 ± 15.0	11.0 ± 6.1
Brefeldin A + Stx1	n.t.	n.t.	43.3 ± 18.0	13.1 ± 7.7
Ileal IEL				
Medium	29.7 ± 11.4	35.5 ± 5.4	34.4	16.2
Actinomycin D (2 μg/ml)	30.2 ± 10.4	41.0 ± 4.1	49.9	44.6
Stx1 (200 CD_{50}/ml)	26.3 ± 9.4	40.9 ± 5.8	32.8	17.9
Stx1 + anti-StxB1 Mab (1.5 μg/ml)	29.1 ± 10.3	39.2 ± 7.4	32.0	17.9
rStxB1 (10 μg/ml)	26.8 ± 12.5	37.6 ± 5.9	32.8	19.0
Gb_3/CD77 cross-linking[a]	27.1 ± 8.9	38.3 ± 5.7	35.8	17.8
LPS (1 ng/ml)	30.1 ± 9.1	36.3 ± 4.9	35.4	16.3
Brefeldin A (10 μg/ml)	27.4 ± 6.4	36.4 ± 6.3	39.5	30.8
Brefeldin A + Stx1	25.2 ± 7.6	37.5 ± 7.7	39.7	29.4

Data presented are arithmetic mean ± S.D. of three independent experiments (one experiment for iIEL incubated 20 h) with duplicate determinations each.

[a] For the cross-linking of Gb_3/CD77, the cells were treated with a rat anti-CD77 MAb (50 μg/ml) pre-incubated with a goat anti-rat IgM (10 μg/ml).

cells were transferred in 96 U-shaped plates (2×10^5 per well), centrifuged 3 min at $400 \times g$, 20 °C and stained for 20 min at 25 °C in the dark with 5 μl of Annexin-V-PE (Coulter, Germany, diluted 1:10 in Ca^{2+}-containing Annexin-V binding buffer) and 100 μl of Annexin-V binding buffer containing 1 μg/ml of 7-AAD (Sigma–Aldrich). Before analysis, cells were centrifuged, washed once in Annexin-V binding buffer and transferred into 200 μl PBS. Single positive (Annexin-V^+) cells were considered as early apoptotic while double-positive (Annexin-V^+, 7-AAD^+) cells were considered as late apoptotic/necrotic. Five thousand events were acquired by a FACSCalibur™ flow cytometer.

2.8. Statistical analysis

Data (Table 2 and Fig. 3 excepted) were analysed statistically by Student's *t*-test for normal distributed data and by Mann-Whitney Rank Sum test for non-normal distributed data by using the SigmaStat (version 2.03) software (SPSS Inc., USA). Results were evaluated as follows: highly significant ($^{***}P \leq 0.001$), significant ($^{**}P \leq 0.01$), weakly significant ($^{*}P \leq 0.05$). Results were considered not significant if $P > 0.05$; in this case the results of the statistical analyses were omitted from the figures.

3. Results

3.1. Immunophenotyping of bovine iIEL

Isolated cells displayed a phenotype as previously described for bovine iIEL (Menge et al., 2004a, b; Wyatt et al., 1999): the majority of cells were $CD8^+$ ($51.4 \pm 11.7\%$; mean ± S.D.), fewer were $CD4^+$ ($19.4 \pm 11.6\%$), and only small numbers expressed

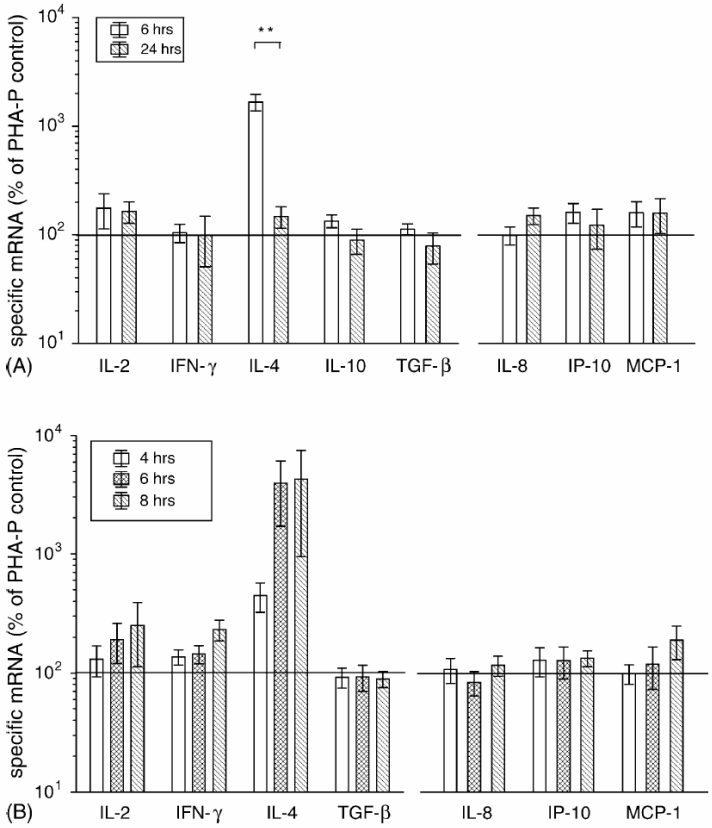

Fig. 1. Relative amounts of gene transcripts harboured by bovine iIEL upon cultivation in the presence of purified Stx1. PHA-P-stimulated (2.5 μg/ml) cells were incubated 6 and 24 h (A), or 4, 6, and 8 h (B) with Stx1 (200 CD_{50}/ml as determined on Vero cells). Subsequently, mRNA was reversely transcribed and quantified by real-time PCR. The transcription of the housekeeping gene GAPDH was used for normalization of the samples. Cells incubated with medium and PHA-P were used as a control (=100% as visualized by the black line). Data represent the arithmetic mean ± S.E.M. of the results obtained with iIEL preparations from five (A) and six (B) different animals. Significant cytokine production was determined by Mann–Whitney rank sum test and levels of significance were depicted if $^{*}P \leq 0.05$. Asterisk above the bracket indicates a significant difference between embraced bars, asterisk above bar indicates significant difference to the medium + PHA-P control.

the B cell marker CD21 (6.4 ± 4.0%); 25.1 ± 4.2% of cells expressed the γδ TCR and 8.1 ± 1.8% expressed Gb_3/CD77, the Stx1 receptor (Menge et al., 1999; Stamm et al., 2002); 61.1 ± 10.4% of cells also expressed CD25, pointing to an activated state of the majority of iIEL *in vivo*.

3.2. Chemokine gene transcripts in iIEL cultured in the absence or presence of Stx1

Bovine iIEL were capable of producing mRNAs for IL-8, IP-10, and MCP-1. Transcripts of the respective genes were detectable by RT-PCR in freshly isolated iIEL (data not shown) as well as in mitogenically stimulated cells cultured for up to 24 h. However, supplementation of PHA-P stimulated iIEL cultures with 200 CD_{50}/ml of purified Stx1 (as quantified on Vero cells) did not significantly alter the relative amounts of specific transcripts for these chemokines within 6 and 24 h of cultivation ($P > 0.05$, Fig. 1A). A more immediate response to the toxin could be ruled out by reducing the cultivation period to 4 h in a separate set of experiments with IEL preparations from six additional animals (Fig. 1B).

3.3. Release of chemoattractants by iIEL cultured in the absence or presence of Stx1

The inability of Stx1 to influence the production of granulocyte chemoattractant factors by iIEL was also confirmed on the protein level (Fig. 2). A culture-supernatant of stimulated PBMC, used as a positive control, efficiently induced the attraction of granulocytes into the lower chamber of the migration system. The release of chemoattractants into the culture supernatants was lower by iIEL compared to the PBMC control. Nevertheless, the migratory activity of granulocytes towards iIEL supernatants significantly exceeded that towards non-conditioned medium ($P \leq 0.01$), indicating that iIEL stimulated by PHA-P for 8 and 18 h did release chemoattractant factors. However, significant differences in the migration rate towards culture supernatants derived from iIEL incubated in the presence or absence of Stx1 (200 CD_{50}/ml) could not be detected ($P > 0.05$).

3.4. Effect of Stx1 on the relative number of cytokine gene transcripts in iIEL

Semi-quantitative RT-PCR revealed that iIEL *ex vivo* also harbour transcripts for the T_H-prototype cytokines IL-2, IFN-γ, IL-4, IL-10 and TGF-β (data not shown).

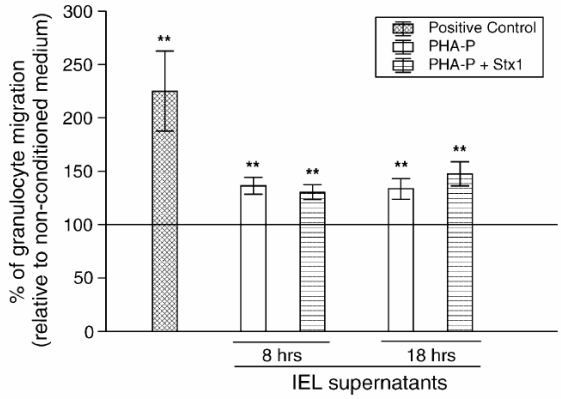

Fig. 2. Migratory activity of bovine granulocytes towards supernatants obtained from bovine iIEL cultures incubated in the absence or presence of Stx1. Bovine granulocytes were allowed to migrate for 2 h at 37 °C towards the lower compartment containing agonists (iIEL supernatants; stimulated PBMC supernatant as positive control), then harvested and counted. The results are expressed relative to the spontaneous granulocyte migration occurring in migration chambers in which the lower compartment was filled with non-conditioned (i.e. fresh) medium (=100% as visualized by the black line). Data represent mean ± S.E.M. of the values from migration assays with six independently obtained iIEL culture supernatants. Statistical analysis revealed that the induced migration towards PBMC and iIEL conditioned supernatants significantly exceeded the spontaneous migration towards the non-conditioned medium ($^{**}P \leq 0.01$).

In order to assess whether Stx1 is able to alter the cytokine gene transcription, iIEL were PHA-P stimulated *in vitro* and simultaneously treated with 200 CD_{50}/ml of Stx1 for up to 24 h. Real-time PCR analysis showed that Stx1 had no influence on the iIELs content of mRNA specific for both, the pro-inflammatory T_H1 cytokines IL-2 and IFN-γ and for the anti-inflammatory T_H3 cytokine TGF-β after all incubation times investigated (Fig. 1A and B). Stx1 likewise did not affect the relative amounts of *il-10* transcripts (Fig. 1A), even though they were detectable in small numbers (data not shown, C_T values 39–40). In contrast, Stx1-treatment led to a significant increase in the relative amounts of mRNA specific for the T_H2-type cytokine IL-4 after 6 h of incubation ($P \leq 0.05$, Fig. 1A). Stx1 specifically increased the number of *il-4* transcripts as early as after 4 h of incubation (4.5-fold the medium control) and this effect was dramatically amplified in the next hours to reach a maximum after 8 h of incubation (42-fold the medium control, Fig. 1B). The effect could be efficiently neutralized by pre-incubation of Stx1 with an anti-StxB1 antibody (Figs. 3 and 5). Although iIEL preparations from several animals differed in their sensitivity to Stx1, even minute concentrations of Stx1 ($P \leq 0.05$; 22 CD_{50}/ml) turned out to be sufficient to induce a prominent increase in

IL-4 specific mRNA after 6 h of incubation (Fig. 3). Nevertheless, this effect was no longer detectable after the iIEL were incubated for 24 h in the presence of 200 CD_{50}/ml Stx1 ($P > 0.05$; Fig. 1A).

Bovine IEL were particularly sensitive to an Stx1-induced increase in IL-4 mRNA since bovine PBMC incubated for 6 h with 33 CD_{50}/ml of Stx1 did not exhibit any significant increase in *il-4* transcripts (data not shown).

3.5. Cytokine synthesis by iIEL in the absence or presence of Stx1

The subsequent production of selected cytokines by unstimulated and stimulated iIEL was quantified by flow cytometry on a single cell level. Since a first series of experiments with iIEL preparations from three animals had revealed that stimulation of the cells with PHA-P failed to detectably facilitate the production of the cytokines under investigation (data not shown), the following experiments were carried out using PMA and ionomycin as stimulating agents. Ileal IEL were cultured 1 and 8 h in the absence or presence of 200 CD_{50}/ml Stx1 and then incubated in the presence of brefeldin A for further 5 and 16 h, respectively, in order to prevent the secretion of newly synthesized protein. The Fig. 4 shows that IL-4 was produced by only a small portion of cells (approximately 3%) after both, short (6 h) and long-term (24 h) incubation and production was not augmented by stimulation of the cells with PMA and ionomycin. Stx1 did not affect the percentage of iIEL expressing IL-4 protein under either condition ($P > 0.05$). Similarly, despite a slight increase after 24 h of cultivation, the percentage of TGF-β producing iIEL was low (approximately 8%) and was not affected

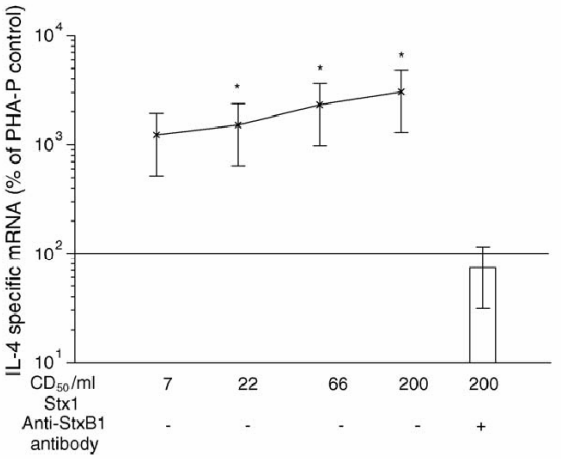

Fig. 3. Effect of purified Stx1 on the amounts of IL-4-specific mRNA in bovine iIEL cultures. PHA-P-stimulated (2.5 μg/ml) cells were incubated for 6 h with different concentrations of Stx1 (7, 22, 66, and 200 CD_{50}/ml as determined on Vero cells) or with 200 CD_{50}/ml of Stx1 and 1.5 μg/ml of anti-StxB1 antibody 13C4. Subsequently, mRNA was reversely transcribed and quantified by real-time PCR. The transcription of the housekeeping gene GAPDH was used for normalization of the samples. Cells incubated with medium and PHA-P were used as a control (=100% as visualized by the black line). Data represent arithmetic mean ± S.E.M. of the results obtained from three different animals. Significant cytokine production was determined by Mann–Whitney rank sum test and levels of significance were depicted if $^*P \leq 0.05$.

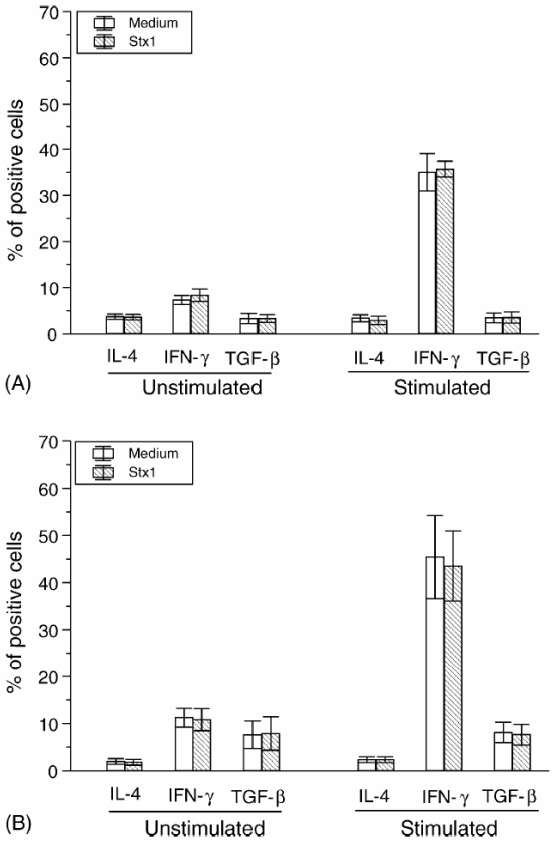

Fig. 4. Percentage of bovine iIEL capable of synthesizing certain cytokines *in vitro* upon incubation in the absence or presence of Stx1 (200 CD_{50}/ml as determined on Vero cells). Cells were left unstimulated or stimulated with PMA (50 ng/ml) plus ionomycin (10 μg/ml) for 6 h (A) and 24 h (B). Brefeldin A (10 μg/ml) was added 1 h (A) and 8 h (B), respectively, after the beginning of the incubation. At the end of the incubation period, cells were fixed, permeabilized, and intracellular cytokines were immunodecorated. Subsequent FACS analysis was performed by acquisition of 5,000 cells for each sample. Gates were defined according to the negative and isotype controls (irrelevant antibodies) defining less than 2% of the cells as positive. Data are arithmetic mean ± S.E.M. of five independent experiments with duplicate determinations. No statistical significant difference was found between cells incubated in the absence or presence of Stx1.

by the toxin ($P > 0.05$). Even though Stx1 is known as a potent protein synthesis inhibitor, iIEL cultures stimulated with PMA and ionomycin and supplemented with 200 CD_{50}/ml of Stx1 still retained their ability to produce IFN-γ protein (approximately 44% after 24 h). Moreover, the toxin had no detectable effect on the percentages of iIEL that synthesized this T_H1-type cytokine within 6 or 24 h ($P > 0.05$). While stimulation of IFN-γ production by PMA/ionomycin resulted in a prominent increase in the mean fluorescence intensity of the cells indicating an increased average cellular content of the cytokine, significant differences in this parameter between cells cultured in the absence and presence of Stx1 could not be observed for either cytokine (data not shown).

3.6. *Stx1-induced increase in il-4 transcripts is apoptosis-independent yet it requires the enzymatic activity of Stx1*

As the induction of apoptosis by other ribosome inactivating toxins is accompanied by an increase in the expression of IL-4 (Stein et al., 2000), we investigated whether the observed increase in IL-4 mRNA in bovine iIEL could be a side effect of Stx1-induced apoptosis. Ramos cells, used as a positive control system (Tétaud et al., 2003), expressed Gb_3/CD77 (data not shown) and addition of Stx1 (200 CD_{50}/ml) or actinomycin D comparably induced apoptosis after 20 h as detected by phosphatidylserine exposure on the cell surface (Table 2). Anti-StxB1 antibody neutralized this effect by reducing the percentage of apoptotic cells to a level comparable to the medium control. Apoptosis by Stx1 reportedly may also originate, independently of the enzymatic activity of the Stx1 holotoxin, from the triggering of the Stx receptor Gb_3/CD77. Indeed, an increase in the percentage of apoptotic cells could be induced in Ramos cells by addition of the B-subunit of Stx1 (rStxB1; 10 μg/ml) lacking the enzymatic activity of the holotoxin. In turn, the pro-apoptotic effect of the holotoxin was partially prevented by the Golgi inhibitor, brefeldin A, suggesting that both, triggering of the receptor by rStxB1-binding and holotoxin uptake contribute to the induction of apoptosis in Ramos cells. In contrast to Tétaud et al. (2003), however, no apoptosis was induced by cross-linking Gb_3/CD77 with anti-CD77 38.13 and anti-rat IgM.

Upon staining with Annexin-V and 7-AAD, 41.9% of bovine iIEL appeared apoptotic and/or necrotic after the isolation procedure (data not shown). Incubation of the cells with PHA-P increased the portion of apoptotic cells to 65.2% (Table 2). Nevertheless, the RNA-synthesis inhibitor actinomycin D, used as a positive control, was still capable of inducing further cellular death within 20 h of cultivation. In contrast, neither supplementation of the iIEL cultures with Stx1 holotoxin nor with rStxB1 subunit accelerated the onset of apoptosis in iIEL cultures within 6 and 20 h of incubation.

Actinomycin D treatment also induced a decrease in the overall mRNA quantity (data not shown), and even significantly decreased the relative amounts of *il-4* transcripts in PHA-P stimulated bovine iIEL after 6 h of incubation ($P \leq 0.01$; Fig. 5). This further supports the notion that the Stx1-induced increase in IL-4 specific mRNA does not result from an onset of apoptosis.

The effect of the holotoxin compulsory relied on its enzymatic activity, as rStxB1 alone failed to alter the relative amount of *il-4* transcripts. Pre-incubation of iIEL with brefeldin A almost completely blocked the effect of the holotoxin further pinpointing the importance of the uptake of the holotoxin into the cells. Neutralization of Stx1 by anti-StxB1 antibody and the failure of the endotoxin LPS to cause comparable effects finally confirmed that the effect was specific for Stx1 (Fig. 5).

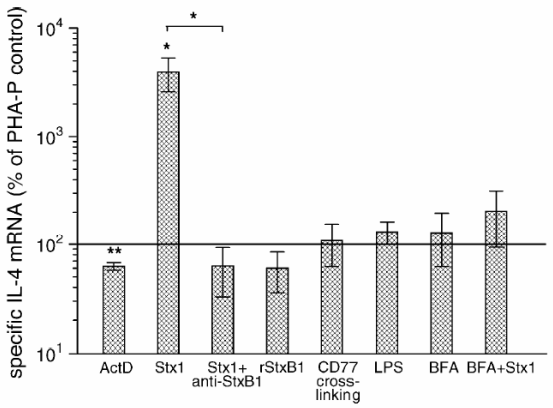

Fig. 5. Effect of different agents on the amounts of IL-4 specific mRNA in bovine iIEL cultures. PHA-P stimulated (2.5 μg/ml) cells were incubated for 6 h in the presence of different agents (for concentrations see Section 2). Subsequently, mRNA was reversely transcribed and quantified by real-time PCR. The transcription of the housekeeping gene GAPDH was used for normalization of the samples. Cells incubated with medium and PHA-P were used as a control (=100% as visualized by the black line). Data presented are arithmetic mean ± S.E.M. from experiments with three iIEL preparations obtained from different animals. ActD: actinomycin D; CD77 cross-linking: incubation with anti-CD77 and anti-rat IgM; LPS: lipopolysaccharide, BFA: brefeldin A. Significant differences to the medium + PHA-P control were determined by Student's *t*-test and levels of significance were depicted if $^{**}P \leq 0.01$ or $^{*}P \leq 0.05$.

4. Discussion

Without causing cellular death, Shiga toxin 1 (Stx1) inhibits the activation of bovine peripheral (Menge et al., 1999, 2003b) and ileal intraepithelial lymphocytes (iIEL) *in vitro* (Menge et al., 2004a) and *in vivo* (Menge et al., 2004b), but the consequences *in situ* are yet to be elucidated. Since Stxs can increase the cytokine production of several cell types (Harrison et al., 2005, 2004; Ramegowda and Tesh, 1996; Tesh et al., 1994; Thorpe et al., 1999, 2001), we speculated that, in cattle, Stx1 has the potential to modulate the lymphocytes' expression of soluble factors that participate in the mucosal immune regulatory network. In support of this hypothesis, the present study revealed that concentrations of Stx1 in the picogram range (Menge et al., 1999) led to an immediate increase in the cellular content of IL-4 specific mRNA. Although peripheral blood mononuclear cells (PBMC) and iIEL transcribed a number of different cytokine and chemokine genes, the effect of Stx1 at low concentrations was considerably specific in that it was restricted to *il-4* and occurred in iIEL only.

Our data provide the first evidence that bovine iIEL produce both chemokine mRNAs and proteins, indicating that these cells actively contribute to the recruitment of immune cells to the bovine intestinal mucosa. However, neither the relative amounts of certain chemokine mRNA transcripts nor the total release of granulocyte chemoattractants were altered by incubation with Stx1. These results supplement observations that Stx1 fails to stimulate bovine intestinal epithelial cells to release granulocyte chemoattractants *in vitro* (Stamm et al., 2006) and to induce intestinal inflammation *in vivo* (Stevens et al., 2002). By contrast, Stx1 strongly induces several chemokines in human epithelial cells (Thorpe et al., 1999, 2001). The subsequent recruitment of neutrophils is part of a severe inflammation during human STEC infections (Hurley et al., 2001). Although comparative experiments with human IEL are pending, Stx1 does not seem to act as a proinflammatory factor in cattle as it does in humans (Proulx et al., 2001). These findings add to our understanding why STEC are able to colonize their bovine host without disturbing the intestinal homeostasis.

In contrast to the other cytokines investigated, Stx1 specifically increased the relative amounts of *il-4* transcripts as early as after 4 h of incubation thereby substantiating preliminary data (Menge et al., 2004a). When the amounts of different transcripts were calculated relative to the amount of transcripts specific for *gapdh* in each sample (data not shown), it became apparent that, after incubation with Stx1, *il-4* transcripts became the most abundant cytokine mRNA species. The amount of IL-4 mRNA even exceeded 10-fold the amount of TGF-β mRNA, the latter of which dominated in PHA-P stimulated iIEL cultures without Stx1 treatment. IL-4 mRNA continued to be the major mRNA species until 8 h of incubation and decreased to baseline levels only after overnight culture. Since Grogan et al. (2001) found that appropriately stimulated murine T cells transcribe *il-4* within hours, the Stx1-induced increase in IL-4 mRNA may result from an elevated transcriptional activity. On the other hand, Stx1 can induce the accumulation of certain cytokine/chemokine mRNA transcripts within 4–8 h by increasing the half-life of the mRNA (Harrison et al., 2004; Thorpe et al., 2001). As the translation-dependent mRNA degradation occurs in a transcript-specific fashion in mammalian cells (Jacobson and Peltz, 1996; Ross, 1995), it remains to be determined whether Stx1 increases the amount of IL-4 mRNA in bovine iIEL by inducing *de novo* mRNA synthesis or by delaying the degradation of pre-existing transcripts. In both instances, the effect of Stx1 appears to be restricted to *il-4* full-length transcripts which were exclusively quantified with the primers used for the real-time PCR (Table 1). Waldvogel et al. (2004) presumed a role of mRNA splicing variants in the regulation of IL-4 production. We also detected IL-4δ2 splice variants in bovine iIEL but semi-quantitative analysis did not reveal any differences between iIEL incubated with or without Stx1 (data not shown).

The inhibitory effect of Stx1 on different subsets of bovine PBMC (Menge et al., 1999) correlates with the varying expression of the Stx1-receptor Gb_3/CD77 on the subpopulations (Menge et al., 2003b, 2001). We previously showed that some cytokines implicated in the pathogenesis of STEC infections in humans were neither able to mimic nor to overcome the effect of Stx1 (Menge et al., 2003b). The results led us to conclude that the inhibitory activity of Stx1 primarily results from a direct effect on Gb_3/CD77$^+$ bovine lymphocytes rather than from altering intercellular signaling events (Menge et al., 2003b). Bovine iIEL consist of a number of different lymphocyte subpopulations each of which contains cells in different states of activation (Menge et al., 2004a). Similar to PBMC, Stx1 predominantly affects the Gb_3/CD77$^+$ cells within the different iIEL subpopulations *in vitro* (Menge et al., 2004a). It is tempting to assume, therefore, that the increase in IL-4 mRNA did not result from altered cellular interactions but occurred in iIEL that were directly affected by the toxin.

Although IL-4 is a cytokine mainly produced by the T_H2 subset of $CD4^+$ T cells under physiological conditions, the cellular source of the Stx1-induced IL-4 mRNA remains to be determined. Stx-receptors of the Gb_3/CD77 type are predominantly expressed by bovine $CD8\alpha^+$ T iIEL *ex vivo*, but the expression of Gb_3/CD77 by bovine $CD4^+$ T iIEL can be induced *in vitro* and these cells are equally sensitive to Stx1 then (Menge et al., 2004a). The proportions of $CD4^+$ T cells varied in our iIEL preparations, but the numbers of these cells did not correlate with the magnitude of the Stx1's effect (data not shown). It cannot be excluded that Stx1 induced the transcription of IL-4 in a CD4-negative subset of iIEL. In fact, mistletoe lectins, type II ribosome-inhibiting proteins like Stx, are able to stimulate the expression of IL-4 in human $CD4^+$ and $CD8^+$ T cells (Stein et al., 2000). This effect is strongly associated with the expression of apoptotic markers (Stein et al., 2000). Actinomycin D but not Stx1 treatment accelerated apoptosis in the bovine iIEL cultures. Elevated levels of IL-4 mRNA are not a side effect of Stx1-induced apoptosis, therefore. Whatever the cellular source of Stx1-induced IL-4 mRNA might be, bovine iIEL responding to Stx1 *in situ* also may not undergo apoptosis but remain integrated in the mucosal network and participate in the interaction between STEC and the bovine mucosa.

The rStxB1 subunit induced apoptosis in Ramos cells, but did not affect the number of *il-4* transcripts in bovine iIEL. Similar to findings in human epithelial cells (Yamasaki et al., 1999, 2004), the induction of cytokine mRNA in bovine iIEL required the enzymatic activity of Stx1. The cellular level of *il-4* transcripts was sensitive to very low concentrations of the toxin (22 CD_{50}/ml). Nevertheless, bovine iIEL retained their ability to produce IFN-γ and to respond to PMA/ionomycin stimulation with a further increase in the synthesis of this cytokine even in the presence of Stx1 at 200 CD_{50}/ml. Bitzan et al. (1998) also observed that Stx1 increased the number of some mRNA species after 12–24 h in bovine endothelial cells at toxin concentrations below those required for a detectable inhibition of protein synthesis. At these concentrations, Stx even increase the production of cytokine proteins in human monocytes without significantly inhibiting the protein synthesis machinery (Ramegowda and Tesh, 1996; van Setten et al., 1996). Moreover, Stx1 induces a "ribotoxic stress response" (Smith et al., 2003; Thorpe et al., 2001), ultimately leading to the production of, e.g. IL-8 or TNF-α after translocation of NF-κB and AP-1 (Sakiri et al., 1998). The discrepancy between the significant increase in *il-4* transcripts in Stx1-treated bovine iIEL and the lack of a detectable increase in IL-4 protein synthesis apparently does not result from the abrogation of protein synthesis.

Bovine γδ T cells (Baldwin et al., 2002) and $CD4^+$ and $CD8^+$ T cells from bovine blood and mesenteric lymph nodes (Seder et al., 1992; Sopp and Howard, 2001) produce IFN-γ upon stimulation. However, PHA-P, a T-cell activator through CD2, was unable to induce a detectable IFN-γ protein production in bovine iIEL after 10 or 18 h of stimulation (data not shown). Protocols previously established by others (Enomoto et al., 1998; Paliogianni et al., 1996), used PMA, a protein kinase C activator, in combination with ionomycin, a Ca^{2+} ionophore, to stimulate lymphocytes for studies aimed at quantifying cytokines on a single cell level. PMA treatment decreases the Gb_3/CD77 expression on THP-1 cells (Ramegowda and Tesh, 1996), but stimulation of bovine iIEL with PMA/ionomycin does not abrogate the cells sensitivity for the activation-inhibiting effect of Stx1 (Menge et al., 2004a). We adapted our stimulation protocol and stimulation by PMA/ionomycin in fact resulted in significant production of IFN-γ. Nevertheless, Stx1 neither influenced the number of IFN-γ nor the number of IL-4 producing cells. Since different stimuli were used to activate the cells prior to mRNA and prior to protein quantitation the findings do not exclude the possibility that Stx1 stimulates the expression of *il-4* in bovine iIEL even down to the protein level under physiological conditions *in situ*.

A Stx1-induced increase of mRNA levels by 100-folds reportedly results in a much lower increase of the concentration of the corresponding protein (Thorpe et al., 2001). The detection method for intracellular IL-4 applied in the present study might not have been sensitive enough to detect distinct increases in the amount of IL-4. Notably, IL-4 producing cells only formed a minority of iIEL while a higher number of cells were capable of producing IFN-γ. The secretion of newly produced proteins was blocked in our experiments by brefeldin A only after at least 1 h of incubation with Stx1 in order to first allow Stx1 to enter the cytosol of the cells. It must be considered, therefore, that the iIEL initially secreted amounts of IFN-γ sufficient to rapidly and reciprocally down-regulate T_H2-cell activation (Rook et al., 2004) thereby hindering a potential Stx1-triggered IL-4 synthesis at the posttranscriptional level as described for other IFN-γ target proteins (Ben-Asouli et al., 2002; Matsukura et al., 2003; Mazumder and Fox, 1999). This may be particularly true for PMA/ionomycin-stimulated iIEL. While stimulation with ionomycin induces the transcriptional activity of

the IL-4 promoter (Paliogianni et al., 1996), stimulation by ionomycin plus PMA greatly enhances the production of IFN-γ but not that of IL-4 (Enomoto et al., 1998).

Most bovine STEC strains have an array of virulence determinants (Geue et al., 2002; Wieler et al., 1998) that allows them to interact with intestinal cells (Bérin et al., 2002). It is unlikely that not at least one of these determinants represents a danger signal that is recognized by the local immune system and generates an immune response (Simmons et al., 2001) terminating STEC colonization even in the bovine host. We hypothesized (Menge et al., 2004b) that STEC have evolved additional strategies that retard intestinal immune responses in cattle, permitting a commensal-like lifestyle (Smith et al., 2002). During bovine infections with *Mycobacterium bovis*, the extend of lung pathology positively correlates with the production of IL-4 by PBMC in response to mycobacterial antigen *in vitro* (Wedlock et al., 2003). However, IL-4 produced in the intestinal mucosa rather represents an anti-inflammatory factor in that it promotes TGF-β secreting T_H3 cell differentiation and oral administration of IL-4 enhances oral tolerance in mice (Inobe et al., 1998). IL-4 specifically down-regulates the responsiveness of human $CD8^+$ IEL without affecting the proliferation of peripheral $CD8^+$ T-cells (Ebert and Roberts, 1996) suggesting a very localized modulation of gut immune cells. As Stx1 is not directly cytolethal for bovine iIEL (Menge et al., 2004a), IL-4 represents an interesting candidate for a mediator implicated in the depletion of $CD8^+$ iIEL during experimental STEC infections in calves (Menge et al., 2004b). Further studies are strongly encouraged to reveal the consequences of the Stx1-induced increase of *il-4* transcripts for the synthesis of IL-4 protein and for the overall responsiveness of bovine iIEL. However, with respect to the prominent role of IL-4 in the intestinal homeostasis (Colgan et al., 1994; West et al., 1996) and in the persistence of some bacterial infections (Hernandez-Pando et al., 2004), it is tempting to assume even at the present state that induction of *il-4* transcription is one mechanism by which human pathogenic Stx-producing *E. coli* accomplish the enduring intestinal colonization of their reservoir host.

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6.11 "Bovine immune response to Shiga-toxigenic *Escherichia coli* O157:H7."

Hoffman, M.A., **C. Menge**, T.A. Casey, W. Laegreid, B.T. Bosworth and E.A. Dean-Nystrom*

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Bovine Immune Response to Shiga-Toxigenic *Escherichia coli* O157:H7[▽]

Mark A. Hoffman,[1,4] Christian Menge,[2] Thomas A. Casey,[1] William Laegreid,[3] Brad T. Bosworth,[1,5] and Evelyn A. Dean-Nystrom[1]*

Enteric Diseases and Food Safety Research, National Disease Center, USDA, Agriculture Research Service, Ames, Iowa 50010[1]; Institute for Hygiene and Infectious Diseases of Animals, Justus Liebig University, D-35392 Giessen, Germany[2]; U.S. Meat Animal Research Center, USDA, Agriculture Research Service, Clay Center, Nebraska 68933[3]; Cemer Corporation, Kansas City, Missouri 64117[4]; and Novartis Animal Health US, Greensboro, North Carolina 27408[5]

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Although cattle develop humoral immune responses to Shiga-toxigenic (Stx$^+$) *Escherichia coli* O157:H7, infections often result in long-term shedding of these human pathogenic bacteria. The objective of this study was to compare humoral and cellular immune responses to Stx$^+$ and Stx$^-$ *E. coli* O157:H7. Three groups of calves were inoculated intrarumenally, twice in a 3-week interval, with different strains of *E. coli*: a Stx2-producing *E. coli* O157:H7 strain (Stx2$^+$O157), a Shiga toxin-negative *E. coli* O157:H7 strain (Stx$^-$O157), or a nonpathogenic *E. coli* strain (control). Fecal shedding of Stx2$^+$O157 was significantly higher than that of Stx$^-$O157 or the control. Three weeks after the second inoculation, all calves were challenged with Stx2$^+$O157. Following the challenge, levels of fecal shedding of Stx2$^+$O157 were similar in all three groups. Both groups inoculated with an O157 strain developed antibodies to O157 LPS. Calves initially inoculated with Stx$^-$O157, but not those inoculated with Stx2$^+$O157, developed statistically significant lymphoproliferative responses to heat-killed Stx2$^+$O157. These results provide evidence that infections with STEC can suppress the development of specific cellular immune responses in cattle, a finding that will need to be addressed in designing vaccines against *E. coli* O157:H7 infections in cattle.

Enterohemorrhagic *Escherichia coli* (EHEC) is a common cause of hemorrhagic colitis (HC) in very young, elderly, and immunocompromised humans (16, 30). In the United States, the EHEC serotype most often associated with bloody diarrhea is O157:H7, but non-O157 EHEC serotypes have caused significant outbreaks in the United States and abroad (30, 35). Up to 10% of patients with EHEC-associated HC develop hemolytic uremic syndrome, with resulting renal and neurological damage and, occasionally, death (21). All EHEC strains produce one or more cytotoxins called Shiga toxins (Stx1 and Stx2) (27), which target endothelial cells, are believed to mediate much of the tissue damage during HC and hemolytic uremic syndrome (29, 37), and can influence the duration of Stx-producing *E. coli* (STEC) shedding by ruminants (7).

Ruminants are important sources of EHEC O157:H7 strains because they frequently shed STEC in their feces (26). Single STEC O157:H7 clones can be repeatedly isolated from a herd (6, 14), implying that these clones persist within that herd. This could result from frequent transmission between and reinfection of different animals (2) or from a true persistence in single animals (3, 7, 8). Experimental infections of calves revealed that several bacterial factors involved in the "attaching and effacing" adherence of *E. coli* O157:H7 to epithelial cells (e.g., intimin [10], other products of the locus of enterocyte effacement [11], and *efa-1* [32]) promote bacterial colonization of the intestinal mucosa. However, recent studies failed to link the induction of attaching and effacing to the establishment of persistent infections in animals (4, 18).

Several lines of evidence support the hypothesis that Stx can suppress the bovine immune response. Stx1 targets bovine peripheral blood (23, 25, 31) and intraepithelial lymphocytes (IEL) (22) in vitro. Stx1 binds to Stx receptor-expressing (Gb_3-/CD77-positive) lymphocytes at early activation stages (31) and blocks the proliferation of distinct lymphocyte subpopulations (CD8α^+ T cells, B cells) in vitro (25). A significant portion of bovine CD8α^+ IEL expresses Stx-receptors in situ (22). Inoculation of ligated ileal loops in 2-week-old calves with Stx1-producing STEC reduces the number of CD8α^+ IEL within 12 h (24). Direct evidence for a suppression of immune cell functions in the course of STEC infections in cattle, as observed during experimental STEC infections in pigs (5), has been lacking. Although bovine lymphocytes are sensitive to minute concentrations of Stx1 and Stx2 (1, 12, 13, 25), Stxs do not completely abolish the development of specific immune responses in naturally and experimentally infected cattle. Antibodies against O157 lipopolysaccharide (LPS), Stx1, and Stx2 are frequently detected in bovine sera and mucosal secretions (colostra) (17, 28). Since Stx1 suppresses bovine lymphocytes in vitro without inducing cell death (25), we hypothesized that in vivo, Stxs primarily reduce or delay the host's cellular immune response, thereby generating an opportunity for STEC bacterial colonization. The objectives of the present study were to monitor the levels and durations of fecal shedding of Stx-producing (Stx$^+$) and Stx-negative (Stx$^-$) *E. coli* and to examine specific cellular and humoral immune responses in calves experimentally inoculated with either human pathogenic Stx2$^+$ or Stx$^-$ *E. coli* O157:H7 bacteria.

* Corresponding author. Mailing address: USDA, ARS, National Animal Disease Center, P.O. Box 70, Ames, IA 50010. Phone: (515) 663-7376. Fax: (515) 663-7458. E-mail: enystrom@nadc.ars.usda.gov.
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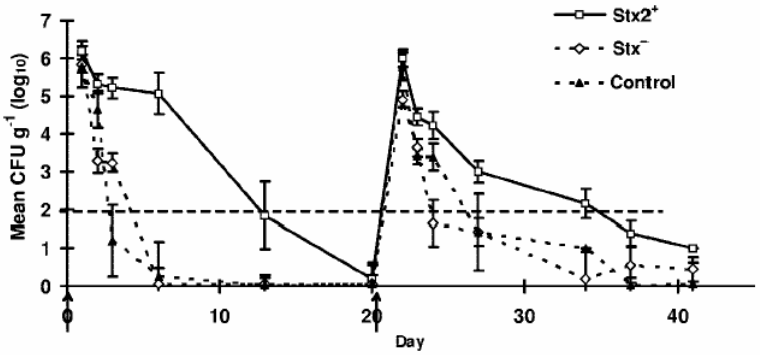

FIG. 1. Fecal shedding of *E. coli* by calves inoculated with different strains. Numbers of inoculum bacteria recovered from feces of calves inoculated with Stx2$^+$ *E. coli* O157:H7 strain 86-24 (Stx2$^+$), Stx$^-$ *E. coli* O157:H7 strain 87-23 (Stx$^-$), or control *E. coli* O43:H28 strain 123 (Control) are shown. Samples which were negative by direct culturing methods (<100 CFU g^{-1}) were tested by enrichment culturing. All values below the dashed line represent enrichment levels. Samples which were positive by enrichment analysis were given an arbitrary value of 10 CFU g^{-1}, and those which were negative were given a value of 1 CFU/g. Arrows indicate dates of inoculation. Data are presented as mean numbers (± standard errors of the means) of CFU g^{-1} for the indicated groups.

MATERIALS AND METHODS

Animals. Holstein calves 6 to 8 weeks old were purchased from local sources. Calves were allowed to acclimate to the diet and conditions at the National Animal Disease Center (NADC) for 3 weeks prior to oral inoculations. During infections, the calves were housed in BL2 containment barns and fed a diet of two-thirds grain and one-third hay. Animal care was in accordance with requirements of the NADC Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care. Calves were euthanized with sodium pentobarbital at the end of the study.

Inoculations. The strains used in this study were EHEC O157:H7 strain 86-24, a streptomycin-resistant mutant of an Stx2-producing strain isolated from an outbreak in Washington state (36); *E. coli* O157:H7 strain 87-23, a nalidixic-resistant mutant of a strain isolated from the same outbreak as 86-24 and lacking both Stx1 and Stx2 genes (19, 36); and, as a control strain, a nalidixic acid-resistant mutant of *E. coli* strain 123 (O43:H28), a porcine isolate which does not produce Stx and is not pathogenic in cattle (9). *Escherichia coli* cultures were prepared and stored at −80°C as previously described (9).

Calves (five calves/group) were inoculated intrarumenally, on day 0 and day 21, with approximately 10^{10} CFU of strain 86-24 (Stx2$^+$O157), 87-23 (Stx$^-$O157), or 123 (control) bacteria in 100 ml of trypticase soy broth (Becton Dickinson, Cockeysville, MD) as previously described (11, 33). Three weeks after the second inoculation (day 42), all calves were similarly inoculated with Stx2$^+$O157. This third inoculation of all calves with the same Stx$^+$O157 strain is referred to as the "challenge" to clearly differentiate it from the two initial inoculations of calves in different groups with different strains.

Bacteriology. Freshly isolated fecal samples (approximately 2 g) were diluted 1:10 and serially diluted in sterile phosphate-buffered saline. Sorbitol-negative STEC O157:H7 bacteria were quantitated on sorbitol-MacConkey agar (Oxoid, Basingstoke, United Kingdom) supplemented with streptomycin (200 μg per ml) or nalidixic acid (20 μg per ml) to select for strains 86-24 or 87-23, respectively. Randomly selected antibiotic-resistant, sorbitol-negative colonies were tested for O157 LPS antigen by slide agglutination (Oxoid). *E. coli* strain 123 bacteria were quantitated on MacConkey agar (Becton Dickinson, Cockeysville, MD) containing 20 μg per ml of nalidixic acid. Enrichment cultures were performed by adding up to 10 g of feces to trypticase soy broth containing 0.01% bile salts 3 (Difco, Detroit, MI) to a final volume of 100 ml. These cultures were incubated overnight, without shaking, at 37°C. Samples (100 μl) from enrichment cultures of feces that were negative by direct plating were incubated on sorbitol-MacConkey agar with the appropriate antibiotic. Enrichment plates were scored as either positive or negative and were not quantitated. Positive enrichment cultures were arbitrarily assigned a value of 10 CFU g^{-1}, and those which were negative were given a value of 1 CFU/g. Student's *t* test was used to determine statistical significance.

Detection of plasma antibodies to O157 LPS and Stx2. Titers against O157 LPS were determined by a blocking enzyme-linked immunosorbent assay (20), using monoclonal antibody MARC 13B3 (38) and highly purified LPS from *E. coli* O157:H7 (strain CDC EDL 933). MARC 13B3 lacks cross-reactivity with other bacteria, such as *Brucella* spp. and *Yersinia enterocolitica*, a problem often found with polyclonal antisera against O157. Endpoints were defined using receiver-operator-characteristic analysis of 1:4 dilutions of known positive and negative serum samples (20).

Vero cell cytotoxicity neutralization assays were performed as previously described (15). Briefly, dilutions of plasma were added to samples containing Stx2. After incubation for 1 h at 37°C, the plasma-toxin mixtures were added to Vero cells and incubated at 37°C for 48 h. The cells were then stained for viability with trypan blue. Antisera obtained from four calves hyperimmunized by subcutaneous injections with 100 μg of a noncytotoxic amino acid substitution mutant of Stx2e (15) were pooled and used as a positive anti-Stx control serum. Antibodies against Stx2e cross-reacted with and neutralized Stx2 in our system.

Preparation of antigen for peripheral blood mononuclear cell (PBMC) stimulation. Heat-killed Stx2$^+$ *E. coli* O157:H7 bacteria (HK-O157) were used for in vitro stimulation. One milliliter containing 10^{10} CFU of *E. coli* O157:H7 strain 86-24 (harvested from a fresh overnight culture) was boiled for 15 min. The cells were pelleted by centrifugation and resuspended in 1 ml of complete media (RPMI; 25 mM HEPES and L-glutamine [Gibco, Carlsbad, CA], 10% fetal calf serum, and 50 μg gentamicin per ml [Sigma, St. Louis, MO]). Aliquots of the cells were tested for sterility by plating them on trypticase soy agar plates and stored at −80°C until needed.

PBMC stimulation. PBMCs were isolated from whole blood collected at weekly intervals. The blood was centrifuged for 25 min at 1,170 × *g*. Plasma samples were removed and stored at −80°C for further analysis. Buffy coat cells were subjected to two rounds of hypotonic red blood cell lysis. PBMCs were then isolated by Percoll (Pharmacia, Piscataway, NJ) density gradient (P = 1.08) centrifugation at 450 × *g* for 40 min. Cells were washed twice in complete media. Cells were then diluted to 6.6×10^6 cells per ml and aliquoted into 96-well plates at 150 μl of cells (10^6 cells) per well. Stimulants (HK-O157, equivalent to 5×10^4 CFU *E. coli* O157:H7 cells, or 1 μg per ml ConA), 50 μl per well, were added to each well. Plates were incubated for 72 h in 5% CO_2 at 39°C. PBMCs were then pulsed with media containing 50 μCi of [^{3}H]thymidine and incubated for an additional 18 h. Cells were harvested with a Harvester 96 (Tomtech, Orange, CN) and analyzed using a 1450 Microbeta Plus scintillation counter (Wallac, Gaithersberg, MD). The stimulation index (SI) is the mean for three wells of cells incubated with stimulant divided by the mean for three wells incubated with media alone. Cell preparations in which the SI for ConA was less than 10 times that for cells incubated with media alone were considered poor and were excluded from further analysis. Statistical analysis was performed using the SAS software package and the analysis of variance technique.

RESULTS

All of the calves remained healthy throughout the study. None of the calves shed detectable levels of *E. coli* O157:H7 prior to the first inoculation. Only one of the calves in the

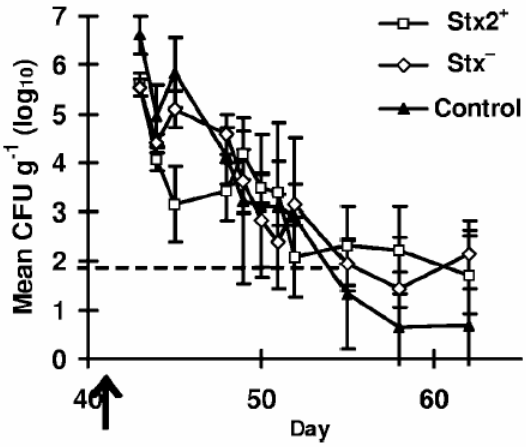

FIG. 2. Fecal shedding of the challenge strain by three groups of calves which were challenged with Stx2$^+$ O157:H7 strain 86-24 following prior inoculations with different strains of *E. coli*. The arrow indicates the date of the challenge. Data are presented as mean numbers (± standard errors of the means) of CFU g^{-1} of sorbitol-negative Str^r O157:H7 bacteria in the feces of the indicated groups. Group designations are same as those in the legend to Fig. 1.

study (one calf in the negative-control group) had detectable antibodies against O157 LPS prior to the first inoculation.

The inoculum strain was recovered in higher numbers and for a longer time from calves initially inoculated with Stx2$^+$ O157:H7 strain 86-24 than from calves inoculated with either of the other two *E. coli* strains (Fig. 1). More than 10^2 CFU of Stx2$^+$O157 bacteria/g of feces were recovered for 9 and 14 days after the first and second inoculations, respectively. Fecal shedding of Stx$^-$O157 decreased at a rate similar to that of the control bacteria, with both strains at $<10^2$ CFU g^{-1} of feces by the fifth day after each inoculation. The differences in fecal shedding between the Stx2$^+$ and Stx$^-$O157 strains were statistically significant ($P < 0.05$ by Student's t test) between days 2 and 13 after the first inoculation and days 22 and 27 after the second inoculation.

The fecal shedding of Stx2$^+$O157 bacteria was similar in all of the calves after they were challenged with Stx2$^+$O157 during week 7 (3 weeks after their second inoculation with Stx2$^+$O157, Stx$^-$O157, or control *E. coli* bacteria) (Fig. 2). Thus, prior exposure to high doses of STEC O157:H7 strain 86-24, Stx$^-$ *E. coli* O157:H7 strain 87-23, or control strain 123 did not reduce the duration or extent of fecal shedding of STEC O157:H7 bacteria after the challenge.

Following the initial two inoculations, all calves inoculated with either Stx2$^+$O157 or Stx$^-$O157 developed antibodies to the O antigen of *E. coli* O157:H7 (Fig. 3). The rates of seroconversion were similar in both groups. Three of the calves inoculated with control bacteria had low titers (≤16) of anti-O157 LPS, and the highest titer in this group was seen at week 4 in the calf which had serum antibodies to LPS O157 prior to the first inoculation. Calves in all three groups developed antibodies to O157 LPS following challenge with Stx2$^+$O157 (Fig. 3). None of the calves in any of the three groups developed antibodies against Stx2.

The in vitro PBMC proliferation assay was used to monitor the lymphoproliferative responses of infected calves to HK-O157 antigens (Fig. 4). Four of the five calves initially inoculated with Stx$^-$O157 developed a high proliferative immune response to HK-O157. This response became apparent at week 3 and reached its strongest level after the challenge. Three of the five calves in this group had SIs greater than 10 for 2 or more weeks. In contrast, calves initially inoculated with either Stx2$^+$O157 or control bacteria did not develop consistent PBMC proliferative responses to HK-O157 at any point during the study. Two of the five calves inoculated with Stx2$^+$O157, and one of the five inoculated with control bacteria, had SIs of >10 in a single assay (at week 4 or 8 for Stx2$^+$O157 calves and at week 10 for the control calf). Trend analysis using the sum of squares technique showed that the cumulative PBMC proliferative response for the calves inoculated with the Stx$^-$ strain was significantly higher than that for the calves which received Stx2$^+$O157 ($P = 0.0001$) or control bacteria ($P = 0.0009$). There were no consistent differences in overall ConA activation (Fig. 4).

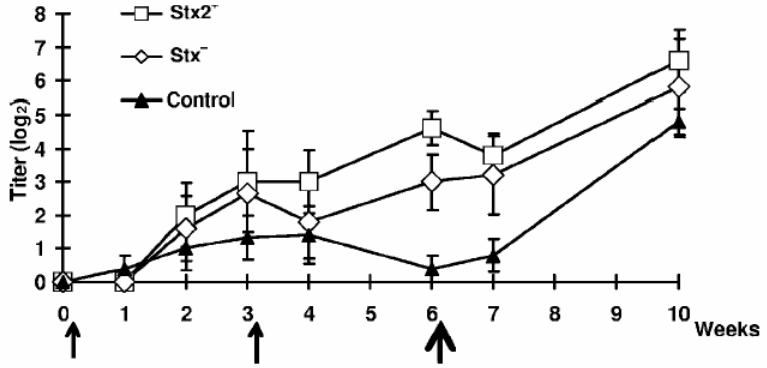

FIG. 3. Development of antibody titers against O157 LPS in calves inoculated with different *E. coli* strains. Data are shown as the means (± standard errors of the means) of $\log_2$ titers of plasma antibodies. For the designations of calf groups, see the legend to Fig. 1. Arrows indicate the dates of the initial inoculations (small arrows) and challenge (large arrow).

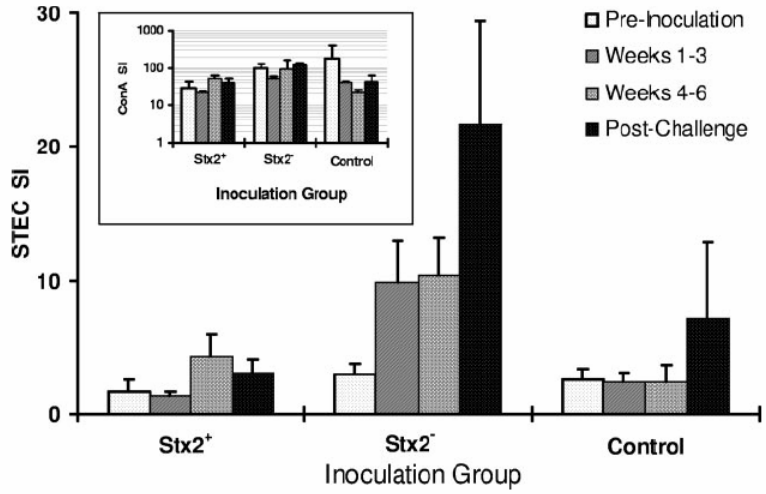

FIG. 4. Comparison over time of the lymphoproliferative responses to heat-killed Stx2$^+$ *E. coli* O157:H7 bacteria (HK-O157) in calves inoculated with different *E. coli* strains. Calves were inoculated twice (on days 0 and 21) with Stx2$^+$ *E. coli* O157:H7 strain 86-24 (Stx2$^+$), non-Shiga-toxigenic *E. coli* O157:H7 strain 87-23 (Stx$^-$), or control *E. coli* O43:H28 strain 123 (Control) prior to a final challenge (at 7 weeks postinoculation) with Stx2$^+$ *E. coli* O157:H7 strain 86-24. Results are shown as the means (± standard errors of the means) of the SIs obtained with PBMCs from five calves per group. The index for an individual animal was determined by dividing the mean counts per minute for three wells stimulated with HK-O157 by the mean counts per minute for three wells of nonstimulated cells. Lymphoproliferative responses to ConA stimulation are shown in the insert. Data obtained with blood samples during weeks 1, 2, and 3, during weeks 4 and 6, and postchallenge (during weeks 7, 8 and 10) were combined within those respective groups.

DISCUSSION

This study provides the first evidence that STEC infections can suppress the development of an antigen-specific cellular immune response in cattle. Following the initial inoculations, calves inoculated with Stx2$^+$ *E. coli* O157:H7 strain 86-24 shed the inoculum strain at higher levels and for longer periods than did calves inoculated with Stx$^-$O157:H7 strain 87-23. Despite this increased colonization, the lymphoproliferative responses to HK-O157 in calves inoculated with Stx2$^+$O157 were significantly lower than the responses in calves inoculated with Stx$^-$O157 and were similar to the responses in calves that received control bacteria. This reduced cellular immune response in Stx2$^+$O157-inoculated calves extends evidence that STEC possesses virulence traits that actively suppress cellular elements of the host immune response (1, 22, 23, 32). The differences observed in the lymphoproliferative responses in calves inoculated with the Stx2$^+$O157 and Stx$^-$O157 strains used in this study are consistent with the suppressive effects of Stxs on bovine peripheral and intraepithelial lymphocytes observed in vitro and in vivo (1, 12, 13, 22–25, 31).

Our studies were not designed to examine the mechanism underlying the observed immunosuppression. However, the observation that the PBMCs obtained from the different inoculation groups did not significantly differ in their proliferative responses to ConA provides preliminary evidence that the effect of STEC infection on the bovine immune system was not generalized. Furthermore, the observed immunosuppressive effects of STEC infections were restricted to antigen-specific in vitro PBMC proliferative responses. This contrasts with a generalized suppression of T cells described for mice and piglets, which is characterized by a drop in the reactivities of lymphocytes to ConA following injection with purified Stx2 and inoculation with Stx1$^+$ *E. coli* strain O111:NM, respectively (5, 34). Differences in levels of systemic Stx may be responsible for the observed differences in the effects of Stx on the immune system in the different studies. The mouse and piglet studies were more likely to involve high systemic concentrations of Stx. Mice were intravenously injected and piglets were orally inoculated with high doses of Stx$^+$ bacteria at 24 h of age, when gut closure may not have been complete and the likelihood of toxin absorption from the intestine was increased. Consequently, the effects of Stx on the immune system in these models may have been high enough to affect "general" functions, like mitogenic responsiveness. The effects of Stx in calves infected at 6 to 8 weeks of age (notwithstanding the high inoculum doses) may better reflect the effects of "physiologic" infections that occur in the field.

Thus, we interpret the present findings as support for our hypothesis that Stxs are immunosuppressive factors which play a role in promoting STEC colonization in the bovine host primarily by reducing or delaying the host's antigen-specific cellular immune response against the bacteria.

As previously postulated (31), the immunosuppressive effects of STEC infections appeared to result from a prevention of the onset of an immune response rather than the downregulation of an established one. The development of an in vitro PBMC response was prevented when Stx2 was present at the time that the naïve O157:H7 *E. coli*-specific immune cells were first stimulated by the initial inoculations (i.e., in calves initially inoculated with Stx2$^+$O157). In contrast, the Stx2$^+$O157 strain did not exert an immunosuppressive effect after the immune response was efficiently induced by two inoculations with Stx$^-$O157. Calves initially inoculated with Stx$^-$O157 showed a continued proliferative response that, similar to an anamnestic

response, was even more pronounced after they were challenged with Stx2$^+$O157.

Remarkably, inoculation of calves with Stx2$^+$O157, on the contrary, did not hinder the development of a humoral response against O157 LPS (17; this study). B-cell responses in calves apparently were less sensitive to STEC-induced suppression than in vitro PBMC proliferative responses (25), particularly when the B cells were directly activated in vivo by T-cell independent B-cell antigens, like LPS. The absence of antibodies against Stx2 in our calves, which is consistent with previous observations after experimental STEC O157:H7 inoculations in cattle (17), can therefore not be explained by a suppression of B cells.

Despite the different immune responses in the three groups of calves following the initial inoculations, the shedding patterns for Stx2$^+$O157 bacteria were similar in all groups in the first weeks after the calves were challenged with the Stx2$^+$O157 strain. Neither the induction of O157 antibodies in the Stx2$^+$O157 group nor the induction of O157 antibodies and HK-O157-specific lymphocytes in the Stx$^-$O157 group reduced the duration or magnitude of shedding after the challenge compared to what was found for the control group during the observation period. Further experiments will be necessary to determine how O157:H7 *E. coli*-specific cellular immune responses affect long-term and intermittent STEC shedding (3, 8).

The effects of STEC infections on the development of specific cellular immune responses must be addressed in designing vaccines aimed at reducing STEC O157:H7 infections in cattle, especially vaccines directed against Stx, which is the only virulence factor shared by all STEC strains.

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Disclaimer: the use of names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of any product, and the use of a name by the USDA implies no approval of the product to the exclusion of others that may also be available.

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7. ZUSAMMENFASSUNG

Persistent mit Shigatoxin-bildenden *Escherichia coli* (STEC) infizierte Rinder sind das wichtigste Reservoir für Infektionen des Menschen mit humanpathogenen STEC-Stämmen (sog. enterohämorrhagischen *E. coli* [EHEC]). Eine erfolgreiche Prävention humaner EHEC-Infektionen muss deshalb die Bekämpfung der STEC im Rind zum Ziel haben. Zur Entwicklung wirksamer Massnahmen fehlte bisher das Verständnis der Persistenz-mechanismen. Mit den Shigatoxinen (Stx) verfügen die STEC über die Fähigkeit zur Hemmung der Proliferation peripherer boviner Lymphozyten. Eine immunsupprimierende Wirkung der Toxine könnte zur Persistenz der STEC-Infektion beitragen.

Um diese Hypothese zu überprüfen, sollte der Mechanismus der Stx-bedingten Immun-modulation aufgeklärt, Zielzellen für Shigatoxine in der Darmschleimhaut des Rindes identifiziert und die pathophysiologische Wirkung der Toxine *in vitro* und *in vivo* bestimmt werden. Dazu wurden Primärkulturen von peripheren und intraepithelialen Lymphozyten und von Epithelzellen und nicht-epithelialen Zellen aus dem Kolon etabliert. Nach Inkubation mit gereinigtem Stx1 wurden die Zellen mit Hilfe der Durchflusszytometrie, der Fluoreszenz-mikroskopie und reverser „real-time“-PCR phänotypisch und funktionell charakterisiert. Der Stx-Rezeptor wurde nach Glykolipid-Extraktion massenspektrometrisch analysiert. Die *in vitro* gewonnenen Erkenntnisse wurden abschliessend in Infektionsversuchen überprüft.

Die strukturelle Aufklärung des funktionellen Stx1-Rezeptors auf peripheren Lympho-zyten zeigte, dass die Wirkung des Toxins durch das Glykosphingolipid Globotriaosylzeramid (Gb_3 syn. CD77; Gal(α1-4)Gal(1-4)Glc(1-1)zeramid) vermittelt wurde. Dieses damit beim Rind erstmalig beschriebene Leukozytenantigen wurde von Lymphozyten *in vitro* und *in vivo* aktivierungsabhängig exprimiert. Dabei existierten verschiedene Isoformen des Gb_3/CD77, die aufgrund unterschiedlicher Fettsäuren im Membran-verankerten Teil der Moleküle in ihrer Affinität für Stx1 differierten. Stx1-bindende Isoformen wurden von peripheren Lymphozyten insbesondere zu Beginn der Aktivierung exprimiert. Die Proliferationshemmung durch Stx1 betraf insbesondere CD8α-positive T-Zellen und B-Zellen. Sie manifestierte sich ohne Auslösung von Zelltod und wurde auch von der enzymatisch inaktiven, die Rezeptor-Bindung vermittelnde B-Untereinheit (StxB1) induziert. Die Wirkung wurde nicht durch Zytokine wie IL-2, IFN-α oder TNF-α vermittelt, sondern beruhte auf einer direkten Einwirkung des Shigatoxins 1 auf Lymphozyten in frühen Aktivierungsstadien. Dies lässt vermuten, dass Shigatoxine nicht generell immunsuppressiv wirken, sondern frühzeitig die Entwicklung einer Antigen-spezifischen Immunität behindern können. Tatsächlich entwickelte sich eine

systemische zelluläre Immunität gegen STEC-Antigene nach Infektionen von Kälbern mit einem Stx2-bildenden EHEC O157:H7 Stamm nur signifikant verzögert.

Zusätzlich wirken Shigatoxine aber auch unmittelbar auf die lokale Immunabwehr der bovinen Darmschleimhaut. Bovine Granulozyten exprimierten, auch wenn sie auf eine Schleimhaut-Oberfläche migriert waren, keine Stx-Rezeptoren und waren gegenüber Stx1 resistent. Mit Subpopulationen von Kolonepithelzellen und mit Makrophagen-artigen Zellen konnten allerdings Zielzellen für Stx1 identifiziert werden, die sich bei der Kolonisation der bovinen Darmschleimhaut in unmittelbarer Nähe zu den STEC-Kolonien befinden. Diese Zellen widerstanden ebenfalls der zytoletalen Wirkung des Shigatoxins 1, reagierten jedoch auf das Toxin mit der vermehrten Transkription bestimmter Chemokin-Gene. Vor allem Lymphozyten stellen aber in der Schleimhaut Zielzellen für Stx1 dar. Bei der Analyse intraepithelialer Lymphozyten (IEL) *ex vivo* exprimierten überwiegend reife, aktivierte $CD3^+CD8\alpha^+$-T-Zellen Gb_3/CD77. Nach Inokulation von Darmschlingen bei Kälbern mit einem Stx1-bildenden O103:H2 Stamm verschob sich im Vergleich zu Kontrollschlingen, die mit einer isogenen Δ*stx1*-Mutante des Stammes inokuliert worden waren, innerhalb von 12 Stunden die Zusammmensetzung der IEL zulasten dieser Subpopulation. *In vitro* blockierte Stx1 bereits im Nanogramm-Konzentrationsbereich die Stimulierbarkeit der IEL. Im Gegensatz zu den Subpopulationen peripherer Lymphozyten unterschieden sich die IEL-Subpopulationen nur geringfügig in ihrer Empfindlichkeit gegenüber der aktivierungs-hemmenden Wirkung des Shigatoxins 1. Darüberhinaus waren bereits wenige Stunden nach Internalisierung enzymatisch aktiven Stx1-Holotoxins in IEL, nicht aber in PBMC, *il-4*-Transkripte signifikant häufiger nachweisbar.

Die Ergebnisse unterstützen die Hypothese, dass Shigatoxine bei der enteralen STEC-Infektion von Rindern über Glykolipid-Rezeptoren immunmodulatorisch wirken. Die Rezeptoren dienen dabei nicht nur der Internalisierung des Holotoxins, sondern scheinen nach Toxinbindung auch Signale von der Zelloberfläche zu vermitteln. Im Gegensatz zur immun-stimulierenden Wirkung anderer bakterieller Toxine, die als Glykolipid-Rezeptor-Agonisten wirken, und auch im Gegensatz zur zytoletalen und pro-inflammatorischen Wirkung der Shigatoxine beim Menschen, steht beim Rind die immunsuppressive Wirkung der Shigatoxine im Vordergrund. Dies könnte das Fehlen von klinischen Erscheinungen bei bovinen STEC-Infektionen ebenso erklären wie den persistierenden Charakter der Infektion. Damit eröffnen diese Ergebnisse neue Möglichkeiten zur Entwicklung effizienter Massnahmen zur Bekämpfung dieser humanpathogenen Erreger beim Rind.

8. SUMMARY

A considerable number of cattle is persistently infected with Shigatoxin-producing *Escherichia coli* (STEC) and thereby represents a source of infections of man with human pathogenic STEC strains (syn. enterohemorrhagic *E. coli* [EHEC]). Intervention strategies to effectively prevent human EHEC infections must be aimed at the limitation of bovine STEC infections, therefore. The mechanisms that form the basis of the persistence are poorly understood. By secreting Shiga toxins (Stxs) STEC are capable of inhibiting the proliferation of peripheral bovine lymphocytes. It is tempting to speculate that an immunosuppressive activity of these toxins contributes to the persistence of STEC infections in cattle.

In an attempt to prove this hypothesis the objectives of the studies were to elucidate the mechanism of the Stx-induced immunomodulation, to identify target cells for Stxs in the bovine intestinal mucosa, and to determine the pathophysiological activity of the toxins *in vitro* and *in vivo*. For this purpose primary cultures of peripheral and intraepithelial lymphocytes and of colonic epithelial and non-epithelial cells were established. Upon incubation with purified Stx1 cells were characterised phenotypically and functionally by flow cytometry, fluorescence microscopy and reverse real-time PCR. The Stx-receptor was analysed by mass spectometry after glycolipid extraction. The immunomodulatory properties of Stxs were eventually verified by infection experiments.

Structural analysis of functional Stx1-receptors on peripheral lymphocytes revealed that the effect of the toxin was mediated by the glycosphingolipid globotriaosylceramide (Gb_3 syn. CD77; Gal(α1-4)Gal(1-4)Glc(1-1)ceramide). This leukocyte antigen, newly discovered in cattle, was expressed by lymphocytes *in vitro* and *in vivo* in an activation-dependent fashion. Evidence is provided for the existence of different isoforms of Gb_3/CD77 that differed in their affinity for Stx1. These differences were based on differences in the fatty acids incorporated in the membrane-anchored part of the Gb_3/CD77 molecules. Stx1-binding isoforms were expressed by peripheral lymphocytes predominantly at early stages of the activation process. The Stx1-induced inhibition of proliferation mainly affected CD8α-positive T-cells and B-cells. This effect occurred independently of cellular death and was induced as well by the enzymatically inactive B-subunit (StxB1) responsible for receptor binding. The effect of Stx1 was not mediated via cytokines as IL-2, IFN-α, or TNF-α but required the direct impact of Stx1 on lymphocytes at early activation stages. These findings let to assume that Stxs have the potency to hinder the development of an antigen-specific immunity in cattle rather than being generally immunosuppressive. In fact, the development of a systemic cellular immune

response specific for STEC antigens was significantly retarded in calves experimentally infected with a Stx2-producing EHEC O157:H7 strain.

Furthermore, Stx1 was found to directly act on the local immune response in the bovine intestinal mucosa. Bovine granulocytes lacked Stx-receptors even after the cells had migrated to a mucosal surface and the cells proved to be resistant to Stx1. However, subpopulations of colonic epithelial cells and macrophage-like cells, presumably residing in the bovine mucosa in proximity to STEC colonies, were identified as target cells for Stx1. These cells resisted the cytolethal effect of Stx1 but responded to the toxin by an elevated transcription of certain chemokine genes. Still, lymphocytes appear to be the most important target cells for Stx1 in the mucosa. When intraepithelial lymphocytes (IEL) were analysed *ex vivo* Gb_3/CD77 was predominantly expressed by mature, activated $CD3^+CD8\alpha^+$ T-cells. Accordingly, inoculation of ligated intestinal loops in calves with a Stx1-producing O103:H2 strain led within 12 hours to a small but significant reduction in this IEL subpopulation when compared to loops inoculated with an isogenic Δ*stx1*-mutant to that strain. *In vitro,* Stx1 inhibited the mitogenic responsiveness of IEL in the nanogram concentration range. In contrast to subpopulations of peripheral lymphocytes, IEL subpopulations only marginally differed in their sensitivity to the activation-inhibiting activity of Stx1. Furthermore, significantly more *il-4*-specific transcripts were detected in IEL but not in peripheral lymphocytes a few hours after internalisation of the enzymatically active Stx1 holotoxin.

The results support the hypothesis that, during bovine STEC infections, Stxs act as immunomodulators via glycolipid receptors. The receptors are not only required for the internalisation of the holotoxins but also appear to mediate signals from the cell surface upon toxin binding. Different from the immunomodulatory activities of other bacterial toxins acting as glycolipid-receptor agonists, and in contrast to the cytolethal and pro-inflammatory activity of Stxs in humans, the Stxs principally act as immunsuppressive virulence factors in cattle. These findings might explain both, the lack of clinical symptoms in the course of bovine STEC infections as well as the persistent character of the infection. The immunosuppressive capacity of the Stxs should be considered during the development of effective strategies ought to lower the magnitude and frequency of shedding by cattle of these human pathogenic bacteria.

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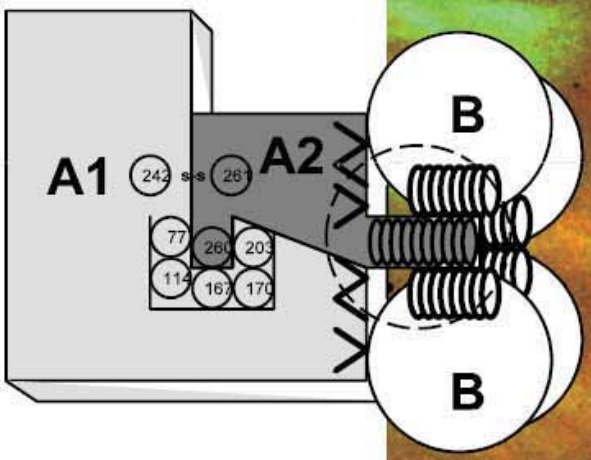

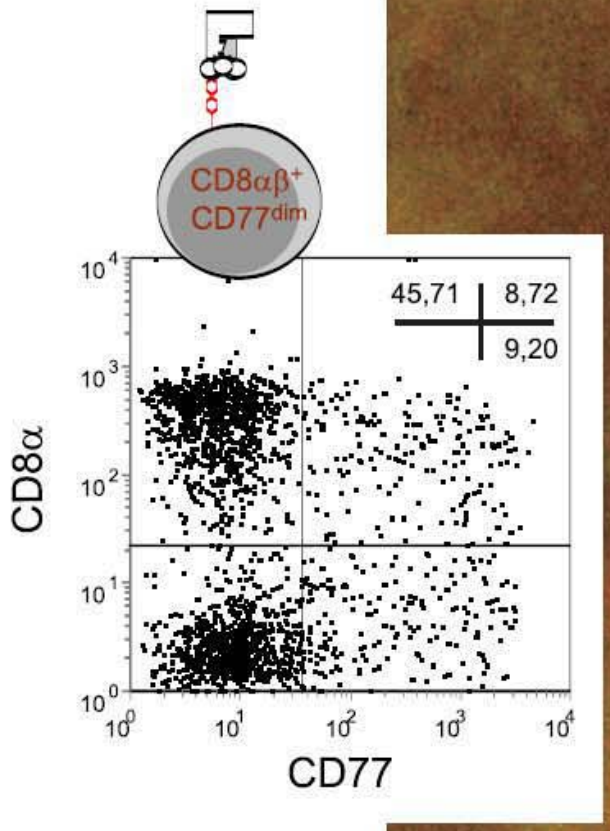

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