Aus dem Institut für Hygiene und Infektionskrankheiten der Tiere
Justus-Liebig-Universität Gießen

Effect of *Escherichia coli* Stx1 on the cytokine profile of bovine ileal intraepithelial lymphocytes

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften
der Justus-Liebig-Universität Gießen

Fachbereich Biologie, Chemie und Geowissenschaften

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Gießen 2006

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Date of the oral examination:	May 09th 2006

My parents

“Decide carefully, exactly what you want in life, then work like mad to make sure you get it!”

Hector Crawford

Publications and posters

Part of the work presented in this doctoral thesis was submitted for publication under the following reference:

Moussay Etienne, Stamm Ivonne, Taubert Anja, Baljer Georg, and Christian Menge
Escherichia coli Shiga toxin 1 enhances *il-4* transcripts in bovine ileal intraepithelial lymphocytes.
Submitted.

And was presented, as posters, at the following congresses:

Etienne Moussay, Ivonne Stamm, Georg Baljer, Christian Menge
Shiga toxin 1 from *E. coli* induces an increase in IL-4 mRNA synthesis in bovine ileal intraepithelial lymphocytes. Poster presented at the 56th Annual meeting of the DGHM (German Society for Hygiene and Microbiology) in Münster (Germany) / Abstract published in the International Journal of Medical Microbiology, Vol. 294S1, Supplement N°39, Sept 2004, p 223-224.

Etienne Moussay, Ivonne Stamm, Georg Baljer, Christian Menge
Shiga toxin 1 from *E. coli* induces an increase in IL-4 mRNA synthesis in bovine ileal intraepithelial lymphocytes. Poster presented at the annual CRWAD (Conference of Research Workers in Animal Diseases) in St. Louis (Missouri, USA), 2005.

Abreviations

7-AAD	7-amino actinomycin D
Abs	Absorbance
ActD	Actinomycin D
A/E	Attaching/effacing
AP-1	Activation protein-1
APC	Antigen-presenting cell
ATCC	American type culture collection
A.U.	Arbitrary units
BCR	B-cell receptor
BCV	Bovine coronavirus
BL-3	Bovine lymphoma-3
BLV	Bovine leukemia virus
bp	base pair
BSA	Bovine serum albumine
CD	Cluster of differentiation
CD_{50}	Cytotoxic dosis for 50% of the cells
ConA	Concanavalin A
DCs	Dendritic cells
DEPC-water	Diethyl-pyrocarbonate treated water
DiO	Dioctadecyloxacarbocyanin-perchlorate
DN	Double negative ($CD4^-$ $CD8^-$ lymphocytes)
DNA	Desoxiribonucleic acid
DP	Double positive ($CD4^+$ $CD8^+$ lymphocytes)
DTT	1.4 Dithiotreitol
EBV	Epstein-Barr virus
ECACC	European collection of animal cell cultures
E. coli	*Escherichia coli*
EDTA	Ethylenediamine Tetraacetic Acid
EF-1 (-2)	Elongation factor-1 (-2)
EHEC	Enterohemoragic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*

ER	Endoplasmic reticulum
Esp	*Escherichia coli* secreted protein
FACS	Fluorescent-activated cell sorting
FAE	Follicle-associated epithelium
f.c.	Final concentration
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat
GALT	Gut associated lymphoid tissue
Gb_3/CD77	Globotrioacylceramid
γ_c	Common γ-chain from interleukine receptors
GM-CSF	Granulocyte and monocyte-colony stimulating factor
hrs	Hours
HIV	Human immunodecifiency virus
HUS	Haemolytic uremic syndrome
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
iIEL	Ileal intraepithelial lymphocyte
IFN-γ	Interferon-gamma
Ig	Immunoglobulin or immunoglobin
IL-	Interleukin-
ILF	Isolated lymphoid follicle
IP-10	Interferon-induced peptide 10
Kb	Kilobase
kDa	Kilodalton
KGF	Keratinocyte growth facotr
LEE	Locus of enterocyte effacement
LFA	Leukocyte factor of adherence
LifA	Lymphostatine A
LPL	Lamina propria lymphocytes
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
MHC I or II	Major histocompatibility complex of class I or -II
min	Minute
MAb	Monoclonal antibody

MIP	Monocyte inflammatory protein
mRNA	Messenger-ribonucleic acid
NF-κB	Nuclear factor κB
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PMA	Phorbol-12-myristate-13-acetate
PFA	Paraformaldehyde
PHA-P	Phytohemagglutinin-P
PKC	Protein kinase C
PP	Peyer's patch
RAG-1 and -2	Recombination-activating gene 1 and 2
RAJ	Recto-anal junction
RBS	Ribosome binding site
RIP	Ribosome inactivating proetin
rRNA	Ribosomal-ribonucleic acid
RT	Reverse transcription
SCF	Stem cell factor
SDGF-3	Spleen-derived growth factor-3
sec	Second
SEC	Staphylococcal enterotoxin C
Stat6	Signal transducer and activator of transcription 6
STEC	Shiga toxin-producing *Escherichia coli*
stx	gene encoding Shiga toxin
Stx1 or 2	Shiga toxin 1 or 2 protein
StxA1	A subunit of Shiga toxin 1
StxB1	B subunit of Shiga toxin 1
syn.	Synonymous
TCR	T-cell receptor
TGF-β	Transforming growth factor-β

TGN	Trans-Golgi network
T_H	T helper lymphocytes (T_H0, 1, 2, and 3)
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
tRNA	Transfer-ribonucleic acid
ZO	Zona occludens

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) infections are a major cause of bloody diarrhoea, hemorrhagic colitis and haemolytic uremic syndrome in humans (23). Following initial major outbreaks (321, 413), 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 toxins (1A:5B) and were identified as main STEC virulence factors (287), that principally target endothelial cells (222), leading to vascular damages in human kidneys, brain, pancreas and intestine. After binding of the B-subunit(s) to the glycosphingolipid Gb_3/CD77 (109), Stx is retrogradly transported (332) and the A_1-subunit translocates to the cytosol, where it exerts its cytotoxicity and rapidly kills the affected cells (334). 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 (88, 90) and eventually block the eEF-1 and eEF-2 dependent elongation process (290). 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 (240, 261). 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 (392, 393), mesangial cells (354) and monocytes and macrophages (139, 140, 390, 401) 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 (390).

In cattle, representing the main source of human infections (137), intestinal STEC-infections are mostly asymptomatic (356) but result in a high percentage of animals shedding STEC for prolonged periods (69, 77). It was hypothesized (247) 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 (356). Indeed, Stx1 suppresses bovine lymphocyte functions (250, 254) and presumably represents a STEC virulence factor even in cattle (252). In contrast to human lymphocytes, bovine B and T cell subsets both express functional Stx-receptors and are affected by the toxin (363). Stx1 blocks the activation and the proliferation of these cells *in vitro* (103, 250, 254) and *in vivo* (151). 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 (247). 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 (247, 252). However, since Stx1 neither induces cellular death nor affects the NK activity of IEL *in vitro* (247) and *in vivo* (252), the consequences of this effect of Stx1 for mucosal immune responses remained to be elucidated.

IEL represent effector cells against bacterial (277) and viral infections (125, 268). 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 (19, 110, 156). IEL are also a potent source of chemokines (e.g. IL-8, MIP-1α and -1β) (35, 227). Based on the 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. These investigations aimed at helping to elucidate the mechanisms by which Stx1-producing *E. coli* colonize the intestine and persist in cattle in a commensal-like lifestyle.

2. Literature review

2.1. Mucosal immune system

The immune system is defined as the molecules, cells, tissues, and organs that function to provide a protection against foreign organisms (1). This system is composed of several compartments as the blood, the mucosal tissues, the thymus, the bone marrow, the body cavities and the skin. The mucosa-associated lymphoid tissue (MALT) is the largest and the most complex compartment of this system. The intestinal mucosa is a huge surface of exchange and is consequently more exposed to microorganisms. The gut-associated lymphoid tissue (GALT), which is a part of the MALT, comprises four distinct compartments: the Peyer's patches (PPs) and other lymphoid follicles associated with the follicle-associated epithelium (FAE), the lamina propria (LP), the intraepithelial lymphocytes (IEL), and the mesenteric lymph nodes (MLN). The small intestinal epithelium of ruminants contains large populations of lymphocytes (298), comparable, in number, to the pool in the spleen. The GALT is a sophisticated system in which food antigens have to be ignored (tolerance) while antigens of pathogenic microorganisms have to induce both strong innate and adaptive immune responses to protect the organism and to prevent further dissemination of the pathogens. Specialised systems such as secretion of immunoglobulin (Ig) A, certain T_H-type cell responses and induction of tolerance are essential mechanisms of the mucosal protection (265). The presence of several compartments and the association of inductive (PPs and MLN) and effector (LP lymphocytes [LPL] and IEL) sites allow a very efficient sampling of antigens from the mucosa, draining towards the lymph nodes and an adequate immune response (40). In addition to cell subsets and lymphoid structures of the immune system, several types of cells are present in the mucosa and constitute a well-integrated network with intense cell-to-cell and cytokine-mediated communication allowing a physical protection of the mucosa of the host (enterocytes [or intestinal epithelial cells, IEC], Paneth cells, and goblet cells). The surveillance of the mucosa and the sampling of exogenous antigens are performed by M cells and dendritic cells (DCs) present in the epithelial monolayer and in the lamina propria, respectively. Effective immune responses toward pathogens mounted in the mucosa are mediated by IEL and LPL. A highly-regulated control of the infected IEC and activated IEL during and after the clearance of the infection allow an efficient homeostasis of

the mucosal immune system. The anatomy of the small intestine is considered as prototype of a mucosal immune tissue and therefore represents a model for studying infectious processes.

2.2. Functions of the intestinal epithelium

2.2.1. Barrier function

The lumen of the small intestine is lined by a monolayer of microvilli-folded epithelial cells, called enterocytes, forming tight junctions (230, 231), which play different structural and functional roles (physical barrier, exchange of molecules, immune response). The enterocytes are first covered by a layer of negatively charged mucin-like molecules and carbohydrates called the glycocalyx which protect them from chemical injuries (243). The enterocytes are then covered by several layers of mucus produced by the goblet cells. The main functions of this viscous fluid are to protect the epithelium against chemical damages and to trap and eliminate particles and micro-organisms by peristalsis in the lumen of the intestine.

2.2.2. Immunological functions

Secretion of antimicrobial peptides

Paneth cells are part of the epithelial layer in the small intestine. Their main function is to produce lysozyme, phospholipase A2 and antimicrobial peptides. Epithelial cells can as well produce antimicrobial peptides to ensure the protection of the mucosa. In vertebrates, the defensins and the cathelicidins are the main products with antibacterial activity (83). Three subfamilies of defensins have been described (α, β and θ). Alpha-defensins, called “cryptdins” in mice, are produced by phagocytes in human, primates and rodents and by Paneth cells of the small intestine in human, mouse, and rat in response to stimulation by bacterial antigens (12). Alpha-defensins are processed to their active form by the matrix metalloproteinase 7 (MMP-7 or matrilysin)(422) and were shown to be involved in clearance of *E. coli* infection in the small bowel. The β-defensins were first described in the tracheal epithelium of cattle and are produced after activation of the Toll-like receptor (TLR) -2 pathway (342). In contrast to β-defensins, the α- and θ-defensins are constitutively expressed. In addition to defensins, the cathelicidin LL-37 which is constitutively produced in the human intestinal tract by

colonic epithelial cells but not in the small intestine (141), binds to LPS and can efficiently attract monocytes and neutrophils (213).

Antigen presentation by IEC and regulation of IEL activation

IEC are considered effective antigen presenting cells (APC) as they express both MHC class I and II molecules like professional APC do (179), and produce the co-stimulatory molecule B7H (ICOS-L) (272) to activate T cells *in vitro* (175). The presence of the HLA-G, -H, (32) and CD1d antigens (33) on the surface of IEC suggests that these cells can also present antigens in the context of MHC class I-like molecules rather than conventional MHC antigens. IEC are thought to maintain and regulate the immune homeostasis in the intestine by selectively activating or suppressing IEL functions (29, 30). In one hand, IEC induce the proliferation of lamina propria lymphocytes without any classic MHC restriction (296). On the other hand, IEC can downregulate functions and inhibit activation of both αβ- and γδ-TCR intraepithelial lymphocytes subsets (335, 430).

A possibility of antigen sampling in the intestine of humans and porcine, described recently, implicates the transport of IgG through the intestinal epithelial barrier using the Fc receptor as a shuttle service (373, 436).

Cytokine and chemokine production by IEC

In addition to the secretion of antimicrobial peptides and the presentation of antigens, IEC have a very important immune function as they can produce large amounts of cytokines and chemokines, including TGF-α, TGF-β, IL-15, IL-6, TNF-α, GM-CSF, IL-8, and MCP-1 (211). In response to bacterial invasion of the mucosa, the production of such mediators is up-regulated (84) and is a critical factor for the regulation of the innate immune response of the mucosal epithelium (85). In addition to the production of cytokines during an infection, the production of chemokines is an important process in physiologic conditions (absence of infection with pathogens) leading to the homing of IEL and will be addressed in another section (see **2.3.4.3**).

2.3. Gut-associated lymphoid tissues (GALT)

2.3.1. Peyer's patches (PPs)

Peyer's patches are specialised complex and well-organised lymphoid structures located in the small intestinal wall containing IgA-producing B lymphocyte follicles with T cell areas surrounding the germinal centres. PPs contribute to the local and systemic immunity against intestinal antigens. Consequently, all types of cells necessary for initiating, regulating, and performing immune responses are present in PPs (macrophages, dendritic cells, and polymorphonuclear cells). After stimulation by TGF-β and IL-10 produced by DCs and T cells, B cells undergo Ig class switch from IgM to IgA (245). Fifty percent of the B cells from the lymphoid follicles express surface IgA. Although IgA are very important in the mammalian gastrointestinal immune response, IgG play a part in immune response to foreign antigens as well. T cells are found in the T-dependent interfollicular areas and are intercalated with B cells in the dome overlying the follicles (92).

In ruminants, two different categories of PPs exist in the small intestine. The ileal and jejunal PPs contain functionally distinct B-cell populations. While ileal PPs mainly produce $CD5^+$ $sIgM^+$ B-cells, jejunal PPs contain IgA-producing plasma-cells. Moreover, ileal PPs' follicles can be repopulated by circulating B-cells if necessary (128, 129).

One specificity of the GALT (and more generally of the MALT) is the constant recirculation of lymphocytes. Naïve lymphocytes originating from the bone marrow, can be activated in the PPs, migrate through the lymphatic draining of the intestine, via the mesenteric lymph nodes and the thoracic duct to the blood, and can finally re-enter the MALT in the lamina propria using specific adhesins. This mechanism allows the spread of the protection all along the whole length of the intestinal immune system (9).

There are evidences that the intestinal immune response is initiated in PPs because they are covered by a specialised follicle associated epithelium (FAE) containing M cells involved in antigen uptake and processing (293). In the epithelial monolayer of the FAE (dome), enterocytes are interrupted by large cells without well-shaped villi, called M cells, that cover small and large intestinal PPs (280). M cells present in the specialised FAE are characterised by the absence of glycocalyx seen on enterocytes, by the lack of a well-organized brush border on their apical surface, and by the presence of a cavity (or pocket) where B and T lymphocytes and small numbers of macrophages are stored. Most of T cells are $CD4^+$ expressing the typical antigen of memory cells CD45RO (99). M cells take up antigens, via

endocytosis by uncoated or clathrin-coated pits and vesicles, and transport them across the FAE. M cells can as well make direct contact with lymphocytes or APC (122). Although MHC class II molecules have been reported on the surface of M cells, no evidence have confirmed their implication in the presentation of antigens yet (6). M cells can also be used as a way of infection by bacteria and contribute to infection of other cells (279). Viruses (reovirus, poliovirus, HIV) and bacteria (*V. cholerae, E. coli, S. thyphimurium, Y. enterocolotica, S. flexneri, and C. jejuni*) can adhere to M cells and then infect the mucosal tissues (171). A rabbit pathogenic *E. coli* strain was found to intimately bind to M cells, to induce effacement of M cell villi, and to trigger the formation of pedestals (161, 162). The capacity to transfer different materials distinguishes M cells from typical enterocytes and underlines both their antigen-sampling function and their vulnerability to infection by pathogens. Consequently, M cells represent attractive candidates for drug and vaccine delivery (169).

2.3.2. Lamina propria

The intestinal lamina propria (LP) is a connective tissue located between the epithelium and the muscularis mucosae. The LP, an effector site of the mucosal immune response, contains several types of cells of various functions and states of activation. Large numbers of B cells are present in the intestinal LP and are a major source of IgA that enter the lumen of the intestine to neutralize bacteria or antigens. Locally synthesized by plasma cells in the LP, IgA can bind pathogens and lead to their excretion through the epithelium into the lumen, or directly bind the antigens in the lumen of the intestine. During their intraepithelial transport to the lumen, IgA are able to bind to viral particles and then inhibit viral production inside IEC (203).

In addition to B cells, both $CD4^+$ and $CD8^+$ T cells are present in the LP at a high level of activation, and can mediate cytotoxicity or produce helper and suppressor cytokines (183). LPL are predominantly of T_H2 phenotype (50 to 70 %) and many $CD4^+$ T cells stimulate the production of antibodies by B cells. The LP contains a smaller proportion of LPL bearing the CD8 antigen (20 to 30 %) capable of cytotoxic-T-lymphocyte (CTL) activity (209). In several species, the LP is a reservoir of memory cells (142, 143), suggesting the LP as a site of secondary responses. Both $CD4^+$ and $CD8^+$ T cell populations of memory phenotype are phenotypically different from peripheral blood memory cells. LPL express CD45 (tyrosine phosphatase) and CD58 (= LFA3, ligand of CD2) antigens at high level (337) but relatively

few adhesion molecules like CD18 (integrin β2), CD29 (integrin β1), and CD44 (142, 337), indicating a lack of functional properties. In addition, $CD4^-$ $CD8^-$ and $CD4^+$ $CD8^+$ T cell subsets have been observed in the LP of pigs. LPL generally show a high degree of activation by expressing IL-2 mRNA, IL-2Rα, and surface expressed MHC class II antigen (183).
The presence of macrophages in the LP also underlines the possibility of primary responses (234). The LP contains as well DCs which process antigens and become mature cells capable of driving a T cell response in the LP.

Dendritic cells (DCs)

The main function of DCs is to capture antigens and to present them to lymphocytes. Three stages of development of DCs have been reported: 1) precursor DCs patrol through the blood and the lymph, 2) tissue-residing immature DCs capture antigens, 3) mature DCs present these antigens locally to LP T cells or within secondary lymphoid organs. Immature DCs are characterized by the expresion of mannose receptors, Fc receptors, and by the absence of molecules involved in T cell activation. Immature DCs express CCR6 and are then attracted by the chemokine CCL20 (MIP-3α)(13) produced by IEC in response to infection by enteric pathogens (281). DCs are able to sample the lumenal antigens directly by extending dendrites across the epithelial cell layer and by expressing tight junction proteins without disturbing the integrity of the monolayer (320). By this mechanism DCs can transport extracellular bacteria from the apical to the basolateral side of the epithelium. Mature DCs do not express Fc or mannose receptors anymore but high level of MHC class II and T cell activation molecules (CD86, ICAM-1, and IL-12). DCs process and present antigens and then orientate the response of T cells and drive their differentiation into helper, regulatory or cytotoxic T cells. For that purpose, DCs may migrate to T cells zones, mesenteric lymph nodes, or interact with memory cells.

2.3.3. Mesenteric lymph nodes (MLN)

Mesenteric lymph nodes, the largest lymph nodes in the body, contain B and T lymphocytes and some APC, such as macrophages and DCs. MLN are divided into a peripheral cortex composed of B lymphocyte-rich follicles and a central medulla. When responding to an antigenic stimulation, B lymphocytes of the follicles are activated, start to proliferate and produce antibodies. T cells expressing CD49d ($\alpha_4\beta_7$ integrin) also accumulate in the follicles,

multiply, differentiate after contact with an antigen, and migrate to the medulla. Cytotoxic and helper T cells then leave the node via efferent vessels towards other nodes and circulate to spread the protection specific for pathogens among the lymphatic net. MLN are considered crossroads between peripheral and mucosal recirculation pathways (265). Under physiological conditions, the presentation of food antigens in MLN leads to the induction of tolerance mediated by the induction of T_H3-type cells secreting TGF-β and IL-10 (58).

2.3.4. Intraepithelial lymphocytes (IEL)

2.3.4.1. General characteristics of IEL

In contrast to Peyer's patches and lamina propria, the small intestinal epithelium contains small amounts of B lymphocytes (271) and relatively few $CD4^+$ T cells. Newborn calves' small intestines have relatively fewer IEL than adult animals. In addition, the B cell number is significantly increased in the ileum of calves compared to adult animals (299). One lymphocyte per approximately five to ten epithelial cells is present in the small intestine, representing 10 to 15 % of the cells in the adult bovine intestinal epithelium. In cattle, IEL often co-express the αβ TCR with a CD8 molecule or the γδ TCR without any co-receptor (427, 428). The IEL, located on the basolateral side of the mucosa between epithelial cells, occupy a unique location at the interface between the epithelium and the gut lumen and are the first lymphoid population that encounter exogenous pathogens/antigens (441) and/or associated toxins in the whole body (266). IEL are distinct from systemic T cells and represent an unusual T cell compartment characterized as a large and heterogeneous population of lymphocytes containing resting, activated and memory cells derived from a limited number of T cell clones (34, 144).

In contrast to spleen, blood and lymph lymphocytes which are subdivided into MHC-class II restricted $CD4^+$ αβ TCR T cells and MHC-class I restricted $CD8\alpha\beta^+$ αβ TCR T cells, IEL comprise mainly $CD8^+$ T cells. Other parameters are required to classify the IEL. For this purpose, most of the investigations were performed in mice. Following a classification based on gene expression profiles and on TCR and co-receptors expression proposed by Hayday *et al.* (144), two major subsets of IEL can be segregated. The "type a" and "type b" IEL can be distinguished functionally as these populations become differently activated. While

"type a" IEL are activated by conventional MHC restriction, the "type b" IEL do not recognize these classical MHC molecules (144).

In addition to activation segregation, the "type a" mucosal T cells express αβ TCR together with CD4 or CD8αβ co-receptors. Relatively little is known about the functions of $CD4^+$ IEL which can be also divided into two subsets possessing both pro- and anti-inflammatory functions ($CD4^+$ $CD45Rb^{high}$ T_H1-like and $CD4^+$ $CD45Rb^{low}$ T_H2-like populations)(310, 311). Additionally, double positive (DP) $CD4^+CD8\alpha\alpha^+$ IEL were reported in the small intestine (110, 111, 206). The "type b" subset includes αβ TCR $CD8\alpha\alpha^+$ IEL and also contains γδ TCR $CD8\alpha\alpha^+$ and γδ TCR double negative (DN, $CD4^-CD8^-$) T cells. DN T cells can represent as 10 % of small intestinal IEL (144).

The bovine IEL population is composed of 3 to 5 times more $CD8^+$ than $CD4^+$ T cells (409). In cattle, IEL are predominantly "type a" cells expressing the αβ TCR and preferentially the CD8αβ heterodimer co-receptor in contrast to other species (31, 60). A high proportion of cells also expresses the bovine activation marker ACT2 ($ACT2^+$ $CD8^+$ αβ TCR and $ACT2^+$ γδ TCR cells)(10, 427). Most of IEL are activated mature T cells expressing CD3 (61.0 %), and CD6 (48.6 %)(248). Bovine IEL also express the MHC class II molecule (271) characteristic of activated cells pointing to an effector function of IEL *in vivo*.

Human IEL also express CD45RO or CD45RB and co-express CD11a, CD29 and CD58 constantly (168), suggesting a previously activated or memory phenotype. Moreover, high expression of Bcl-2^+ and the absence of CD95 (Fas) on human small intestinal IEL is supposed to render the cells relatively resistant to the activation-induced cell death (350). Freshly isolated murine IEL have a high level of MAP kinase-2 pointing to an *in vivo* activation state independent of exposure to bacteria (381). Investigations in mice revealed that the generation of the IEL repertoire is random and not governed by viable microbial flora, although certain food antigens could play a role (319). In other terms, all intestinal IEL subpopulations display characteristics of 'activated yet resting' immune cells (60).

2.3.4.2. Gammadelta (γδ) TCR intraepithelial lymphocytes

Described in the past as non-classic T cells, γδ TCR lymphocytes have gained in consideration in the recent years. A characteristic of mucosal surfaces is the relative abundance of "type b" IEL bearing the γδ TCR. This TCR is expressed by more than 37 % of human IEL, 30 to 50 % of murine IEL (309, 437), and the bovine intestinal IEL compartment

contains 13.4 to 25.1 % of γδ TCR bearing cells (248)(Moussay et al., submitted). The great majority of these γδ TCR cells express the co-receptor CD8 in calves (427). Analysis of $CD8^+$ γδ T cells demonstrated that they are involved in promoting quiescence, consistent with a role of sentinel in the mucosa. More generally, γδ TCR cells are highly conserved and are the first T cells to develop (104). They can differentiate from late foetal liver and adult bone marrow precursors and require the presence of IL-7, which has been shown to be a critical factor for the rearrangement and the expression of the TCRγ genes in mice and for survival of γδ TCR cells in the periphery (202). In cattle, γδ TCR cells generally do not express CD2, CD4, and CD6 (229) but are the only cell type in the organism to express the WC1 antigen, a member of the scavenger receptor cysteine rich (SRCR) family (418). $WC1^+$ γδ TCR cells have a T_H1-like cytokine profile characterised by production of IFN-γ and the lack of IL-4 (16).

Functionally, γδ TCR IEL respond to a relatively small range of stimuli, do not significantly proliferate, and do not recognize antigens presented in the context of classical MHC (62, 264). However, they can directly interact with self-MHC molecules not loaded with processed antigens (MHC class I-related molecules)(366).

Bovine blood γδ TCR $WC1^+$ cells stimulated 24 hrs by concanavalin A produce mRNA for multiple cytokines (IL-2, -4, -6, -7, -10, -12, -15, IFN-γ, TNF-α, TGF-β and GM-CSF)(297) and some cytokine proteins (IFN-γ and TGF-β)(324). Recently, bovine and human γδ TCR T cells of adults and neonates were shown to strongly respond to Pathogen-Associated Molecular Patterns (PAMPs) and to produce large amounts of cytokines (MIP-1α, 1-β, TNF-α, IFN-γ) and chemotactic factors for activated neutrophils, thus confirming the implication of γδ T cells in innate immunity. The TLR2 and 4, Nucleotide-binding oligomerization domain (NOD), in cooperation with CD11b/CD18, have been proposed to be at the origin of this strong response of γδ T cells (146). In addition, the interpretation that γδ TCR IEL use their TCR as a pattern recognition receptor led to suggest that these IEL act as a bridge between the innate and the adaptative immune systems (152).

2.3.4.3. Origin of IEL

Thymic and extra-thymic origin of IEL

After the migration of IEL precursors (TCR^-CD8^-) from the foetal liver and later the bone marrow, the process of IEL maturation is not fully understood. Several studies, performed in mice, underlined both thymic and extra-thymic origin of gut IEL subsets (134, 212, 218, 323). Most of αβ TCR DP IEL ($CD4^+CD8\alpha\alpha^+$) and αβ TCR $CD8\alpha\alpha^+$ IEL subsets appear to be of thymic origin (219). "Type a" IEL seem to be the progeny of circulating conventional T cells that had been positively selected in the thymus and already exposed to antigens in the GALT (9). Moreover, in absence of MHC class I, these IEL are not produced since the positive selection is blocked (115). Because the adult intestine is not a significant site for αβ TCR T cell development in normal conditions (81), αβ and some γδ IEL precursors from thymic origin were found to mature in the periphery and then home in the gut (208). Even if the majority of IEL is antigenically distinct from peripheral blood lymphocytes (PBMC)(350, 409), some "type a" IEL are phenotypically identical to mature thymus-derived T cells. In addition, T cell subsets of the ileal mucosa of naïve neonatal calves are different from those of adult cattle (300). Calf IEL also produce mRNA for the recombinases RAG-1 and RAG-2, which are critical for DNA recombination events that form functional Ig and TCR (228), indicating that a postnatal maturation of the gut mucosal IEL occurs *in vivo*. In any case, the development of intraepithelial T lymphocytes is regulated by the cytokines IL-2, IL-7, and IL-15, which bind to their respective receptors sharing the common gamma-chain (γ_c). The different activities of these cytokines assure the development of phenotypically diverse subsets of intestinal IEL (307, 308).

In chicken, only the thymus is an effective source of αβ and γδ TCR intestinal IEL and "type b" IEL originate in the thymus but acquire the expression of CD8αα homodimers in the gut microenvironment. The negative selection might occur in the intestine and then IEL colonize the gut where they are able to survive for months (157).

Murine and human small intestine IEL and rat γδ TCR IEL were reported to develop from cells of extrathymic origin (226, 315). Murine $CD8^+$ IEL can undergo selection in the absence of thymus as well. Interestingly, studies about the effects of stress, aging and thymus involution have shown that the extra-thymic production of αβ TCR IEL can supplant the thymic cell production in certain conditions. However, even if γδ TCR IEL are from extra-

thymic origin, they need to undergo a self-antigen β2 microglobulin-dependent selection process. A recent report indicated that the differentiation and maturation of γδ TCR IEL requires the presence of immature αβ TCR thymocytes (351).

The epithelium has been early proposed as a primary lymphoid organ (105) and to be a site of development for γδ TCR and CD8αα lymphocytes in adult animals (134). In fact, all prerequisites for cell development, maturation, and activation can be produced by IEC. Several hints reinforce the existence of distinct IEL origins. Epithelial cells produce the stem cell factor (SCF), express MHC-class I and II molecules (179), and the co-stimulatory molecule B7H (272). SCF binds to the tyrosine kinase membrane receptor c-Kit expressed only by the "type b" IEL (γδ TCR IEL and αβ TCR CD8αα$^+$ IEL)(201, 313). IL-7, produced by IEC, is also absolutely required for the development of all γδ TCR IEL.

In contrast to αβ TCR CD8αβ IEL, the development of murine αβ TCR CD8αα IEL in the intestine is promoted, following a Gram-negative bacteria infection, by the production of IL-15 by IEC in a TLR4-dependent manner (177).

A distal site to the intestine, such as the bone marrow, cannot be excluded from the list of potential site of extra-thymic T-cell development (210). Specifically reported in mice, the lamina propria can contain cryptopatches, clusters of lymphoid cells in the basal LP, rich in IL-7R$^+$ cells supposed to be progenitor T cells for extra-thymic descendants which can migrate to the IEL compartment (176, 327). In contrast to cryptopatches, lymphoid aggregations such as isolated lymphoid follicles (ILF) and Peyer's patches (PP) are not indispensable for the generation of IEL (294).

IEC chemokine secretion and IEL homing

IEL are thought to reside in the LP and MLN and to be attracted by epithelial-produced-IL-8 or MCP-1 in response to injury and bacterial or parasitic infections. Moreover, cultured intestinal IEL migrate into a polarized human epithelial monolayer while PBMC are incapable to do so (345). IEC are capable of producing a large panel of chemokines in order to attract IEL (434). In parallel, IEL are known to express several chemokine receptors (CCR1, CCR2, CCR5, CXCR3), mainly in the ileum (224). However, due to the redundancy of ligand-receptor, both α- and β-chemokines commonly use a limited number of receptors expressed by IEL (322). Consequently, IEL migrate upon antigen stimulation (186), and are attracted to the site of infection in response to both α- and β-chemokines produced by IEC. Alpha (α)-chemokines (IL-8, GRO family) are characterised by a C-X-C sequence while β-chemokines

have a C-C motif (MCP-1, MIP family, and RANTES). IEC also produce the chemokine CXCL10 (10 kDa-IFN-γ inducible protein, IP-10) and the monokine induced by IFN-γ (MIG) CXCL9 (79) which bind to CXCR3 expressed by freshly isolated IEL (349). In addition, CCR5 is an important component of the migration of intraepithelial $CD8^+$ T cells in response to parasite infection (224). Virtually, all IEL co-express the $\alpha_E\beta_7$ integrin (CD103) and the CCR9 (receptor for CCL25, or thymus-expressed chemokine [TECK] produced by IEC of the small intestine)(380, 425, 440). The association CCR9/CCL25 promotes the expression of CD103 on $CD8^+$ cells (91).

IEL can reside in the MLN where they express the $\alpha_E\beta_7$ integrin. After activation by PPs or MLN DCs (170, 260, 362), IEL down-regulate the expression of the $\alpha_E\beta_7$ integrin and then migrate towards the epithelium, via the bloodstream, by using the $\alpha_4\beta_7$ integrin. The $\alpha_4\beta_7$ heterodimer binds to an Ig-like domain in the mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) expressed by the endothelium in PPs and LP (25). After homing in the epithelium, IEL express $\alpha_E\beta_7$ again which then binds to E-cadherin expressed by IEC (56). Integrins are primordial components of IEL behaviours. After the migration, the interaction of IEC and IEL is important for the maintenance of IEL in the epithelium. More than 90 % of IEL express $\alpha_E\beta_7$ (55) and the intercellular adhesion molecule-1 (ICAM-1). The expression of the integrin $\alpha_E\beta_7$ is another marked difference between IEL and PBMC. In fact, 95 % of intestinal IEL express this heterodimer while only 5 % of PBMC bear this integrin. This molecule is significantly up-regulated on IEL following the production of TGF-β by IEC (348, 379). Similarly, the homing of IEL into the epithelium induces an up-regulation of MHC class I and II, ICAM-1, CD44, and increases the production of IL-8 and IP-10 by IEC (347). After homing, IEL are thought to be closely bound to epithelial cells and this cell-to-cell interaction down-regulates the proliferation of both αβ and γδ TCR IEL which stay in the mucosa as resting cells. In addition, the interaction with IEC down-regulates both T_H1- (IFN-γ and IL-2) and T_H2- (IL-4 and IL-5) cytokine production of IEL. In contrast, co-incubation of IEC has no effect on splenic αβ T cell proliferation, indicating very specific cell-to-cell interactions between IEC and IEL (430).

2.3.4.4. Principal functions of IEL

The several subsets of IEL express different surface antigens and possess special features in order to maintain homeostasis and to develop an adequate immune response in the gut. While

some IEL are dedicated to perform the immune surveillance of the epithelium (166) and to mount efficient immune response against pathogens, other contribute to the regeneration of the epithelial monolayer (homeostasis, tumor surveillance) and to the removal of damaged epithelial cells and/or cells exposed to toxic agents (257, 291)(Natural Killer (NK) properties).

IEL as effector cells of the immune response

Even if IEL are considered as part of the adaptative immune system, αβ TCR IEL are thought to be involved in the development of innate immune response to lumenal antigens (383). All the diverse populations of IEL show a phenotype of antigen-experienced lymphocytes (61, 204) characterized as "activated yet resting" immune cells (60). Mucosal $CD4^+$ T cells mainly secrete TGF-β and IL-10 (116). In addition to regulatory functions of αβ TCR $CD4^+$ and γδ TCR IEL, several studies confirmed that αβ TCR CD8αβ IEL possess cytolytic functions, have a memory function and are protective against bacterial (277, 306), viral (125, 187) and parasitic infections (133, 216). IEL from the small intestine have a more pronounced cytolytic activity *in vitro* than IEL from the large intestine (49). Through an enhanced cytotoxicity, IEL are more involved in protection against a challenge infection than against primary infection of *Cryptosporidium* (133). Alphabeta TCR CD8αβ IEL are also more potent cytolytic cells than CD8αα, exert an efficient protective activity *in vivo* against acute viral infection, and produce perforin and granzyme B mRNA in the small bowel 6 days post-infection (268).

Similarly to other species, bovine intestinal IEL possess cytotoxic activity against bovine coronavirus (BCV)-infected target cells and are able to inhibit the viral replication. This activity was enhanced by IL-2 and TNF-α. Ileal IEL (iIEL) have a better cytotoxic activity than lymphocytes isolated from other sites in the GALT or systemic immune system (124, 125). As murine IEL, bovine iIEL respond to *Cryptosporidium parvum* antigen *in vitro* and are then important in the host's response towards enteric infections (408).

Gammadelta TCR IEL of several species have cytolytic activity and are involved in the host response against a variety of intracellular pathogens (2, 167, 270). Gammadelta TCR IEL activation is not restricted by classical MHC molecules but can be induced by small bacterial antigens, MHC-class I-like molecules and several other ligands (174).

Soluble factors produced by IEL

An important feature of IEL is to locally produce cytokines and chemokines. In opposition to cytolytic functions, some IEL subsets possess suppressive functions. By producing IL-10, $CD4^+$ $CD8\alpha\alpha^+$ intestinal IEL can suppress a T_H1-induced intestinal inflammation (66). Activated γδ TCR IEL can secrete a large array of cytokines (IFN-γ, TNF-α, IL-2, IL-3, TGF-β, IL-4, IL-5, and IL-10)(19, 442). They perform surveillance and regulate the epithelium homeostasis (158) by producing keratinocyte growth factor (KGF), which stimulates proliferation (36) and regulates differentiation of epithelial cells (198). Moreover, γδ IEL regulate autoimmunity (267) and are a significant protective T cell population against colitis in a mouse model by aggregating at sites of epithelial cell damage, by producing TGF-β and IL-4, and by down-regulating $CD4^+$ $CD8\alpha\beta^+$ cells (suppression of T_H1-type immune response)(57, 158). Human small intestinal IEL spontaneously secrete the cytokines IFN-γ and IL-4 (53). These cytokines are probably involved in the normal homoeostasis of the intestinal mucosa. Disturbances in their secretion could play a role in the pathogenesis of gastrointestinal diseases.

Freshly isolated normal human IEL express mRNA encoding IL-1β, IL-2, IFN-γ, TNF-α and approximately 10 % of IEL produce IFN-γ protein, suggesting that IEL are immunologically active *in vivo*. Human IEL could be stimulated *in vitro* to secrete IL-10, TNF-α, and TGF-β proteins pointing to suppressive and cytolytic functions for IEL (19, 227).

Ileal IEL of neonatal calves express TNF-α and IFN-γ mRNA but no transcripts for the anti-inflammatory cytokines IL-4 and IL-10 were found (428). As PBMC, iIEL of 4-week old-calves produced IFN-γ in response to mitogens. Ileal IEL and especially γδ T cells also produced T_H1-type cytokines at the early steps of infection with *Listeria monocytogenes* in mice and rats, underlining the important role of iIEL in the host immune response.

Ileal IEL are also a potent source of chemokines (IL-8, MIP-1α, -1β, RANTES and lymphotactin) (35, 227) and are consequently thought to act as sensors to infections and to induce attraction of both cells of the innate immune response (monocytes, neutrophils) and lymphocytes.

Natural killer (NK) properties of IEL

Representing around 15 % of intestinal IEL in mice, some IEL subsets have natural killer activities. These cells are not restricted by the classical MHC system (52), and become cytolytic *in vivo* after exposure to antigens (207). Both murine αβ and γδ TCR IEL express

NK receptors and mediate cytotoxicity through perforin and Fas (135) against enteric murine coronavirus infected cells (52) and against enterocytes in a model of graft versus host disease (328). The rat IEL compartment harbours a large population of $CD3^-$ cells that function as NK cells, but display an activated phenotype and an unusual cytokine profile that clearly distinguishes them from splenic NK cells. In fact, all NK IEL express CD25 and spontaneously secrete IL-4 or/and IFN-γ whereas splenic NK cells do not (394). Several groups already reported the presence of a $CD3^-$ $CD7^+$ subset of IEL in the human small intestine representing around 10 % of the cells and expressing the NK markers CD161 (P-selectin glycoprotein ligand, PSGL-1) and CD122 (IL-2Rβ) (87, 168). Variable percentage of IEL express CD94, CD56 (cell adhesion), CD16 (Fcγ RIII), and contain perforin granules. However, these IEL do not express CD18 (integrin β2) and CD44 (cell adhesion) indicating limited capacity of migration (86). Similarly to other species, bovine iIEL have NK activity against the bovine lymphoma cell line BL-3 (248).

Implication of IEL in the regeneration of the epithelium

In contrast to αβ TCR IEL, γδ TCR IEL perform regulation of the homeostasis of the epithelium (106, 158, 166), but as well tumor surveillance, and removal of epithelial cells exposed to toxic agents (257, 291). Ileal IEL promote the repair of epithelial lesions to maintain intestinal integrity. Gammadelta TCR IEL possess electron-dense granules and are closely associated with apoptotic enterocytes in the small intestine of cattle (229). Damaged or infected intestinal epithelial cells express the MHC-like molecules MIC-A and MIC-B (131) which are ligands for the type C lectin NKG2D expressed on the surface of γδ TCR IEL in the GALT. Thereafter IEL lyse damaged cells in the mucosa (22, 131).

Several other types of IEL are involved in regeneration of the epithelium. $CD4^+$ $CD8\alpha\alpha^+$ IEL are able to respond to self-MHC class I expressed on epithelial cell surface, and to express CD178 (Fas ligand) which induces apoptosis of IEC via Fas-dependent pathway (159, 437). Both αβ and γδ TCR IEL were also recently described as a potent source of angiotensin converting enzyme (ACE)(419), which is known to have a significant role in promoting apoptosis in epithelial cells (355, 407).

After induction of apoptosis by IEL, epithelial cells are eliminated by LP dendritic cells to the mesenteric lymph nodes. However, the induction of IEC apoptosis implies to maintain the selective permeability of the epithelium. A recent study reported a novel function of IEL which contribute to the barrier function of the epithelial surface (160). Murine IEL from the

small intestine express junctional molecules like IEC. Zonula occludens (ZO)-1, occludin and junctional adhesion molecule (JAM), β-catenin and E-cadherin mRNA were found in IEL, which constitutively expressed occludin and E-cadherin at the protein level (160).
Activated γδ TCR IEL regulate the homeostasis of the epithelium by producing the keratinocyte growth factor (KGF, also known as SDGF-3 in cattle (378)) and TGF-β. KGF is a specific mitogen for many epithelial cells but not for fibroblasts and endothelial cells. KGF stimulates the proliferation (36) and regulates the differentiation (198) of epithelial cells. TGF-β is a well-known growth factor for intestinal epithelial cells (18). The proliferation of IEC *in vivo* is reduced in the absence of γδ TCR IEL (198).

2.3.4.5. IEL apoptosis

The intestinal mucosa is chronically exposed to an abundance of dietary antigens and exogenous pathogens, and this continuous antigen challenge should constantly activate IEL and provide a constant state of inflammation. As IEL are activated *in situ*, the homeostasis of the mucosal immune system is strongly regulated (263). A high level of apoptosis contributes to maintain IEL homeostasis by limiting the proliferation of activated T cells and clearing primed lymphocytes. In physiological conditions, 25 % of human duodenal IEL were quantified as apoptotic (71).
Investigations relative to IEL functions are further complicated by the high apoptosis rate of freshly isolated and *in vitro* cultured cells. The close association of IEL with epithelial cells appears to be the key mechanism of IEL survival *in vivo*. In absence of any stimulation, 60 ± 16 % of IEL were apoptotic (annexin-V^+) after 6 hrs of incubation and very few cells survived overnight (70). Cytokines, such as IL-7 and IL-15, produced by epithelial cells are known to enhance IEL survival *in vitro* (159), and to regulate IEL-T_H1 cytokine production and cytotoxicity (70). However, stimulation of IEL by mitogens or activation through the TCR can compensate the lack of these survival promoters by increasing the expression of Bcl-$\times_L$. In addition, the induction of IEL death has been shown in mice to be regulated, at least partially, by endogenously produced glucocorticoids (46).

2.4. Shiga toxin-producing *Escherichia coli* (STEC)

The STEC denomination for Shiga-toxin-producing *Escherichia coli* (*E. coli*) was adopted following the proposed classification of Calderwood *et al.* (47), due to the biological relation of the *E. coli* Shiga-like toxin to the Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1. More than 400 serotypes of *E. coli* can produce Shiga toxins and are called Shiga toxin-producing *E. coli* (STEC) or Verocytotoxin-producing *E. coli* (VTEC) due to a high toxicity of the Shiga toxin to Vero cells (199, 284, 439). The term EHEC (enterohemorrhagic *E. coli*) denotes a subset of STEC and includes a clinical connotation. EHEC is used to denote strains, as *E. coli* O157:H7, that cause haemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC), express Stx1 or 2, and cause Attaching and Effacing (A/E) lesions on epithelial cells (273). In contrast to STEC, all EHEC strains are believed to be pathogens for humans. STEC strains are found in the intestinal and faecal flora of several animals including sheep, cattle, goats, pigs, cats, dogs, chickens, and gulls (273, 301), and are often at the origin of food or waterborne infections of humans.

2.4.1. STEC infections in humans

STEC infections are a major cause of bloody diarrhoea, HUS, and HC in humans. HUS is characterised by an acute renal failure and a risk of persistent kidney damage (23) but other organs can be affected such as the brain and the pancreas. Due to lack of curative therapy to these diseases (395), diarrhoeagenic *E. coli* have been recently included on the Biodefense Research Priority Pathogens Category B list of the National Institute of Allergy and Infectious Disease (NIAID, USA) and are so considered to be of important public health concern. STEC were categorized as human pathogens following two major outbreaks (321, 413). The dominant STEC type most commonly implicated in large outbreaks in the United States, Canada, and the United Kingdom is O157:H7 but the serogroups 026, O103, O111, and 0145 are also prominent in many european countries (42, 178, 416). STEC strains can be transmitted by water, vegetables or directly from person to person. Fruits (apples and cider)(343), vegetables (melon, lettuce, radish sprouts) and drinking water were also reported as causes of infections in humans. Person-to-person and contamination after farm and zoo visitations are common causes of EHEC infections as well (178). A potential airborne transmission after exposure to a contaminated building was also recently reported by Varma *et al*. (402). Due to the relative long time of *E. coli* survival in the environment (more than 10

months), there is still a significant risk of infection for humans even a long time after the initial contamination of an environment. In addition, it has been shown that *E. coli* 0157:H7 can multiply within house-flies, which can then spread the bacteria in the environment (194, 336). However, most of the cases reported are caused by ingestion of unpasteurized milk and undercooked contaminated meat, mainly of bovine origin (273).

2.4.2. STEC infections in cattle

2.4.2.1. Epidemiology

Cattle appear to be the main reservoir of Stx-producing O157:H7 (37) and is the most important source of STEC strains pathogenic for humans (27, 114, 233). More than 120 different O:H types have been isolated in cattle. A recent survey of healthy cattle in Switzerland reported that the majority of bovine STEC strains isolated (90 %) belonged to five serotypes previously reported in association with HUS, including the O157:H7 serotype (28). In addition, Ont:H^- and ONT:H25 (non-typable) are present at high frequency in healthy cattle in Australia and North America (346).

In adult ruminants, STEC are part of the normal gastrointestinal flora of healthy cattle, sheep, and deers. A high prevalence of STEC in cattle herds is reported from different countries worldwide (69, 77, 214). The reasons of the wide distribution of STEC in ruminants are not known but eventual benefits arising from colonisation by STEC are possible. Ferens and Hovde (103) reported that STEC strains, by producing Shiga toxins, can slow down the Bovine Leukemia Virus (BLV) -induced proliferation of bovine PBMC. Additionally, Stx1 is implicated in diarrhoea of calves (233)(see also **2.5.5.**).

2.4.2.2. Tropism, persistence and shedding

O157 and non-O157 strains appear to have strikingly different tropisms. After an experimental challenge by STEC strains, of whom O26, bacteria could be recovered from all intestinal sites (399). In opposition, the initial binding of *E. coli* O157:H7 occurs in the follicle-associated epithelium of PPs within the small bowel in humans (302) and at the recto-anal junction in cattle (274). This tropism for the mucosal epithelium of lymphoid follicles of

the recto-anal junction enables a longer colonization and a prolonged duration of shedding in the faeces (223, 274).

STEC infections in cattle show two different age-dependent patterns. The infection of calves or neonates by STEC strains (O5, O26, O111, 0123, O157) may lead to development of diarrhoea (256), production of mucopurulent exudate in calves' ileum and colon (331), and enterocolitis with formation of A/E lesions in small and large intestines (68, 374). STEC O5, O26, and O118 strains are often found to attach to the epithelium of the large intestine (372) (extensive adherence and A/E lesion), in part explaining the implication of these strains in diarrhoea in farm animals (399). However, O157:H7 strains are not pathogenic in adult animals in which the infection is persistent but mostly asymptomatic. In addition to A/E lesions observed in the intestine of experimentally infected calves (68, 69), *E. coli* O157:H7 adhere to and form A/E lesions on the intestinal mucosa of adult bovine biopsies *in vitro* (302) and of naturally colonized adult animals *in vivo* (274, 275).

Three different patterns of shedding were observed in ruminants: 1 week, 1 month, and 2 months or more, suggesting that O157:H7 can persist a long time in the gastro-intestinal tract of ruminants (127). STEC serotypes involved in human diseases, including O26, O91, O103, and O111, can survive a long time in bovine faeces after shedding too and are a possible source of further contamination (113).

2.4.2.3. Colonization factors

Virulence factors differentially utilized by O157:H7 and O26

STEC strains from different serotypes possess different virulence factors involved in the colonization. Non-O157 STEC strains (i.e. O26) utilize different virulence factors but apparently no type I fimbriae to colonize the intestine of calves (399). In contrast to O157 strains in which the removal of the large plasmid containing the enterohemolysin EhxA and the *E. coli* secreted protein EspP has no effect on the pathogenesis of O157 in piglets (396), EhxA plays a role in O26 infection of calves (399). O26 strains interact with IEC, inducing an acute inflammatory response. In order to resist to the immune cells, O26 strains produce type III secreted proteins (EspA, EspD) and cytotoxins (EhxA, serine protease PssA) to inactivate the host immune response (399).

Several genes were recently reported to be commonly used by *E. coli* strains and to be particularly important for STEC colonization and survival in the bovine intestinal tract and persistence in water. In fact, mutations of *ecf* operon (*E. coli* attaching and effacing gene-positive conserved fragments) and *lpxM* gene (lipid A myristoyl transferase) altered the motility and the survival of the bacteria in the bovine intestine (435). The putative structural component of the type III secretion apparatus (EscN), the non-LEE-encoded type III secreted effector D (NleD), and EspI/NleA (non-LEE-encoded effector A) were identified as essential for intestinal colonization (80, 132, 269). The locus of enterocyte effacement (LEE) is a pathogenicity island (PAI) located on a 35-45 kb fragment of the chromosomal DNA. LEE possesses more than 50 genes and ORF coding for translocated proteins and a type III secretion system and associated chaperones and is highly controlled by the quorum sensing-regulated protein Ler (360).

The *E. coli* factor for adherence (Efa1) is then required for an efficient colonization of the bovine intestinal tract by O26 STEC, since *efa1* deletion and insertion mutants are shed in the faeces in significantly lower numbers (372). The *efa1* gene is identical in size and 99.9 % identical in nucleotide sequence to the lymphostatin *lifA* gene of enteropathogenic *E. coli* (EPEC). The EPEC *lifA* and STEC *efa1* genes encode predicted proteins of 366 kDa that are 97.4% identical at the amino acid level. The EPEC LifA inhibits the proliferation and the mitogen-induced cytokine synthesis (IL-2, IFN-γ, IL-4) of human PBMC (192) and of human and murine IEL, indicating an influence in intestinal colonization by modulating mucosal immunity in the gut.

While *E. coli* O157:H7 lacks the full-length *efa1* gene, a large gene (*toxB*/*l7095*) with significant homology to *efa1* also exists on the *E. coli* O157:H7 pO157 virulence plasmid. Efa-1 and ToxB indirectly influence the adherence by modulating the production and secretion of LEE-encoded type III secreted proteins EspA and Tir (372) that are required for the formation of A/E lesions.

2.4.2.4. Establishment of Attaching and Effacing (A/E) lesions

The initial step of the infection is the adherence of the bacteria to the surface and to the microvilli of the host epithelial cells followed by the development of the characteristic Attaching and Effacing (A/E) lesion. STEC are considered extracellular pathogens which intimately attach to the epithelial cell surface. The colonisation of the gut, formation of A/E

lesions and establishment of the disease in calves require the presence of a bacterial protein, the intimin. O157:H7-infected calves developed watery diarrhoea after 18 hrs and the enteritis could lead to the death of animals. The primary factors required for the initial adhesion to the cells of the mucosa are still unknown (233) but trigger the expression of several genes located on the LEE on the chromosome. The type III secretion system induces rearrangement of the cytoskeleton of the target cell, then leading to effacement of the microvilli, and polymerisation of actin. The bacteria create a more intimate adhesion to the epithelial cells by expressing and injecting specific proteins into host cells. STEC then induce the activation of the phosphatidylinositol (IP_3) cascade in epithelial cells leading to an increased concentration of intracellular Ca^{2+}, an important second messenger in cell signalling (163). The intimin encoded by the LEE gene *eae* is the major factor of this tight attachment (74) and is required to allow the persistence of a non-toxigenic O157:H7 *E. coli* strain in the bowel of sheep (424). The intimin is a type II outer membrane protein (OMP) binding to its receptor Tir (Translocated intimin receptor), a bacterial protein injected into the host cells by the type III secretion system. STEC translocate both their own receptor (Tir) and an Nck-like protein called TccP (Tir-cytoskeleton coupling protein) to facilitate the polymerization of actin (117). The binding intimin-Tir amplifies cell cytoskeleton modifications leading to the formation of a pedestal enabling the settlement of the bacteria.

The A/E lesion caused by EHEC is not directly responsible for the diarrhoea in humans. However, the cytokine-induced inflammation, the opening of the tight junctions, the increase of paracellular permeability, or modification of ions absorption have been showed *in vitro* as consequences of bacterial adherence and proposed as more potent causes of the diarrhoea *in vivo*.

2.5. Shiga toxins (Stx)

Even if an asymptomatic carriage of STEC O157:H7 was eventually found in specific cases of farmers or their relatives (352), STEC strains are pathogenic for humans due to the presence of the Shiga toxins (Stx), which are the main virulence factors of STEC. The *E. coli* Stx was first identified in 1983 by Karmali *et al.* (181) in stools of sporadic HUS patients. STEC can produce several toxins designated Stx1, Stx1c, Stx1d, Stx2, Stx2c, Stx2d, Stx2e, Stx2f. A total of 11 variants of Stx2 have been described yet. Several subvariants of Stx2d

have also been identified. In particular, *E. coli* Shiga toxin 1 (Stx1) has been identified as a virulence factor both in human (287) and in cattle (251).

2.5.1. *stx* genes structure

The *stx1* and *stx2 A* and *B* subunits genes exist as a tandem Open Reading Frame (ORF) coding for A and B subunits transcribed from a 5' iron-regulated promoter to the *stxA* gene (339, 412) in the central position of lambdoid bacteriophages in *E. coli* STEC strains (153, 164, 276, 288). These phages are present in two different states, alternatively "lysogenic" and "lytic", and possess transcription units for various functions, such as replication and lysis. During the lysogenic period, the genome in incorporated into the bacterial chromosome and is replicated by the host machinery. In contrast, during the lytic phase, the phage excises itself, replicates and produces the Shiga toxin. The entry into the lytic phase induces the death of the bacteria spreading Stx in the lumen of the intestine. The toxin production is coupled with the phage release during lytic growth because *stx* genes are found in the late operons of the phages (305) along with lysis genes downstream of the Q transcription activator which modifies transcription complexes initiating at the late promoter p_R' (276). In addition, several promoters can contribute to *stx1* transcription, such as the iron-regulated promoter p_{Stx1} and the phage promoters p_R regulated by prophage induction (404). Phage-inducing agents such as mitomycin C are known to increase Stx production by *E. coli* (4).

2.5.2 Stx 1 protein structure

Shiga toxin 1 from *E. coli* is part of a family of heterodimeric toxins composed of the Heat-labile enterotoxin of *E. coli*, the cholera toxin of *Vibrio cholerae*, ricin, and several other plant toxins. Stx1 and Stx from *Shigella dysenteriae* are the most similar members of the family (99 %)(286). Their B subunit is identical (48, 67) and only one amino acid in their A subunit differs between these two toxins (Ser45 for Stx *vs* Thr45 for Stx1)(375). Stx1 and Stx2 proteins have been reported to be 56 % identical in amino acid sequence and are immunologically distinct (286).

Each molecule of Stx is composed of one A subunit (315 amino acids) non-covalently linked to a doughnut-shaped pentamer of B subunits (89 amino acids each)(67). Studies to understand the regulation of the stoichiometry of Stx subunits (1A:5B) revealed that this

regulation takes place on both transcriptional and translational levels. A second promoter for the B subunit, a Ribosome Binding Site (RBS)(136), and secondary structures in the mRNA surrounding the RBS lead to the over-expression of the B-subunit.

The StxA subunit (32 kDa) is, after cleavage, enzymatically active in the cytosol of the target cells. The processing of StxA is performed by a serine protease, the enzyme furin (118, 119) which is located in the Golgi apparatus and the endosomes. The proteolytic cleavage of Stx is important for the activation of the toxin and facilitates rapid intoxication of target cells (334). StxA is processed into a 27.5 kDa A1 fragment (enzymatically active) and a small A2 fragment (3 kDa) that blocks the catalytic centre in the intact A subunit and mediate the A-B association (11).

Each B subunit of Stx1 (7.5 kDa) is composed of two three-stranded anti-parallel β-sheets which are linked together through hydrogen bonds and form a β-barrel. This β-barrel is then capped by an α-helix located between β-strands 4 and 5 of each monomer (365). The B subunit of Stx1 is responsible for the binding of the toxin to the glycosphingolipid Gb_3/CD77. The affinity of binding of Stx is related to the presence of a phenyl ring of phenylalanine-30 in the StxB subunit and to the presence of a terminal galactose in Gb_3/CD77 (221).

Ling *et al.* (220) and Bast *et al.* (20) showed that three biologically relevant Gb_3-binding sites are present on each StxB monomer (3 × 5 for the holotoxin). Even if the site 1 and 2 are more physiologically significant, this multiplicity enhances the avidity of the B pentamer for the cell membrane surface (358). Functional studies confirmed that the uptake of the toxin mediated by sites 1 and 2 is important for both toxicity and cytokine production, while site 3-mediated uptake plays an auxiliary role (423).

The release of Stx by the bacteria happens in the lumen of the intestine. However, StxA1 has the same toxic effect on bacterial ribosomes (Inhibitory concentration for 50 % of the ribosomes IC_{50} = 0.8 nM) than on eukaryotic ribosomes (IC_{50} = 1 nM)(377). Consequently, Stx is found in vesicles released by *Shigella dysenteriae* (78). Similarly, *E. coli* 0157:H7 produce spherical vesicles (20-100 nm) composed of an intact membrane bilayer carrying DNA and proteins (197). This way of secretion of Stx1 allows a protection of other bacteria, from exogenous proteases, and can be an effective mechanism for the transfer of the toxin directly to target cells of the mucosa. Consequently, Stx can be detected in faecal samples of STEC-infected human (180, 386) and cattle (17, 156). After being released, the toxin is absorbed through the mucus and the intestinal epithelial barrier.

2.5.3. Gb_3/CD77 as a receptor and cellular processing of the toxin

The toxin binds to the globotriaosylceramide Gb_3/CD77 (αGal(1-4)βGal(1-4)βGlc-ceramide), a neutral glycosphingolipid without a cytoplasmic domain (195) expressed on the surface of different types of mammalian cells (109). Gb_3/CD77 is a marker for the germinal centre stage of B cell development (14) and is also a Burkitt's lymphoma associated antigen (285). The expression of glycosphingolipids and consequently the susceptibility to Stx occur as a function of the cell cycle. Pudymaitis and Lingwood showed that stationary phase cells are resistant to the toxic effects of Stx (314).

All the physiological functions of Gb_3/CD77 *in vivo* are not yet known. However, glycosphingolipids are involved in signal transduction pathways and cell surface-associated mechanisms. Gb_3/CD77 seems to be specifically required for the interferon-induced inhibition of B cell growth (236). It has also been suggested that Gb_3/CD77 may act in the intracellular transport of proteins such as CD19 (185), a member of a complex involved in modulating the signal transduction through the BCR. CD19 possesses StxB-like regions potentially explaining the interaction with Gb_3/CD77 (220, 237) and the retrograde transport into the cells (185). As a Stx-like amino acid sequence was found on the β-chain of human and murine MHC class II molecules, Gb_3/CD77 is thought to be involved in the interaction of the antigen and the MHC molecule. Gb_3/CD77 could therefore play a functional or regulatory role in MHC class II-mediated functions specifically related to antigen presentation by B-cells to T helper cells in humans (121).

In humans, Stx mainly exerts its cytotoxicity on renal glomerular and vascular endothelial cells (289). Both cell types express the toxin receptor Gb_3/CD77 on the cell surface (38). The Stx-induced damage of renal endothelial cells and the induction of apoptosis by decreasing the expression of anti-apoptotic protein (93, 94) are the central steps of the pathogenesis. To reach target cells so far from the primary site of infection, Stx can circulate in the human blood bound to B-cells, monocytes, polymorphonuclear leukocytes (PMN), and erythrocytes (318). The potential implication of PMN was suggested by an increase in PMN number during the HUS. Stx bind to PMN and can be then transferred to human glomerular endothelial cells (389).

Stx are also able to cross through human intestinal epithelial Caco-2 and T84 cell lines without destroying or altering the functions of the thigh junctions (3, 154, 155). However, Stx can indirectly increase the permeability of the tight junctions by inducing chemokine production by IEC leading to intense migration of PMN. Other bacterial strains are able to

induce such a trafficking of PMN across polarized intestinal epithelial cells. *Shigella flexneri* increases the migration of PMN and this plays an important role in the early stages of infection by opening the paracellular pathway (244).

In cattle, the Stx receptor is present on the surface of intestinal epithelial cells, kidney cells (tubules and collecting ducts)(148), bovine peripheral lymphocytes, and intestinal IEL (248, 254). However, bovine endothelial cells from kidney glomeruli and intestine and bovine granulocytes lack Gb_3/CD77 expression (312)(Menge et al., submitted). This observation contributes to explain the discrepancies between human and cattle concerning the disease and the absence of vascular damages due to the toxin during STEC infections in adult cattle.

The presence of cholesterol in the lipid bilayer significantly enhances the binding of Stx1 to Gb_3/CD77. Interestingly, human intestinal epithelial cells which lack Gb_3/CD77 can still take up Stx1. Then a transcellular mode of transport was confirmed by observation of the toxin in endosomes and associated with specific targets including the trans-Golgi network (TGN), the endoplasmic reticulum (ER), and the nuclear membrane (303). Thus, a Gb_3/CD77-independent retrograde transport route exists in T84 cells for Stx1 that does not induce cell damage (340).

After binding to Gb_3/CD77, Stx can be endocytosed by the clathrin-coated pits pathway (333) or independently of clathrin (98) and can reach the Golgi apparatus by a non-conventional retrograde transport via the endocytic early/recycling compartment and then through structures containing the γ-adaptine AP-1 (235). By this mechanism, Stx avoid to cope with the degrading environment of the late endosomes. Stx was the first molecule to be shown to be transported from the cell surface to the Golgi apparatus and the endoplasmic reticulum by retrograde transport (332) related with cholesterol-rich microdomains (lipid rafts) in HeLa cells (98).

The StxB subunit can also use a Rab6-dependent COP-I-independent retrograde transport to be delivered from the TGN to the ER (123). Once in the ER, Stx binds to HEDJ (Hsp40 chaperone), Sec61 (central core of the ER translocation channel), and to BiP (ER-localized chaperone), composing the translocon which is involved in retrotranslocation of proteins to gain access to the cytoplasm (97, 438).

However, in some resistant cells, Stx1 can be differently processed. In contrast to what is observed in human IEC which lack Gb_3/CD77, bovine crypt epithelial cells of the small and large intestine express Gb_3/CD77 on the cell surface (148) but escape Stx-mediated cell toxicity by routing the endocytosed toxin to the lysosome rather than to the endoplasmic reticulum (149).

2.5.4. Mode of action of Stx

2.5.4.1. Damage of nucleic acids

Using the transmembrane protein complex, Stx is released from the ER in the cytosol, cellular compartment in which the StxA1 subunit exerts its cytotoxic activity by catalysing the same depurination reaction as ricin does (90). Stx target the 28S rRNA of the 60S ribosomal subunit (89) by cleaving the N-glycosidic bond and by specifically removing the adenine A-4324 in ribosomal RNA (90). In addition, Stx inhibit the elongation factor (eEF-1 and eEF-2) -dependent binding of aminoacyl-tRNA to ribosomes (in particular of Phe-tRNA), and consequently irreversibly inhibit the protein elongation (290).
Recent evidences also indicate that Stx can directly damage DNA (removal of adenine moieties), inhibit the DNA repair, and can sometimes cause transformation-like changes in mammalian cells (344).

2.5.4.2. Induction of apoptosis

Apoptosis is very important physiological process indispensable to allow a coherent development and to maintain the homeostasis of the organism. This highly regulated process is first composed of three manifestations as the loss of mitochondrial membrane potential, the activation of the caspases, and the internucleosomal DNA fragmentation. Then the so-called “flipping” of phospatidylserine from the internal to the external side of the cellular membrane happens. The last steps of the apoptotic process are the permeabilization of the nuclear membrane, the vacuolation of the cytoplasm, the collapse of the cytoskeleton, the blebbing of the cell membrane, and the final formation of apoptotic bodies (59).
In contrast to necrosis, no inflammation is observed during apoptosis. Consequently, some pathogenic agents or associated toxins can specifically induce apoptosis of target cells to decrease or slow down the immune response in order to ease the bacterial infection. Stx1 shares this ability with several toxins from bacteria. These toxins are part of the large group of RIPs (ribosome inactivating proteins) which share similarities in cell processing (entry and transport) and mode of action to exert their cytotoxicity.

The ability of Stx1 holotoxin or StxB1 subunit to induce apoptosis has already been extensively studied on several cell lines as Burkitt's lymphoma cell lines (182, 189, 240, 241, 261, 382, 391), ACHN, THP-1, and astrocytoma cells (8, 196, 384). In Ramos cells, Stx1 holotoxin induced a caspase-dependent apoptosis very efficiently after 18 hrs of incubation (241). Stx1-mediated apoptosis is Fas- and TNFR-independently mediated. Anti-Fas antibody or TNF-α do not affect the Stx1 mediated apoptosis of Ramos cells. Stx1 can induce apoptosis by triggering the signaling cascade linked to Gb_3/CD77 (189) in a caspase and mitochondria-dependent pathway (391). StxB1 and the cross-linking of Gb_3/CD77 with antibody trigger as well apoptosis in Burkitt's lymphoma B cells (240) but not in Vero cells (420).

Stx also induce apoptosis in a dose- and time-dependent manner of the human epithelial cell lines Hep-2, Caco-2, HeLa expressing Gb_3/CD77 (63, 112, 172). Apoptosis was shown to be induced by the caspase 3 and the activation of stress-activated kinase cascade JNK/SAPK and p38 (357).

Stx induce apoptosis of endothelial cells isolated from various sites, which appear to be very sensitive to low amounts of toxin (10-100 fM). The Stx holotoxin inhibits the expression of the anti-apoptotic FLICE-like inhibitory protein (FLIP, inactive homologous of caspase 8) and of Mcl-1, a member of the anti-apoptotic Bcl-2 family (93, 94). The Stx-induced cell death also involves the pro-apoptotic Bak factor (421). The StxB subunit also induces apoptosis of endothelial cells in the nanomolar range of concentration (304).

Human endothelial cells are resistant to LPS-induced apoptosis. However, a pre-treatment with reduced amounts of Stx1 increases the sensitivity of the cells to apoptosis mediated by LPS (94). In addition, the fact that pro-inflammatory cytokines such as TNF-α also contribute to the vascular damages (304, 316) suggests that cytokines, Stx1 and LPS may act in concern *in vivo* to induce endothelial cell death in humans.

2.5.4.3. Induction of ribotoxic stress response and cytokine expression

Stx1 treatment of monocytic THP-1 cells induced a ribotoxic stress response and the activation of the transcriptional factors NF-κB and AP-1 (329). IL-1β and TNF-α are produced by macrophages when stimulated with purified Stx *in vitro* (317, 390, 401). Without inducing apoptosis, Stx enhances the production of TNF-α and GM-CSF from human peripheral blood monocytes in a p38 MAP kinase (mitogen-activated kinase) and ERK (extracellular-regulated kinase) dependent pathway. Investigations with the

monocyte/macrophage THP-1 cell line *in vitro* revealed that the enzymatic activity of Stx1 was required (140). Several chemokine mRNAs were induced by Stx1 in THP-1 cells but a poor correlation was found with the amount of secreted proteins (IL-8, MIP-1α, -1β, and GRO-β) confirming a post-transcriptional regulatory effect (139). Stx1 increased both TNF-α mRNA and protein production *in vitro* by triggering the transcription factors NF-κB and AP-1 complex pathways (329) but was a poor inducer *in vivo* in mice (423).

Human vascular endothelial cells are the main targets of Stx1 in the course of the disease in humans. The surface expression of the receptor Gb_3/CD77 can be increased by endogenous cytokines including TNF-α, -β and IL-1β (398). However, renal and intestinal endothelial cells do not produce TNF-α themselves.

As Stx does not induce apoptosis in human PMN (356), activated PMN and monocytes produce cytokines (IL-1, TNF-α) inducing the expression of Gb_3/CD77, VCAM-1, and ICAM-1 by glomerular endothelial cells (262), then leading to the adhesion of PMN to the endothelium and inflammation. Similarly to Stx1, Stx2 triggers leukocytes' adhesion and transmigration via a NF-κB dependent up-regulation of IL-8 and MCP-1 (443). An enhanced adhesion to endothelial cells and degranulation of PMN has also been described *in vivo* in HUS patients, associated with increased levels of IL-6, IL-8 and TNF-α in serum. Stx increase the formation of superoxide in PMN but fail to increase the formation of oxygen radicals. Moreover, PMN exposed to Stx1 have both reduced bacterial phagocytosis and responsiveness to mitogens (188).

In addition to endothelial cells and PMN, glomerular epithelial cells are early targets of Stx and release pro-inflammatory cytokines, vasoactive factors and enzymes contributing to spread the damages in humans (258). Stx induce the production of the chemokine IL-8, GRO-(α, β, γ) mRNA, and both ENA-78 mRNA and protein by intestinal epithelial cell lines (393). The interaction between Stx and its receptor induces the activation of the protein kinase p38 (392), and expression of the primary stress response genes *c-jun* and *c-fos* in IEC (357), indicating the importance of the ribotoxic stress response pathway. Stx1 also induces the stabilization of IL-8 and GRO-α mRNA in epithelial cells and this induction by Stx require the enzymatic activity of the toxin (432).

Taken together, in addition to clearly inducing cytotoxic effects on target cells, Stx1 triggers other cell types to produce mediators which then participate to up-regulate the endothelial expression of Gb_3/CD77 (259) and to spread the vascular damages.

2.5.5. Effects of Stx in cattle

The majority of strains isolated from calves with diarrhoea are positive for *stx1* (233). Several reports confirmed that Stx is produced in the intestine of cattle and can be recovered in faeces from bovine (17, 156) and is consequently thought to influence the colonization of the intestine by STEC strains (148). In opposition to the implication of Stx in the development of diarrhoea observed in humans, pigs, and rabbits, the significance of Stx in the intestine of cattle is different. In fact, Stx is not indispensable for the ability of STEC strains to colonize (399) and to induce diarrhoea in neonatal calves (69). Moreover, Stx-negative O157:H7 strains could induce diarrhoea and colonic oedema in neonatal calves independently of A/E-lesion, intimin, or Stx1 (368). In addition, cattle lack the vascular receptor for *E. coli* Shiga toxins and this leads to a lack of vascular damage in adult cattle (312). Stx1 does not bind to blood vessels of intestinal organs and viable *E. coli* O157:H7 or Stx-containing bacterial extracts are not enterotoxic in ligated ileal loops in newborn calves *in vivo*.

Nevertheless, Stx1 has been confirmed as a virulence factor in adult cattle (251) and to suppress functions of bovine lymphocytes which express a functional receptor (249, 253, 363). In fact, Stx1 holotoxin and B subunit bind to stimulated bovine PBMC (B and $CD8^+$ T cells) which are highly sensitive to Stx1 (363). In contrast to human, both B and T cells are affected by the toxin in cattle, suggesting a more broad impact of Stx on the bovine immune system. Stx1 blocks the mitogen-induced activation and proliferation of bovine lymphocyte subpopulations without inducing apoptosis (254), independently of IL-2, TNF-α and IFN-α *in vitro* (249), and *in vivo* (151). Stx1 also specifically blocks bovine leukaemia virus (BLV) - dependent initiation of bovine lymphocyte proliferation in culture (103). BLV-infected B cells are selectively eliminated from cultures and this effect of Stx1 requires the enzymatic activity of StxA1 (102) which reduces the viral protein synthesis, interrupts virion assembly, and induces cell death (21). Stx1A inhibits the production of the viral p24 protein by bovine PBMC. Both Stx1 and 2 exert an antiviral activity and limit the BLV infection in cattle by slowing the progression of the infection to its malignant end stage (21).

Recently, intestinal intraepithelial lymphocytes, the first immune cells to gain contact with the toxin in the whole body, have been shown to express Gb_3/CD77 and to be the main target of Stx1 in the bovine gut (248, 251). Ileal IEL are strongly affected by Stx1 which reduces the percentage of mitogen-induced-transformed blast cells within all subpopulations identified. An *in vivo* model of ligated ileum (calf) revealed that Stx1 specifically depletes the ileal mucosa of a certain subset of $CD8\alpha^+$ Gb_3/CD77 expressing T lymphocytes (251). The rStxB1

subunit binds to iIEL *ex vivo* and in particular to CD8α^{+} iIEL and to iIEL co-expressing TcR1-N12, a pan γδ TCR cell marker in cattle. However, the consequences of this effect on bovine iIEL for the mucosal immunity remain to be elucidated since Stx1 does not affect the NK activity of iIEL *in vitro* and *in vivo* against a bovine lymphoma cell line (BL-3 cells)(248).

3. Materials and methods

3.1. Isolation of bovine ileal intraepithelial lymphocytes (iIEL)

Ileal IEL were isolated following the procedure described by Menge *et al.* (248) with slight modifications. Briefly, the isolation was based on incubation of ileal mucosa with EDTA and mechanical detachment followed by density gradient centrifugation. Gut specimen (distal ileum) were obtained from freshly slaughtered adult cattle older than 24 months from the local slaughterhouse (Giessen, Hessen, Germany). The ileum was incubated for 25 min in erlenmeyer flask with phosphate-buffered saline (PBS; see **8.2.** for all recipes of buffers and cell culture media) supplemented with 1 mM of 1,4-Dithiotreitol (DTT, Carl Roth GmbH, Karlsruhe, Germany) at 37°C under constant shaking (100 rpm). After the examination of the mucosal surface and the removal of eventual parasitic nodules, specimen were cut into strips (0.5 - 1 cm), transferred to 50-ml centrifugation tubes (Greiner, Frickenhausen, Germany) containing 35 ml of PBS-EDTA-AB (PBS supplemented with 2 mM of Ethylenediamine Tetraacetic Acid [EDTA], 100 IU/ml of penicillin, 100 µg/ml of streptomycin [PAA Laboratories GmbH, Pasching, Austria] and 50 µg/ml of gentamicin [Biochrom AG, Berlin, Germany]), and incubated for 20 min at 37°C under constant shaking (100 rpm). The tissues were vortexed for 2 min at full speed and supernatants were passed through nylon wool (Biotest AG, Dreieich, Germany) disposed into 50 ml-Multistepper tips (Eppendorf, Hamburg, Germany). The cells were collected by centrifugation (250 × g, 8 min, 20°C), resuspended in 25 ml of Percoll solution (Sigma-Aldrich Chemie, Steinheim, Germany, density adjusted to δ=1.0500 g/ml with PBS-EDTA 1X (PBS supplemented with 5.4 mM EDTA), and layered on 10 ml of Percoll solution (δ=1.0816 g/ml). After centrifugation (652 × g, 20 min, 20°C, Ominifuge 2.0 RS, rotor type 2251, Heraeus Holding GmbH, Hanau, Germany), the cells were carefully recovered from the Percoll-Percoll interface, washed once with 20 ml of PBS-EDTA 1X (202 × g, 7 min, 20°C) and once with 20 ml of PBS 1X. The cell number was estimated by live-dead exclusion in a Neubauer chamber after a dilution 1:20 with trypan blue solution. The iIEL (2×10^7) were transferred to reaction tubes (Eppendorf), centrifuged (350 × g, 5 min, 20°C) and directly lysed in 600 µl of RLT buffer (RNeasy® Mini Kit, QIAGEN, Hilden, Germany) supplemented with 1 % of β-mercaptoethanol (Fluka, Taufkirchen, Germany), or were resuspended and cultured in IEL-medium (78 % RPMI 1640 with 2 mM stabilized L-glutamin and 2.0 g/l $NaHCO_3$ [PAN™ BIOTECH GmbH,

Aidenbach, Germany], supplemented with 20 % of heat-inactivated foetal calf serum [FCS, Biowest, Essen, Germany], 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 2.5 µg/l of amphotericin B [PAA Laboratories GmbH] and 2.5 µg/ml of gentamicin [Biochrom AG]).

3.2. Immunophenotyping of iIEL

Ileal IEL were immunolabeled and analysed by flow cytometry to control the quality of the preparation. Freshly isolated iIEL were washed twice by centrifugation with 10 ml of PBS 1X (202 × g, 7 min, 20°C) and transferred into wells of a V-shaped microtiter plate (Greiner) at the density of 4×10^5 per well, and centrifuged (400 × g, 3 min, 20°C, in Eppendorf 5403 centrifuge [rotor type 16 M2-MT, Hamburg, Germany]). Ileal IEL were stained for several surface-expressed antigens by incubation 20 min in the dark with the antibodies listed in table 1, as previously described (248). Some of the antibodies used in this study were produced by hybridoma cell lines kindly provided by J. Naessens (International Livestock Research Institute [ILRI], Nairobi, Kenya). Some antibodies were also purchased from VMRD, Inc. (Pullman, WA, USA)(see table 1). The anti-human CD77 antibody (clone 38.13) was purchased from Beckman-Coulter GmbH (Krefeld, Germany).

The cells were next centrifuged (400 × g, 3 min, 20°C) and washed once with PBS 1X and resuspended 20 min in the dark in 50 µl of secondary antibody. A goat anti-mouse IgG (Heavy and Light chains) conjugated with fluorescein isothiocyanate (FITC) was purchased from Dianova (Hamburg, Germany) and was used diluted 1:400 in PBS 1X. An anti-rat IgM conjugated with phycoerytrin (PE) was purchased from Beckman-Coulter GmbH (Krefeld, Germany) and was used diluted 1:200 in PBS 1X. One microgram of 7-amino actinomycin D (7-AAD, Sigma) was added per ml of secondary antibody suspension. The staining with 7-AAD, which is capable to bind DNA, indicates an increased permeability of the cell membranes and can be used as a death stain (338). The cells were next centrifuged (400 × g, 3 min, 20°C), washed twice with PBS 1X, resuspended into 100 µl of PBS 1X and transferred into 5 ml-FACS tubes containing 200 µl of PBS 1X. Five thousand events were acquired by a FACSCalibur™ flow cytometer (Becton-Dickinson [BD], Heidelberg, Germany) using the acquisition software Cell Quest Pro (BD).

The analysis of the data was performed with the software FCS express 2 (De Novo software, Thornhill, Ontario, Canada). The gating of the cell populations allowed the exclusion of

apoptotic/necrotic cells which took up the death dye 7-AAD and to analyse the expression of antigens on viable iIEL only.

Table 1. Antibodies specific for bovine antigens used for iIEL immunuphenotyping

Antigen specificity	Hybridoma cell line	Isotype	Cell distribution	Supplier	Condition of use
CD4	IL-A11	IgG_{2a}	T helper	ILRI	50 µl, pure [1)]
CD8α	IL-A105	IgG_{2a}	T cytotoxic	ILRI	50 µl, pure [1)]
CD8β	BAT82A	IgG_1	T cytotoxic	VMRD	50 µl, 1:800 [2)]
CD21	IL-A65	IgG_{2a}	B	ILRI	50 µl, pure [1)]
CD25	IL-A111	IgG_1	T and B	ILRI	50 µl, pure [1)]
CD77 [4)]	38.13	IgM	various	Coulter	50 µl, 1:20 [3)]
TcR-N12	CACT61A	IgM	γδ T	VMRD	50 µl, 1:200 [2)]
WC1	IL-A29	IgG_1	γδ T $CD2^-$	ILRI	50 µl, pure [1)]

CD = Cluster of Differentiation, WC = Workshop Cluster, ILRI = International Livestock Research Institute, VMRD = Veterinary Medical Research & Development. [1)] hybridoma cell line supernatant, [2)] diluted in PBS + 1 % BSA, [3)] diluted in PBS, [4)] anti-human CD77 antibody.

3.3. Isolation of bovine peripheral blood mononuclear cells (PBMC)

Bovine PBMC were isolated following the method of Bøyum (39) modified as described by Menge *et al.* (254). PBMC were isolated by density gradient 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 ("Oberer Hardthof") with 60 ml syringes pre-filled with 12 ml of citrate solution (20 % of 3.8 % sodium-citrate and 80 % of blood). Twenty millilitres of citrated blood were diluted with 17 ml of PBS-EDTA 1X in a 50 ml-centrifugation tube in order to reduce the tendency of erythrocytes to aggregate and to trap lymphocytes. The diluted blood was then carefully layered onto 12 ml of Ficoll-Paque™ Plus that was previously placed at room temperature for an optimal cell separation (density of Ficoll-Paque™ Plus is 1.077 g/ml). A centrifugation was performed during 45 minutes at $800 \times g$, and 20°C with smooth deceleration to keep the different phases separated. The plasma was then removed and the

interphase PBMC-Ficoll-Paque™ Plus was recovered and transferred to a 50 ml-centrifugation tube. PBS-EDTA 1X was added up to 50 ml to disrupt the gradient and a centrifugation was performed to pellet the cells at 249 × g during 8 minutes at 20°C. In order to remove the eventual contaminating erythrocytes, the cell pellet was then resuspended in 10 ml of erythrocyte-lysis buffer (8.26 g NH_4Cl, 1.09 g $NaHCO_3$, and 0.037 g Na_3EDTA per 1 litre of distilled water) and incubated for 5 minutes at room temperature. Ten millilitres of PBS-EDTA 1X were added to restore the osmolarity and the cells were centrifuged at 202 × g during 7 minutes at 20°C. The PBMC were next washed once with 10 ml PBS-EDTA 1X and once with 10 ml PBS 1X. The pellet of PBMC was resuspended in 3 ml of PBMC-medium containing RPMI 1640 with stabilized L-glutamine and 2.0 g/l $NaHCO_3$ (PAN™ BIOTECH GmbH), 10 % fetal calf serum (Biowest) and 3 µM β-mercaptoethanol (Fluka, Taufkirchen, Germany). The cell number was estimated by live-dead exclusion in a Neubauer chamber after a dilution 1:10 with a trypan blue solution.

3.4. Cultivation of bovine lymphocytes for RNA isolation

Freshly prepared iIEL were seeded in 6-well plates in IEL-medium (2×10^7 in 9 ml). For lymphocytes' stimulation, the medium was supplemented with phytohemaglutinin-P (2.5 µg/ml, PHA-P, Sigma-Aldrich). The cells were then incubated in absence or presence of purified Stx1 (7, 15, 22, 30, 66, 90, 180, or 200 CD_{50}/ml, as determined on Vero cells (254) with an equivalence of about 1 CD_{50}/ml to 1 pg/ml (292). The Shiga toxin 1 was purified in our institute by Dr. Menge and Dr. Stamm, and the methods for Stx1 preparation and purification were published by Menge *et al.* (254). Briefly, Stx1 was obtained from the STEC strain 2403 (rough, H^-), purified by affinity chromatography (FPLC®, Amersham Biosciences Europe GmbH) (resin Cibacron blue 3G-A linked to agarose beads), dialyzed against 10 mM sodium phosphate buffer, and purified by immunoaffinity chromatography (MAb 13C4 anti-StxB1 coupled to protein A/G agarose). Finally, the preparation was passed through a Detoxi-Gel™ column (Pierce) to reduce the amount of endotoxin, and analysed by SDS-PAGE. The titration of the preparation by a cytotoxic assay using Vero cells (ATCC CRL 1587) established the concentration at 65,000 CD_{50} per ml. The *Limulus* amoebocyte lysate assay indicated a concentration of 0.17 ng of endotoxin per ml. The iIEL were incubated for 4, 6, 8, 18 or 24 hrs at 37°C in 5 % CO_2 and 95% humidity, and then resuspended in the wells, transferred to 50 ml-centrifugation tubes, washed once with PBS (202 × g, 7 min, 20°C) and

lysed in 1.5 ml-reaction tubes with 600 µl of RLT buffer (RNeasy® Mini Kit, QIAGEN) supplemented with 1 % β-mercaptoethanol and stored at -70°C. Supernatants of 8 and 18 hrs cultures were recovered, aliquoted in 2 ml-reaction tubes and immediately frozen at -20°C. These supernatants were later used for ELISA and neutrophil migration assays.

Based on previous results that the effect of Stx1 on iIEL could not always be neutralized by the anti-StxB1 antibody 13C4 (31), iIEL (2×10^7 in 9 ml) were stimulated with PHA-P (2.5 µg/ml), and several concentrations of Stx1 were applied to the cultures (7.5, 15, 30, 90, or 180 CD_{50}/ml Stx1, as determined on Vero cells) with a fixed concentration of 1.5 µg/ml of anti-StxB1 monoclonal antibody 13C4. As controls, the cells were incubated only with either a) IEL-medium (= visualized as 100 %), b) Stx1 (180 CD_{50}/ml), c) an anti-StxB1 antibody 13C4 (1.5 µg/ml). The iIEL were harvested after 6 hrs of cultivation, transferred to 50 ml-centrifugation tubes, washed twice by centrifugation with PBS 1X (202 × g, 7 min, 20°C), and lysed in 600 µl of RLT-buffer (Qiagen).

The monoclonal antibody 13C4 (376) was used at the concentration of 1.5 µg per ml to neutralize the effect of Stx1 on the different types of cells used in this study. The 13C4-containing supernatant was obtained by culturing the hybridoma cell line. The preparation was then purified by affinity chromatography (Protein G, FPLC®) and titrated by neutralization of the cytotoxic effect of Stx1 on Vero cells (363).

Bovine PBMC were seeded in 6-well plates in PBMC medium (2×10^7 in 9 ml) supplemented with 5 µg/ml PHA-P, incubated for 6 or 20 hrs at 37°C in the absence or presence of purified Stx1 (33 CD_{50}/ml) or with Stx1 pre-incubated at 37°C with the anti-StxB1 antibody 13C4 (f.c. 1.5 µg/ml) during 90 minutes. The PBMC were then resuspended, washed twice in PBS, and lysed as described for iIEL in 600 µl RLT buffer and stored at -70°C.

3.5. Quantitation of cytokine/chemokine mRNA *in vitro* from bovine lymphocytes

3.5.1. Isolation of total RNA from lymphocytes

The samples (lymphocytes lysed in RLT buffer) 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, Hilden, Germany) was used following the instructions of the manufacturer (RNeasy® Mini Handbook, Third Edition, June 2001) with slight modifications. Six hundred microlitres of ethanol 70 % (Merck, Darmstadt, Germany) were added to each

sample which were then loaded onto the QIAGEN columns and centrifuged for 30 sec at 16,000 × g (MIKRO 20, rotor type 2073, Hettich ZENTRIFUGEN, MAGV, Rabenau-Londorf, Germany). Briefly, the RNA extraction was done by using a column containing a silica-gel membrane which trapped total RNA. All the next centrifugations were performed 30 sec at 16,000 × g, except when noted. Several washes and treatments were required to eliminate DNA, proteins and others elements that would be contaminants for the next steps. A first DNA digestion was made in the QIAGEN column with 70 µl of RDD buffer (RNase-Free-DNase Set, QIAGEN) and 10 µl of DNase I (27 units, RNase-Free-DNase Set, QIAGEN) during 20 min at room temperature. After a wash by centrifugation with 350 µl of RW1 buffer (QIAGEN), RNA was eluted twice by centrifugation with 2 × 40 µl of RNase-free DEPC-treated water (Carl Roth GmbH, Germany). A second DNA digestion was performed in the reaction tubes with 30 units of DNase I (Amersham Biosciences Europe GmbH) for 20 minutes at 37°C. RNA was then protected by 80 units of Ribonuclease Inhibitor (RNasin, Fermentas GmbH). The volume of the reaction was adjusted to 100 µl by addition of 10 µl of DNase buffer and 5 µl of DEPC-treated water. The DNase was then inactivated by chemical (10 µl of 3 M sodium acetate, pH 4.6) and physical denaturations (repeated vortexing).
Total RNA was precipitated with 200 µl of 99 % ethanol two hours at -70°C. After centrifugation (12,000 × g, 60 min, 4°C, Eppendorf centrifuge 5804R, rotor type F45-30-11), the RNA was washed twice with 250 µl of ethanol 70 %, air-dried and finally resuspended in 40 µl of DEPC-treated water supplemented with 40 units of RNasin.

The nucleic acid content was then estimated spectrophotometrically. For this purpose, 5 µl of RNA solution were diluted 1:20 in Tris-EDTA and the absorbance (Abs) was measured against Tris-EDTA (blank) at 260 nm and 320 nm by using a spectrophotometer (DU® 640, Beckman). RNA quantity was calculated by using the following formula:

$$(Abs_{260\ nm} - Abs_{320\ nm}) * 40 * 20 = \text{ng of RNA / µl of sample}$$

The quality of the isolated RNA was checked by agarose gel electrophoresis. Depending on the amount of RNA harvested, 200 or 400 ng of RNA were mixed in a final volume of 10 µl with DEPC-treated water and 2 µl of loading-dye (Fermentas GmbH), and loaded into an 1 % agarose gel (Serva Electrophoresis GmbH, Heidelberg, Germany, in 40 mM Tris, 40 mM

glacial acetic acid, 1 mM EDTA, 10 ng/ml ethidium bromide) and the migration was performed 45 min at 90 volts.

The figure 1 shows RNA of 6 and 24 hrs mitogen-stimulated iIEL after electrophoresis in an 1 % agarose gel. RNA appears in the characteristic 3-bands-shape: the upper band representing the 28S rRNA, the middle band the 18S rRNA and the lower band representing the 5.8S rRNA. The mRNA, representing only 1 % of total cellular RNA, cannot be visualized as a single band because the different transcripts are of variable sizes.

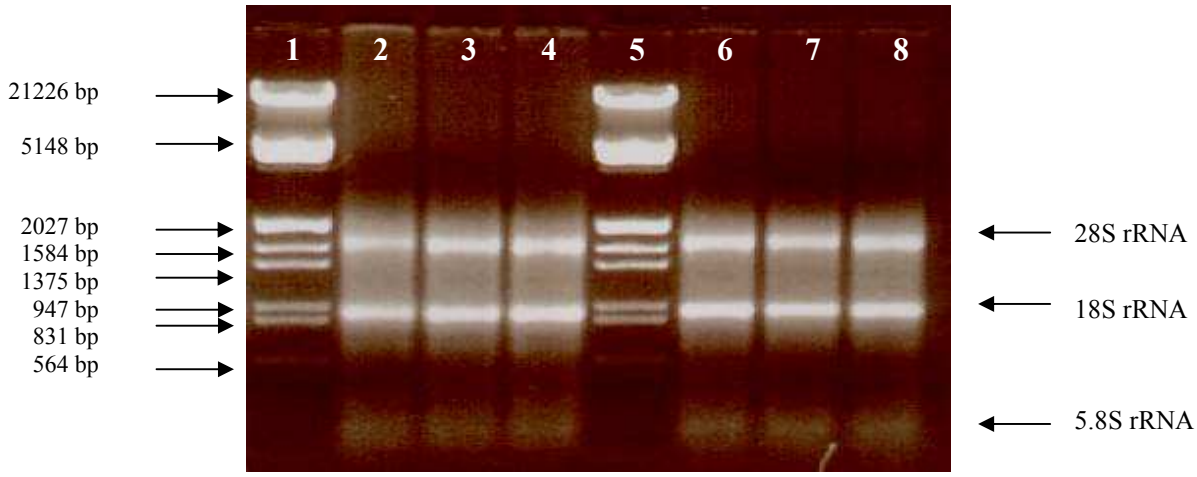


Fig. 1. Electrophoresis of 400 ng of RNA isolated from 6 and 24 hrs PHA-P stimulated ileal IEL.

Legend of the figure 1:

Lanes 1 and 5: Lambda DNA/Eco RI + Hind III marker (Fermentas GmbH)

Lane 2 : RNA from 6 hrs-stimulated iIEL incubated with medium

Lane 3 : RNA from 6 hrs-stimulated iIEL incubated with 200 CD_{50}/ml of Stx1

Lane 4 : RNA from 6 hrs-stimulated iIEL incubated with 200 CD_{50}/ml of Stx1 and 1.5 µg/ml of anti-StxB1 antibody

Lane 6 : RNA from 24 hrs-stimulated iIEL incubated with medium

Lane 7 : RNA from 24 hrs-stimulated iIEL incubated with 200 CD_{50}/ml of Stx1

Lane 8 : RNA from 24 hrs-stimulated iIEL incubated with 200 CD_{50}/ml of Stx1 and 1.5 µg/ml of anti-StxB1 antibody

The absence of genomic DNA in the RNA preparation was checked by Polymerase Chain Reaction (PCR) for a housekeeping gene encoding the glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) performed with the reagents listed in the table 2. The primers purchased from MWG Biotech AG (Ebersberg, Germany) are listed in the table 5.
After successive addition of the reagents, the tubes were vortexed, centrifuged 10 sec at 10,000 × g, and placed in a Perkin Elmer gene Amp PCR System 2400 thermocycler. After a denaturation step of 2 min at 94°C, the amplification was performed in 40 sequential cycles (94°C, 15 sec; 60°C, 30 sec; 72°C, 30 sec) followed by a post-elongation step for 2 min at 72°C. PCR products were mixed with 2.5 µl of loading dye, loaded into a 2 % agarose gel (Serva Electrophoresis GmbH, in 40 mM Tris, 40 mM glacial acetic acid, 1 mM EDTA, 10 ng/ml ethidium bromide), and the migration was performed 60 min at 100 volts.

Table 2. Reagents used to perform the GAPDH control PCR

Reagent	Volume (in µl)	Quantity or final concentration	Supplier
Bi-distilled water	11.6	-	
10 X PCR buffer II	2.0	1 X	Applied Biosystems
25 mM $MgCl_2$	1.2	1.5 mM	Applied Biosystems
5 µM Forward primer	1.0	0.25 µM	MWG Biotech AG
5 µM Reverse primer	1.0	0.25 µM	MWG Biotech AG
dNTP (4 mM each)	1.0	0.2 mM	PAN Biotech GmbH
AmpliTaq® DNA polymerase	0.2	1 unit	Applied Biosystems
RNA (1:4 in water)	2.0	≈ 50 ng	
Final Volume	20.0		

3.5.2. Reverse transcription (RT) of mRNA

Complementary DNA (cDNA) was obtained from mRNA by reverse transcription of 1 µg of total RNA per sample by using an oligo d(T) primer (Table 3) in a 40 µl reaction volume according to the instructions of the manufacturer of the reverse transcriptase (Promega, Mannheim, Germany). This reaction was performed following a two-step method. In a first step, 1 µg of RNA was diluted in 23 µl of DEPC-treated water and 2 µl of 50 µM oligo $d(T)_{16}$

primers were added (corresponding to 0.5 µg; Applied Biosystems, Darmstadt, Germany). The annealing was performed during 5 min at 70°C in a thermocycler (Perkin Elmer gene Amp PCR System 2400). In a second step, each sample (1 µg RNA) was reverse transcribed by 200 units of Moloney-Murine Leukemia Virus (M-MLV) Reverse Transcriptase RNase H Minus purchased from Promega (Mannheim) at 40°C for 60 min, followed by 94°C for 2 min. The cDNA was then stored at -20°C or used immediately for real-time PCR.

Table 3. Reagents used to perform the reverse transcription

Reagent	Volume (in µl)	Quantity or final concentration	Supplier
RNA (in water)	23.0	1 µg	-
Oligo $d(T)_{16}$	2.0	0.5 µg	Applied Biosystems
5X M-MLV buffer	8.0	1 X	Applied Biosystems
dNTP (5 mM each)	4.0	0.5 mM each	PAN™ Biotech GmbH
Bi-distilled water	1.0	-	-
RNasin	1.0	40 units	Fermentas GmbH
M-MLV RTase	1.0	200 units	Promega
Final volume	40.0		

3.5.3. Cytokine/chemokine-specific semi-quantitative or real-time PCR

For freshly isolated iIEL, a semi-quantitative PCR was performed to establish a cytokine and chemokine mRNA profile that indicates which mRNA are harboured by iIEL *in vivo*. RT-PCR were performed to detect mRNA encoding the following cytokines: Interleukine (IL) -2, IL-4, IL-10, interferon-γ (IFN-γ), transforming growth factor-β (TGF-β), and the three chemokines CXCL8 (syn. IL-8), CXCL10 (syn. 10 kDa-Interferon-Inducible Protein or IP-10), and CCL2 (syn. Monocyte Chemotactic Protein-1 or MCP-1). The presence of the Tumor-Necrosis Factor-α (TNF-α) mRNA was also investigated. The conditions of the amplification were the same as described for GAPDH (see **3.5.1** and **Table 2**). The housekeeping gene GAPDH was used as a control for constitutive gene expression. The primers purchased from MWG Biotech AG are listed in Table 5.

For mitogen-stimulated iIEL and PBMC a real-time PCR was performed (Table 4); primers (purchased from MWG Biotech AG) and probes (purchased from Eurogentec, Liège, Belgium) are shown in Table 5. The qPCR™ Mastermix purchased from Eurogentec (Seraing, Belgium) already contains dNTP, $MgCl_2$, and a Hot Goldstar DNA polymerase. The TaqMan® 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). For real-time PCR, 1.5 µl of cDNA (corresponding to 37.5 ng of total RNA) was used in a 25 µl PCR reaction mixture containing 12.5 µl qPCR™ MasterMix (Eurogentec), 300 nM of each primer and 200 nM of probe. Twenty-three microlitres of this mix were transferred to the 96-well optical reaction plates (Micro Amp Optical Reaction Plate, Applied Biosystems) which was then closed with an adhesive cover (ABI PRISM Optical Adhesive Cover Starter Kit, Applied Biosystems). The PCR amplification was performed on an automated fluorometer (ABI PRISM™ 5700 Sequence Detection System, Applied Biosystems). Each sample was analysed in duplicates.

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 sec and at 60° C for 60 s.

Table 4. Reagents used to perform the real-time PCR

Reagent	Volume (in µl)	Quantity or final concentration	Supplier
Bi-distilled water	7.0	-	-
2 X qPCR™ MasterMix	12.5	1 X	Eurogentec
5 µM TaqMan® probe	1.0	0.2 µM	Eurogentec
5 µM Forward primer	1.5	0.3 µM	MWG Biotech AG
5 µM Reverse primer	1.5	0.3 µM	MWG Biotech AG
cDNA	1.5	≈ 37 ng	
Final Volume	25.0		

In order to compare the mRNA expression of one cytokine in iIEL incubated in medium in the absence or presence of Stx1, quantitative analyses were used (comparative C_t method [$\Delta\Delta C_t$ method]) according to the instructions of the manufacturer of the ABI PRISM™ 5700 Sequence Detection System and reported as differences in comparison to the medium control

Table 5. Sequences of primers and probes used for the amplification of cDNA by semi-quantitative RT-PCR and real-time PCR

Specificity	Primers (5´- 3´) and probes (5´- 3´)	**Size of products (in bp)**	**Reference**
IL-2 Forw	GGA TTT ACA GTT GCT TTT GGA GAA A		
IL-2 Rev	GCA CTT CCT CTA GAA GTT TGA GTT CTT	165	(217)
TaqMan® probe	CGT GCC CAA GGT TAA CGC TAC AGA ATT GAA		
IL-4 Forw	CAT GCA TGG AGC TGC CTG TA		
IL-4 Rev	AAT TCC AAC CCT GCA GAA GGT	83	(405)
TaqMan® probe	TGC TGC CCC AAA GAA CAC AAC TGA GAA G		
IL-8 Frow	CAC TGT GAA AAA TTC AGA AAT CAT TGT TA		
IL-8 Rev	CTT CAC CAA ATA CCT GCA CAA CCT TC	113	(217)
TaqMan® probe	AAT GGA AAC GAG GTC TGC TTA AAC CCC AAG		
IL-10 Forw	CCA AGC CTT GTC GGA AAT GA		Moussay
IL-10 Rev	GTT CAC GTG CTC CTT GAT GTC A	91	*et al.*,
TaqMan® probe	AGC CTG TGG CAT CAC CTC TTC CAG GTA A		(submitted)
IFN-γ Forw	CAG CTC TGA GAA ACT GGA GGA CTT		
IFN-γ Rev	TGG CTT TGC GCT GGA TCT	77	(405)
TaqMan® probe	AGC TGA TTC AAA TTC CGG TGG ATG ATC T		
TGF-β Forw	GGC CCT GCC CTT ACA TCT G		Moussay
TGF-β Rev	CGG GTT GTG CTG GTT GTA CA	74	*et al.*,
TaqMan® probe	CCT GGA TAC ACA GTA CAG CAA GGT CCT GG		(submitted)
TNF-α Forw	TCT TCT CAA GCC TCA AGT AAC AAG T		
TNF-α Rev	CCA TGA GGG CAT TGG CAT AC	103	(387)
TaqMan® probe	AGC CCA CGT TGT AGC CGA CAT CAA CTC C		
IP-10 Forw	AAG TCA TTC CTG CAA GTC AAT CCT		
IP-10 Rev	TTG ATG GTC TTA GAT TCT GGA TTC AG	103	(388)
TaqMan® probe	CCA CGT GTC GAG ATT ATT GCC ACA ATG A		
MCP-1 Forw	CGC TCA GCC AGA TGC AAT TA		
MCP-1 Rev	GCC TCT GCA TGG AGA TCT TCT T	77	(388)
TaqMan® probe	CCC AAG TCG CCT GCT GCT ATA CAT TCA A		
GAPDH Forw	GCG ATA CTC ACT CTT CTA CCT TCG A		
GAPDH Rev	TCG TAC CAG GAA ATG AGC TTG AC	82	(388)
TaqMan® probe	CTG GCA TTG CCC TCA ACG ACC ACT T		

(which was set as 100 %) after normalizing the samples referring to the housekeeping gene GAPDH. PCR data are presented in the results section as relative percentage of expression compared to the medium control. The calculation procedure was performed as follows:

Step 1: Calculate the arithmetic mean of the Ct duplicate values (mCt)

Step 2: Normalize the values by using a housekeeping gene (GAPDH):

$$\Delta Ct = mCt_{\text{(target gene)}} - mCt_{\text{(housekeeping gene)}}$$

Step 3: Normalize the values by using a calibrator (= condition "Medium"):

$$\Delta\Delta Ct = \Delta Ct_{\text{(lymphocytes incubated with Stx1)}} - \Delta Ct_{\text{(lymphocytes incubated with Medium)}}$$

Step 4: Calculate the relative quantity of the target gene:

$$\text{Relative quantity} = 2^{(-\Delta\Delta C_t)}$$

Step 5: Transform the relative quantity in relative percentage of expression:

$$\text{Relative \% of expression} = \text{Relative quantity} \times 100$$

In addition to this calculation based on the ΔΔCt values used to compare the mRNA expression of one cytokine in different conditions (Med *vs* Stx1), an alternative calculation method was used to compare the amount of several chemokine mRNA in iIEL incubated in the same condition. To do so, the step 3 was omitted; thus allowing to directly compare mRNA amounts (GAPDH values set to 100 %).

3.5.4. IL-4 splice variants PCR

Ileal IEL were isolated as previously described, and incubated 6 hrs with 2.5 µg/ml of PHA-P. The cells were harvested, washed once in PBS and mRNA was isolated from 2×10^7 IEL using the RNeasy Mini Kit (QIAGEN). The same procedure was also performed for freshly isolated iIEL. Reverse transcription and semi-quantitative PCR were performed to detect the production of IL-4 mRNA. GAPDH was used as a housekeeping gene for constitutive gene expression. The presence of IL-4 full length (408 bp) and splice variants (IL-4δ2 [360 bp] and IL-4δ3 [282 bp]) was investigated by RT-PCR in iIEL incubated in the presence or absence of Stx1 and with or without anti-StxB1 antibody by using the following primers published by Waldvogel *et al.* ((406), Forward 5'- ATG GGT CTC ACC TAC CAG CTG -3' and Reverse 5'- CAC TTG GAG TAT TTC TCC TTC ATA ATC G -3'). Complementary DNA was amplified by 35 cycles (15 sec at 94°C, 30 sec at 60°C, 1 min at

72°C) followed by a final post-elongation step for 5 min at 72°C. The PCR product were analyzed on a 2 % agarose gel.

3.6. Analysis of the blast transformation and of the expression of Gb_3/CD77 of iIEL

The impact of Stx1 on several parameters of iIEL biology was investigated by flow cytometry. Ileal IEL were seeded (5×10^6 per well) in 6-well plates (Nunc, Wiesbaden, Germany) in 2.5 ml of IEL-medium. For stimulation, the culture medium was supplemented with phorbol-12-myristate-13-acetate (PMA, Phagoburst® Kit, ORPEGEN Pharma; Heidelberg, Germany, 50 ng/ml), with ionomycin (Sigma, 1 µg/ml), and the cells were cultivated in the absence or presence of purified Stx1 (200 CD_{50}/ml). For neutralization the latter was pre-incubated for 90 min at 37°C with purified anti-StxB1 antibody 13C4 (1.5 µg/ml). The iIEL were then incubated for 6 or 18 hrs at 37°C in 5 % CO_2 and 95 % humidity. At the end of the incubation time, iIEL were harvested, washed twice by centrifugation with 10 ml of PBS 1X (202 × g, 7 min, 20°C), and then transferred to V-shaped microtiter plates (Greiner) at the density of 4×10^5 cells per well.

The detection of Gb_3/CD77 on the cell surface was performed by an indirect immuno-staining. In order to quantify the total amount of Gb_3/CD77 present in the cells, iIEL in some wells were fixed for 10 min in the dark with paraformaldehyde (PFA 1 % in PBS 1X, Merck, Darmstadt, Germany), washed once by centrifugation (150 × g, 7 min, 4°C) with PBS 1X, and permeabilized 5 min in the dark with PBS 1X supplemented with 0.1 % of saponin (Merck) and 0.1 % of sodium azide (Merck). Ileal IEL in other wells were left in their native form to quantify the amount of the Stx1-receptor present on the cell surface.

The cells were centrifuged (150 × g, 7 min, 4°C) and incubated then 20 min in the dark at room temperature with 50 µl of an anti-human CD77 antibody (Beckman-Coulter GmbH) diluted 1:20 in PBS 1X. As a control for the specificity of the staining, iIEL in some wells were also incubated with 1 µg/ml of unspecific rat IgM (CAMON, Wiesbaden, Germany). After two washes with PBS 1X by centrifugation (150 × g, 7 min, 4°C), the cells were incubated 20 min in the dark at room temperature with 50 µl of secondary antibody diluted 1:200 in PBS 1X (anti-rat IgM (µ-chain specific)-PE conjugated antibody [Beckman-Coulter GmbH]). The iIEL were washed twice in PBS 1X, and transferred into 5 ml-FACS tubes containing 200 µl of PBS 1X.

The morphology of the iIEL, the total cellular content of the glycosphingolipid Gb_3/CD77, and its expression on the cell surface were investigated with the FACSCalibur™ flow cytometer and the Cell Quest Pro software (BD). Five thousand events were acquired per sample. The data were then analyzed with the software FCS express 2 (de Novo software).

3.7. Cytokine and chemokine protein expression by bovine PBMC and iIEL *in vitro*

3.7.1. Intracellular detection of cytokine proteins in PBMC and iIEL by flow cytometry

The intracellular detection of cytokine proteins was performed by flow cytometry concerning three T_H-type specific cytokines. IFN-γ, IL-4, and TGF-β were chosen to represent the T_H1, T_H2, and T_H3-type immune responses, respectively. Specific antibodies were purchased from Serotec GmbH (Düsseldorf, Germany).

3.7.1.1. Titration of the anti-human TGF-β antibody

In order to detect the production of TGF-β in iIEL, an anti-human TGF-β antibody was used because the human and the bovine forms of mature TGF-β proteins are 100 % identical (5, 400). Before being suitable for iIEL analysis, this antibody required a titration to find an appropriate concentration leading to a reduced unspecific binding of the antibody to unstimulated cells and to a specific detection of the antigen in stimulated cells.
Bovine PBMC were isolated as described (see **3.3**) and were incubated 6 hrs or overnight (37°C, 5 % CO_2, and 95 % humidity) with brefeldin A (10 µg/ml) in the presence or absence of mitogens (50 ng/ml of PMA [ORPEGEN Pharma] and 1 µg/ml of ionomycin [Sigma]). The brefeldin A is a fungal metabolite obtained from *Penicillium brefeldianum* which reversibly disassembles the structure and disrupts the functions of the Golgi complex and also stops the intracellular trafficking of vesicles (endocytosis and exocytosis)(72). In the context of this study, the addition of brefeldin A to the culture blocks the secretion and induces the storage of the cytokines in the cell cytoplasma allowing the intracellular detection of cytokine after fixation and permeabilization of the cells. The cells were harvested, washed twice with PBS 1X by centrifugation (202 × g, 7 min, 20°C), transferred to wells of V-shaped microtiter plates (Greiner). The cells were centrifuged (150 × g, 7 min, 4°C) and fixed by incubation in

100 µl of paraformaldehyde (1 % in PBS, Merck) for 10 min in the dark at room temperature. The cells were next washed with 100 µl of the "washing buffer" (PBS supplemented with 0.1 % saponin [Merck] and 0.1 % sodium azide [Merck]) and then permeabilized 5 min at room temperature in 100 µl of "washing buffer". The cells were stained in duplicates for 20 min on ice in the dark with several dilutions of the anti-human TGF-β antibody (from 1:50 to 1:2050 in "Ab-buffer" [PBS supplemented with 1 % bovine serum albumin, 0.1 % saponin, and 0.1 % sodium azide]). A goat anti-mouse IgG (heavy and light chain specific)-FITC conjugated purchased from Dianova (Hamburg, Germany), was used diluted 1:400 (final concentration: 3.4 µg/ml) in "Ab-buffer".

As seen on the titration curve (Fig. 2A), the utilization of a high concentration of antibody (dilutions 1:50 to 1:400) led to a strong binding to unstimulated cells comprised between 61.3 and 17.3 % respectively. Reduced concentrations of antibody (dilutions 1:600 to 1:1400) led to a reduced binding (7.6 to 1.1 %).

The second part of the titration performed with mitogen-stimulated PBMC (Fig. 2B) showed a plateau when the dilutions of the antibody between 1:600 and 1:900 were used. This titration led to choose the dilution 1:800 as a working dilution for the intracellular detection of this cytokine in iIEL.

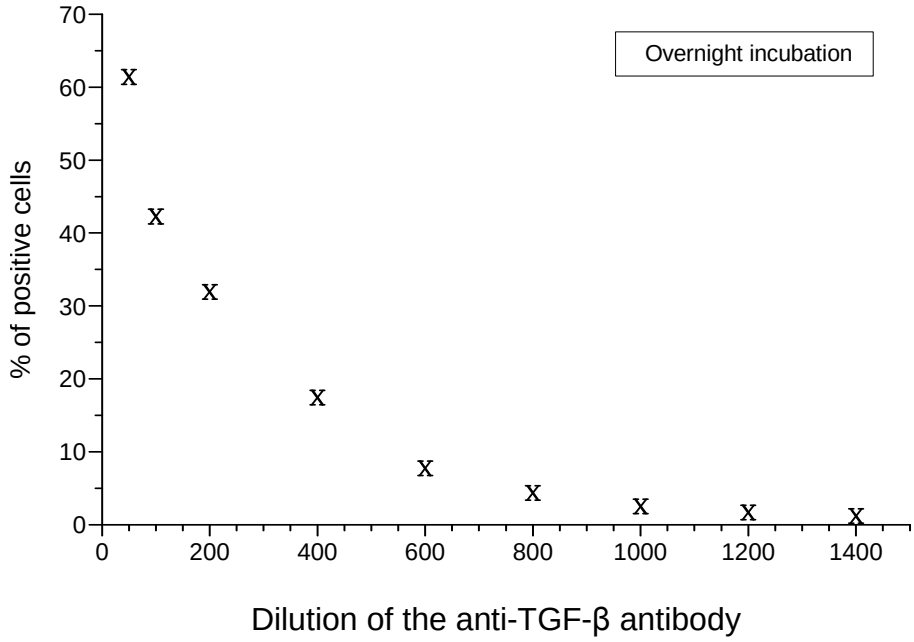
3.7.1.2. Intracellular detection of cytokine proteins in bovine PBMC and iIEL

The cells (5×10^6) were seeded in 6-well plates (Nunc) in 2.5 ml of PBMC-medium or IEL-medium, stimulated by mitogens (see **3.7.1.1**), and incubated in the absence or presence of 200 CD_{50}/ml Stx1. For neutralization the latter was pre-incubated for 90 min at 37°C with purified anti-StxB1 antibody 13C4. The cells were then incubated 6 or 24 hrs at 37°C in 5 % CO_2 and 95 % humidity, in the presence of brefeldin A (Sigma, 10 µg/ml) which was added 1 h or 8 hrs after the beginning of the incubation, respectively.

At the end of the incubation period, cells were resuspended, transferred to a 50 ml-centrifugation tube and washed once with 10 ml PBS-EDTA 1X by centrifugation ($202 \times g$, 7 min, 20°C), and once with 10 ml PBS 1X ($202 \times g$, 7 min, 20°C). The cells were then resuspended in PBS at the density of 4×10^5 cells per 100 µl and transferred to V-shaped microtiter plates (Greiner) for immunostaining. Briefly, the cells were centrifuged ($150 \times g$, 7 min, 4°C), fixed and then permeabilized. The antibodies listed in the table 6 directed against

bovine IL-4 (Serotec), bovine IFN-γ (Serotec) and human TGF-β (Serotec) were diluted in "Ab-buffer".

A



B

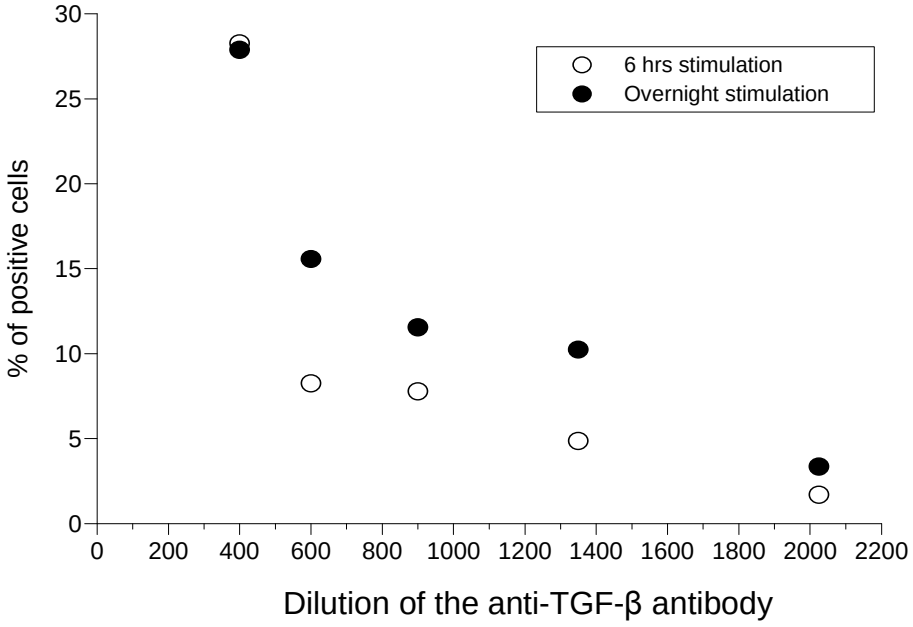


Fig. 2. Titration of the anti-human TGF-β antibody with unstimulated (A) and mitogen-stimulated (B) bovine PBMC. Cells were incubated 6 hrs or overnight with brefeldin A (10 µg/ml), then harvested, washed, fixed, permeabilized, and stained 20 min on ice with several dilutions of the antibody. Five thousand events were acquired by a FACSCalibur™ flow cytometer.

The cells were also incubated with irrelevant antibodies (1F9 and 2-4A5 C5D7) directed against *Clostridium perfringens* phospholipase C and β2-toxin as IgG isotype controls. These antibodies were produced in the supernatant of hybridoma cell lines cultured in RPMI 1640 supplemented with 2 % Ultroser® HY (CYTOGEN, Sinn, Germany), 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (PAA Laboratories GmbH). Five thousand events were acquired for each sample. The gates were defined according to the negative control (PBS) and the respective isotype controls defining less than 2 % of the cells as positive.

Table 6. Antibodies used for intracellular detection of cytokines by flow cytometry

Specificity	Bovine IL-4	Bovine IFN-γ	Human TGF-β	PLC of *C. perfringens*	β2-toxin of *C. perfringens*
Clone	CC 303	CC 302	TB 21	1F9	2-4A5 C5D7
Dilution	1:400	1:800	1:800	1:4	1:4
Isotype	IgG_{2a}	IgG_1	IgG_1	IgG_{2a}	IgG_1

C. pefringens = *Clostridium perfringen*s, PLC= phospholipase C

The intracellular detection of cytokines was also performed in bovine PBMC which were isolated as described, and incubated 8.5 or 21 hrs at 37°C with 5 % CO_2 in the absence or presence of Stx1 (33 CD_{50}/ml). Brefeldin A (10 µg/ml f.c.) was added respectively 1 h or 9.5 hrs after the beginning of the incubation and then the cells were cultivated for 7.5 or 11.5 additional hours, respectively. The PBMC were then harvested, washed, fixed, permeabilized, and the cytokines were intracellularly immunodecorated as described for iIEL.

3.7.2. Establishment of a polymorphonuclear neutrophil (PMN) migration assay

In order to assess whether Stx1 modifies the release by iIEL of total chemoattractant substances for PMN, a biological assay was performed with bovine PMN. Dyed cells were used to count migrating PMN by flow cytometry.

3.7.2.1. Generation of a positive control supernatant

Bovine peripheral blood lymphocytes were isolated as previously described (see **3.3**) and were seeded in 5 ml of PBMC-medium at the density of 1×10^7 per well into 6-well plates. The cells were stimulated with 5 µg/ml of concanavalin A (Sigma) and 200 IU/ml of recombinant-human interleukin-2 (rhIL-2). The cells were split every 3 days during three weeks and the cell-culture supernatants were saved in 50 ml-centrifugation tubes (Greiner), centrifuged (202 × g, 7 min, room temperature) to eliminate dead cells and debris and then stored at 4°C. Fresh culture medium supplemented with 100 units of rhIL-2 per ml was then added to the cultures to continue the proliferation and activation of the lymphocytes. This supernatant was later used as a positive control for induction of the migration of bovine PMN in the system.

3.7.2.2. Comparison of two calibrators to count bovine leukocytes

Isolation of bovine leukocytes

Bovine leukocytes were prepared from 4 ml-aliquots of whole blood by lysis of erythrocytes with 18 ml of bi-distilled water 50 seconds at room temperature. The osmolarity was restored by adding 2 ml of 10 X-concentrated PBS-EDTA. The leukocytes were washed once with PBS 1X by centrifugation (202 × g, 7 min, at 20°C) and counted by trypan blue exclusion after dilution 1:10. The cells were finally resuspended in PBS supplemented with 1 % of BSA at the density of 1×10^6/ml.

Preparation of fluorescent beads

Fifty microliters of a commercial suspension of Fluoresbrite calibration grade 3.0-micron YG microspheres (Polysciences Europe GmbH, Eppelheim, Germany) were washed twice in 1.450 µl of PBS 1X by centrifugation (16,000 × g, 3 min, at 20°C) in 2 ml-reaction tubes and finally resuspended in PBS 1X.

Staining of BL-3 cells

The bovine B lymphoma BL-3 cells (ECACC 86062401) were counted by trypan blue exclusion. The density of 1×10^6 cells per ml of suspension was adjusted by addition of BL-3 medium (RPMI 1640 with 2 mM of stabilized glutamin, 15 % of Lebowitz 15 medium, 10 % of FCS, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, 1 mM of β-Mercaptoethanol. The dye 3-3´dioctadecyloxacarbocyanine perchlorate (DiO, Molecular Probes, Leiden, The Netherlands) was added to the cell suspension at a final concentration of 1.5 µg/ml. After 1 h of incubation at 37°C in the dark under constant shaking (100 rpm/min), the cells were centrifuged (202 × g, 7 min, 20°C) and then washed twice in PBS 1X. The DiO-BL-3 cells were counted again and resuspended in a certain volume of PBS 1X to establish a concentration of 1×10^6 cells per ml. The DiO-BL-3 cells were fixed with the same volume of paraformaldehyde 4 % (dilution 1:2; final concentration 2 %), and incubated for 30 min in the dark at 20°C. Until the end of the procedure, DiO-BL-3 cells were protected from light and stored on ice. Finally, the cells were centrifuged (202 × g, 7 min at 20°C), wash once in PBS 1X and resuspended in PBS 1X.

The morphology and the fluorescence of cells were checked by flow cytometry. Twenty microliters of stained cells were added to 80 µl PBS 1X and acquired with the FACSCalibur™ flow cytometer and the acquisition software Cell Quest Pro (Becton-Dickinson). After verifying the morphology and fluorescence, the cells were frozen at –20°C in PBS 1X supplemented with 10 % of FCS at the density of 1.25×10^6 cells/ml in 2 ml-reaction tubes for later use. Data were analyzed with the software FCS Express 2.

Counting of leukocytes

Different quantities of leukocytes to be counted and of counting particles were mixed in 5 ml-FACS tubes and 1,000 fluorescent events were acquired by the flow cytometer. In order to compare the efficiency of the counting with the two systems, in some cases, the number of leukocytes was kept constant (5×10^5) and variable numbers of counting particles were added per tube (1.56 to 50 µl of bead suspension, or 12.5 to 500 µl of DiO-BL-3 cell suspension). In other cases, the number of counting particles was kept constant (12.5 µl of beads or 100 µl of DiO-BL-3 cell suspension) and the number of leukocytes to be counted varied (2.5 to 7.5×10^5 leukocytes). The acquisition by FACS stopped after 1,000 fluorescent particles were counted in R5. Each sample was acquired in triplicates. The arithmetic mean of these three

determinations was considered as the number of leukocytes counted per 1,000 beads or DiO-BL-3 cells.

Figure 4 shows the comparison of the counting of leukocytes performed by flow cytometry by using fluorescent beads (Fig. 4A, C) or stained DiO-BL-3 cells (Fig. 4B, D). The counting of bovine leukocytes with BL-3 cells stained with the fluorescent dye DiO was found to be as precise as with the beads. Consequently, the release of chemoattractants for PMN by iIEL in supernatants was assessed by using DiO-BL-3 cells in the subsequent experiments.

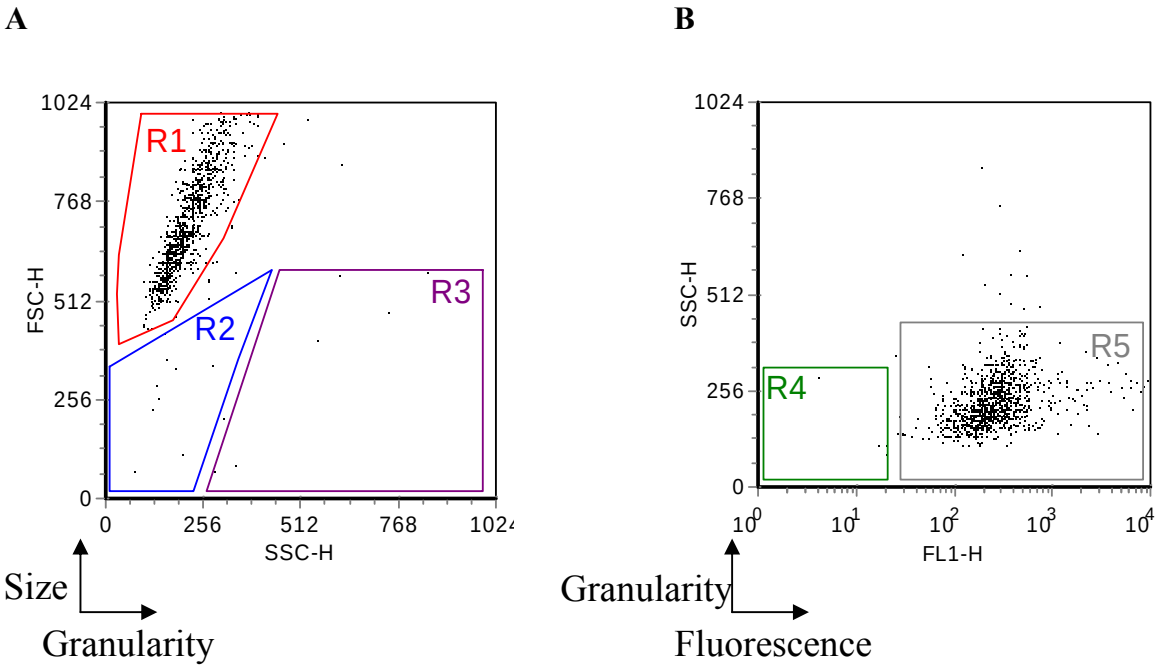


Fig. 3. Morphology (A) and fluorescence (B) of DiO-BL-3 cells. The cells were stained for 1 h with DiO, fixed with PFA 2 %, washed with PBS 1X and were analyzed by flow cytometry. One thousand events were acquired using a FACSCalibur™ flow cytometer.

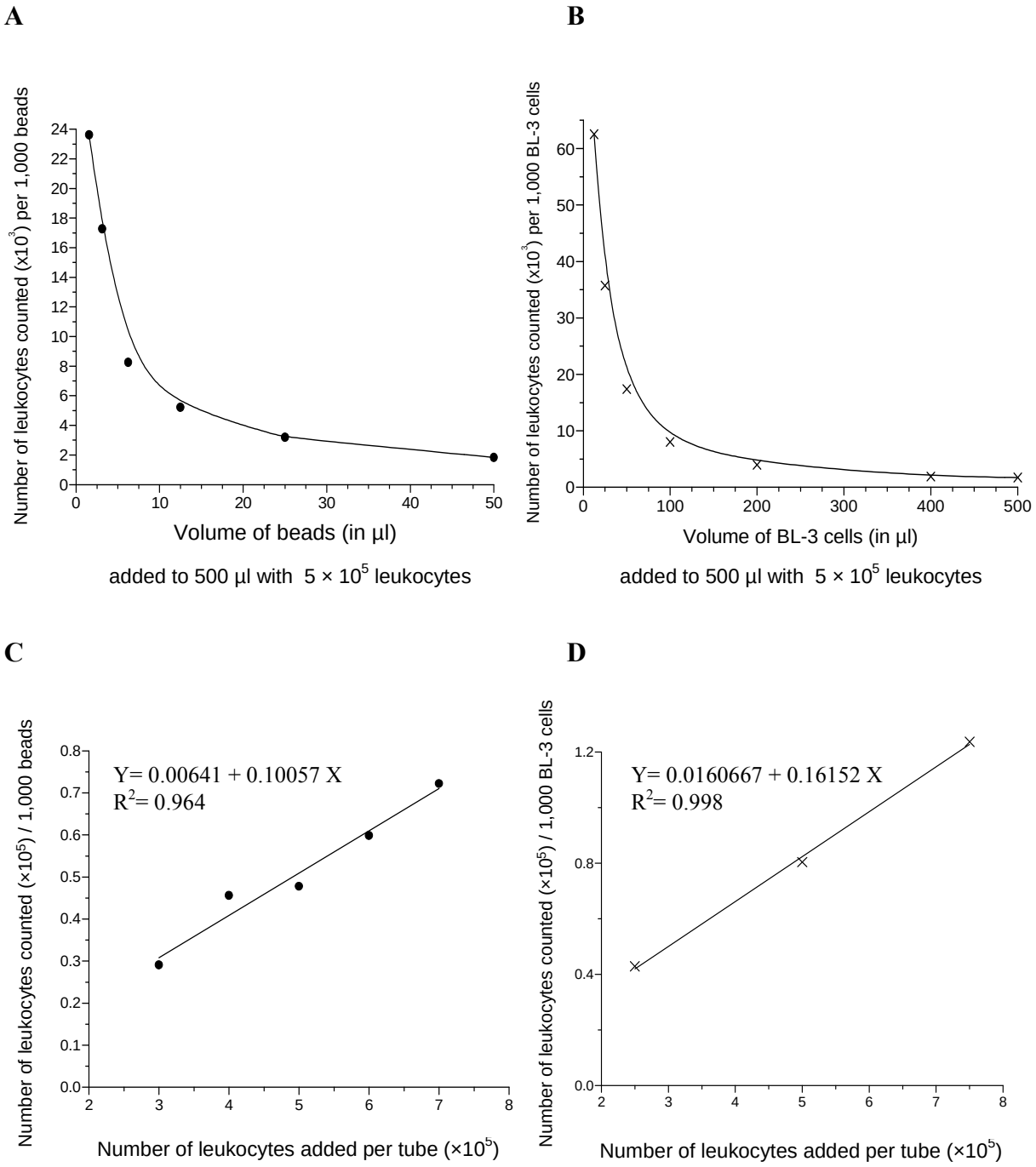


Fig. 4. Counting of bovine leukocytes by flow cytometry. A suspension of fluorescent beads (A, C) or in house-stained DiO-BL-3 cells (B, D) were used as a calibrator. The acquisition stopped after 1,000 fluorescent events were acquired by the FACSCalibur™.

3.7.2.3. Isolation of bovine PMN from whole blood

PMN were prepared from whole blood by density gradient centrifugation, as described for PBMC (see **3.3**). After centrifugation (45 minutes, 800 × g at 20°C), the plasma and the interphase PBMC-Ficoll-Paque™ Plus were removed and discarded. Around half of the red sediment (≈ 4 ml), containing erythocytes and PMN, was then recovered with a plastic pipette and distributed to two 50 ml-centrifugation tubes (≈ 2 ml per tube). This sediment was diluted with 2 ml PBS-EDTA 1X and erythrocytes were lysed. Bi-distilled water was added (27 ml per tube) and mixed by intense swivelling. After 50 seconds, 3 ml of 10X-concentrated PBS-EDTA solution were added and mixed intensively to restore osmolarity. The tubes were next filled with RPMI 1640 (PAN™ BIOTECH GmbH) and were centrifuged 7 minutes at 202 × g at 20°C. The pellet was once washed with PBS-EDTA 1X and the PMN were finally resuspended in 2 ml of PBMC-medium.

The purity of PMN preparations was confirmed by flow cytometry. Fifty microlitres of cell suspensions were diluted with 500 µl of PBS and transferred into 5 ml-plastic tubes, and were analyzed by the FACSCalibur™ flow cytometer with help of the Cell Quest Pro software (acquisition software). Five thousand events were acquired and then preparations with purity greater than 90 % PMN were pooled. Data were treated with the analysis software FCS Express 2 (plot presented in figure 5). PMN constituted a dense and homogenous population located in the region R3, as defined in the FSC (size of cells) *vs* SSC plot (granularity of cells). In the example given (Fig. 5.), PMN represented 97 % of the events acquired by FACS; mononuclear cells located in R1 represented 0.2 %, debris located in R2 1.6 %. Around 1 % of the cells did not fit into the defined regions.

The cell number was next estimated by live-dead exclusion with trypan blue solution in a Neubauer chamber.

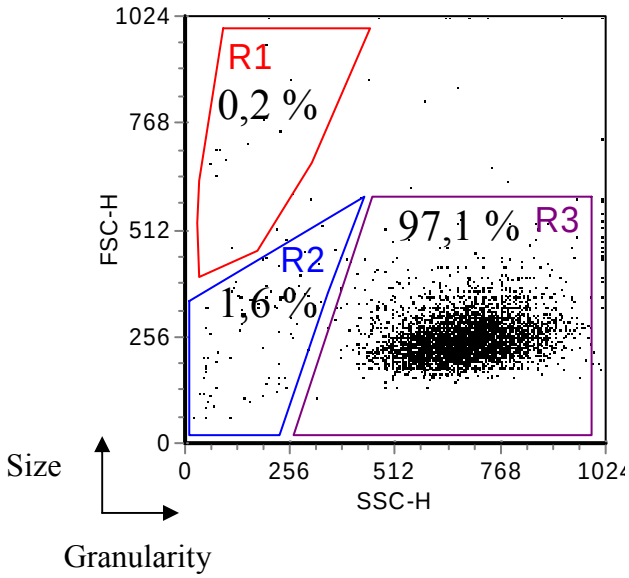


Fig. 5. Morphology of bovine PMN after the procedure of isolation. After a density gradient centrifugation and lysis of erythrocytes, the cells were washed, and analyzed by flow cytometry. Five thousand events were acquired by a FACSCalibur™ flow cytometer (BD).

3.7.2.4. Bovine PMN migration assay

A cell culture non-conditioned IEL-medium (1.5 ml) was used as a negative control and a supernatant of stimulated-PBMC cultures (see **3.7.2.1**) (1.5 ml) was used as a positive control to induce the migration of bovine PMN.

Culture supernatants of iIEL to be tested (agonists, see **3.4**) were centrifuged 10 min at 10,000 × g (Centrifuge Eppendorf 5804R) to eliminate dead cells and debris and then 1.5 ml was transferred to the wells of a 12 well-flat bottom plates (the lower compartment of the migration system). The upper compartment, cell culture inserts with 3 µm pores (12 mm diameter, Transwell clear, Corning Costar, Germany), were carefully placed in the wells and immediately filled with 500 µl of PBMC medium containing 5×10^5 neutrophils. The multiwell-plates were incubated 1 h 45 min at 37°C. After the incubation, the plate was placed 15 minutes at 4°C to stop the migration. During this time, DiO-BL-3 cells were thawed, centrifuged 5 min at 350 × g and washed once in PBS 1X, counted by trypan blue exclusion, and the cell concentration was established at 1.5×10^4 per 50 µl of PBS 1X. During the whole procedure, DiO-BL-3 cells were protected from light and stored on ice.

The filters were carefully removed from the wells, placed in free wells and the upper surface was washed with 500 µl of PBS-EDTA 1X. This cell suspension was transferred to 5 ml-tubes containing 55 µl of PBS 1X-EDTA 10X. The tubes which were placed on ice to decrease the cell adhesion.

The cell suspensions of the lower compartments (migrating PMN) (1.5 ml) were transferred to FACS tubes and the wells were washed with 166 µl PBS 1X-EDTA 10X. The cells were resuspended by pipetting and 500 µl of these suspensions from the lower compartment were transferred to 5 ml plastic FACS-tubes placed on ice. To avoid the adhesion of neutrophils to the wall of the tubes, 55 µl of PBS 1X-EDTA 10X were added per tube.

In order to count the PMN, 1.5×10^4 DiO-dyed BL-3 cells in 50 µl of PBS-1X were added per tube. A specific document for acquisition was created with the acquisition software Cell Quest Pro to discriminate the different populations of DiO-BL-3 cells, PMN and lymphocytes. Each sample was measured in triplicates.

Data were exported and analysed with the analysis software FCS Express 2. The plots presented in figure 6 originate from a single neutrophils' preparation. The gating strategy displayed on the following FACS plots discriminate the different cell types in presence. The DiO-BL-3 cells are displayed in R1. As the neutrophils' preparation could contain up to 10 % of mononuclear cells and debris (R1 and R2), the gating strategy was not elaborated based on the morphology of the cells. As seen on the plot 6B, the BL-3 cells were dyed and were the only fluorescent events (R5). Consequently, the acquisition stopped exactly after 1,000 DiO-BL-3 cells were counted in the region R5.

The plots A and C (Fig. 6) also demonstrate that the lymphocytes present in the preparation do not migrate in this system and stay in the upper compartment. These lymphocytes are found in the region R4, as they have a low granularity and express a low fluorescence.

Polymorphonuclear eosinophils were identified as a granulocytic subpopulation displaying a high autofluorescence (54). In absence of any evidence, it can be hypothesized that the cells located in the region R8 (right part of R6) are some eosinophils.

In order to calculate the percentage of migration induced by IEL culture supernatants of each condition, the number of migrating PMN incubated with non-conditioned IEL medium was used as a reference and defined as 100 %.

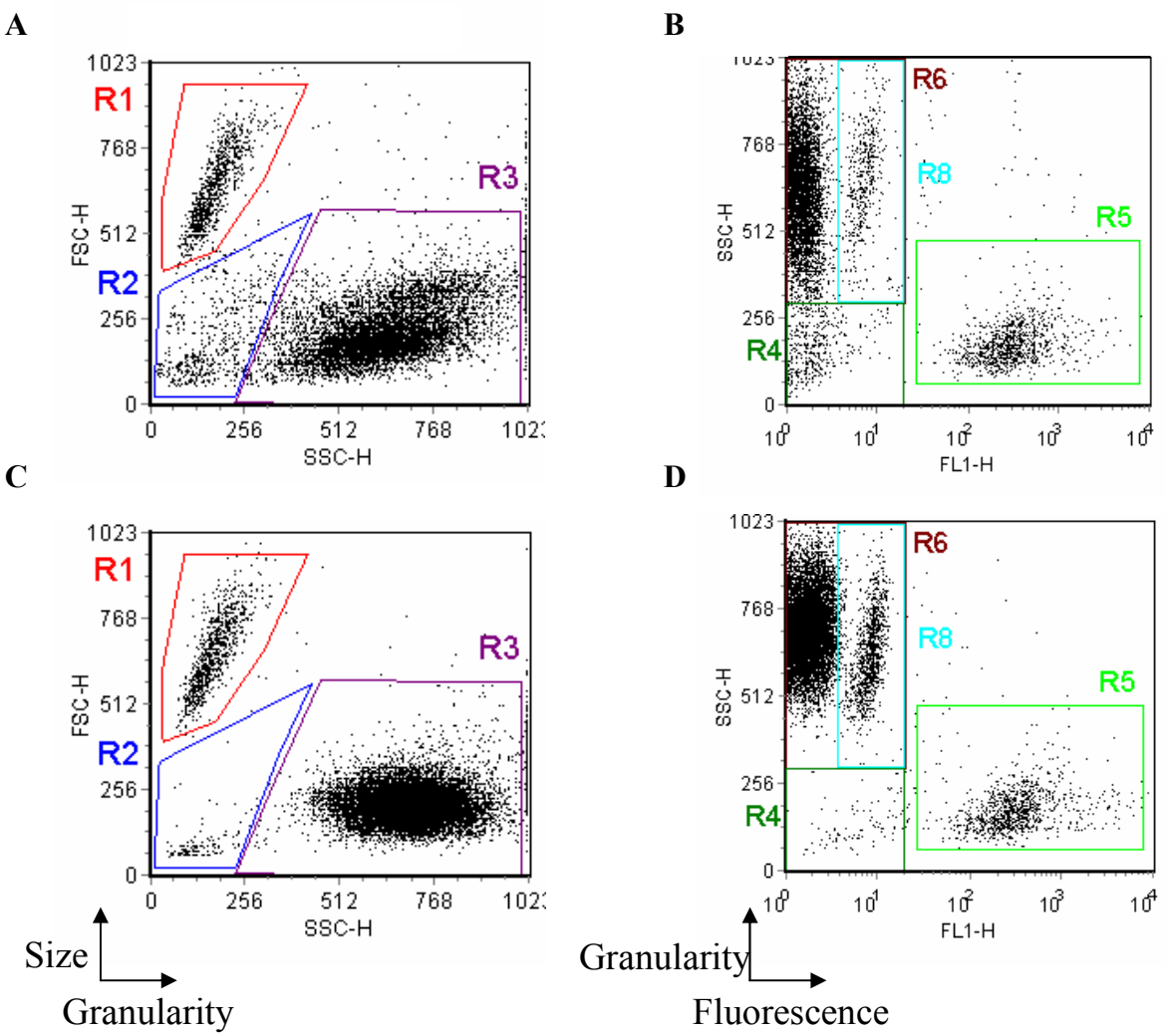


Fig. 6. Counting of PMN of both upper (A, B) and lower (C, D) compartments with DiO-BL-3 cells by a FACSCalibur™ flow cytometer. The cell counting stopped after 1,000 DiO-BL-3 cells were acquired by the cytometer. The figures display the morphology (A, C) and the fluorescence (B, D) of the different cell types in presence (migration induced by the positive control). The gating strategy allowed the discrimination of the different cell types. R1: DiO-BL-3 cells, R2: debris, R3: Neutrophils, R4: Lymphocytes, R5: DiO-BL-3 cells (1,000 events acquired), R6: Neutrophils to be counted.

3.8. Analysis of the importance of the enzymatic activity of Stx1 and induction of apoptosis of iIEL

3.8.1. Cultivation of bovine PBMC, Daudi and Ramos cells

Daudi and Ramos cells were used as positive controls for Stx1- and StxB1-induced apoptosis and to confirm the suitability of the detection systems. Daudi cells obtained from the European Collection of Cell Cultures (ECACC Nr. 85011437) and Ramos cells (ATCC. Nr 91030710, RA1 cells), a kind gift of Prof. Dr. M. Oppermann (Georg-August University, Göttingen, Germany), were cultured in RPMI 1640 supplemented with 2 mM of glutamine (PAN™ Biotech GmbH), 100 U/ml of penicillin, 100 µg/ml of streptomycin (PAA Laboratories GmbH), and 10 % of FCS (Biowest).
Ramos and Daudi cells were incubated 20 or 24 hrs, respectively, in the presence of the components listed in the table 7.

Table 7. Conditions of incubation for the study of apoptosis.

Substance	Concentration	Origin / Manufacturer
Actinomycin D	2 µg/ml	Sigma
Stx1	200 CD_{50}/ml	In house-prepared
Stx1 + anti-StxB1 MAb	200 CD_{50}/ml; 1.5 µg/ml	Both in house-prepared
rStxB1 subunit	10 µg/ml	In house-prepared
rStxB1 subunit + anti-StxB1 MAb	10 µg/ml; 1.5 µg/ml	Both in house-prepared
anti-CD77 antibody (clone 38.13)[1)]	50 µg/ml	Dr. J. Wiels, France
LPS	1 ng/ml	Sigma
Brefeldin A	10 µg/ml	Sigma
Brefeldin A + Stx1	10 µg/ml; 200 CD_{50}/ml	Sigma; In house-prepared

[1)] pre-incubated 90 min at 37°C with a goat anti-rat IgM (µ-chain specific)(Dianova, 10 µg/ml)

The recombinant rStxB1 subunit was prepared in our institute by Dr. I. Stamm from the *E. coli* DH5α [pSU108] strain as described in Stamm *et al*. (2002). The preparation was purified by affinity chromatography (FPLC®, Amersham Biosciences Europe GmbH), passed through a Detoxi-Gel™ column (Pierce) to reduce endotoxin contaminants. The analysis by

MALDI-TOF mass spectrometry was performed by Dr. M. Wuhrer (Institute for Biochemistry, Justus-Liebig University, Giessen). The rStxB1 subunit preparation was mainly composed of monomers containing the signal peptide (9750.3 m/z) and dimers (19468.0 m/z) (Fig. 7) which could also multimerize *in vitro*. The StxB1-subunit preparation was analyzed by SDS-PAGE, and titrated (162 µg/ml) with the BCA protein assay® (Pierce, Old Beijerland, The Netherlands). The *Limulus* amoebocyte lysate assay indicated a concentration of 0.93 ng of endotoxin per ml.

For functional assays, the 38.13 antibody was kindly provided by Dr J. Wiels (Institut Gustave Roussy, CNRS, Villejuif, France), as sodium azid-free ascites.

In addition to Burkitt's lymphoma cell lines, the apoptosis was also induced on primary cultures of bovine PBMC. The cells were isolated as previously described and incubated 6 hrs in 9 ml of PBMC-medium (2×10^7), supplemented with either actinomycin D (2 µg/ml, Sigma), or 200 CD_{50}/ml of purified Stx1.

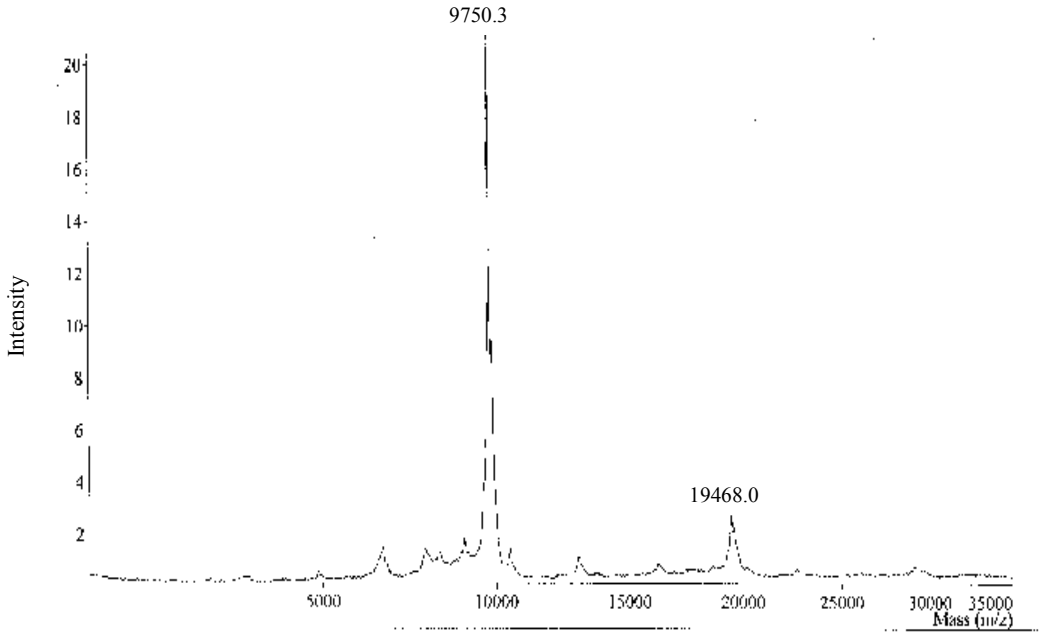


Fig. 7. Mass spectrometric analysis of the StxB1 subunit preparation.

3.8.2. Cultivation of bovine iIEL

For stimulation, the IEL-medium was supplemented with 2.5 µg/ml of PHA-P. The iIEL were isolated as previously described, and 2.5×10^7 iIEL were cultivated 6 or 20 hrs at 37°C and 5 % CO_2 in 9 ml of IEL-medium supplemented with the components described above (see table 7). As a control, apoptosis was also quantified in iIEL freshly isolated from the intestinal mucosa.

At the end of the 6 or 20 hrs of cultivation, iIEL were centrifuged for 5 min at 350 × g at 20°C. In the meanwhile, the wells were washed with 5 ml of ice-cold PBS-EDTA 1X. This suspension was then used to resuspend the cell pellet in the 50 ml-centrifugation tubes. The lymphocytes were washed once with PBS 1X. The isolation of mRNA from 2×10^7 iIEL was performed by using the RNeasy Mini Kit (QIAGEN). Reverse transcription and real-time-PCR were performed to detect the production of IL-4 mRNA, as previously described. The remaining iIEL (5×10^6) were used for investigation of apoptosis by flow cytometry as described beneath.

3.8.3. Detection of mitochondrial membrane potential

In order to quantify the mitochondrial membrane potential, 2×10^5 Daudi cells, Ramos cells, bovine PBMC or iIEL were seeded per well in a 96 U-shaped plates, centrifuged at 400 × g, 3 min and 20°C, resuspended in 100 µl of JC-1 dye (Biocarta Europe GmbH, Hamburg, Germany, diluted 1:400 in JC-1 buffer 1 X), and incubated for 15 min in the dark at 37°C. For analysis, the lymphocytes were washed once in JC-1 buffer 1X and transferred into 200 µl of PBS. JC-1 is a cationic dye that exhibit potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (monomer detected in FL-1 channel) to red (aggregates detected in FL-2 channel) in the conventional flow cytometer channels, respectively. According to the manufacturer protocol, the cells showing a decreased red fluorescence and/or an increased green fluorescence were considered as apoptotic and/or necrotic. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Five thousand events were acquired by a FACSCalibur™ flow cytometer and the acquisition software Cell Quest Pro. An appropriate layout was created with the analysis software FCS Express 2 which allowed the discrimination of cell populations with different viability (Fig. 8 and 9). Viable cells were localized in the region 4 (R4), while cells with altered mitochondrial

potential were located in R5, R6, and R7. The percentages of cells located in R5, R6, and R7 were summed and this number represented the percentage of cells with altered mitochondrial potential (see results' section). The same gating strategy, using four distinct regions, was utilized to analyse bovine PBMC (data not shown).

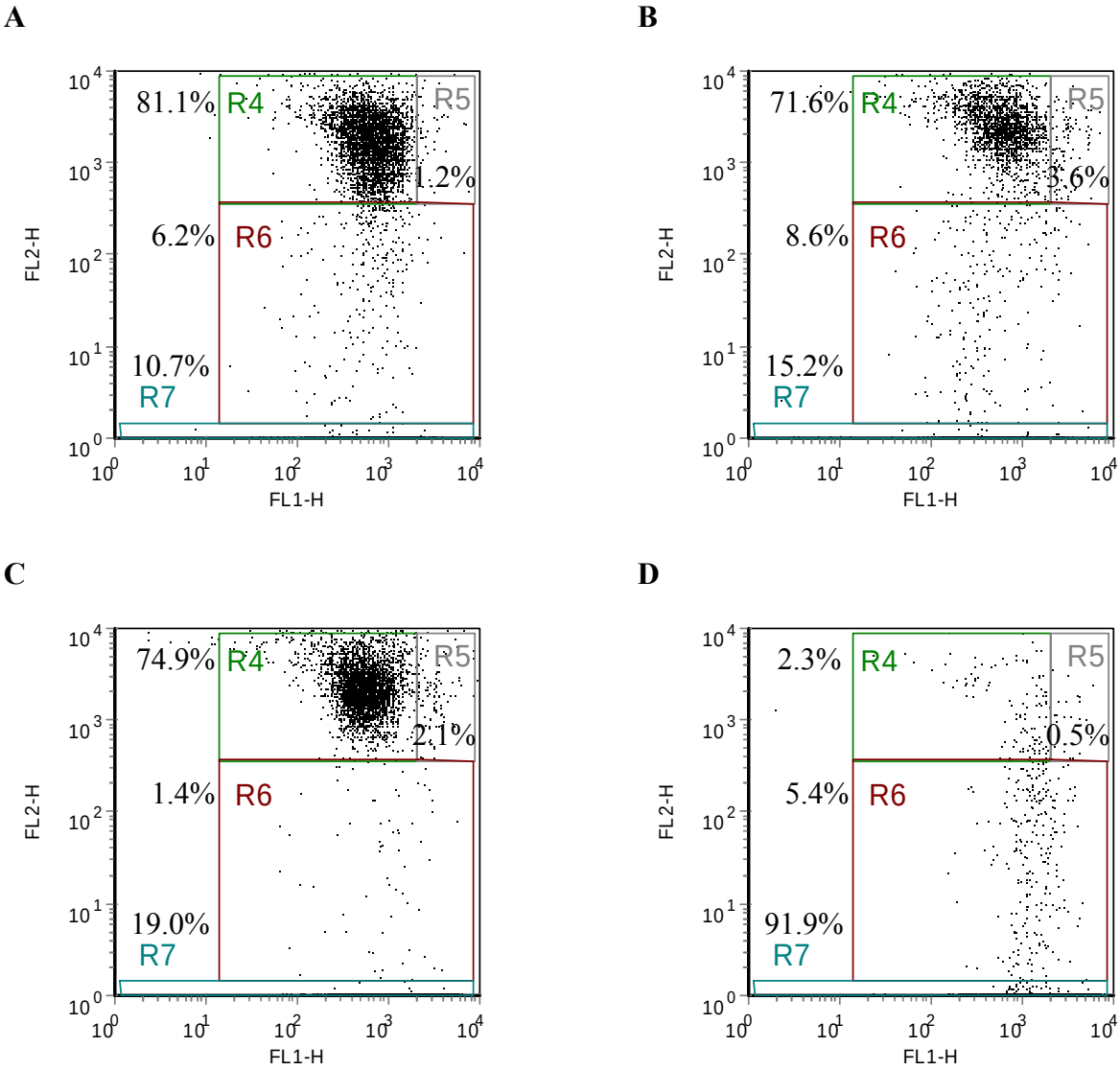


Fig. 8. Bi-colour JC-1 analysis of mitochondrial membrane potential in Daudi and Ramos cells by flow cytometry. Daudi (A, B) and Ramos (C, D) cells were incubated 20 and 24 hrs respectively in culture medium (A, C) or in medium supplemented with 2 µg/ml of actinomycin D (B, D). Daudi and Ramos cells were stained with the JC-1 dye and 5,000 events were acquired with a FACSCalibur™ flow cytometer.

Concerning iIEL, the gating strategy was different because the cells did not fit into the four regions described for the investigation with Daudi and Ramos cells. Consequently, only two regions were created with the analysis software FCS Express 2 (Fig. 9). The viable cells could be visualised in the upper region (R4) while subvital, apoptotic and necrotic cells were localised in the lower region (R5).

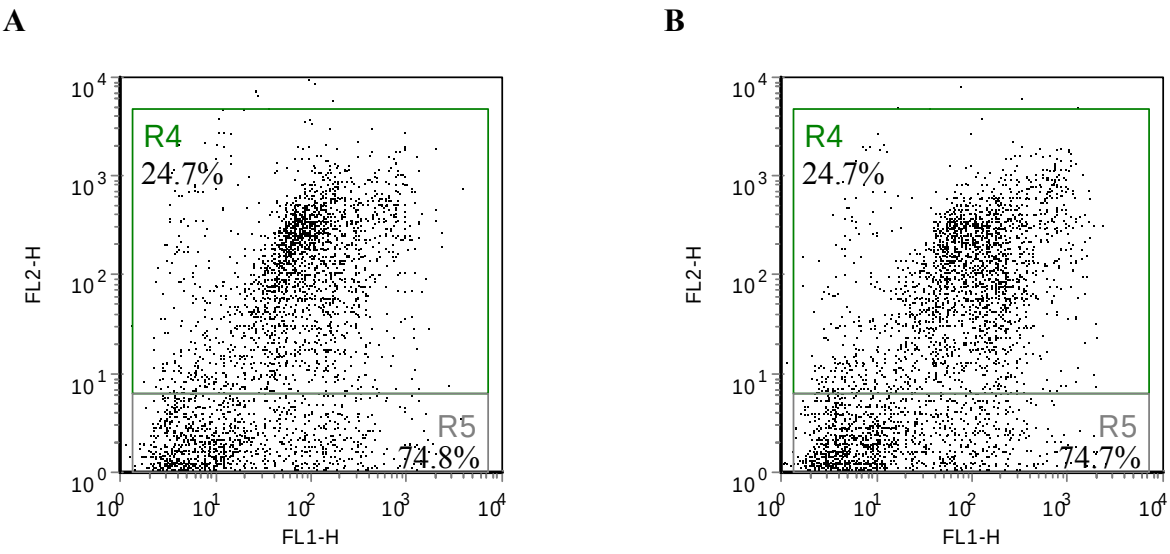


Fig. 9. Bi-colour JC-1 analysis of mitochondrial membrane potential in iIEL by flow cytometry. The cells were incubated 6 hrs in culture medium (A) or in medium supplemented with 2 µg/ml of actinomycin D (B). Ileal IEL were stained with the JC-1 dye, washed and analyzed with a FACSCalibur™ flow cytometer.

3.8.4. Detection of phosphatidyl serine exposure and staining of DNA

Phosphatidylserine redistribution from the internal to the external side of the cellular membrane of cells is a sign of early apoptosis that triggers specific recognition and removal of lymphocytes by macrophages (95). A vascular anticoagulant ubiquitous protein capable of binding phospholipids in the presence of calcium called annexin (403) was used for this assay. PE-conjugated human recombinant Annexin-V was purchased from Caltag Laboratories (Hamburg, Germany). The iIEL incubated in different conditions (Table 7) were seeded in 96 U-shaped plates at the density of 2.5×10^5 per well, centrifuged at 400 × g for 3 min and 20°C and incubated 20 min in the dark at 20°C in 5 µl of Annexin-V-PE (diluted 1:10 in PBS) and 100 µl of Ca^{2+}-containing Annexin-V binding buffer 1X containing 1 µg/ml

of 7-amino actinomycin D (7-AAD, Sigma). The cells were centrifuged, washed once in 100 µl of Annexin-V binding buffer, and transferred to test tubes containing 200 µl PBS. Single positive (Annexin-V^+) cells were considered as apoptotic while double-positive (Annexin-V^+, 7-AAD^+) cells were considered as necrotic. Five thousand events were acquired by a FACSCalibur™ flow cytometer (Becton Dickinson). An appropriate analysis-plot was created with the software FCS Express 2 (De Novo software) which allowed the discrimination of cell population viability (Fig. 10 and 11).

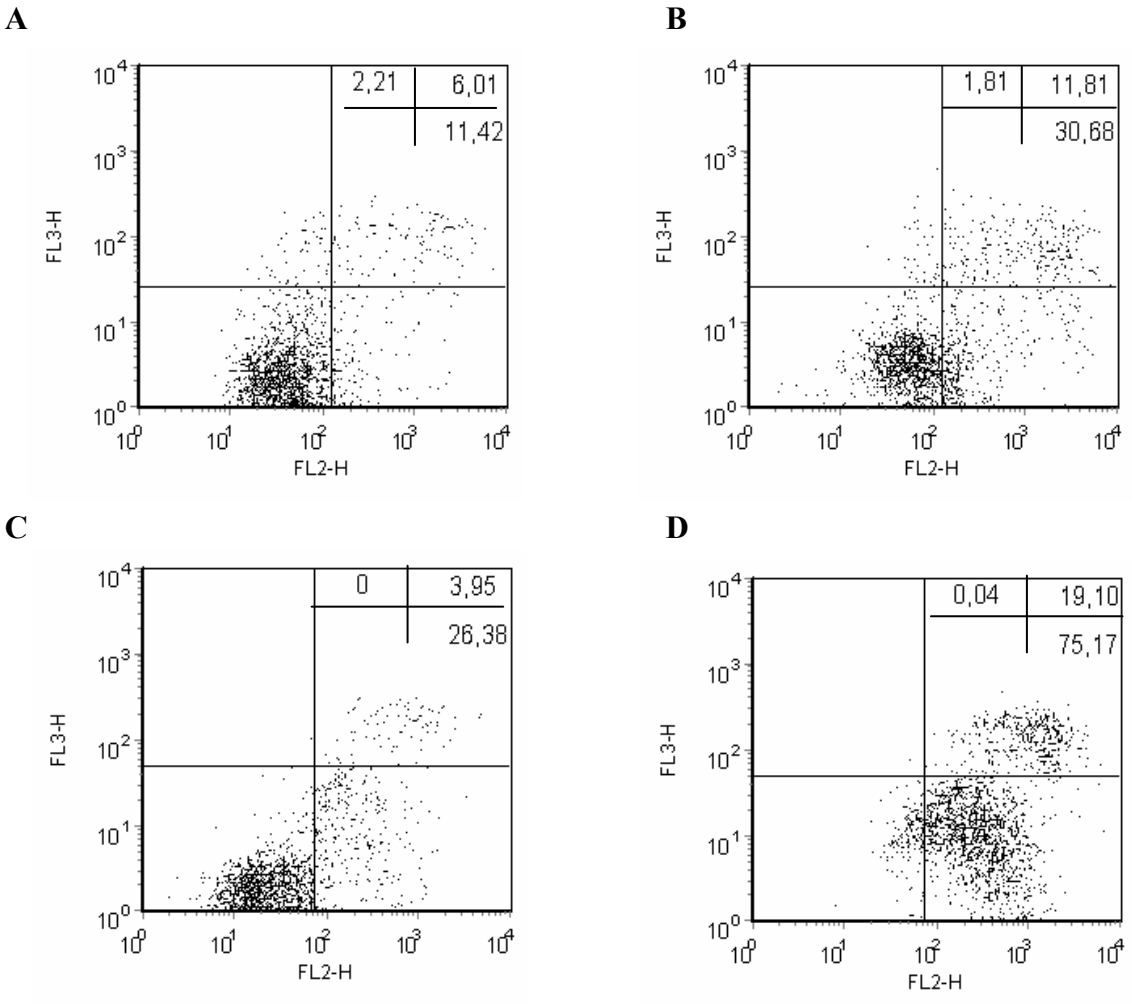


Fig. 10. Investigation of Daudi (A, B) and Ramos (C, D) cells viability by staining with Annexin-V and 7-AAD. The cells were incubated 20 (Daudi) or 24 hrs (Ramos) in culture medium only (A, C) or in medium supplemented with 2 µg/ml of actinomycin D (B, D). The cells were stained with Annexin-V-PE and 7-AAD, and analyzed with a FACSCalibur™ flow cytometer. Viable cells were localized in the lower left quadrant, early apoptotic cells in the lower right quadrant, and late apoptotic and necrotic cells in the upper right quadrant.

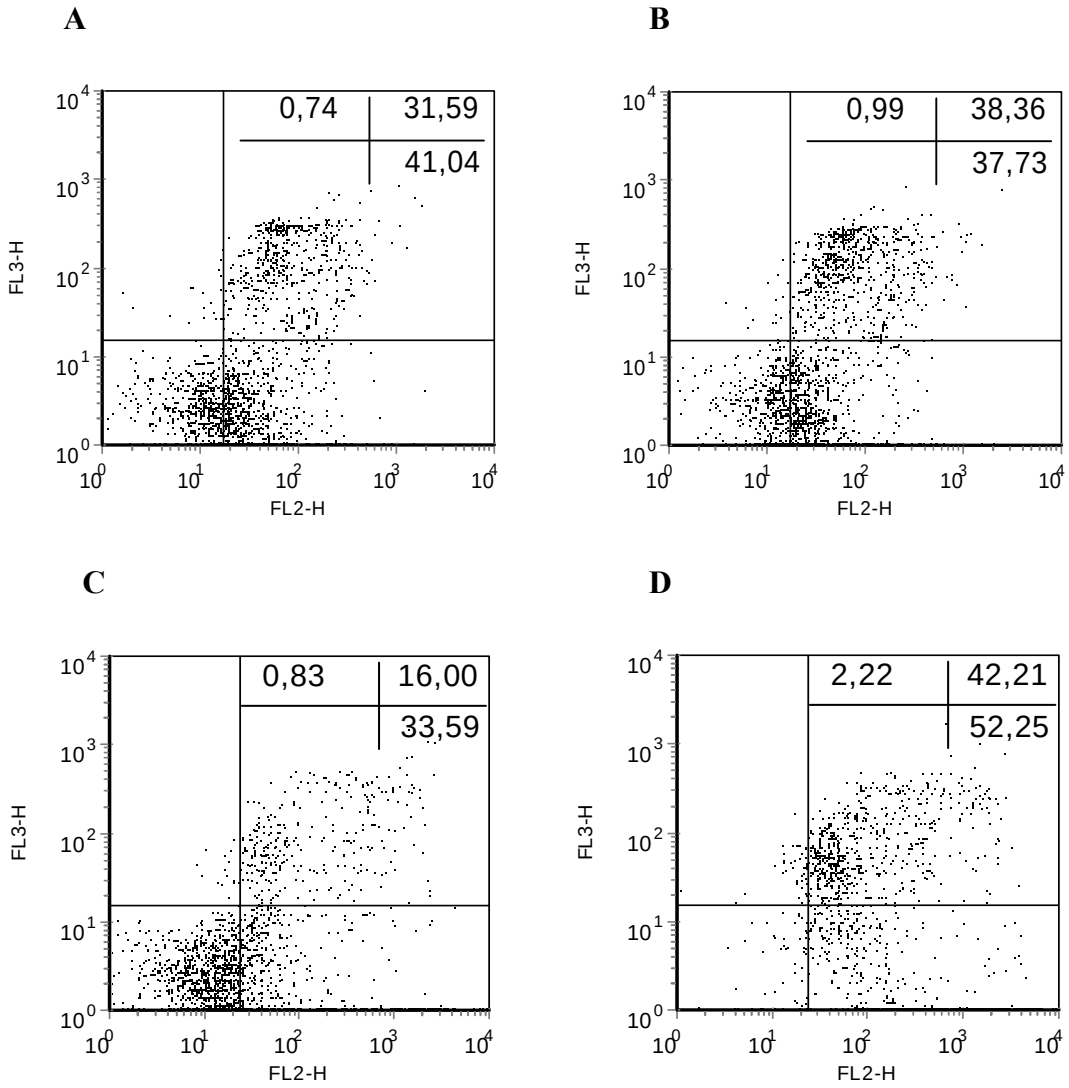


Fig. 11. Investigation of iIEL viability by staining with Annexin-V and 7-AAD. Cells were incubated 6 hrs (A, B) or 20 hrs (C, D) in culture medium (A, C) or in medium supplemented with 2 µg/ml of actinomycin D (B, D). Ileal IEL were stained with Annexin-V-PE and 7-AAD, and analyzed with a FACSCalibur™ flow cytometer.

3.9. Statistical analysis

All data presented in the results part of this study, the tables 8, 9 and the figure 17, 18, 28 excepted, were statistically analysed by Student's *t*-test for normally distributed data and by Mann-Whitney Rank Sum test for non-normally distributed data by using the SigmaStat (version 2.03 and 3.11) software (SPSS Inc., USA). Results evaluated as highly significant ($P \leq 0.001$, ***), significant ($P \leq 0.01$, **), or weakly significant ($P \leq 0.05$, *), are depicted on the figures. Results were evaluated as not significant if $P > 0.05$.

4. Results

4.1. Immunophenotyping of iIEL

The results presented here are the average of duplicate determinations from iIEL isolated from 4 different animals. Most of freshly isolated viable iIEL (absence of staining by 7-AAD) were found to express the CD8α antigen (51.4 ± 11.7 %, Mean ± SD), less cells were $CD4^+$ (19.4 ± 11.6 %), and only a low number of cells expressed the specific B cell marker CD21 (6.4 ± 4.0 %). The γδ TCR (TcR N12) was expressed by 25.1 ± 4.2 % of the cells and 11.1 ± 7.1 % also expressed the WC1 antigen specific of some γδ TCR T cell subsets. The glycosphingolipid Gb_3/CD77, the receptor of Stx1, was found on the surface of 8.1 ± 1.8 % of the iIEL. As 61.1 ± 10.4 % of the cells expressed CD25, the majority of the iIEL showed an activated state *in vivo*.

4.2. Morphology of mitogen-stimulated iIEL

After isolation, the iIEL were seeded in IEL-medium, incubated 6 or 18 hrs and stimulated by mitogens in order to investigate their potential to transform to blast cells. The figure 12 depicts a representative analysis of mitogen-stimulated iIEL

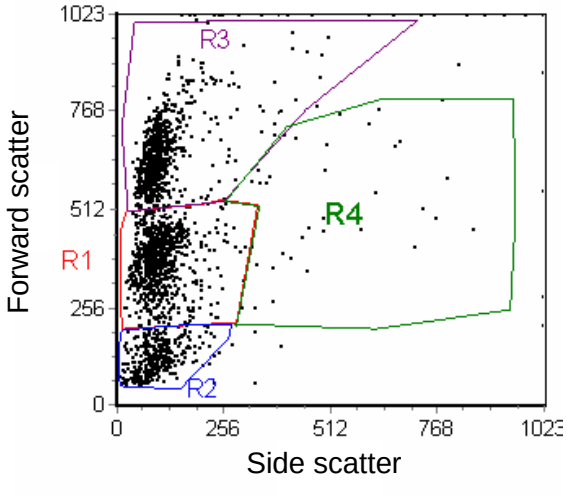


Fig. 12. Morphology of iIEL stimulated 6 hrs by PMA and ionomycin. Five thousand events were acquired with the FACSCalibur™ flow cytometer. The side scatter indicates the granularity of the cells and the forward scatter indicates the size of the cells.

Viable but not yet transformed cells can be observed in the region R1, while debris and subvital cells are localized in the region R2. The stimulation of iIEL with mitogens for 6 hrs did also induce the transformation of certain subsets of cells and the presence of blast cells could be consequently observed in the region R3.

4.3. Effect of Stx1 on the blast transformation of iIEL

In order to assess whether the effect of Stx1 on the iIEL activation previously reported (31, 248) could be reproduced, the blast transformation was quantified by flow cytometry. Ileal IEL were incubated in culture medium supplemented with mitogens, in the absence or presence of Stx1, and for the latter with or without anti-StxB1 antibody. As shown by the figure 13, iIEL treated with Stx1 showed a significant decrease in the proportion of blast cells ($P \leq 0.01$), accompanied by a significant increase in the percentage of untransformed viable cells ($P \leq 0.01$) in comparison to the cells incubated with medium.

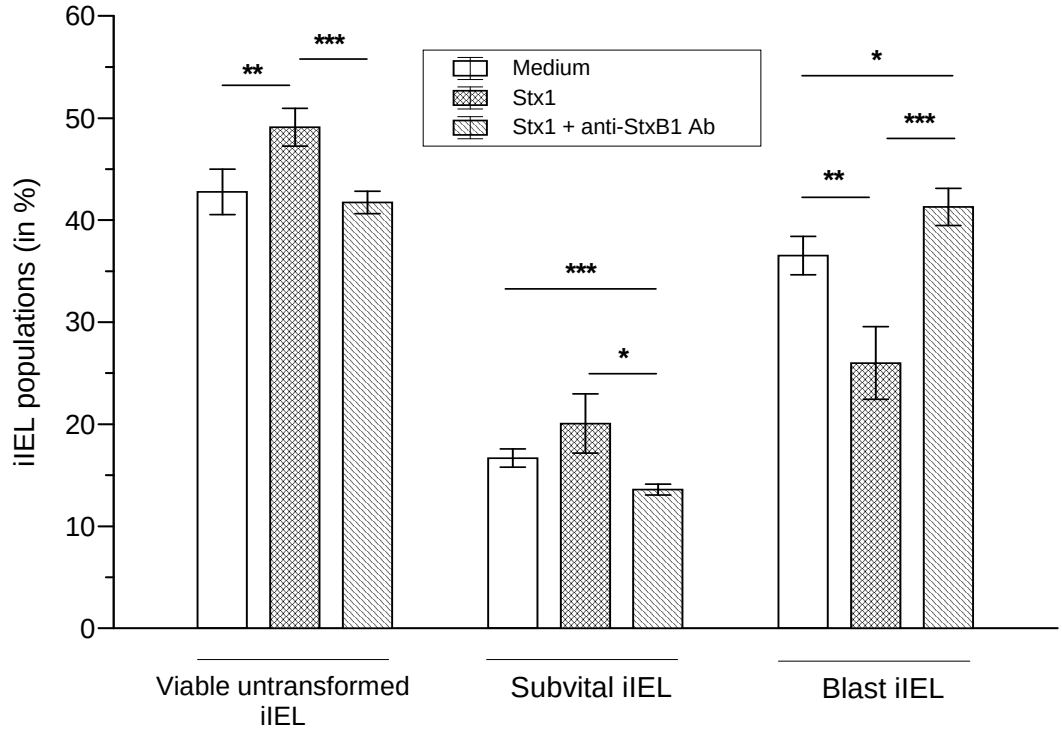


Fig. 13. Effect of Stx1 on the blast transformation of iIEL. Ileal IEL were stimulated with PMA and ionomycin and incubated 18 hrs in absence or presence of 200 CD_{50}/ml Stx1 and with or without 1.5 µg/ml anti-StxB1 antibody. Data are arithmetic means and standard deviations of 4 independent experiments with duplicates. Five thousand events were acquired by the flow cytometer. Significant effect of Stx1 was determined by Student's t-test and depicted if $P \leq 0.001$ (***), $P \leq 0.01$ (**), or $P \leq 0.05$ (*).

This effect of Stx1 was neutralized by the anti-StxB1 antibody which restored the number of blast cells ($P \leq 0.001$) to a level comparable though slightly different ($P \leq 0.05$) from the medium control.

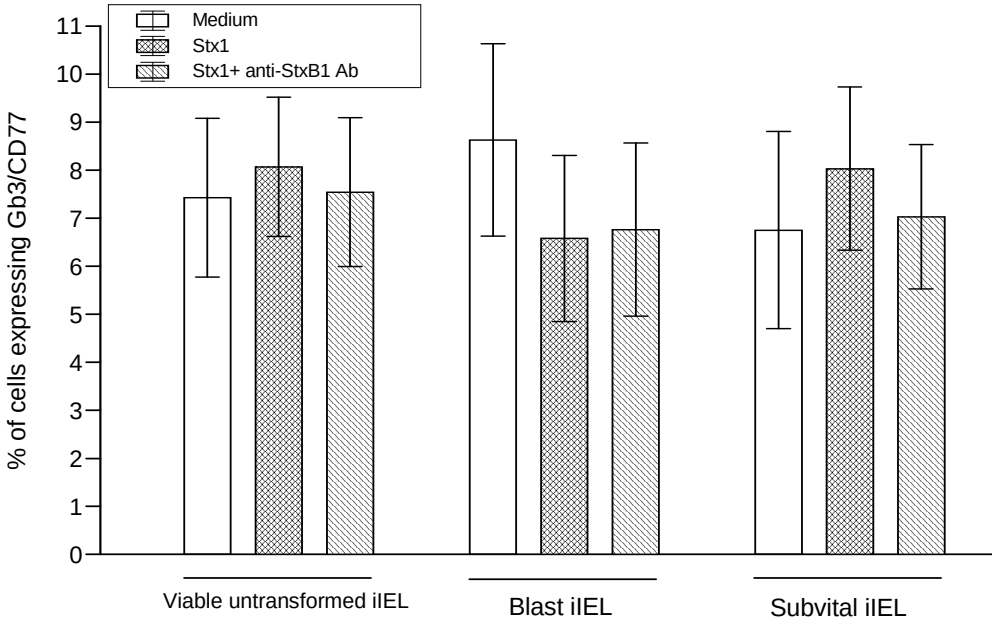
4.4. Effect of Stx1 on the expression of Gb_3/CD77 by iIEL

As a consequence of treatment with Stx1, a decrease in the number of iIEL expressing Gb_3/CD77 was reported (31). Consequently, the effect of Stx1 on iIEL was investigated here on 18 hrs-mitogen-stimulated cells to determine whether this observation was due to the elimination of the cells from the culture or from a redistribution of the receptor from the cell surface to the intracellular compartments. The analysis of native iIEL indicated that Stx1 slightly reduced both the percentage of blast cells expressing Gb_3/CD77 (Fig. 14A) and the level of its expression per cell (i.e. the fluorescence intensity of Gb_3/CD77 detection, Fig. 14B). However, this effect could not be blocked by the anti-StxB1 antibody. In contrast to blast iIEL, both the percentage of expressing cells and the level of Gb_3/CD77 expression were increased in subvital cells, from 6.7 to 8.0 % and from 39.1 to 69.0 A.U. (arbitrary units), respectively. The expression of the toxin receptor by viable cells was not affected by the toxin.

However, evidence was provided that native and fixed/permeabilized cells showed an important difference in Gb_3/CD77 expression. While not all iIEL expressed the receptor on the cell surface, after permeabilization of the cells, Gb_3/CD77 became detectable in an higher number of iIEL. Both the percentage of cells expressing Gb_3/CD77 and the level of expression increased. As only 7.4 ± 1.6 % of the viable iIEL expressed the receptor on the cell surface, 30.7 ± 2.2 % of viable iIEL incubated with medium possess Gb_3/CD77 intracellularly. This tendency was also true concerning the other iIEL populations (blast and subvital iIEL). In addition, Stx1 had a moderate effect on the percentage of subvital iIEL expressing Gb_3/CD77 (Fig. 14C, from 22.2 to 28.2 %) but no effect on viable and blast cells was observed (Fig. 14C and D). The total content in Gb_3/CD77 of the cells was not changed.

Taken together, the data indicate that, upon treatment with Stx1, the receptor is redistributed from the surface to the intracellular compartments but Stx1-affected cells appear to remain present (viable) in the culture, a prerequisite for further investigations on the effect of Stx1 on the molecular level.

A



B

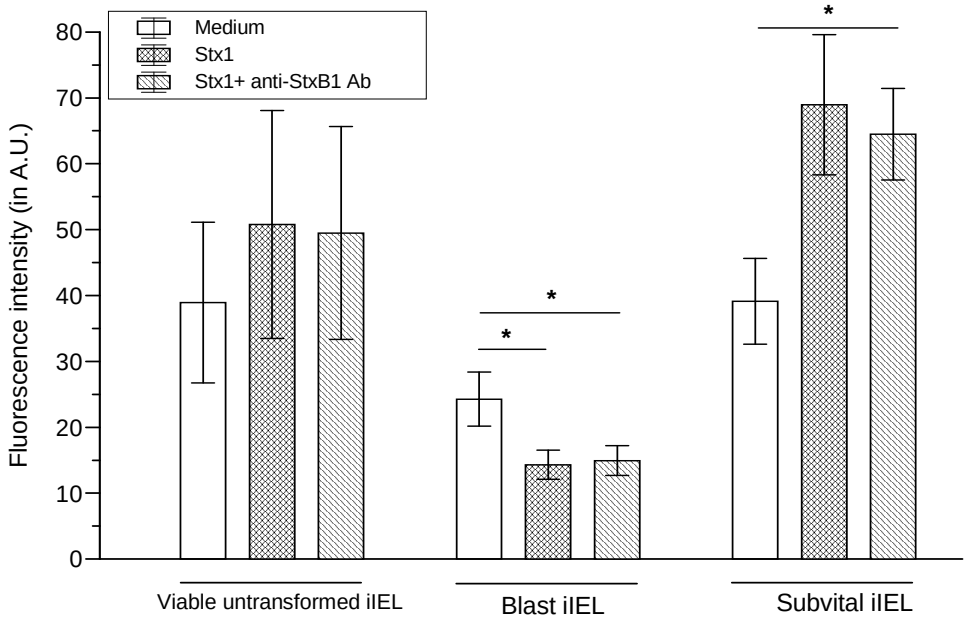
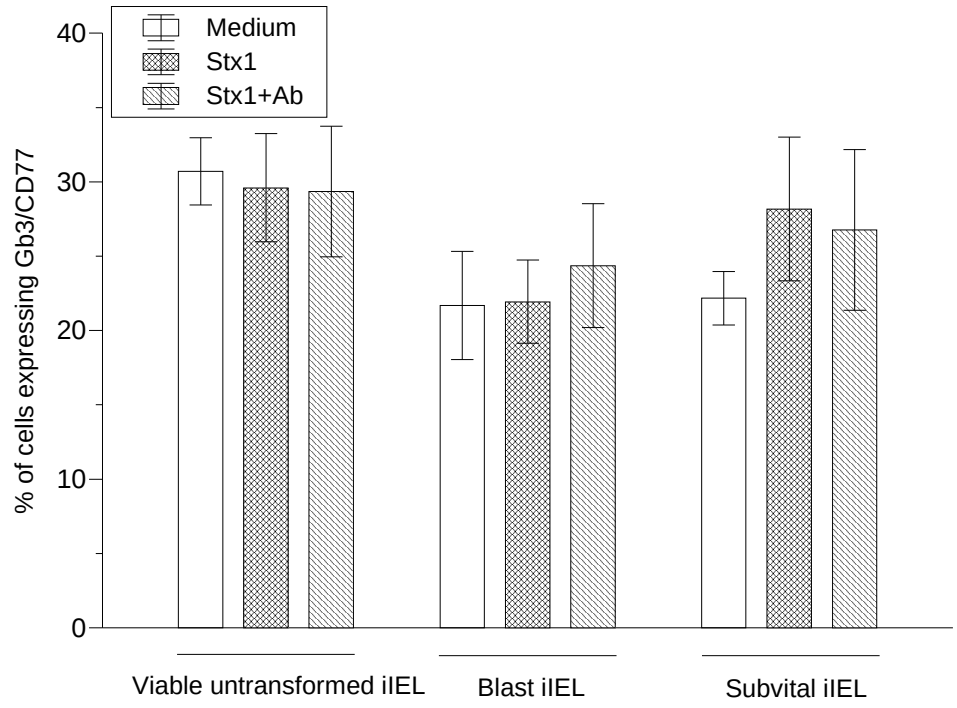


Fig. 14. Effect of Stx1 on the expression of Gb_3/CD77 by iIEL. PMA and ionomycin-stimulated cells were incubated 18 hrs in presence or absence of 200 CD_{50}/ml of Stx1 or with Stx1 and 1.5 µg/ml of anti-StxB1 antibody. The quantitation of the percentage (A and C) and intensity of fluorescence (B and D) of Gb_3/CD77 detection by iIEL was performed by flow cytometry (5,000 events) on native (A and B) or fixed and permeabilized cells (C and D). Data are arithmetic means and SEM of 4 determinations with duplicates. Significant effect of Stx1 was determined by Student's t-test or by Mann-Whitney Rank Sum test, and depicted if $P \leq 0.001$ (***), $P \leq 0.01$ (**), or $P \leq 0.05$ (*).

C



D

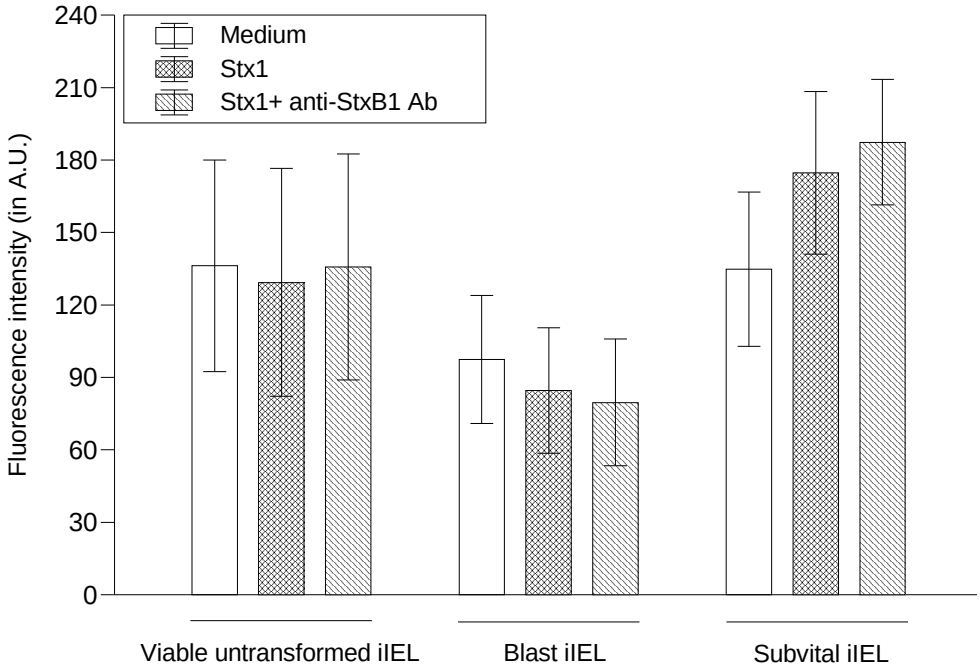


Figure 14 (continued).

4.5. Cytokine and chemokine mRNA expression by freshly isolated bovine iIEL

In order to evaluate the relative amounts of transcripts specific for cytokines and chemokines present in bovine iIEL after isolation and prior to any further stimulation *in vitro* (addition of mitogens or Stx1), the mRNA profile of freshly isolated iIEL was assessed by semi-quantitative RT-PCR. The figure 15 is a representative picture of what was observed in determinations with iIEL originating from 3 different animals. Freshly isolated iIEL were found to harbour transcripts for the chemokines (IL-8, IP-10, and MCP-1) and for the T_H1 (IL-2, IFN-γ), T_H2 (IL-4, IL-10), and T_H3-type (TGF-β) cytokines of the selected panel. In addition, transcripts for the proinflammatory TNF-α were also detected in the cells.

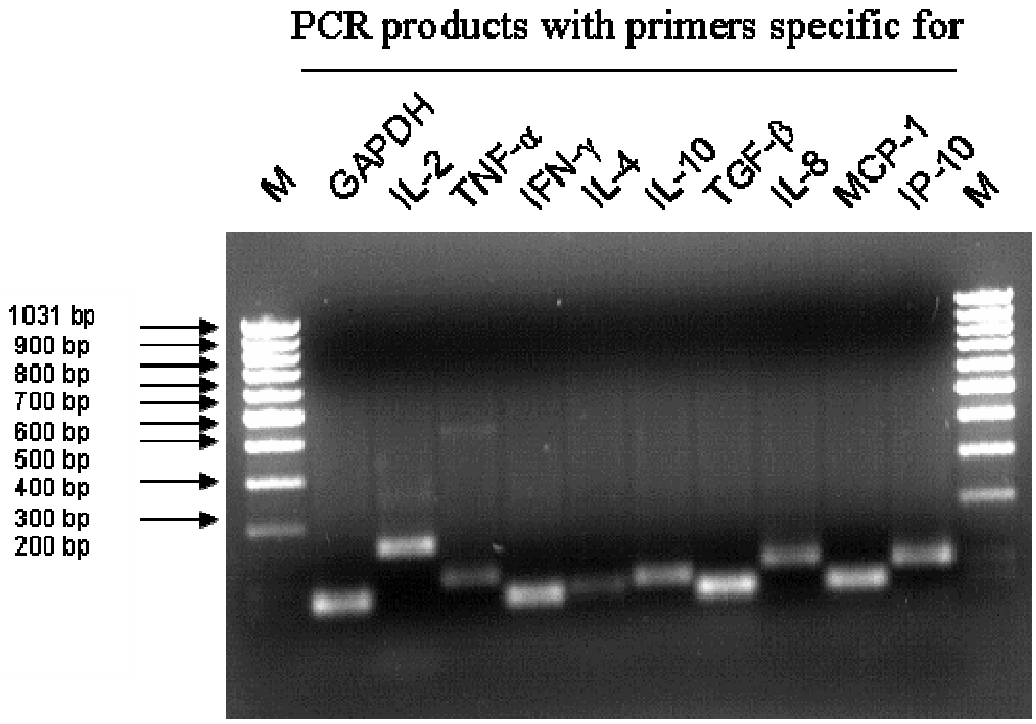


Fig. 15. Cytokine and chemokine mRNA profile of freshly isolated iIEL. Products of semi-quantitative RT-PCR were loaded into a 2 % agarose gel (M = GeneRuler™ 100 bp Marker, Fermentas GmbH). PCR products from a single representative iIEL preparation (n=3).

4.6. Chemokine expression in iIEL in the absence or presence of Stx1

4.6.1. Chemokine mRNA expression

After incubation of iIEL in medium supplemented with PHA-P for 6 hrs, the use of the real-time PCR ΔC_t values brought information concerning the amounts of chemokine transcripts present in the cells relative to *gapdh* transcripts. IP-10 mRNA was very rare in the iIEL (≈ 0.01 % of the *gapdh* transcript content), while transcripts of *mcp-1* were the most abundant (≈ 3 %). Transcripts of *il-8* were constantly harboured by the cells at a moderate level (≈ 1 %).

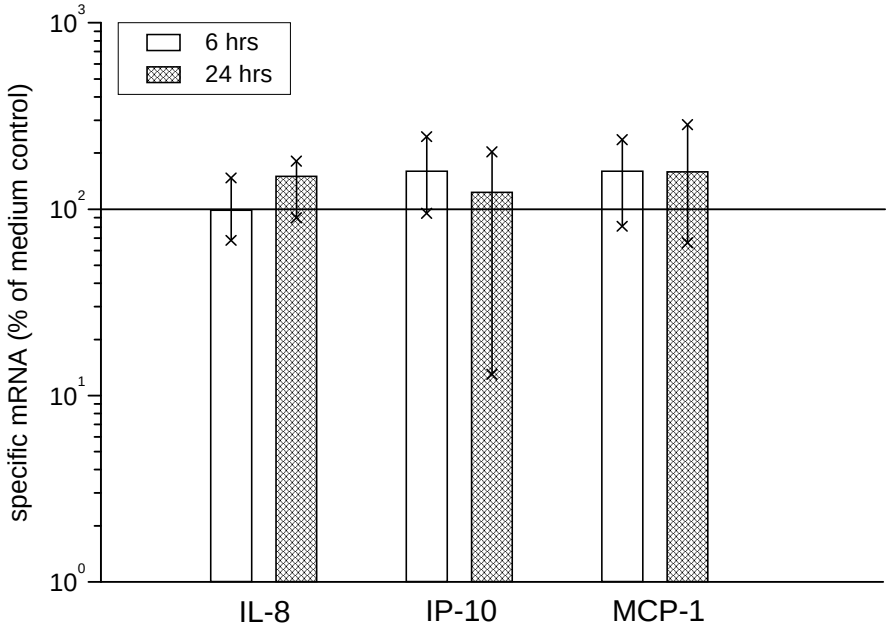
In order to evaluate whether Stx1 has an impact on the production of chemokines, the respective mRNA was quantified in mitogen-stimulated cells incubated in presence or absence of the toxin. As shown in figures 16A and 16B, the gene transcription of the three chemokines investigated (IL-8, IP-10, and MCP-1 mRNA) was not significantly altered by 200 CD_{50}/ml of Stx1 ($P > 0.05$) after several hours of incubation (up to 24 hrs).

4.6.2. Release of chemoattractant factors by iIEL

To rule out that Stx1 did not induce any post-transcriptional modifications and to assess whether the incubation of iIEL with Stx1 could influence the release of other chemokines not investigated by RT-PCR, a functional assay was performed (Fig. 17). The chemoattractant activity of culture supernatants was tested with bovine polymorphonuclear neutrophils. Used as a positive control, concanavalin A plus rhIL-2-stimulated PBMC culture supernatant efficiently induced the attraction of neutrophils to the lower part of the migration system ($P \leq 0.01$). The release of chemoattractive substances by iIEL into the culture supernatant was lower than in the PBMC control ($P \leq 0.05$).

Nevertheless, the migration rate of neutrophils towards iIEL-conditioned media was always significantly superior to the non-conditioned-medium control ($P \leq 0.01$), indicating that 8 and 18 hrs PHA-P-stimulated iIEL released chemoattractant factors in the culture. However, no variation could be observed in the migration rate of neutrophils between the different conditions indicating that Stx1 failed to influence the total release of chemoattractants by iIEL after 8 or 18 hrs of cultivation.

A



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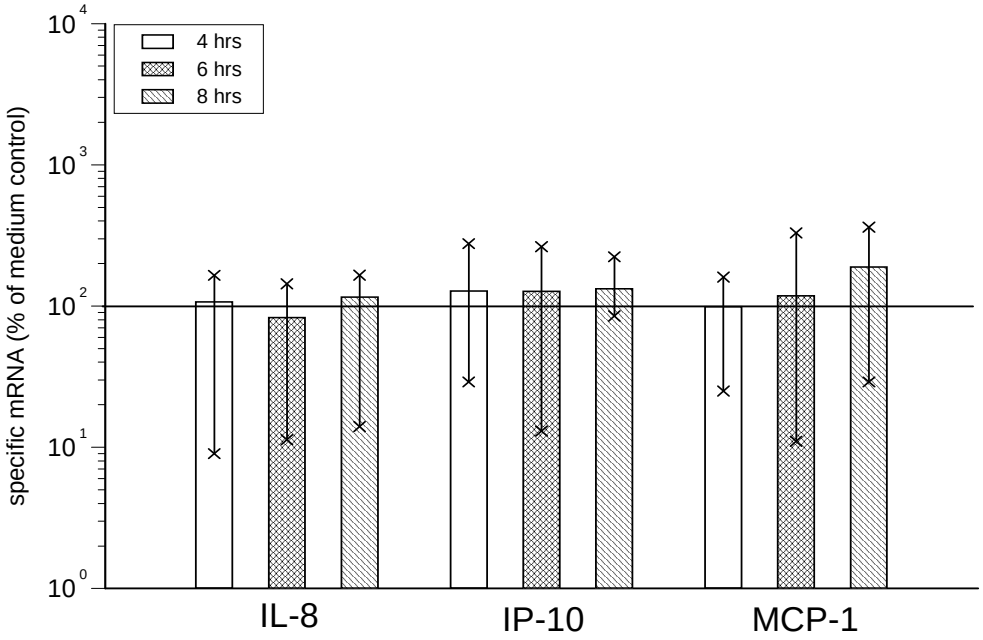


Fig. 16. Relative amounts of chemokine gene transcripts harboured by iIEL upon cultivation in presence of purified Stx1. PHA-P-stimulated cells were incubated 6 and 24 hrs (A), or 4, 6, and 8 hrs (B) with Stx1 (200 CD_{50}/ml as determined on Vero cells). After RT and quantitation by real-time PCR, the transcription of the housekeeping gene *gapdh* was used for normalization of the samples. Cells incubated with medium were used as a control (=100 % as visualized by the black line). Data presented are arithmetic means, minimal and maximal values of results obtained with iIEL preparations from 5 (A) and 6 (B) different animals.

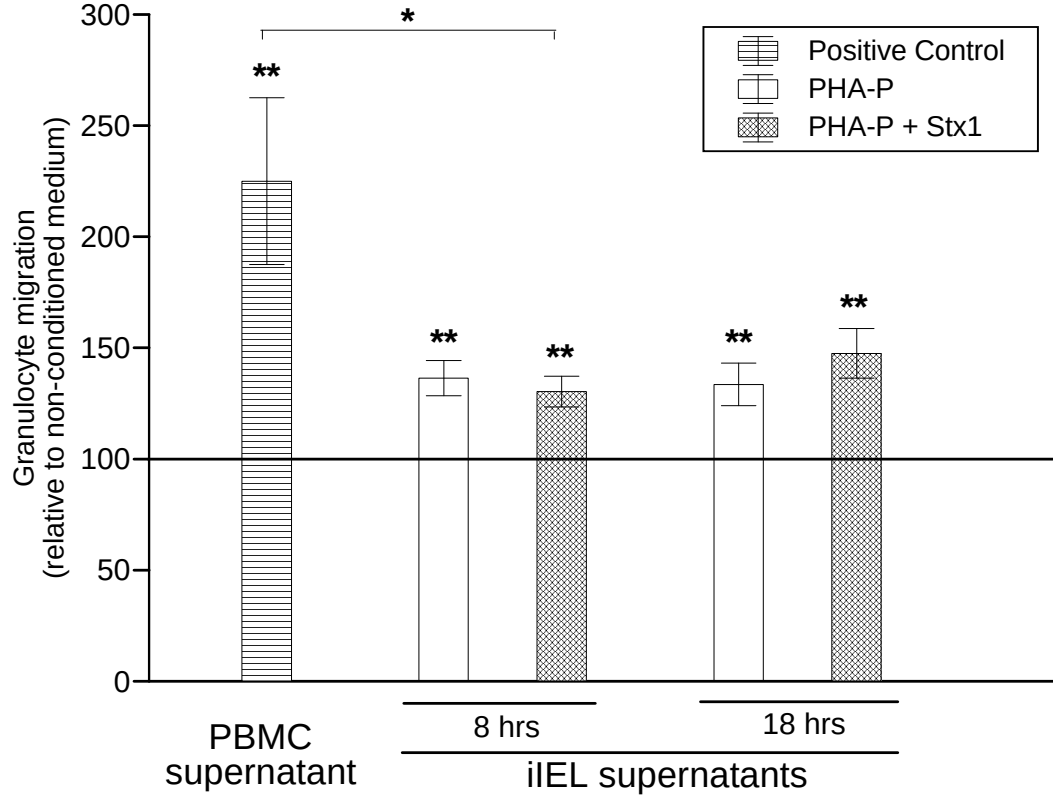


Fig. 17. Migratory activity of bovine neutrophils towards supernatants obtained from iIEL cultures incubated in absence or presence of Stx1. Bovine neutrophils were allowed to migrate for $1^{3/4}$ h at 37°C towards the lower compartment containing agonists (iIEL supernatants; stimulated PBMC supernatant as positive control), then harvested and counted. The results are expressed using a non-conditioned medium as a reference (=100 % as visualized by the black line). Data represent means ± SEM of values from migration assays with 6 independently obtained iIEL culture supernatants. Statistical analysis revealed that all conditions were significantly different from the non-conditioned medium ($P \leq 0.01$, **).

4.7. Effect of Stx1 on cytokine gene transcription in bovine lymphocytes

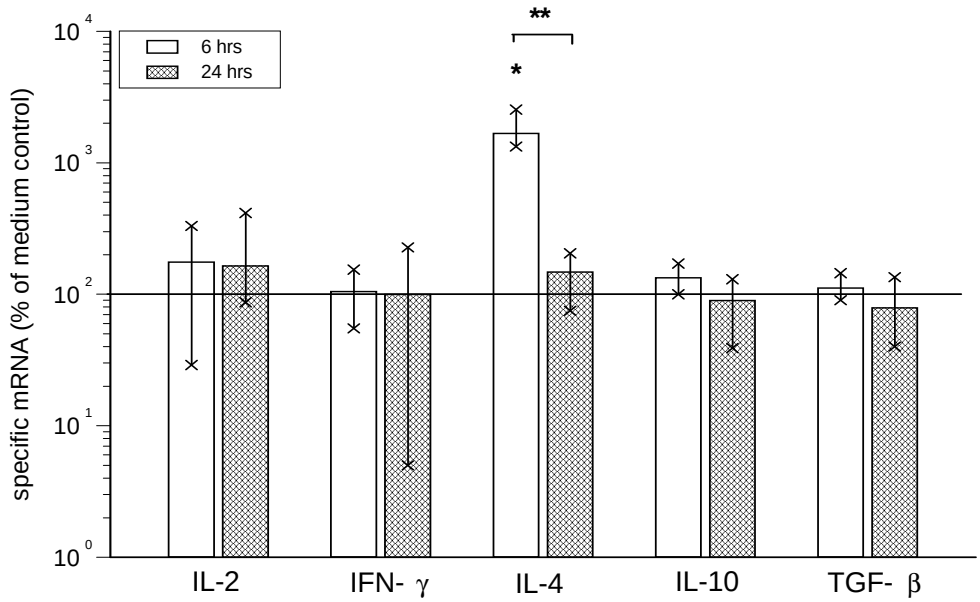
4.7.1. Investigations with bovine iIEL

To assess whether the toxin modified the production of cytokine transcripts after both short and long term incubations, iIEL were stimulated with mitogens *in vitro* and treated with Stx1 for 6 and 24 hrs. Real-time PCR indicated that Stx1 had no influence on the amounts of

transcripts of both pro-inflammatory T_H1-cytokines IL-2 and IFN-γ after both incubation times ($P > 0.05$, Fig. 18A). The level of the anti-inflammatory T_H3-cytokine TGF-β was as well not influenced by the toxin after the incubation times. Stx1 also did not affect the relative amounts of the *il-10* transcripts (Fig. 18A), even though they were detectable in only small numbers (Ct values 39-40) compared to other cytokines (data not shown). However, Stx1 significantly influenced the level of transcripts of the T_H2-type cytokine IL-4 within 6 hrs of cultivation ($P < 0.05$). This enhancement of *il-4* transcripts on iIEL was not detectable in cells incubated 24 hrs with the toxin (Fig. 18A). A more detailed analysis (Fig. 18B) with iIEL incubated for 4 to 8 hrs revealed that, despite the variability of sensitivity between different iIEL preparations, Stx1 enhanced the level of *il-4* transcripts as early as after 4 hrs of incubation (4.5 fold compared to the medium control) and this effect was dramatically increased in the next hours to reach a maximum after 8 hrs of incubation (42 fold compared to the medium control). As described above for 6 and 24 hour-incubations, the amounts of the other cytokine transcripts were not altered by 4-to-8 hour-incubations with the toxin.

This effect of Stx1 on bovine iIEL could be efficiently neutralized by pre-incubating the toxin 90 min at 37°C with 1.5 µg/ml of anti-StxB1 13C4 antibody (Fig. 19). In contrast to the effect of Stx1 on the Gb_3/CD77 surface expression, even the effect of 180 CD_{50}/ml could be perfectly neutralized by pre-incubation of the toxin with the anti-StxB1 antibody.
In addition, even if iIEL preparations from several animals strikingly differed in their sensitivity to the toxin, minute concentrations of Stx1 (7, 22, and 66 CD_{50}/ml), corresponding to the low range of picograms per millilitre, still induced a prominent enhancement of the amount of *il-4* transcripts in iIEL within 6 hrs of cultivation (Fig. 20).

A



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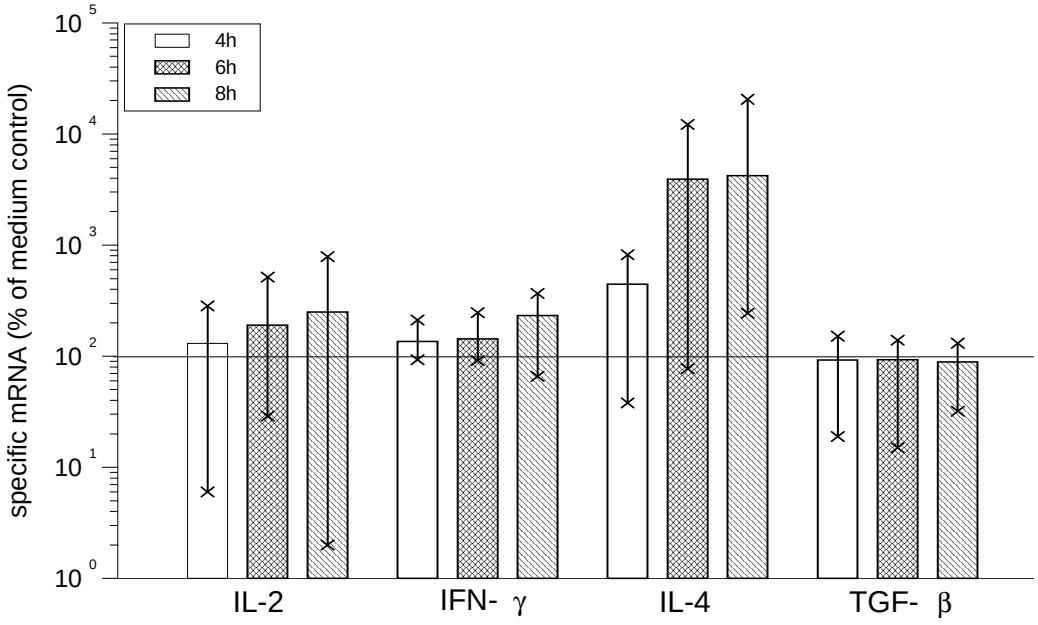


Fig. 18. Relative amounts of cytokine gene transcripts harboured by iIEL upon cultivation in presence of purified Stx1. PHA-P-stimulated cells were incubated 6 and 24 hrs (A), or 4, 6, and 8 hrs (B) with Stx1 (200 CD_{50}/ml as determined on Vero cells). After RT and quantitation by real-time PCR, the transcription of the housekeeping gene *gapdh* was used for normalization of the samples. Cells incubated with medium were used as a control (=100 % as visualized by the black line). Data presented are arithmetic means, minimal and maximal values of results obtained with iIEL preparations from 5 (A) and 6 (B) different animals. Statistically significant differences were determined by Mann-Whitney Rank Sum test and depicted if $P \leq 0.05$ (*) or ≤ 0.01 (**).

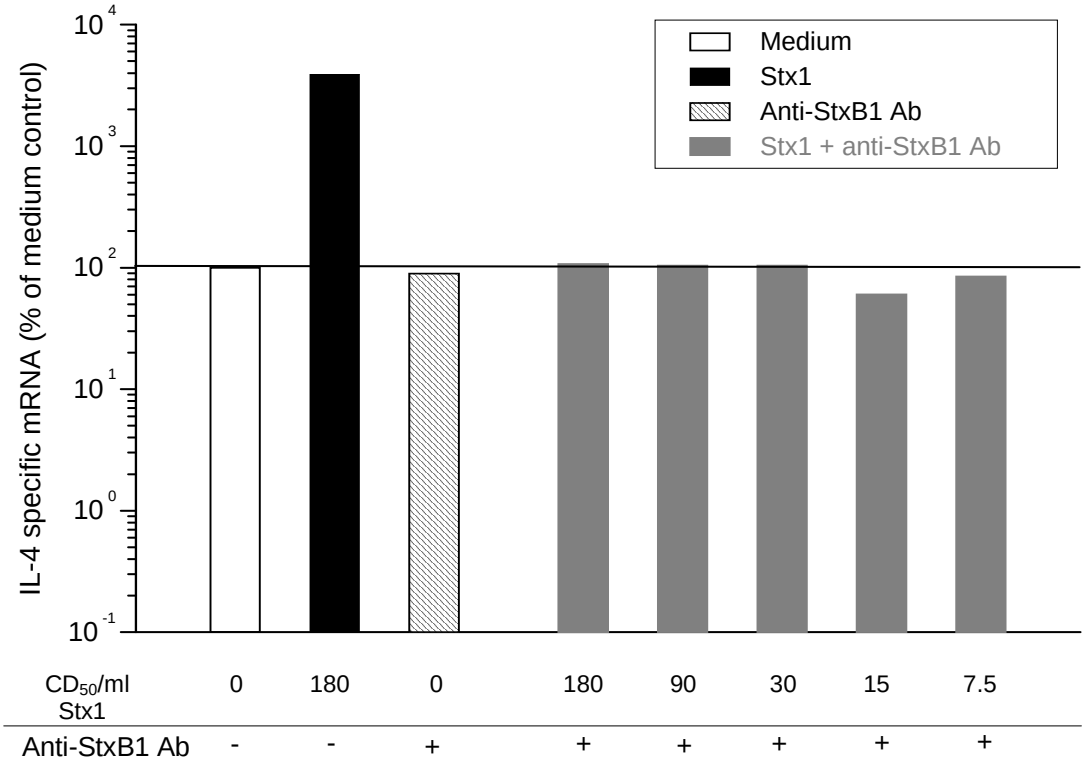


Fig. 19. Neutralization of the Stx1-induced enhancement of *il-4* transcript's level on iIEL. PHA-P-stimulated (2.5 µg/ml) cells were incubated 6 hrs with different concentrations of Stx1 (7.5, 15, 30, 90 and 180 CD_{50}/ml) or 180 CD_{50}/ml of Stx1 and 1.5 µg of anti-StxB1 antibody. After RT and quantitation by real-time PCR, the transcription of the housekeeping gene *gapdh* was used for normalization of the samples. Cells incubated with medium were used as a control (= 100 % as visualized by the black line). N=1 determination analysed in duplicates.

Bovine iIEL produced small amounts of transcripts encoding the IL-4δ2 splice variant (360 bp), as described in bovine peripheral lymphocytes (406). A shown by the figure 21, the IL-4δ2 splice variant was produced by both freshly isolated iIEL (lane 2) and by 6 hrs-mitogen-stimulated iIEL as well (lanes 3 to 5). Although the production of *il-4* full length (408 bp) transcripts was strongly increased by a 6 hrs-incubation with Stx1 (lane 4), semi-quantitative RT-PCR analysis did not provide evidence that Stx1 influenced the production of the splice variant δ2 when the cells were cultivated in presence of 200 CD_{50}/ml of the toxin. The splice variant δ3 could not be detected in iIEL.

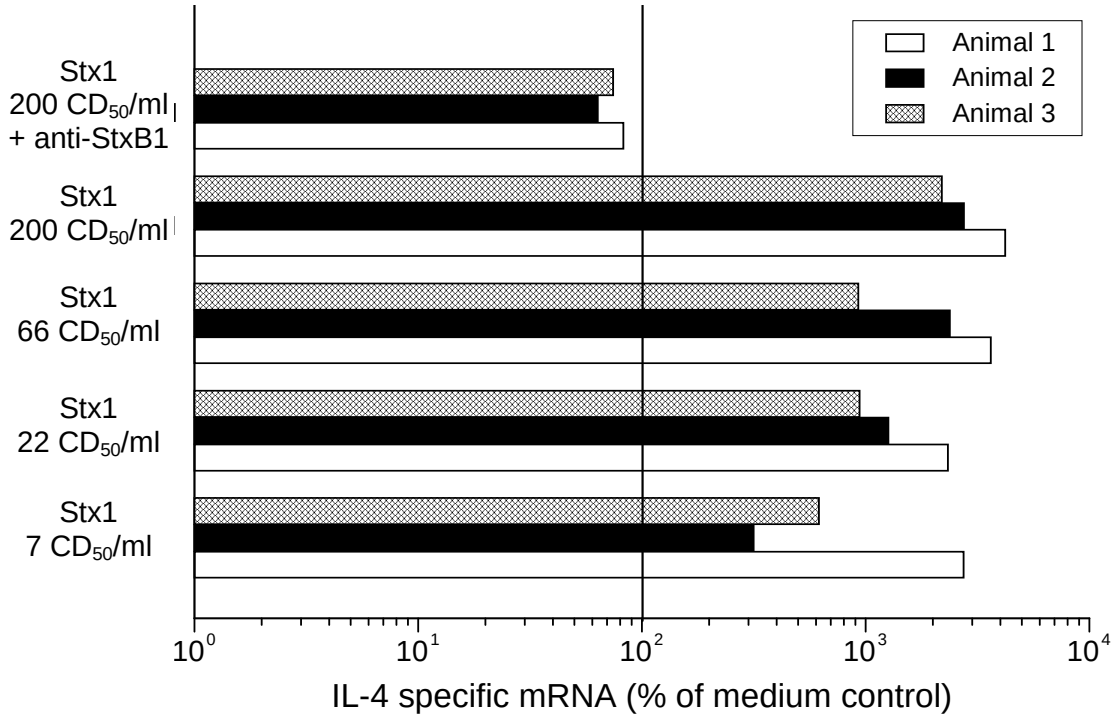


Fig. 20. Effect of purified Stx1 on the amounts of IL-4-specific mRNA in bovine iIEL cultures. PHA-P-stimulated (2.5 µg/ml) cells from 3 different animals were incubated for 6 hrs with different concentrations of Stx1 (7, 22, 66, and 200 CD_{50}/ml as determined on Vero cells) or with 200 CD_{50}/ml of Stx1 and 1.5 µg/ml of anti-StxB1 antibody 13C4. Subsequently, mRNA was reversely transcribed and quantified by real-time PCR. GAPDH mRNA expression was used as a standard. Cells incubated with medium only were used as a control (= 100 % as visualized by the black line). N=3.

4.7.2. Investigations with bovine PBMC

This enhancement of IL-4 mRNA cell content by Stx1 was specific for iIEL. Bovine PBMC incubated 6 hrs with the toxin (33 CD_{50}/ml of Stx1) did not produce more IL-4 transcripts than cells incubated with medium only. Stx1 influenced only slightly the cytokine and chemokine mRNA transcription compared to the medium control (lower than 2 fold, no statistical significance, $P > 0.05$) (Fig. 22).

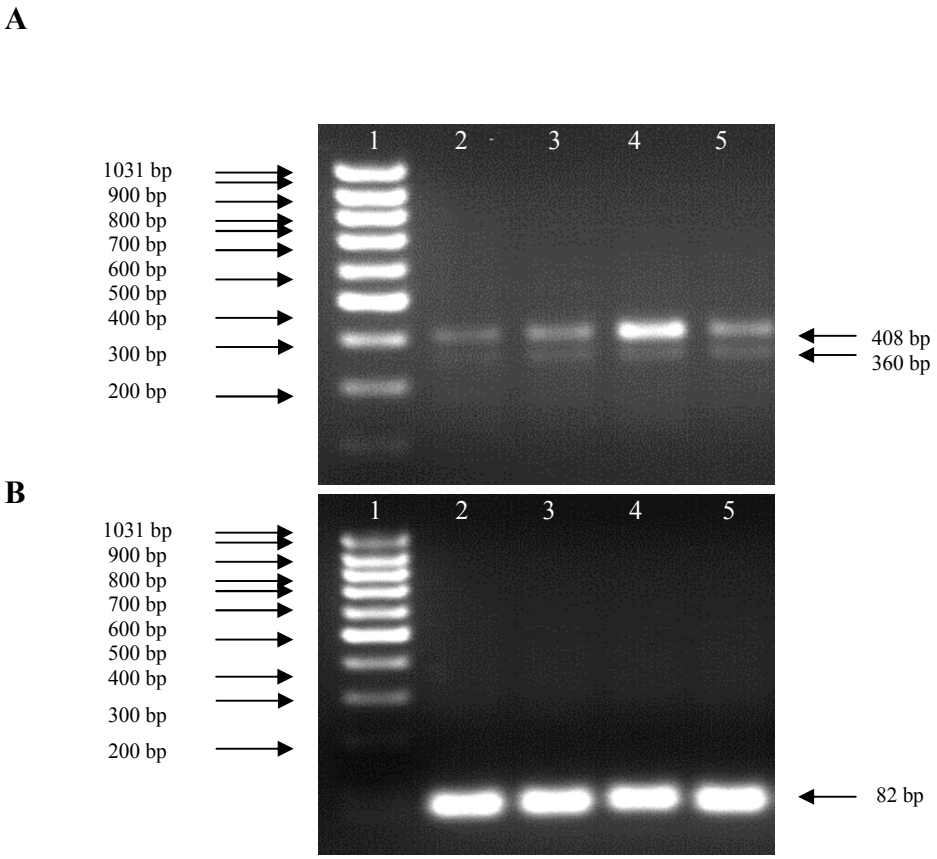


Fig. 21. Production of *il-4* full length (408 bp) and *il-4δ2* (360 bp) splice variants mRNA by iIEL. PHA-P-stimulated (2.5 µg/ml) cells were incubated 6 hours with 200 CD50/ml of Stx1 (as determined on Vero cells) or with 200 CD_{50}/ml of Stx1 and 1.5 µg of anti-StxB1 13C4 antibody. IL-4 mRNA was detected by semi-quantitative RT-PCR and PCR products were analyzed on a 2 % agarose gel (A). *gapdh* was used as a housekeeping gene for constitutive gene expression (B). Data presented are representative of 3 repetitions with different iIEL preparations.

Lane 1: GeneRuler™ 100 bp Marker

Lane 2: Amplification of cDNA from freshly isolated iIEL

Lane 3: Amplification of cDNA from iIEL incubated 6 hrs with IEL-medium

Lane 4: Amplification of cDNA from iIEL incubated 6 hrs with 200 CD_{50}/ml of Stx1

Lane 5: Amplification of cDNA from iIEL incubated 6 hrs with 200 CD_{50}/ml of Stx1 and 1.5 µg/ml of anti-StxB1 antibody 13C4

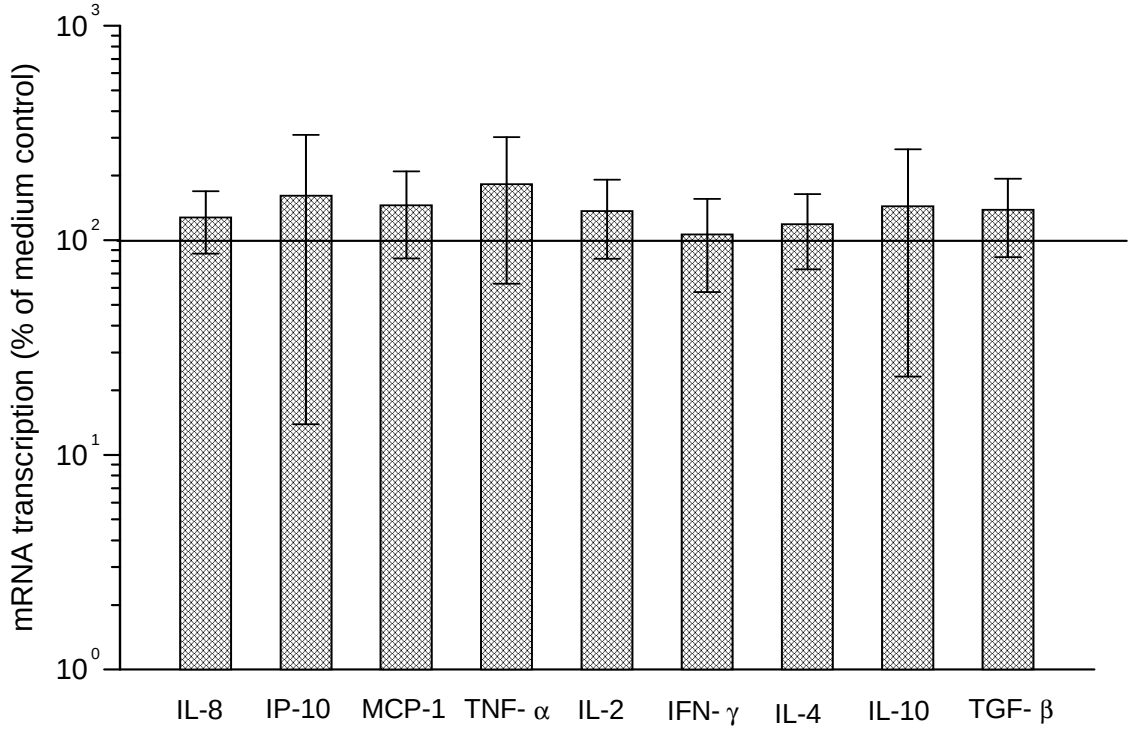


Fig. 22. Effect of purified Stx1 on chemokine and cytokine gene transcription by PBMC. PHA-P-stimulated (5 µg/ml) cells were incubated 6 hrs with 33 CD_{50}/ml of Stx1. mRNA were subjected to reverse transcription and cDNA to real-time-PCR. The transcription of the housekeeping gene *gapdh* was used for normalization of the samples. Cells incubated with medium were used as a control (= 100 % as visualized by the black line). Data presented are the arithmetic means, the minimal and maximal values of 5 different animals with duplicates. Statistical analysis did not show any significant difference between cells incubated in absence or presence of Stx1.

4.8. Cytokine synthesis in the absence or presence of Stx1

4.8.1. Investigations with bovine iIEL

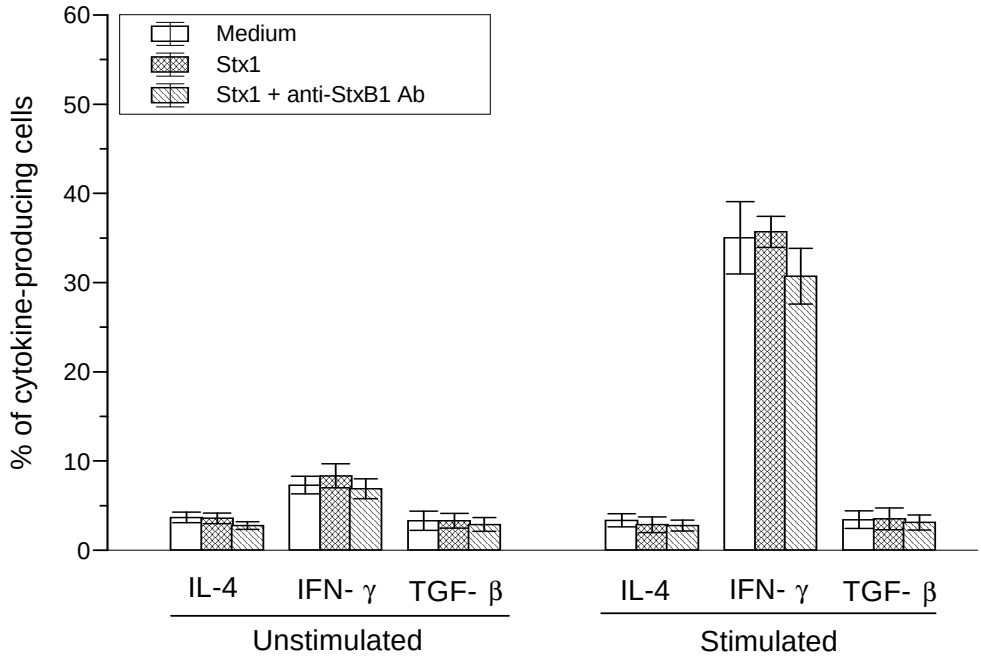
In order to confirm the PCR results at the protein level, the intracellular production of selected cytokine proteins was investigated by flow cytometry on the single cell level. Ileal IEL were unstimulated or stimulated with the mitogens PMA/Ionomycin and cultured for 1 h or 8 hrs with 200 CD_{50}/ml of Stx1 and then incubated additional 5 or 16 hrs, respectively, in the presence of brefeldin A. The figures 23 and 24 show the percentage of cells expressing the

cytokines IL-4, IFN-γ and TGF-β, and the intensity of this expression after a total incubation time of 6 hrs (Fig. 23A and 24A) and 24 hrs (Fig. 23B and 24B).

The IL-4 protein was shown to be produced by only a small portion of iIEL within 6 hrs of incubation (nearly 3 %). Moreover, Stx1 did not affect the percentage of iIEL expressing IL-4 protein at this time points ($P > 0.05$). Concerning TGF-β, the production was low and Stx1 did not influence the protein level too ($P > 0.05$). After 6 hrs of cultivation, IFN-γ-producing cells were the most abundant among iIEL. However, Stx1 did not influence the percentage of cells producing IFN-γ. The mitogenic stimulation dramatically increased the proportion of IFN-γ-producing cells but Stx1 still did not affect this production *in vitro*. The percentage of cells producing IL-4 and TGF-β was not altered upon cultivation with Stx1. The mean fluorescence intensity (average protein content/cell) was identical for the three tested cytokines after 6 hrs of cultivation, independently of the stimulation by mitogens or cultivation with Stx1 (Fig. 24A).

After 24 hrs of incubation, independently of the incubation with mitogens or with Stx1, the number of IL-4-producing cells was low (Fig. 23B). Similarly, even if a slight increase after overnight cultivation could be observed, the production of TGF-β was still low. Upon mitogenic stimulation, the number of cells producing IFN-γ increased after 24 hrs of cultivation. Nevertheless, no statistical significant differences of IL-4 and TGF-β protein production were found between cells incubated in absence or presence of Stx1 ($P > 0.05$). Even though PMA and ionomycin-stimulated iIEL produced dramatically increased amounts of IFN-γ protein, the toxin did not have an effect on this T_H1-type cytokine production after 24 hrs of incubation ($P > 0.05$).

A



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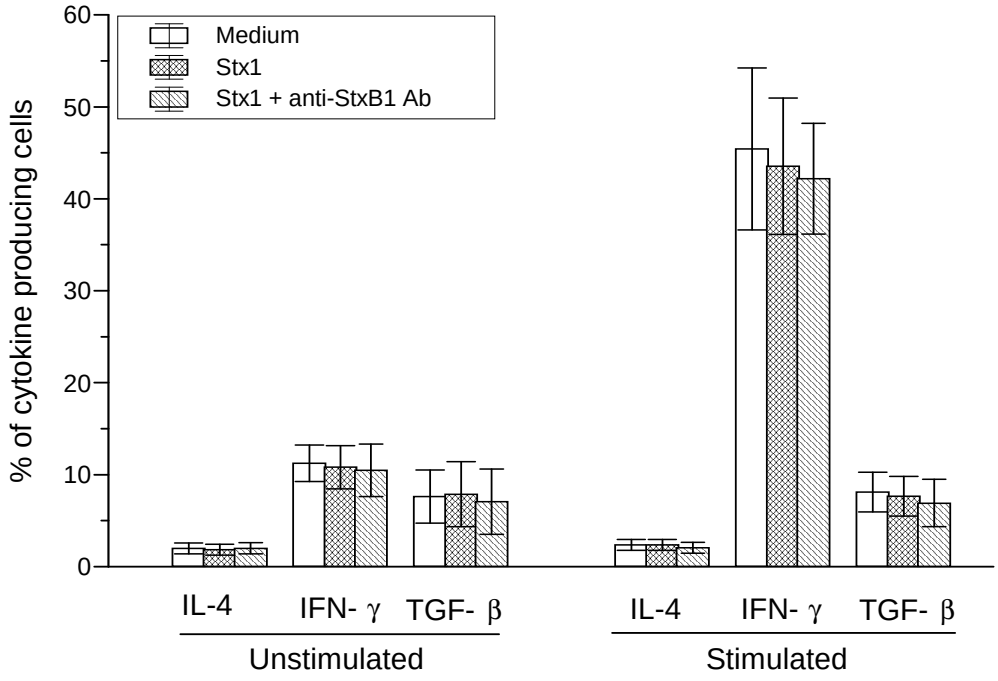
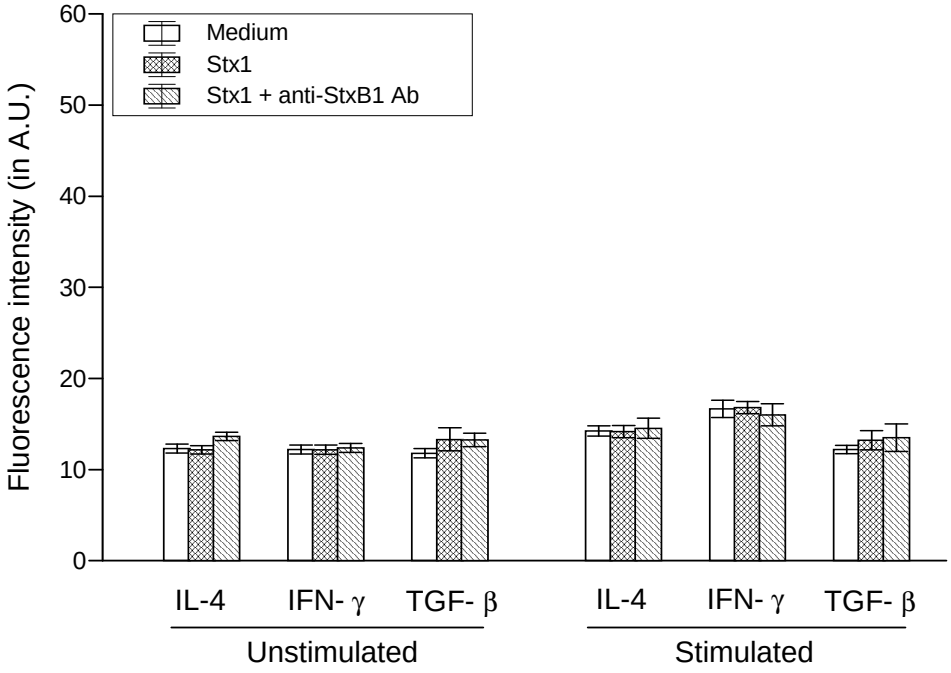


Fig. 23. Percentage of iIEL synthesizing certain cytokines in vitro in absence or presence of Stx1. Cells were left unstimulated or stimulated with PMA (50 ng/ml) and ionomycin (10 µg/ml) for 6 hrs (A) and 24 hrs (B). Brefeldin A (10 µg/ml) was added 1 h and 8 hrs, respectively, after the incubation begun. The cells were fixed, permeabilized, and intracellular cytokines were immunodecorated. Data presented are arithmetic means ± SEM of percentage of cytokine producing cells of 5 independent experiments with duplicate determinations.

A



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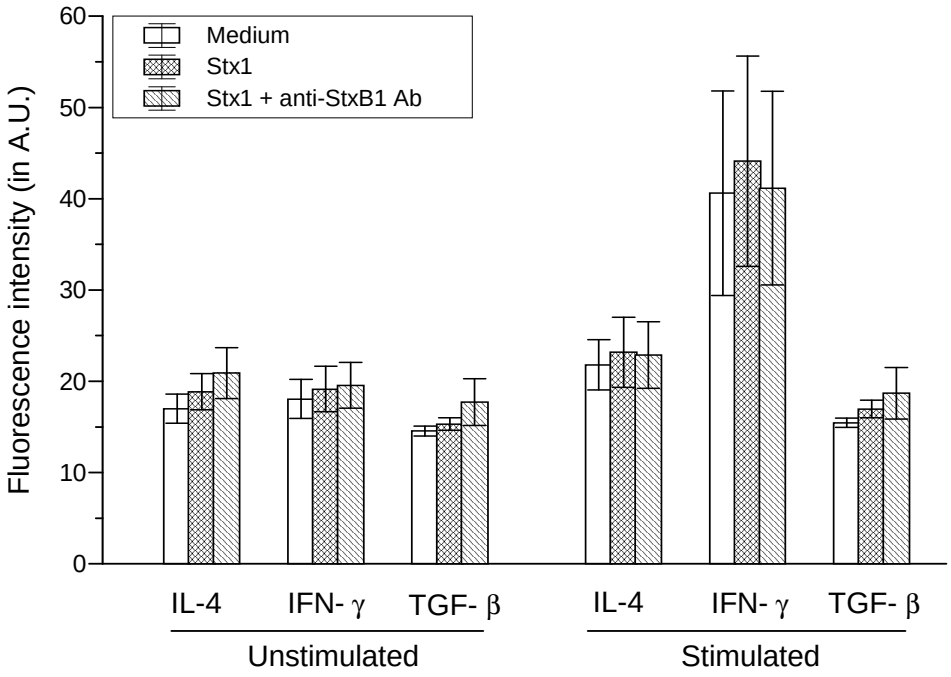


Fig. 24. Fluorescence intensity for the detection of cytokine proteins in iIEL in vitro in absence or presence of Stx1. Cells were left unstimulated or stimulated with PMA (50 ng/ml) and ionomycin (10 µg/ml) for 6 hrs (A) and 24 hrs (B). Brefeldin A (10 µg/ml) was added 1 h and 8 hrs, respectively, after the incubation begun. The cells were fixed, permeabilized, and intracellular cytokines were immunodecorated. Data presented are arithmetic means ± SEM of fluorescence intensities (arbitrary units) of cytokine detection of 5 independent experiments with duplicate determinations.

4.8.2. Investigations with bovine PBMC

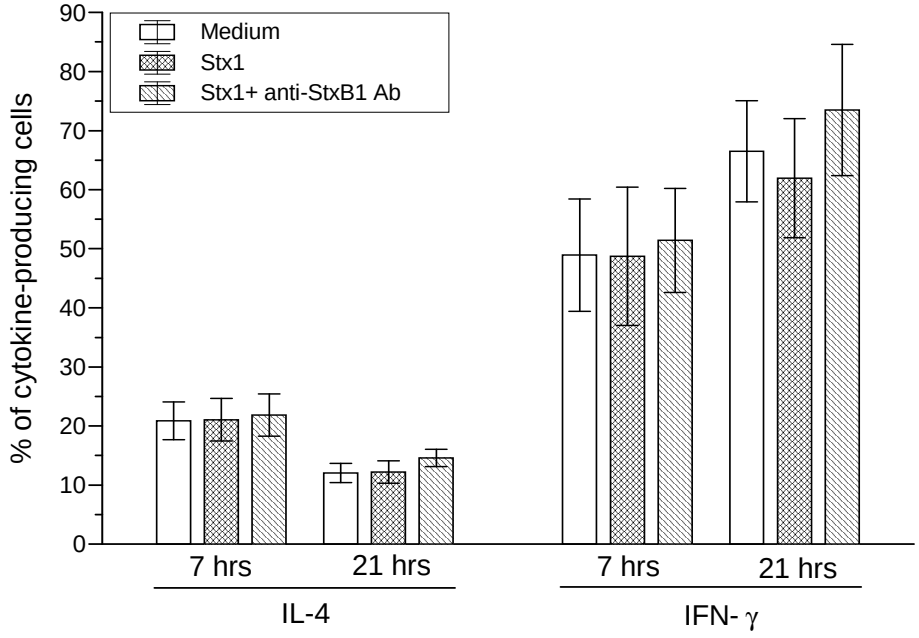
In addition to iIEL, the intracellular production of cytokines was investigated in bovine peripheral lymphocytes. Upon cultivation with mitogens, PBMC produced both IL-4 and IFN-γ proteins within 7 and 21 hrs. After 7 hrs of incubation, half of the cells had produced IFN-γ while only 20.9 ±5.5 % (mean ±SEM) of the cells had synthesized IL-4. After 21 hrs of incubation, the percentage of cells producing IL-4 decreased while the average amount of cytokine produced per cell increased (19.0 ±2.1 A.U. and 36.2 ±10.2 A.U. after 7 and 21 hrs, respectively). Concerning IFN-γ, both the proportion of PBMC producing the cytokine and the amount of cytokine produced per cell (27.4 ±1.3 A.U. and 53.9 ±10.2 A.U. after 7 and 21 hrs, respectively) increased.

Stx1, at the concentration of 33 CD_{50}/ml, slightly decreased the production of IFN-γ by PBMC after 21 hrs of incubation. Stx1 reduced both the total percentage of cells (Fig. 25A) and the average amount of cytokine produced per cell (Fig. 25B). PBMC preparations from several animals strikingly differed in their sensitivity but always showed the same effect of Stx1.

Similarly to what was observed with IFN-γ, the toxin had an effect on the production of IL-4 by PBMC only after 21 hrs of incubation (Fig. 25B), as the average amount of cytokine produced per cell was significantly decreased ($P < 0.05$).

The figure 26 shows the example of a PBMC preparation particularly affected by the toxin. The percentage of cell producing IFN-γ (defined by the range M1) was reduced from 69.9 to 55.5 % after 21 hrs of cultivation with Stx1. The addition of anti-StxB1 antibody restored the IFN-γ production. No effect was observed on the IFN-γ production for this PBMC preparation after 7 hrs of incubation (data not shown). In addition, a detailed examination revealed that Stx1 also reduced the percentage of cells which produced high amounts of IFN-γ (defined by the range M2) after 21 hrs of incubation. This IFN-γ-high-producing population of PBMC was reduced from 13.6 % to 7.0 % in presence of the toxin. An additional pre-incubation of Stx1 with the anti-StxB1 antibody 13C4 restored and even increased the production of IFN-γ.

A



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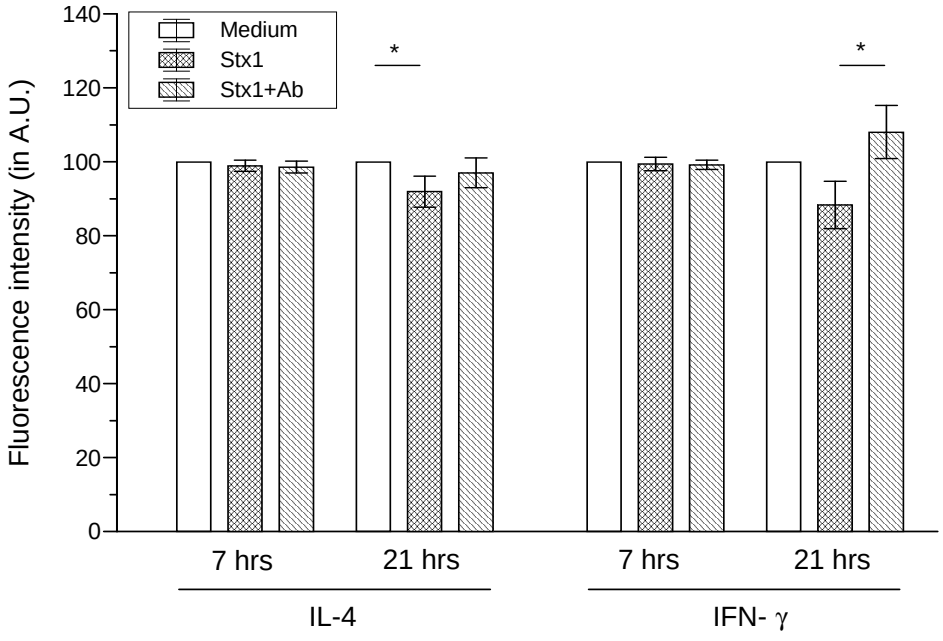


Fig. 25. Effect of Stx1 on intracellular protein expression of IL-4 and IFN-γ by PBMC. The cells were stimulated with PMA (50 ng/ml) and ionomycin (10 µg/ml) for 7 or 21 hrs with or without 33 CD_{50}/ml of Stx1. Brefeldin A (10 µg/ml) was added respectively 1 h or 8 hrs after the incubation begun. Data presented are arithmetic means ± SEM of percentages of cytokine-expressing cells (A) and intensities of fluorescence (B, normalized by setting the condition Medium as 100 %) obtained from 3 independent experiments with duplicate determinations.

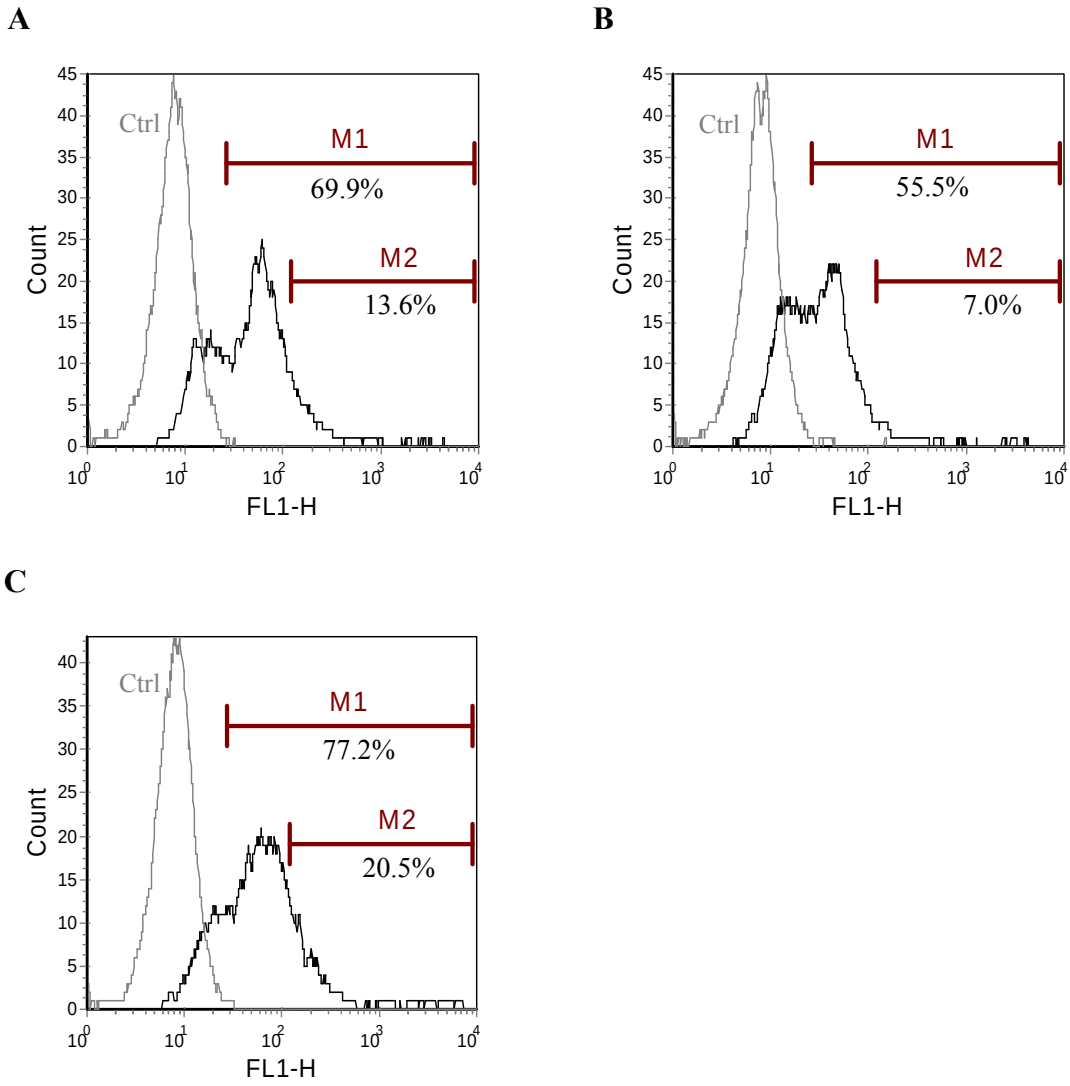


Fig. 26. Effect of Stx1 on the production of IFN-γ by PBMC. Cells were incubated 21 hrs with either medium (A), with 33 CD_{50}/ml of Stx1 (B), and with 33 CD_{50}/ml Stx1 plus 1.5 µg/ml anti-StxB1 antibody (C). FACS analysis plots present the intensity of the fluorescence (FL-1 channel) after a specific staining with an anti-IFN-γ antibody (Ctrl represents the isotype control).

4.9. Investigation of Stx1-induced apoptosis in several cell types

Stx1 and StxB1 were previously shown to induce apoptosis in several cell types as monocyte-like THP-1 cells (138) or Burkitt's lymphoma cell lines (240). The triggering of Gb_3/CD77 by monoclonal antibodies also induces apoptosis in Burkitt's lymphoma cell line (382, 391). In addition, mistletoe lectins (plant toxins) induce apoptosis in $CD8^+$ T cells and this coincided with an increase in IL-4 production (364). Consequently, as most of $Gb_3/CD77^+$ iIEL express the CD8 co-receptor (248), the hypothesis emerged that the enhancement of *il-4* transcripts

induced by Stx1 could be linked with the induction of apoptosis in iIEL by the toxin or by the triggering of Gb_3/CD77 by the rStxB1 subunit.

In order to establish the method of investigation of apoptosis induction, Burkitt's lymphoma cells (Daudi and Ramos cells) were used as positive controls for Stx1- and StxB1-induced apoptosis. As shown in table 8, Daudi cells were found to be relatively resistant to induction of apoptosis either by actinomycin D or by Stx1. The effect of Stx1 could be neutralized by a 90 min-pre-incubation of the toxin with 1.5 µg/ml of the anti-StxB1 antibody. The StxB1 subunit alone did not induced apoptosis neither did the cross-linking of the Stx1 receptor by an anti-CD77 antibody. The addition of the Golgi-inhibitor brefeldin A reduced the effect of the toxin, indicating the importance of the processing of the toxin intracellularly in this cell model. The relative low level of Stx1-induced apoptosis in Daudi cells led to the use of another cell line to obtain an efficient induction of apoptosis. The presence of Gb_3/CD77 on Ramos cells surface was assessed by flow cytometry. All Ramos cells expressed Gb_3/CD77 on their cell surface at a relatively high level, as shown in the figure 27.

Table 8. Induction of apoptosis in Daudi cells

Condition of incubation	Early apoptotic	Late apoptotic and necrotic	Altered mitochondrial membrane potential
Medium	11.8	6.4	15.5
Actinomycin D	31.2	11.6	31.0
Stx1	29.2	19.8	47.2
Stx1 + anti-StxB1 13C4 MAb	16.9	8.6	26.0
Anti-StxB1 13C4 MAb	11.3	4.9	20.6
rStxB1	17.0	7.3	31.2
rStxB1 + 13C4 Ab	15.5	7.0	32.4
CD77 cross-linking	14.9	7.7	30.9
Brefeldin A	12.9	4.8	37.9
Brefeldin A + Stx1	13.8	5.0	34.4

Daudi cells were incubated 24 hrs at 37°C. Apoptosis and necrosis levels were assessed by detection of Annexin-V binding to phosphatidylserine, staining of DNA by 7-AAD, and modification of the mitochondrial membrane potential (JC-1 assay). Data presented are the arithmetic means of percentages obtained from duplicates from 1 experiment.

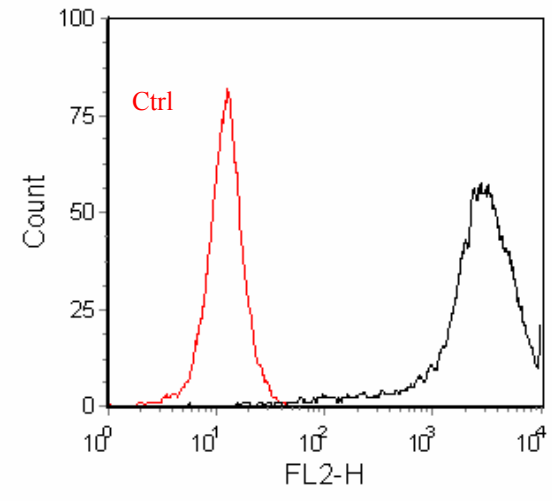


Fig. 27. Expression of CD77 on the surface of Ramos cells. The FACS plot presents the intensity of the fluorescence (FL-2 channel) after a specific staining with an anti-human CD77 antibody. The grey line depicts the isotype control (Ctrl).

As shown in table 9, the addition of 200 CD_{50}/ml of Stx1 to Ramos cell cultures very efficiently induced apoptosis after 20 hrs of cultivation as detected by the exposure of phosphatidylserine on the cell surface and by the loss of mitochondrial membrane potential. The anti-StxB1 13C4 antibody neutralised this effect by considerably reducing the apoptosis to a level comparable to the medium control. Moreover, the addition of a high concentration of the StxB1 subunit (10 µg/ml) also induced apoptosis, confirming a role of the Gb_3/CD77 signaling pathway in Ramos cells. However, the latter effect could not be neutralized by pre-incubation with the anti-StxB1 antibody 13C4. The addition of the Golgi inhibitor brefeldin A slightly reduced the effect of the toxin suggesting that both toxin processing and triggering of the receptor are important in this cell system. However, no apoptosis was observed by cross-linking Gb_3/CD77 by the 38.13 antibody and an anti-rat IgM in Ramos cells.

Before assessing whether Stx1 induced the apoptosis of iIEL, the JC-1 assay was applied to bovine PBMC incubated 6 hrs with 2 µg/ml of actinomycin D or with 200 CD_{50}/ml of Stx1. In comparison to the medium control (apoptosis 7.1 % and necrosis 37.1 %), actinomycin D efficiently reduced the cell viability (Fig. 28), mainly by inducing necrosis of PBMC

(69.8 %). In contrast, a 6 hrs-incubation with Stx1 had no effect on PBMC apoptosis (7.8 %) and necrosis levels (37.9 %).

Upon staining with Annexin-V and 7-AAD, 41.9 % of iIEL were detected as apoptotic and/or necrotic after the isolation procedure from the ileum. Even in the presence of the mitogen PHA-P, a 6 h-incubation in medium increased this level to 65.2 % (table 9). In contrast to Ramos cells, neither Stx1 holotoxin nor the StxB1 subunit induced apoptosis in iIEL after 6 hrs of incubation. 1 ng/ml of LPS also failed to induce any apoptosis and the cross-linking of Gb_3/CD77 with antibodies did not have any effect on the cell viability. The RNA-synthesis inhibitor actinomycin D, used as a positive control, did not induce iIEL apoptosis after short term-incubation but very efficiently did after 20 hrs of incubation. Not any other substance of the panel did induce any iIEL apoptosis after 20 hrs of incubation at 37°C.

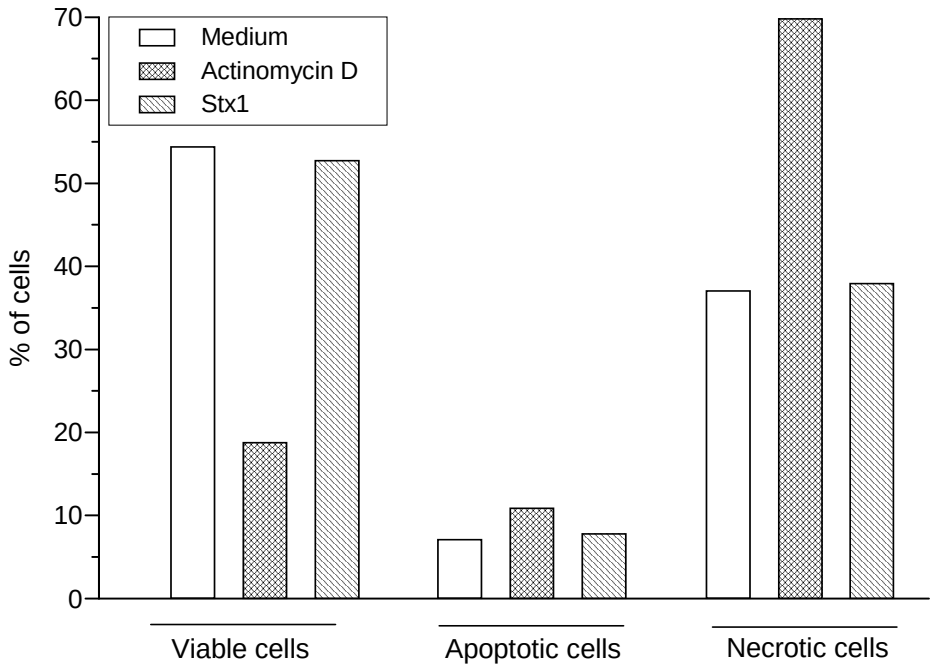


Fig. 28. Induction of apoptosis in bovine PBMC. Cells were incubated 6 hrs at 37°C. Apoptosis and necrosis levels were assessed by FACS by detecting any modification of the mitochondrial membrane potential (JC-1 assay). Five thousand events were acquired per sample with a FACSCalibur™ flow cytometer. Data presented are the arithmetic means of percentages obtained from one PBMC preparation with duplicate determinations.

Table 9. Induction of apoptosis in Ramos cells and bovine iIEL

Cell type and condition of incubation	6 h incubation			20 h incubation		
	Early apoptotic	Late apoptotic and necrotic	Altered mitochondrial potential	Early apoptotic	Late apoptotic and necrotic	Altered mitochondrial potential
Ramos cells						
Medium	n.t	n.t	n.t	17.9 ±8.7	3.3 ±1.4	20.2 ±11.8
Actinomycin D	n.t	n.t	n.t	77.2 ±2.5	15.8 ±2.0	98.0 ±0.8
Stx1	n.t	n.t	n.t	78.8 ±4.1	14.5 ±4.0	98.2 ±0.4
Stx1 + anti-StxB1 Ab	n.t	n.t	n.t	21.2 ±9.1	3.5 ±1.2	20.8 ±11.0
Anti-StxB1 MAb	n.t	n.t	n.t	20.2 ±8.6	4.0 ±2.0	26.8 ±17.5
rStxB1	n.t	n.t	n.t	52.7 ±7.2	8.2 ±4.3	65.7±12.0
rStxB1 + anti-StxB1 Ab	n.t	n.t	n.t	59.2 ±13.1	7.3± 3.7	61.8±12.7
CD77 cross-linking	n.t	n.t	n.t	17.6 ±9.2	5.2 ±3.1	22.3 ±12.2
Brefeldin A	n.t	n.t	n.t	34.0 ±15.0	11.0 ±6.1	65.5 ±15.1
Brefeldin A + Stx1	n.t	n.t	n.t	43.3 ±18.0	13.1 ±7.7	77.3 ±12.0
Bovine iIEL						
Medium	29.7 ±11.4	35.5 ±5.4	74.1 ±1.8	34.4	16.2	n.t
Actinomycin D	30.2 ±10.4	41.0 ±4.1	72.7 ±4.2	49.9	44.6	n.t
Stx1	26.3 ±9.4	40.9 ±5.8	74.3 ±4.5	32.8	17.9	n.t
Stx1 + anti-StxB1 Ab	29.1 ±10.3	39.2 ±7.4	76.7 ±3.2	32.0	17.9	n.t
rStxB1	26.8 ±12.5	37.6 ±5.9	75.7 ±2.7	32.8	19.0	n.t
rStxB1 + anti-StxB1 Ab	27.6 ±10.7	39.4 ±5.2	73.7 ±4.2	33.4	20.4	n.t.
CD77 cross-linking	27.1 ±8.9	38.3 ±5.7	75.6 ±4.2	35.8	17.8	n.t
LPS	30.1 ±9.1	36.3 ±4.9	75.4 ±4.1	35.4	16.3	n.t
Brefeldin A	27.4 ±6.4	36.4 ±6.3	75.5 ±4.4	39.5	30.8	n.t
Brefeldin A + Stx1	25.2 ±7.6	37.5 ±7.7	78.6 ±2.2	39.7	29.4	n.t

Ramos cells, used as positive control for Stx1- and StxB1-induced apoptosis, were incubated 20 hrs at 37°C (for concentrations, see Table 7). Data are means ± SD of 3 determinations with duplicates. Bovine iIEL were incubated 6 or 20 hrs at 37°C. Apoptosis and necrosis levels were assessed by flow cytometry by Annexin-V binding, staining of DNA by 7-AAD, and modification of the mitochondrial membrane potential. Data presented are arithmetic means ± SD of percentages obtained from duplicate determinations with 3 (6 hrs) or 1 (20 hrs) preparation. "n.t." for "not tested".

4.10. Contribution of the enzymatic activity of Stx1 to the enhancement of *il-4* transcripts

In order to assess whether the enzymatic activity of the toxin was required to induce the increase in *il-4* transcripts, iIEL were incubated with the substances listed in table 7. Stx1 holotoxin induced a very strong increase in the amount of *il-4* transcripts within 6 hrs of cultivation in PHA-P-stimulated iIEL (Fig. 29). This effect was perfectly neutralized by 1.5 µg/ml of anti-StxB1 antibody 13C4. However, StxB1 alone did not induce any increase in *il-4* transcripts level, indicating the preponderant role of the enzymatic activity of Stx1 in bovine iIEL. The Golgi inhibitor brefeldin A almost completely removed the effect due to Stx1, confirming the importance of the uptake and processing of the toxin into the cells.

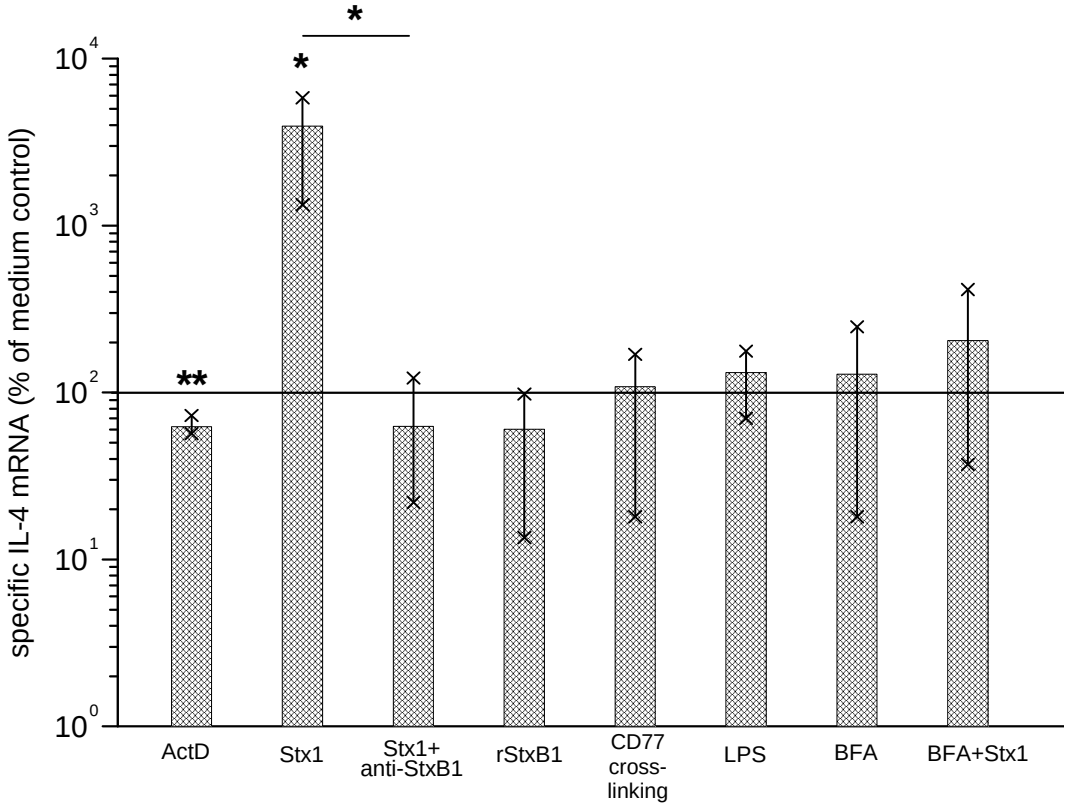


Fig. 29. Effect of different agents on the amount of IL-4-specific mRNA in bovine iIEL cultures. PHA-P-stimulated (2.5 µg/ml) cells were incubated for 6 hrs in presence of different agents (for concentrations, see table 7). After RT and quantitation by real-time PCR, the transcription of the housekeeping gene *gapdh* was used for normalization of the samples. Cells incubated with medium were used as a control (=100 % as visualized by the black line). Data presented are arithmetic means, minimal and maximal values from 3 preparations from different animals. Significant cytokine production was determined by Student's t-test and depicted if $P \leq 0.01$ (**) or $P \leq 0.05$ (*).

The endotoxin LPS from *E. coli*, used at an overdose to what was present in the Stx1 preparation, did not induce any IL-4 mRNA transcription in iIEL, neither did the cross-linking of Gb_3/CD77 by specific antibodies. The treatment with Actinomycin D induced a decrease in the overall mRNA quantity in iIEL (data not shown), and also reduced the relative amount of *il-4* specific transcripts.

5. Discussion

Shiga toxins (Stxs) affect the cytokine production of several cell types involved in inflammatory processes in the course of human infections with Stx-producing *E. coli* (STEC) (139, 140, 317, 392, 393). In adult cattle, the chronic carriage of STEC in the intestine coincides with the ability of Stx1 to affect the immune response by acting on bovine lymphocytes from both systemic compartment and intestinal mucosa. Stx1 blocks the activation and proliferation of blood lymphocytes without inducing cell death (249, 254). It equally affects the lymphoblast transformation of iIEL *in vitro* (248) and partially depletes the bovine ileal mucosa of $CD8^+$ iIEL *in vivo* (251). Ileal IEL, the first immune cells to gain contact with the toxin, are therefore considered the main target cells of Stx1 in cattle (248). These observations prompted to hypothesize that Stx1 may disturb the mucosal cytokine regulatory network in the bovine´s intestine. Therefore, the objectives of the present study were to investigate the cytokine and chemokine gene transcript and protein expression profiles of bovine iIEL upon cultivation in the absence and presence of purified Stx1. Although peripheral blood mononuclear cells (PBMC) and iIEL transcribed a number of different cytokine and chemokine genes, the effect of Stx1 at minute concentrations was considerably specific in that it was restricted to *il-4* and occurred in iIEL only. It is tempting to speculate that, by secreting Stx1, STEC trigger the immune response in the intestine of the bovine host towards a T_H2-like pattern.

5.1. Chemokine production by bovine iIEL cultured in presence and absence of Stx1

Human IEL are a potent source of chemokines (IL-8, MIP-1α, -1β, RANTES and lymphotactin) (35, 227) and are thought to induce the attraction of cells of the innate immune response (monocytes, neutrophils) as well as of lymphocytes. The present study showed, for the first time, that bovine iIEL also produce chemokines mRNA and proteins, indicating that iIEL in this species also actively contribute to the migration of immune cells to the mucosa.

In contrast to preliminary observations with bovine iIEL (248), investigations by real-time PCR proved that the iIEL chemokine mRNA levels were not altered by incubation of the cells with Stx1. This finding was further supported by results from a neutrophil migration assay to investigate the overall release of chemoattractant proteins. While a ConA-stimulated PBMC culture supernatant efficiently induced the migration of neutrophils, supernatants of PHA-P-stimulated iIEL cultures induced migration to a lower extent. No difference could be

observed, however, between the supernatants of iIEL incubated with or without Stx1. Stx1 strongly induces the production of several chemokine´s mRNA and proteins in human intestinal epithelial cells (IEC) (392, 393). During human STEC infections, the subsequent recruitment of PMN, as part of an inflammatory process, leads to an increased uptake of Stx1 which is thought to be the most important prerequisite for significant organ damage to occur (155). Although comparative experiments with human IEL are pending, the results presented here clearly show that Stx1 does not represent a proinflammatory factor for bovine iIEL. Thereby, these results supplement the observation that bovine IEC are as well not stimulated by Stx1 to release chemoattractants for neutrophils *in vitro* (M. Mohr, I. Stamm, E. Schröpfer, G. Baljer, and C. Menge, manuscript in preparation). Together with the observation that Stx1 is not involved in the induction of intestinal inflammation *in vivo* in experimentally STEC-infected calves (368), these *in vitro* findings help to understand why STEC are able to colonize their bovine host without disturbing the intestinal homeostasis.

5.2. Cytokine mRNA and proteins profiles of bovine iIEL

By producing a broad range of cytokines, IEL are involved in the regulation of the mucosal homeostasis in various species (106). Bovine iIEL freshly isolated from the intestinal mucosa and iIEL incubated with PHA-P-supplemented culture medium were also found to produce a number of different cytokine mRNAs. Although *tgf-β* transcripts were found more abundantly than other transcripts, all T_H-type cytokine transcripts from the panel selected for the investigation were found in bovine iIEL. These findings are consistent with previous observations that human freshly isolated IEL harbour cytokine mRNAs and underline the activated state of the mucosal lymphocytes (227).

PHA-P stimulation was unable to induce a detectable IFN-γ and IL-4 protein production in bovine iIEL (data not shown). Bovine γδ T cells (15), as well as $CD4^+$ and $CD8^+$ T cells from bovine blood and mesenteric lymph nodes (341, 359), were previously reported to produce IFN-γ protein upon stimulation. PHA-P is a T-cell activator through CD2, however, and bovine γδ T cells do not express CD2 (229), partially explaining the lack of responsiveness upon PHA-P stimulation. Even if the treatment of the human monocytic cell line THP-1 with PMA decreases the membrane expression of the Stx-receptor Gb_3/CD77 (317), stimulation of bovine iIEL with PMA/ionomycin does not abrogate the cells´ sensitivity for the activation-inhibiting effect of Stx1(248). The mitogenic stimulation of iIEL in the present study was changed, therefore, in favour of PMA, a PKC activator, supplemented by ionomycin, a Ca^{2+} ionophore, which activate non-specifically T cells.

Indeed, IFN-γ protein was very strongly synthesised by more than 40 % of the iIEL upon this kind of stimulation.

Although both $CD4^+$ IEL and murine primed CD8αβ IEL can secrete TGF-β (150, 255), the TGF-β protein was detected in only a minority of bovine iIEL and at a low level independently of the application of mitogens. Moreover, the constant presence of high numbers of mRNA led to speculate that iIEL constitutively generate *tgf-β* transcripts and probably stock them, as NK T cells can do, waiting for an eventual activation (242, 367). Similarly to TGF-β, the IL-4 protein was produced by the cells at a low level, independently of the stimulation of the cells.

A T_H1 vs. T_H2 bias is rarely observed in cattle (44, 45) and bovine T cells can express the cytokines IL-2, IFN-γ, and IL-4, independently and in combination, indicating possible intermediate patterns of cytokine production (43). The incubation of bovine iIEL with mitogens in the present study also led to different mRNA and protein T_H-profiles, with predominance of TGF-β mRNA and of IFN-γ protein, respectively. The simultaneous production of IL-4, IFN-γ, and TGF-β protein points to an unrestricted T_H0-like pattern or to a mixed T_H profile in the unsorted preparations of bovine iIEL. Although it must be considered that the various iIEL subpopulations in the preparations have responded differently, a simultaneous production of these cytokines was also observed in human intestinal IEL (53, 110) and in activated human peripheral blood $CD4^+$ and $CD8^+$ T cells (7, 295). The predominant production of IFN-γ by bovine iIEL (35.0 ± 4.0 % positive cells after 6 hrs, 45.4 ± 8.8 % after 24 hrs) is in accordance with recent results that normal human IEL mainly produced IFN-γ (43 ± 18 %) and only small amounts of IL-4 (< 1 %) after 12 hrs of stimulation with PMA and ionomycin (215). Although mucosal immune responses are often T_H2 dominated (145, 278), IEL, therefore, do not appear to be generally restricted to a certain T_H profile.

5.3. TGF- β production by bovine iIEL cultured in the presence of Stx1

TGF-β represents a cytokine significantly implicated in mucosal homeostasis. Produced by IEL (19, 227), TGF-β is an important growth factor for IEC (18). It had been shown previously, that prolonged STEC shedding by cattle correlates with a decreased proliferation rate of the intestinal epithelial cells (232). It appeared plausible to hypothesize, therefore, that Stx1 may have decreased the production of TGF-β by bovine iIEL in these experiments. However, Stx1 did not modify both *tgf-β* transcript and TGF-β protein levels, suggesting that

STEC may alter the epithelial proliferation rate (232) by a mechanism independent of the TGF-β production by iIEL.

5.4. Effect of Stx1 on the T_H1/T_H2 balance in bovine iIEL

In this study, freshly isolated iIEL were found to express a low quantity of *il-4* transcripts. This finding is in agreement with the literature concerning other species which indicated that *il-4* transcripts were undetectable (107) or present only in low numbers in human IEL (53, 397). No *il-4* transcripts could be detected in IEL of calves by Canals *et al.* (51) and by Wyatt *et al.* (426). In extension of preliminary observations (248), the present study unequivocally confirmed that Stx1 significantly increased the number of *il-4* transcripts in iIEL as early as after 4 hrs of cultivation to levels even easily detectable by semi-quantitative PCR. The analysis of real-time PCR Ct values further revealed that after incubation with Stx1, *il-4* transcripts became rapidly predominant in the cells (data not shown), even exceeding the *tgf-β* transcripts' level which was not modified by the toxin. The *il-4* transcripts represented the majority of the analysed transcripts after 4, 6, and 8 hrs of incubation but declined to baseline levels after overnight culture. While the protein machinery of iIEL was not affected by incubation with 200 CD_{50}/ml of Stx1, the increase in *il-4* transcripts was inducible with concentrations of toxin even as low as 7 CD_{50}/ml. Furthermore, Stx1 predominantly affected the amount of full-length *il-4* transcripts. Waldvogel *et al.* (406) suggested an important role of splice variants in the regulation of IL-4 production, although the biological significance of the IL-4δ2 variant is not fully elucidated yet. However, the *il-4δ2* splice variant was equally detected in iIEL incubated in absence or presence of Stx1, strongly implying that bovine iIEL are remarkably sensitive to a Stx1-induced rapid and highly specific increase of IL-4 translation templates.

PBMC respond differently from iIEL with the production of cytokines after stimulation by different bacterial superantigens underscoring differences in activation requirements (361). The results presented in this study are other examples for these differences. PBMC did not display significant modifications of the cytokine mRNA profile after cultivation with Stx1. Moreover, again in contrast to what was observed in iIEL, cultivation of bovine PBMC with Stx1 (33 CD_{50}/ml) slightly decreased the production of IFN-γ protein. The effect of Stx1 was not found to be statistically significant due to the variability between the PBMC preparations from several animals. Nevertheless, the findings resemble those made with Stx2 at 30 CD_{50}/ml, which as well reduced the IFN-γ synthesis in bovine PBMC (Menge and Dean-Nystrom, personal communication). Despite the differences in the immunomodulatory effects

Stxs exert on bovine PBMC and iIEL, the results led to conclude, that Stxs induce an overall shift of the bovine immune response towards a T_H2-like pattern.

Further investigations are required to identify the iIEL subtype which is stimulated by Stx1 to produce *il-4* transcripts. The expression of Gb_3/CD77 by $CD4^+$ cells can be induced *in vitro* and these cells are equally sensitive as $CD8\alpha^+$ cells to Stx1 then (248). $CD4^+$ IEL can produce IL-4 and provide help for B cells (110). Nonetheless, preliminary data indicate that the magnitude of *il-4* transcripts' level fails to correlate with the percentage of $CD4^+$ cells in the iIEL preparations tested (data not shown). It was reported that rat splenic $CD8^+$ T cells produce 4 times more *il-4* transcripts than $CD4^+$ T cells and that IL-4 regulates the differentiation and the growth of cytotoxic $CD8^+$ T cells (283). Under some circumstances, $CD8^+$ T cells can increase the proliferation of and provide help to B cells, mainly by producing IL-4 (65). Since murine $CD8^+$ T cells also can be primed *in vitro* to produce IL-4 (341), the cellular source of Stx1-induced *il-4* transcripts within bovine iIEL preparations may not be restricted to the $CD4^+$ population.

5.5. Possible mechanisms underlying the Stx1-induced increase in *il-4* transcripts

In addition to the cytosolic effects of the Stxs, many cellular functions are known to be affected by triggering Gb_3/CD77, as MHC-II-mediated functions related to B cell-antigen presentation. The cross-linking of Gb_3/CD77 in Ramos cells leads to the activation of the BCR signaling cascade (261) and binding of the StxB1 subunit induces an increase in cytosolic calcium concentration and release of ceramide. The activation of the Stx-receptor and the release of second messengers eventually induces apoptosis (382). However, high concentrations of rStxB1 induced neither an increase in *il-4* transcripts nor apoptosis of bovine iIEL (containing few B cells). This confirms previous results (432, 433) indicating that the Stx1 holotoxin must enter the cells and that the enzymatic activity is indispensable to induce cytokine transcripts' production in target cells.

In this context it needs to be considered that the Stxs holotoxins may affect the numbers of cytokine gene transcripts even prior to or in the absence of a complete abolishment of protein synthesis (393). Stx1 can increase selected transcripts' levels within 12-24 hrs in bovine endothelial cells at concentrations of toxins that have no effects on protein synthesis (26). In THP-1 cells (140), the Stx1-induced elevation of transcript levels is associated with a ribotoxic stress response involving JNK and p38 MAP (Mitogen-activated protein) kinases (50, 108, 357) and the activation of NF-κB (329). The activation of p38 in monocytes is associated with an enhanced stability of cytokine transcripts (407) and Stx1 in fact induces the

accumulation of certain mRNA transcripts within 4-8 hrs by increasing the half-life of mRNA (alteration of decay rates). It may also be that Stx1 modulates the translation-dependent mRNA degradation in a transcript-specific fashion (165, 326). It remains to be determined whether Stx1 increased the *il-4* transcript numbers in bovine iIEL as well by inhibiting the degradation of pre-existing transcripts. Since Grogan *et al.* (130) showed that stimulated murine T cells transcribe *il-4* within hours, it also appears plausible that the Stx1-induced increase in *il-4* transcripts is the result of an elevated transcriptional activity.

Ribosome-inhibiting proteins (as mistletoe lectins) stimulate the intracellular expression of IL-4 in human PBMC after 24 hrs of cultivation in coincidence with an increased expression of the mitochondrial marker Apo2.7, and with a decreased level of the anti-apoptotic Bcl-2 protein. This has prompted the interpretation that the toxins simultaneously induced the production of IL-4 and apoptosis in these cells (364). IL-4 in turn is known to induce apoptosis in human eosinophils (410) and in stimulated monocytes (239) by activating the proteolytic caspases cascade (96). These findings raised the possibility that the increase in *il-4* transcripts in bovine iIEL could be a side effect of Stx1-induced apoptosis.

The ability of Stx1 holotoxin or rStxB1 subunit to induce apoptosis has been extensively studied on several Burkitt's lymphoma cell lines (182, 189, 240, 241, 261, 382, 391). Consequently, two lymphoma cell lines were used in a first approach to prove the ability of the holotoxin and B subunit preparations used in the present study for their capability to induce apoptosis. The Stx1 preparation had a moderate effect on Daudi cells. Daudi cells are infected with Epstein-Barr virus (EBV) and this may alter the phenotype of the cells leading to a decreased expression of Gb_3/CD77 on the cell surface and an over-expression of the anti-apoptotic Bcl-2 protein. Daudi cells are consequently less sensitive to apoptosis induced by the Shiga toxins than Ramos cells (241) which are not subject to EBV-mediated phenotypic drift (193). Indeed, Ramos cells proved to be particularly sensitive to the Stx1 preparation after 20 hrs of incubation. The effect was completely neutralized by the anti-StxB1 antibody, and partially reduced by pre-incubation of the toxin with brefeldin A as already reported in THP-1 cells (196). The rStxB1 subunit preparation also efficiently induced apoptosis in Ramos cells. This effect could not be neutralized by the anti-StxB1 antibody but this can likely be explained by the considerable high concentration of rStxB1 subunit used which, on a molar basis, exceeded the concentration of the antibody by several magnitudes.

When applied to bovine iIEL, Stx1 reduced the percentage of cells that transformed to blast cells within 18 hrs of incubation. Stx1 thus affects the biology of bovine iIEL more

profoundly as assumed before, when it was discovered that Stx1 exerted this inhibitory effect within 72 hrs of incubation (248). Within 18 hrs, Stx1 particularly lowered the percentage of blast cells that expressed Gb_3/CD77 on their surface. However, this effect vanished when the cells were permeabilized prior to Gb_3/CD77 detection, indicating that the toxin induced a redistribution of the receptor from the cell surface to intracellular compartments rather than eliminating Gb_3/CD77-expressing cells from the cultures. The possibility that Stx1 induces apoptosis in bovine iIEL cultures could be finally ruled out when the Stx1-treated cells were submitted to staining with Annexin-V and 7-AAD. Although Actinomycin D efficiently induced apoptosis in bovine iIEL, Stx1 failed to induce apoptosis in bovine iIEL within 6 or 20 hrs of incubation. These results are in line with previous reports that Stx1 did not induce apoptosis in bovine PBMC (254) and bovine colonic epithelial cells (149) (M. Mohr, I. Stamm, E. Schröpfer, G. Baljer, and C. Menge, Manuscript in preparation). In conclusion, the Stx1-induced elevation in *il-4* transcripts does not seem to be linked to the induction of apoptosis in bovine iIEL. Even if the underlying mechanism is not yet known (for a preliminary model of the Stx1´s cellular activity see fig. 30), bovine iIEL, which show an enhanced quantity of *il-4* transcripts in response to Stx1, are fully viable *in vitro* and presumably remain well integrated in the mucosal network *in vivo*.

5.6. IL-4 production by bovine iIEL cultured in the presence and absence of Stx1

After incubation with PMA and ionomycin, a strong synthesis of IFN-γ protein was detected by flow cytometry. This IFN-γ response was not modified by additional supplementation of the medium with Stx1. As iIEL therefore generally retained their ability to produce cytokines in response to mitogens even in the presence of Stx1, the protein machinery of targeted cells seemed not to be significantly affected by a 6-to-24h-incubation with 200 CD_{50}/ml of Stx1. Nevertheless, the Stx1-induced increase in the numbers of *il-4* transcripts in iIEL was not followed by a detectable increase of the corresponding protein. A number of different factors may account for this obvious discrepancy.

Although Stx1 strongly stimulated the increase in *il-4* transcripts in PHA-P-stimulated iIEL within 8 hours, no IL-4 could be detected in the corresponding culture supernatants even after 18 hrs (data not shown). IL-4 protein only became detectable when it was detected intracellularly on a single cell level by flow cytometry. Upon strong mitogenic stimulation (PMA and ionomycin) and blocking of the protein secretion pathway (brefeldin A, BFA), IL-4 could be detected and only a relative low number of cells was found to be positive (≈ 3 %). As the cellular source of the increased amounts of *il-4* transcripts in Stx1-treated

iIEL cultures remains to be discovered, it cannot be excluded that the population of Stx1-responding cells is identical to this small population of IL-4-producing cells. Even if this is not the case, the correlation between IL-4 mRNA production and secretion of the protein has already been reported by several groups to be difficult to establish (43, 184). Similarly, an increase of the transcript levels specific for *il-8* and *gro-α* by 100 fold due to Stx1 can lead to an increase of the protein to a much lower extent (393).

The detection of small increases in IL-4 are particularly difficult when it is considered that iIEL may express IL-4 receptors (IL-4R) that rapidly remove newly produced IL-4 from the cultures. Indeed, a mitogenic stimulation of T cells, comparable to that in the present study, was shown to increase the accumulation of IL-4R mRNA (73). Notably, binding of IL-4 to IL-4R does not solely result in consumption of the ligand but also induces a negative feed-back mechanism. Naive T cells produce IL-4 and then undergo further T_H2 differentiation (282). This differentiation in turn requires IL-4-induced Stat6 signalling and involves an increase in intracellular-Ca^{2+} concentration. Thereby the autocrine release of IL-4 results in a down-regulation of the IL-4 promoter establishing a self regulation of the IL-4 production (75). It is tempting to assume that similar events occurred in the iIEL cultures investigated in the present study. Since the ionomycin used to stimulate the cells also induces an intracellular increase in Ca^{2+}, the addition of this reagent may even have potentiated the negative feedback signal induced by IL-4 itself.

The lack of an increased IL-4-production in Stx1-treated iIEL cultures may be, at least in part, also due to the fact that stimulation of the mixed iIEL preparations caused a significant production of IFN-γ by a considerable number of cells. Although protein secretion was blocked by addition of BFA at a later time point, the cells were left for up to 8 hrs in the presence of Stx1 before BFA was added. It is highly likely therefore, that IFN-γ was initially secreted into the cell culture medium causing a rapid reciprocal down-regulation of T_H2-cell proliferation, further depleting an already low level of IL-4 producing cells (325).

Consequently, although a direct proof is missing, the results presented in this study argue in favour of the possibility that bovine iIEL do produce IL-4 protein in response to Stx1.

5.7. Biological significance of an increased IL-4 synthesis by bovine iIEL

In several murine models of infection that eventually result in clinical manifestations of the infection, IEL were shown to produce IFN-γ after parasitic (205) or bacterial stimulations (429, 431). IEL from calves infected with *Cryptosporidium parvum* also expressed increased levels of IFN-γ mRNA while no difference was noted for IL-10, IL-2, and IL-4 (51). *Babesia*

bovis-specific $CD4^+$ T cell clones from immune cattle expressed either the T_H0 or T_H1 profile of cytokines (45). By contrast, $CD4^+$ T-cell clones obtained from cattle chronically infected with *Fasciola hepatica* and specific for adult worm antigen displayed both unrestricted and T_H2 cytokine profiles (43). While T_H1 profiles apparently are more oftenly found in acute inflammation and protective immunity, the induction of T_H2 cytokines in bovines hinders the immune clearance of many microbial pathogens (101). Accordingly, an enhanced pathogenicity of bovine tuberculosis may be linked to an increased ratio of T_H0 (IL-4^+ IFN-γ^+) versus T_H1 (IL-4^- IFN-γ^+) T cells (414) since the extend of chronic lung pathology positively correlates with the production of IL-4 by PBMC in response to *Mycobacterium bovis* antigen *in vitro* (411). With further respect to the prominent role of IL-4 in the maintenance of intestinal homeostasis (64, 415) it is tempting to assume that a Stx1-induced IL-4 secretion by bovine iIEL also contributes to the prolonged survival of STEC in the intestine of cattle.

Indeed, several bacterial antigens are involved in immunomodulatory mechanisms presumably leading to a host immunosuppression (101). Some bacterial toxins have immunomodulatory effects on T cells and IEL similar to the effect of Stx1 in bovine iIEL cultures. The staphylococcal enterotoxin C (SEC1) inhibits the full activation of bovine T cells, and induces the production of *il-4* and *il-10* transcripts (100, 101). A production of IL-4, but not IFN-γ, was also induced by minute concentrations (1 ng/ml) of TSST-1 (Toxic-shock syndrome toxin-1) and SEB (Staphylococcal enterotoxin B) in human PBMC, LPL, and IEL after 48 hrs (361). Ferens *et al.* therefore suggest that superantigens induce T_H2 responses and facilitate immunosuppression in cattle and contribute to the chronicity of infections (101). IL-4 is potentially an early player in the events that determine whether the infection progresses or becomes latent. IL-4 down-regulates the inducible nitric oxide synthase (iNOS) (173) and TLR-2 expression (200) in bovine macrophages, and hinders the activation of the cells (126). In mice, IL-4 induces the production of IL-4, IL-10 and TGF-β by iIEL (120). IL-4 is further known to inhibit the secretion of IL-8 by human intestinal epithelial cells (225), thus altering the recruitment of immune cells and contributing to the absence of inflammatory processes in the enterocytes' monolayer. IL-4 also diminishes the epithelial barrier function and increases the paracellular permeability of human intestinal epithelial monolayers (330, 444), which then potentially leads to an increased uptake of toxins from the intestinal lumen (155), thereby establishing a positive feedback circuit. Notably, IL-4 particularly down-regulates the responsiveness of human $CD8^+$ iIEL without affecting the proliferation of $CD8^+$ T cells from the peripheral blood (82), suggesting a very localized immunomodulation

of the gut immune cells. In the light of these considerations, further investigations are strongly encouraged to elucidate whether the depletion of $CD8\alpha^+$ iIEL from the bovine mucosa in the course of experimental STEC infections (251) can be traced back to a Stx1-induced production of IL-4.

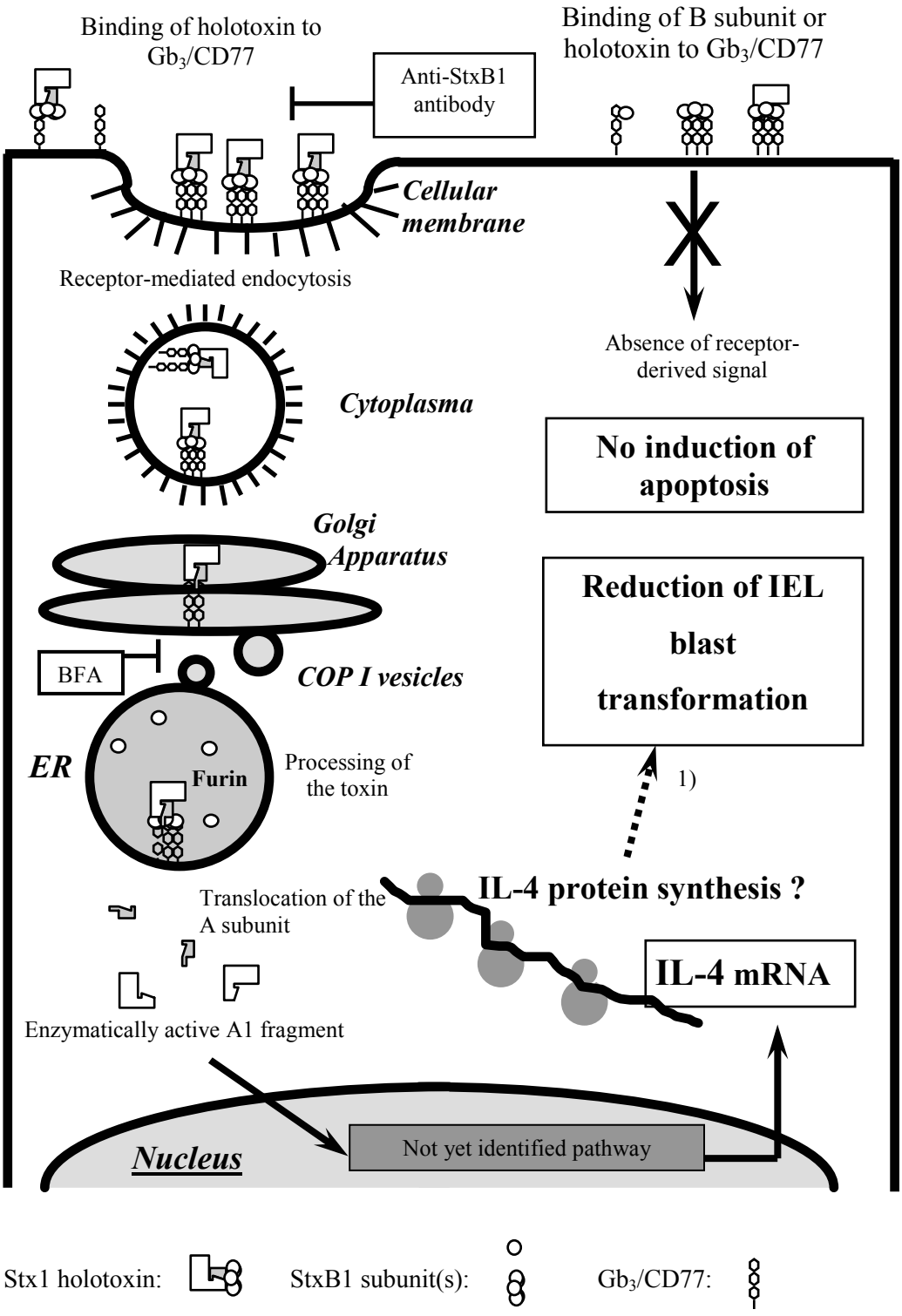
5.8. Conclusions and outlook

The molecular mechanisms leading to a long-term colonization of the bovine intestine by STEC are still not fully understood. Most STEC strains possess an array of virulence determinants (417) that allow them to interact with intestinal cells (24). Although it appears unlikely that none of these factors is recognized as a danger signal that activates mucosal defences (353, 369), STEC are not reported to induce inflammation in the intestine of ruminating cattle. Consequently, STEC must have evolved strategies to actively suppress the immune response and to allow a persistent colonisation of the intestine. Lymphostatin, encoded by the gene *lifA* in enteropathogenic *E. coli* (which is similar to *efa-1* in non-O157 STEC) inhibits the proliferation of human and bovine lymphocytes, and the mitogen-induced IFN-γ and IL-2 synthesis of human PBMC, LPL (190-192), and murine IEL (238). Lymphostatin seems to modulate the gut mucosal immunity and favours the intestinal colonization by STEC O5 and O111 strains in cattle (371). However, the homologous factors in STEC O157:H7 (products of *toxB* and a truncated form of *efa-1*) fail to influence the intestinal colonization in sheep and cattle (370). The data presented here support the notion that in adult cattle, Stx1 acts in a subtle way as an immunomodulatory factor by perturbing cytokine production. Even if the functional consequences of such disturbance are not fully known yet, it can be assumed that Stx1, probably in concert with other STEC factors (51, 62), favours the establishment of a T_H2 response allowing the development of an asymptomatic and persistent STEC infection (Fig. 31). In that, Stxs are the only immunomodulatory STEC factors recognized to date, that, by definition, are common to all STEC strains. Stxs, therefore, are highly interesting targets to be included in vaccination strategies aimed at limiting intestinal colonization by STEC.

It was recently hypothesized that STEC have evolved strategies to permit a commensal-like lifestyle (356). By increasing the amount of *il-4* transcripts, STEC might generate a particular ecological niche. Interestingly, oral treatment of pigs with the probiotic *E. coli* Nissle 1917 also reportedly led to an increase in *il-4* transcripts in the duodenum (76). This raises the possibility that other bacteria might be able to compete with the STEC for the same niche. Such a strategy, known as competitive exclusion, has been succesfully applied to

E. coli O157 infections in cattle, as feeding of cattle with *L. acidophilus* decreased the faecal shedding of O157:H7 (41). Probiotic strains, such as *L. rhamnosus*, tested in animal or cell models, inhibited the adhesion of *E. coli* O157:H7 on Caco-2 cells (147) and reduced the mortality of mice by 50 % after infection (385). In the economic environment of modern animal production, such strategies can be considered a realizable alternative (or complement) to vaccination measures in order to lower the STEC prevalence in adult cattle.

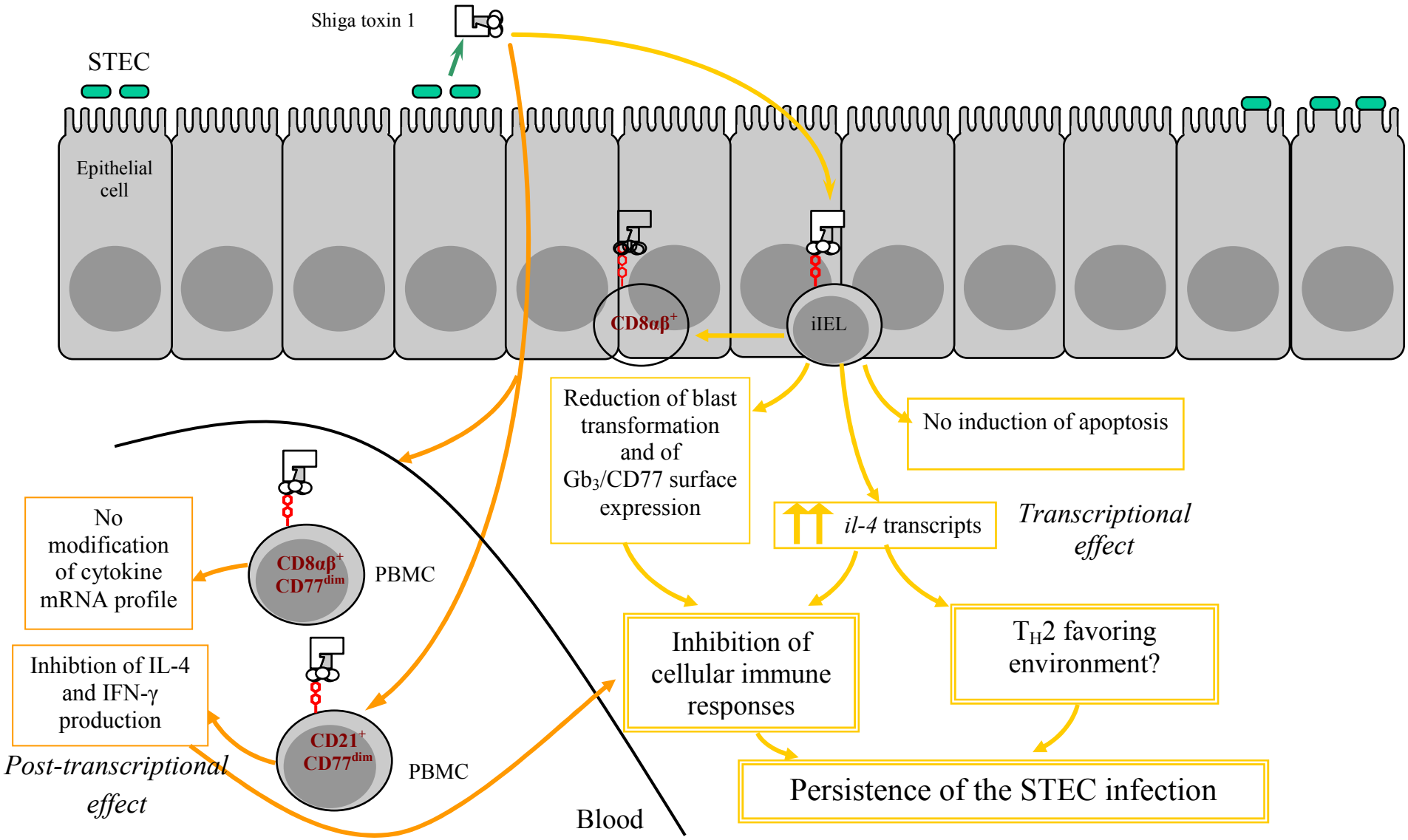
Fig. 30. Proposed cellular model depicting the effects of *E. coli* Shiga toxin 1 on bovine ileal intraepithelial lymphocytes (iIEL) (original cellular frame from Stamm *et al.* (363))



BFA: Brefldin A

[1)] Ebert and Roberts, 1996, Clin Exp Immunol: IL-4 down-regulates the responsiveness of human intraepithelial lymphocytes.

Fig. 31. Proposed model depicting the effects of *E. coli* Shiga toxin 1 on bovine PBMC and iIEL from the intestinal mucosa (adapted from the original mucosa scheme from Menge C. (246))



6. Summary

Escherichia coli Shiga toxin 1 (Stx1) blocks the activation of bovine peripheral (PBMC) and ileal intraepithelial lymphocytes (iIEL). The objectives of the study were to assess the impact of Stx1 on the expression of selected chemokine and cytokine genes in bovine lymphocytes (iIEL and PBMC) *in vitro* by real-time RT-PCR and by quantitation of intracellular cytokine proteins by flow cytometry.

Freshly isolated iIEL were shown to harbour transcripts of chemokines and of T_H1, T_H2, and T_H3 cytokines. While Stx1 did not alter the amount of mRNA specific for IL-2, IL-10, IFN-γ, TGF-β, IL-8, IP-10, and MCP-1 in cultured iIEL, minute concentrations of Stx1 led to an up to 40-fold increase of *il-4* transcripts within 6-8 hrs of incubation. In addition, the effect of Stx1 on *il-4* transcripts was specified to require the enzymatic activity of the holotoxin, as the StxB1 subunit alone did not affect iIEL. Nevertheless, in the presence of Stx1, iIEL retained their ability to synthesize proteins: 40 % of iIEL could be mitogen-stimulated to synthesize IFN-γ while less than 10 % of the cells expressed IL-4 or TGF-β proteins. The enhancement of *il-4* transcripts in iIEL was not accompanied by apoptosis. Although Stx1 significantly impaired the iIEL blast transformation induced by mitogens *in vitro* and induced a marked redistribution of the Stx- receptor from the cell surface to intracellular compartments, Stx1-treated iIEL neither showed an increased exposure of phosphatidylserine on the cell surface nor an altered mitochondrial membrane potential. Comparative experiments with PBMC revealed that the effect on *il-4* transcripts was specific for iIEL. Moreover, Stx1 hindered the translation of IFN-γ in PBMC within 21 hrs.

Mucosal immune cells in cattle were found to be considerably sensitive to a cytokine-stimulating effect of Stx1. The results led to assume that the distinct induction of *il-4* transcripts contributes to the establishment of a commensal-like colonization of the bovine intestinal mucosa by STEC.

7. Zusammenfassung

Escherichia coli Shiga toxin 1 (Stx1) blockiert die Aktivierung boviner peripherer (PBMC) und ilealer intraepithelialer Lymphozyten (iIEL). Ziel dieser Untersuchung war es, den Einfluss von Stx1 auf die Expression ausgewählter Chemokin- und Zytokin-Gene *in vitro* mit Hilfe der „real-time RT-PCR“ und der intrazellulären Quantifizierung zweier Zytokin-Proteine in der Durchflusszytometrie zu bestimmen.

Frisch isolierte iIEL enthielten sowohl Transkripte für eine Reihe von Chemokin-Genen als auch von Genen für T_H1-, T_H2- und T_H3-Zytokine. Während Stx1 den Gehalt an mRNA für IL-2, IL-10, IFN-γ, TGF-β, IL-8, IP-10 und MCP-1 in kultivierten iIEL nicht veränderte, führten bereits kleinste Konzentrationen von Stx1 zu einer bis zu 40-fachen Steigerung der Zahl der *il-4*-Transkripte innerhalb von 6-8 Stunden. Dieser Effekt des Stx1 war von der enzymatischen Aktivität des Holotoxins abhängig, da die rezeptorbindende StxB1-Untereinheit nicht die gleiche Wirkung entfaltete. Trotzdem war auch in Anwesenheit von Stx1 die Fähigkeit der iIEL zur Proteinsynthese erhalten. Vierzig Prozent der iIEL konnten durch Mitogene zur Synthese von IFN-γ stimuliert werden, wohingegen weniger als 10 % der Zellen IL-4- oder TGF-β-Proteine bildeten. Die Stx1-induzierte Vermehrung der *il-4*-Transkripte in iIEL war nicht von Apoptose begleitet. Stx1 beeinträchtigte zwar erheblich die Fähigkeit der iIEL zur Blastentransformation *in vitro* und induzierte eine deutliche Umverteilung der Stx-Rezeptoren von der Zelloberfläche in intrazelluläre Kompartimente. Stx1-behandelte iIEL zeigten aber weder eine verstärkte Exposition von Phosphatidylserin auf der Zelloberfläche noch war ihr mitochondriales Membranpotential verändert. Bei vergleichenden Untersuchungen mit PBMC zeigte sich, dass der Effekt des Stx1 auf *il-4*-Transkripte spezifisch für iIEL ist. Im Gegensatz zur Wirkung des Stx1 auf iIEL reduziert Stx1 bei PBMC innerhalb von 21 Stunden die Translation von IFN-γ.

Schleimhautimmunzellen des Rindes sind damit aussergewöhnlich sensibel gegenüber einem Zytokin-stimulierenden Effekt des Stx1. Dies lässt vermuten, dass die gezielte Induktion von *il-4*-Transkripten zur Kolonisierung der bovinen Mukosa durch STEC in einer Saprophyten ähnlichen Weise beiträgt.

8. Reagents, media, and buffers

8.1. Reagents

Reagents	Company	Catalogue number
1,4 Dithiothreitol (DTT)	Carl Roth GmbH	6908.2
3,3'-Dioctadecyloxacarbocyanin-Perchlorate (DiO)	Molecular Probes	D-275
7-Aminoactinomycin D	Sigma-Aldrich	A-9400
Actinomycin D	Sigma-Aldrich	A-1410
Agarose D-1 Low EEO	Serva Electrophoresis GmbH	8016
Amplitaq® DNA Polymerase, 10X buffer and 25mM $MgCl_2$	Applied Biosystem	N808-0161
Anti-bovine CD8β	VMRD	BAT82A
Anti-human CD77, Clone 38.13	Coulter Immunotech Diagnostic	IM0175
Anti-bovine TcR-N12	VMRD	CACT61A
Anti-bovine IFN-γ	Serotec GmbH	Clone 303
Anti-bovine IL-4	Serotec GmbH	Clone 302
Anti-human TGF-β	Serotec GmbH	Clone TB21
BSA	Serva Electrophoresis GmbH	11930
Brefeldin A	Sigma-Aldrich	B-6542
Collagenase (Type 1 CLS)	Biochrom AG	C 1-22
Concanavalin A	Sigma-Aldrich	C-2010
DNase I and 10X buffer	Amersham Biosciences	27-0514-02
dNTP (4 nmol each)	PAN™ BIOTECH GmbH	739026
EDTA	Serva Electrophoresis GmbH	11280
Ethanol 99 %	Merck	1.00983.1011
Ethidium bromide	Serva	21251
Fetal calf serum	Biowest	S1810
Ficoll-Paque™ Plus	Amersham Pharmacia Biotech	17-0840-03
GeneRuler™ 100 bp DNA ladder	MBI Fermentas	SM0241

Gentamycin	Biochrom AG	A2710
Goat anti-mouse IgG (H+L) – FITC conjugated	Dianova	115-095-062
Goat anti-rat IgM (µ-chain specific)	Dianova	112-005-075
Ionomycin calcium salt	Sigma-Aldrich	I-0634
Lambda DNA/EcoRI+HindIII marker	MBI Fermentas	SM0191
Leibowitz's L-15 medium	Life Technologies GmbH	41300-021
Lipolysaccharide	Sigma-Aldrich	L-4130
Mercaptoethanol PlusOne 98 %	Amersham Biosciences	17-1317-01
M-MLV RTase (H^-)	Promega	M 3683
M-MLV RTase Buffer Pack	Promega	M 5313
Nylon wool	Biotest AG, Dreieich	830034
Oligo $d(T)_{16}$ primer	Applied Biosystems	N808-0128
Paraformaldehyde	Merck	104005
PCR tubes	Nerbe	04.022.1100
PCR primers	MWG Biotech AG	-
PCR TaqMan™ probe	Eurogentec	-
Penicillin / Streptomycin (100X)	PAA GmbH	P11-010
Percoll®	Sigma-Aldrich	P-1644
Phorbol-Myristat-Acetat (PMA)	Orpegen-Pharma	34008480
Phytohematogglutinin P (PHA-P)	Sigma-Aldrich	L-8754
Rat IgM Kappa myeloma protein	CAMON	PRP08
qPCR ™ MasterMix	Eurogentec	RT-QP2X-03
Recombinant human Annexin-V-PE conjugated	Caltag	ANNEXINV04
Ribonuclease Inhibitor (RNasin)	MBI Fermentas	E00312
RNase-free DEPC-treated water	Carl Roth GmbH	T 143.2
Rnase-free DNase Set	QIAGEN	79254
RNeasy® Mini Kit	QIAGEN	74106
RPMI 1640 medium with 2mM stabilized L-glutamin and 2.0 g/l $NaHCO_3$	PAN™ BIOTECH GmbH	P04-18500
Saponin	Merck	7695

Sodium azide	Merck	106688
Tris-Hydroxymethyl-Aminomethan	Carl Roth GmbH	4855
Ultroser® HY	CYTOGEN	66029-018

8.2. Buffers and solutions

Annexin-V binding buffer 10X-concentrate

Hepes/NaOH (pH=7.4)	2.283 g
NaCl	8.182 g
$CaCl_2$	0.277 g
Bi-distilled water	100.0 ml

Annexin-V binding buffer 1X

Annexin-V binding buffer 10X-concentrate	10.0 ml
Bi-distilled water	90.0 ml

Erythocytes lysis buffer

NH_4Cl	8.26 g
$NaHCO_3$	1.09 g
Na_2-EDTA * $2H_2O$	0.037 g
Bi-distilled water	1,000 ml
Sterilization by filtration (Millipore 0.22 μm)	

Electrophoresis loading-dye

Bromophenol blue (Sodium salt)	0.25 g
Xylene Cyanole FF	0.25 g
Glycerin	30 g
Bi-distilled and autoclaved water	100 ml
Aliquoted (1 ml) and stored at –20°C	

NaCl solution

NaCl	0.89 g
Bidistilled water	100.0 ml

10X-concentrated PBS buffer (pH= 7.4)

Na_2HPO_4 * 2 H_2O	18.0 g
KH_2PO_4	2.5 g
KCl	2.5 g
NaCl	100.0 g
Bi-distilled water	1,000 ml

PBS 1X buffer (pH= 7.4)

PBS 10X-concentrate buffer	100.0 ml
Bi-distilled water	900.0 ml

Sterilization by filtration (Millipore 0.22 μm)

PBS 1X – 1 % BSA buffer (pH 7.4)

Bovine serum albumine (Fraction V)	10.0 g
1X PBS (pH 7.4)	1,000 ml

Sterilization by filtration (Millipore 0.22 μm)

Storage at 4°C

10X-concentrated PBS – EDTA buffer (pH= 7.4)

Na_2HPO_4 * $2H_2O$	14.2 g
KH_2PO_4	2.0 g
KCl	2.0 g
NaCl	80.0 g
Na_2-EDTA * $2H_2O$	20.0 g
Bi-distilled water	1,000 ml

Sterilization by filtration (Millipore 0.22 μm)

PBS – EDTA 1X buffer (pH= 7.4)

PBS – EDTA 10X concentrate buffer	100.0 ml
Bi-distilled water	900.0 ml

Sterilization by filtration (Millipore 0.22 μm)

PBS 1X – EDTA 10X buffer (pH 7.4)

Na_2-EDTA * $2H_2O$	20.0 g
PBS 1X	1,000 ml

Sterilization by filtration (Millipore 0.22 µm)

PBS-EDTA-AB buffer (pH 7.4)

Na_2HPO_4 * $2H_2O$	1.42 g
KH_2PO_4	0.20 g
KCl	0.20 g
NaCl	8.00 g
Na_2-EDTA * $2H_2O$	0.75 g
Glucose	1.00 g
Penicillin / Streptomycin	100000 U Penicillin; 100 g Streptomycin
Gentamycin (20 mg/ml)	500 µg
Bi-distilled water	1,000 ml

Sterilization by filtration (Millipore 0.22 µm)

PBS – PFA 4 % (paraformaldehyde)

Paraformaldehyde	4.0 g
1X PBS (pH 7.4)	100.0 ml

Allow to dissolve 30 min in water-bath at 75-80°C. Shake every 5 min. Store at 4°C.

Saponin solution 5 %

Saponin	5.0 g
Bidistilled water	100.0 ml

Sterilize by filtration (0.2 µm), store at 4 °C.

Sodium azide solution (10 %)

NaN_3	5.0 g
NaCl solution (150 mM)	50.0 ml

Sterilize by filtration (0.2 µm), store at 4 °C.

Sodium citrate solution (3,8%)

Na-citrate x 2 H_2O	3.80 g
Bidistilled water	100.0 ml

Sterilize by filtration (0.2 µm), aliquot (100 ml), store at RT

Trypan blue solution

Trypan blue	0.20 g
NaCl solution	1,000 ml

8.3. Cell culture media

BL-3 medium

RPMI 1640 with stabilized glutamin	617.0 ml
Lebowitz 15 medium	150.0 ml
Fetal calf serum	100.0 ml
Penicillin / Streptomycin	1000 IU / 1.0 g in 10.0 ml
β-Mercaptoethanol (1 mM)	3.0 ml

Sterilization by filtration (Millipore 0.22 µm)

Gentamycin

Gentamycin-sulfat	10.0 g
Bi-distilled water	500 ml

Sterilization by filtration (Millipore 0.22 µm)

IEL-medium

RPMI 1640 with stabilized glutamin	780.0 ml
Fetal calf serum	200.0 ml
Penicillin (10,000 U/ml)/ Streptomycin (10,000 µg/ml)	10.0 ml
Amphotericin B (250 µg/ml)	10.0 ml
Gentamycin (20 mg/ml)	2.5 µg/ml

Sterilization by filtration (Millipore 0.22 µm)

PBMC-medium

RPMI 1640 with stabilized glutamin	897.0 ml
Fetal calf serum	100.0 ml
β-Mercaptoethanol (1 mM)	3.0 ml
Sterilization by filtration (Millipore 0.22 µm)	

Ramos cell-medium

RPMI 1640 with stabilized glutamine	890.0 ml
Fetal calf serum	100.0 ml
Penicillin (10,000 U/ml)/ Streptomycin (10,000 µg/ml)	10.0 ml

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Acknowledgment

I would like in this section to thank all the persons who supported me during the whole period of my doctoral thesis.

Prof. Dr. M. Martin is greatly acknowledged for having directed my thesis, and for the discussions about the progress of this work.

I am also very thankful to Prof. Dr. Dr. habil G. Baljer for the co-direction of my thesis, for having welcomed me in the Institute for Hygiene and Infectious Diseases of Animals, and for his constructive remarks concerning the present work.

I would like to thank as well Dr. Christian Menge, who established the subject of the thesis and led my study within the working group. He is also greatly acknowledged for the stimulating discussions we had about the complexity of mucosal immunity, IEL biology and STEC infections. His way of sharing his experience in the field of flow cytometry really helped me to learn a lot about this method. His experience both helped me and encouraged me to continue to study the mechanisms of infectious diseases.

My warm thanks are going to Dr. Ivonne Stamm for her availability and her patience. She introduced me to the world of flow cytometry and shared her impressive knowledge of cell culture. She also taught me so many things that I cannot consequently list everything here!

I also thank the doctoral students (former and present) of the institute for their warm welcome in this so particular “Doktorandenzimmer”, and for the daily friendly atmosphere. I will remember my stay in the place as a period during which reciprocal help, technical competences, and friendship were of important value.

I would like also to thank Melanie Mohr for her good mood when joining me to the farm, to the slaughterhouse, and to the several SFB seminars we had to attend. I do not forget all the personal of the institute for their help, and in particular Gabriele Köpf for her good mood, her presence in the S2 laboratory and her support in molecular biology.

I also thank everybody for the huge patience that you show concerning my total ignorance of the German language in the first months after my arrival in Giessen. Thanks to everybody who contributed to my learning of this “not so easy” language.

The Deutsche Forschungsgemeinschaft and the Sonderforschungsbereich 535 are greatly acknowledged for the financial support of my thesis.

I thank my brother, my family, my friends for their support during my studies in France and in Germany, although we were most of time faraway.
Finally, I thank very much my parents for their comprehension, encouragement, and for their financial support during my studies. Their presence helped me during the last days before the final examination.

“Gratitude is not only the greatest of virtues, but the parent of all others.”
Cicero, 106 B.C- 43 B.C.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen Hilfsmittel als die angegeben benutzt habe. Die Stellen, die ich anderen Untersuchungen dem Wortlaut oder Sinn entsprechend entnommen habe, sind durch Quellenangaben gekennzeichnet. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

Gießen, den 12.01.2006