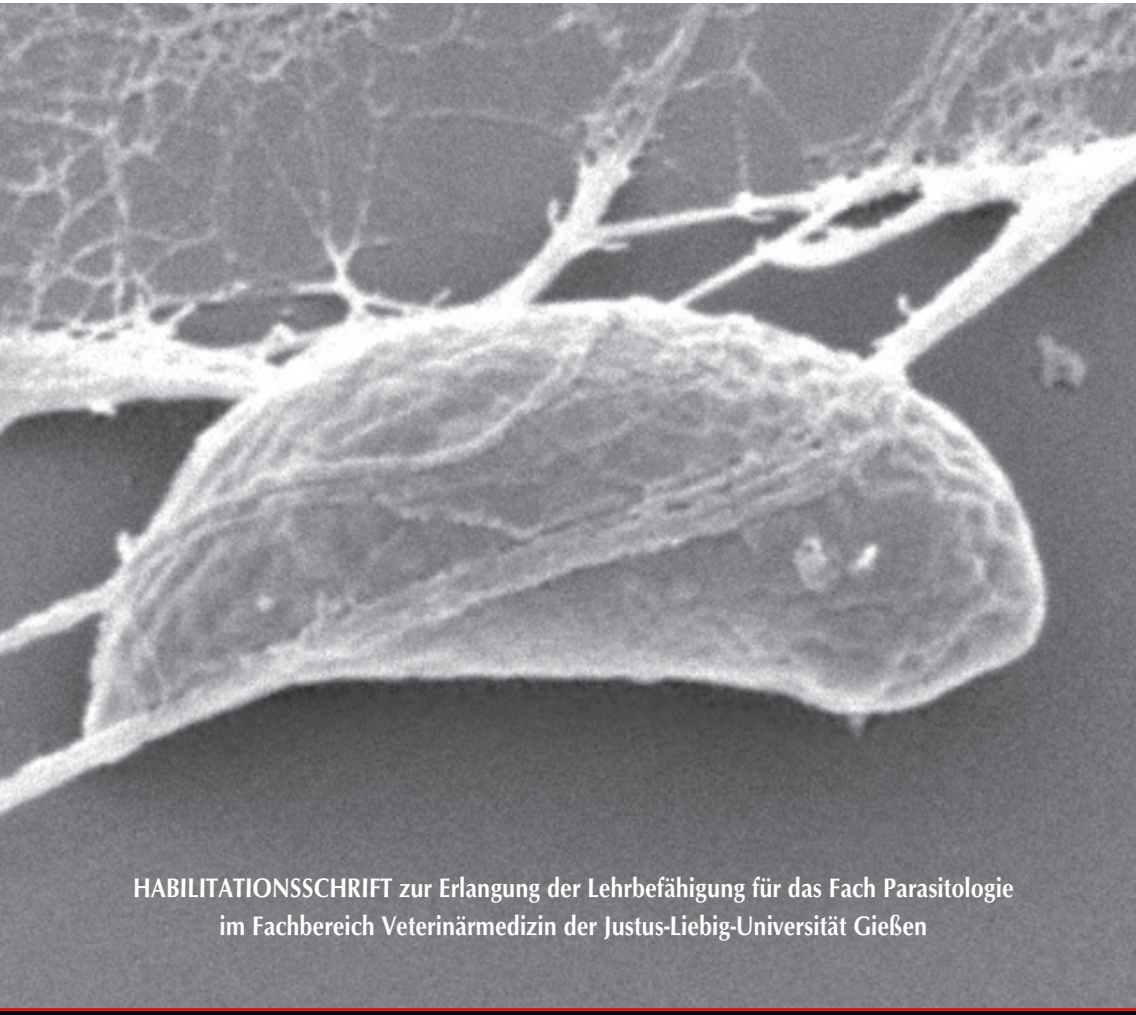
UNTERSUCHUNGEN ZUR ZELLULÄREN IMMUNANTWORT SOWIE ZU WIRTSZELLGEBUNDENEN REAKTIONEN GEGEN *EIMERIA BOVIS*-INFEKTIONEN

ANJA TAUBERT



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Untersuchungen zur zellulären Immunantwort sowie zu wirtszellgebundenen Reaktionen gegen *Eimeria bovis*-Infektionen

Habilitationsschrift
zur Erlangung der Lehrbefähigung für das Fach Parasitologie
im Fachbereich Veterinärmedizin
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vorgelegt von

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gewidmet

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1 EINLEITUNG

Eimeria bovis ist eine der häufigsten bovinen *Eimeria*-Arten in Mitteleuropa und zählt zu den pathogensten Kokzidien des Rindes. Infektionen mit diesem intrazellulären Protozoen können beim Kalb unter bestimmten Haltungsbedingungen (vgl. [50]), sowie bei Ko-Infektionen, beispielsweise mit viralen Erregern [152], große wirtschaftliche Bedeutung erlangen. Die klinischen Erscheinungen sind dann von einer schweren, hämorrhagischen Typhlocolitis geprägt [76]. Gleichwohl besteht in der Regel ein ausgeprägtes, von einer belastungsfähigen Immunität der Tiere getragenes, enzootisches Gleichgewicht, das z. T. klinische Konsequenzen der Infektion verhindert (vgl. [50]). Die Mechanismen, die bei der Kontrolle der Erstinfektion und der Immunität gegen Reinfektionen mit *E. bovis* zum Tragen kommen, sind nur unzureichend bekannt. In Übereinstimmung mit anderen *Eimeria*-Infektionen bei Säugern besteht zwischen dem Antikörperprofil und einer schützenden Immunität im Kalb kein klarer Zusammenhang, auch wenn die Antikörperspiegel mit der Schwere einer *E. bovis*-Infektion korrelieren [110, 115].

Zelluläre Immunreaktionen des Rindes auf *E. bovis*-Infektionen sind bisher nur in geringem Umfang untersucht worden. Insbesondere auf Ebene der angeborenen Immunreaktionen, die entscheidend beim ersten Zusammentreffen von Parasit und Wirt sein müssen, liegen keinerlei Untersuchungen vor, obwohl Daten zu anderen *Eimeria*-Infektionen auf die Relevanz dieser Reaktionen hindeuten [25, 24, 222, 316, 325, 327, 372]. Bezüglich adaptativer Immunreaktionen kann aus Untersuchungen zu anderen *Eimeria*-Infektionen, vorwiegend in Nagermodellen, geschlossen werden, dass sowohl die Begrenzung einer erstmaligen *Eimeria*-Infektion als auch der Schutz gegen eine Reinfektion zum Großteil T-Zell-abhängig sind (zusammenfassend unter [395]). Aus der Mehrzahl der Untersuchungen lässt sich annehmen, dass im ersteren Fall eher $CD4^+$-T-Zellen eine wichtigere Rolle spielen [291, 333, 367], offensichtlich mit Th1-Zellen über IFN-γ als entscheidendem Mediator und Induktor von zytotoxischen Komponenten wie N- und O-Intermediaten bzw. -Radikalen [260, 382]. Bei Reinfektionen dagegen scheinen $CD8^+$-T-Zellen die entscheidenden Effektorzellen zu sein [111, 259, 325, 334]. Diese v. a. im Maussystem durchgeführten Untersuchungen sind jedoch nur bedingt auf das Rind übertragbar, da adaptativ-zelluläre Immunreaktionen im bovinen System nicht zwingend dem für die Maus gültigem Schema der Th1/Th2-Dichotomie folgen [45].

Aus bisherigen Untersuchungen an *E. bovis*-infizierten Tieren ist im Rahmen der Primärinfektion eine gesteigerte Proliferationsleistung von PBMC als Reaktion auf *E. bovis*-Antigen sowie eine Vermehrung des Anteils der $CD4^+$- und $CD8^+$-T-Zellen ersichtlich [144, 157, 158]. Dagegen scheinen $\gamma\delta$-TCR^+-T-Zellen in die Prozesse nicht involviert zu sein [144]. Die spezifische Reaktivität von T-Zellen aus lymphatischen Organen ist gleichfalls über die Patenz hinaus erhalten, vor allem in denjenigen Lymphknoten, die die betroffenen Gebiete drainieren. In diesen Lymphknoten ließen sich deutlich mehr IL-2- als IL-4-Transkripte nachweisen [144]. Daten zu IFN-γ oder zu anderen Zytokinen im Verlauf einer *E. bovis* Primär- oder Reinfektion sind jedoch fragmentarisch.

Das Ziel der vorliegenden Arbeit war es, sowohl Reaktionen von Zellen des angeborenen Immunsystems auf *E. bovis*-Stadien zu überprüfen, als auch adaptativ-zelluläre Immunreaktionen im Verlauf der Primär- und Reinfektion mit *E. bovis* zu charakterisieren. Zudem waren *E. bovis*-induzierte Modulationen der infizierten Wirtszellen auf Transkriptom- und Proteom-Ebene Gegenstand der Untersuchung.

2 SCHRIFTTUM

2.1 *Eimeria bovis*: Entwicklungsbiologische Aspekte im Bezug auf Immunitätsentwicklung

Eimeria bovis ist ein streng wirtsspezifischer und obligat intrazellulärer Parasit. Er entwickelt sich im Rind über zwei Merogonien, wobei im Zuge der ersten Teilung im Endothel der zentralen Lymphkapillaren der Zotten des distalen Ileums innerhalb von 14-18 Tagen Makromeronten mit >100.000 Merozoiten I entstehen. Die zweite Merogonie sowie die Gamogonie finden danach in rascher Folge in Epithelzellen von Zäkum und Kolon statt, so dass die Präpatenz 18-21 Tage beträgt [133, 135, 134].

Bezüglich der Fragestellung, welche Parasitenstadien in immunen Tieren die Ziele der Immunität sind, ist bei *E. bovis* wenig bekannt. *E. bovis*-Sporozoiten infizieren nicht wie andere bovine *Eimeria* spp. Epithelzellen des Darms, sondern müssen stattdessen durch die mukosale Schicht des Ileums wandern, um Endothelzellen der zentralen Lymphkapillaren der Darmvilli zur Infektion zu erreichen. Es ist bisher nicht bekannt, ob Endothelzellen von basal oder apikal infiziert werden (in der Zellkultur erfolgt die Invasion von apikal). *In vitro* wird ein z. T. wiederholtes Verlassen bereits infizierter Wirtszellen mit nachfolgendem Aufsuchen neuer Zellen beobachtet, ein Phänomen, das auch für andere *Eimeria* spp. belegt ist [74, 75]. Als Folge ihres Invasionsverhaltens sowie ihrer Wanderung sollten freie Sporozoiten im Interstitium und in der Lymphe als Ziele von Immunzellen erfassbar sein. Sobald jedoch Sporozoiten adäquate Wirtszellen invadiert haben und die intrazelluläre Weiterentwicklung begonnen hat, sind *E. bovis*-Stadien bis zur Freisetzung der Merozoiten I, die dann wieder frei zugänglich wären, nur über die Eliminierung der endothelialen Wirtszellen angreifbar. Dies trifft ebenso auf spätere Stadien, wie Meronten II oder Gamonten zu.

Daten aus anderen, nicht-bovinen Systemen sprechen dafür, dass protektive Immunreaktionen früh nach der (Re-)Infektion greifen und bereits Sporozoiten bei immunen Tieren beeinträchtigt werden. Diesbezüglich wird diskutiert, ob Sporozoiten anderer *Eimeria* spp. vor der Invasion von Enterozyten [286] erfasst werden oder ob über Immunreaktionen vorgeschädigte Sporozoiten zwar noch zur Infektion, nicht aber mehr zur intrazellulären Weiterentwicklung befähigt sind [195, 227]. Rose et al.

[296] zeigten im murinen System, dass nach Übertragung von Lymphozyten aus immunen Tieren auf naive Mäuse und anschließender Primärinfektion die Anzahl der Sporozoiten in der Mukosa deutlich reduziert war. Auch im Falle von *E. tenella* sind Sporozoiten in immunen Tieren z. T. in apikalen Epithelzellen, v. a. aber in Enterozyten der Zäkumkrypten in ihrer Zahl vermindert [294]. Interessanterweise scheint hier v. a. der Transfer von Sporozoiten aus als Transportmedium dienenden intraepithelialen Lymphozyten in Enterozyten der Krypten beeinflusst zu werden.

Sollten tatsächlich Sporozoiten die Ziele adaptativer Immunreaktionen sein, stellt sich die Frage ob solche, spezifische Reaktionen zusätzlich auch spätere Stadien wie Merozoiten erfassen könnten. Bezüglich *E. bovis* sprechen kreuzreagierende Antikörper von Tag 10 p. i., die mit Sporozoiten und Merozoiten gleichermaßen reagieren [158], zumindest für einige gemeinsame antigene Determinanten. Proteinanalysen zeigten, dass mindestens vier Proteine in beiden Stadien vorhanden sind [277], so dass auf zellulärer Ebene eine gegen Sporozoiten gerichtete Reaktivität spätere Stadien wie Merozoiten grundsätzlich erfassen könnte.

Neben Sporozoiten sprechen v. a. histopathologische Untersuchungen bei der Maus (*E. falciformis*: [228]; *E. vermiformis*: [296]), der Ratte (*E. separata*: [327]) und im Huhn (*E. tenella*: [294, 195]) zusätzlich für die sich entwickelnden, frühen ersten Meronten als betroffene Entwicklungsstadien protektiver Immunreaktionen. Dabei weisen beispielsweise bei *E. tenella* deutlich höhere Reduktionsraten in immunen Tieren bezüglich entwickelter Meronten I versus Sporozoiten auf zusätzliche, gegen Meronten gerichtete Mechanismen hin [294].

Ältere Daten zu Immunisierungen von *E. bovis*-infizierten Kälbern weisen sowohl über eine geringere Anzahl von Merozoiten im Darminhalt als auch über eine verminderte Anzahl von Meronten I in der Dünndarmschleimhaut eher auf frühe Meronten als Zielstadien der Immunität hin [132]. In der entsprechenden Arbeit fehlen jedoch Aussagen über Sporozoiten.

Interessanterweise ließen sich auf frühen, *in vitro* entwickelten Meronten I von *E. bovis* immunreaktive Komponenten nachweisen, die mit Seren immuner Kälber reagieren [15]. Das Reaktionsmuster der Immunseren wies dabei einerseits auf merozoitenspezifische Antigene und andererseits auf ein Auftreten dieser Moleküle auf der Oberfläche infizierter Wirtszellen frühestens ab Tag 7 p. i. hin, also zu einem

Zeitpunkt, zu dem die Proliferation des Parasitens zwar begonnen hat, jedoch noch keine reifen Merozoiten vorhanden sind. Da *in vivo* die entsprechenden Wirtszellen von Endothelzellen repräsentiert werden, erscheint es im Hinblick auf NK-Zell- oder adaptativ-zellulär-vermittelte Reaktionen von besonderem Interesse, dass Endothelzellen grundsätzlich Antigen-präsentierende Eigenschaften besitzen [20, 35, 176, 379] und z. B. T-Zellen aktivieren können [63, 102, 268]. Im Prinzip wäre ein solcher Mechanismus aufgrund der oben genannten Lokalisation von Parasitenantigenen auch in *E. bovis*-infizierten Kälbern denkbar.

2.2 Reaktionen von Zellen des angeborenen Immunsystems gegen Eimerien

Es besteht kein Zweifel, dass Reaktionen des angeborenen Immunsystems die frühestmögliche und damit schnellste Abwehrfront des Wirts repräsentieren und grundsätzlich entscheidend für den weiteren Verlauf einer Infektion und der Entwicklung des Krankheitsgeschehens sind. Wie von P. Parham treffend resümiert, ist die Tatsache, dass Menschen (und Tiere) nicht permanent krank sind, auf Reaktionen des nicht-adaptativen Immunsystems zurückzuführen, die letztendlich die meisten Infektionen unterdrücken und somit inapparent bleiben lassen [265]. Zellen des nicht-adaptativen Immunsystems wie NK-Zellen, neutrophile Granulozyten (PMN) oder Makrophagen und ihre Immunreaktionen wurden jedoch über viele Jahre insbesondere in tiermedizinischer Sicht kaum wissenschaftlich untersucht.

Auch im Falle der Eimerien und anderer Apikomplexa beschäftigen sich die meisten Untersuchungen mit Reaktionen des adaptiven, spezifischen Immungeschehens. Der individuell stark variierende Krankheitsverlauf bei *E. bovis* primär infizierten Kälbern, der letztendlich über die Wirtschaftlichkeit der betroffenen Tiere entscheidet, könnte jedoch auch als Ausdruck unterschiedlich effektiv agierender, nicht-adaptativer Immunreaktionen gewertet werden. Somit erscheinen detailliertere Untersuchungen zum nicht adaptativen Immungeschehen essentiell für das Verständnis der Immunogenese der Kokzidiose des Rindes.

2.2.1 Neutrophile Granulozyten (PMN)

PMN stellen Immunzellen der vordersten Verteidigungslinie dar und verfügen über multiple Effektormechanismen. Sie sind zur Phagozytose befähigt und können Pathogene intrazellulär über Sauerstoff-abhängige („oxidative burst“, Bildung reaktiver Sauerstoffspezies) oder Sauerstoff-unabhängige (Inhalte der PMN-Granula: Defensine, Lysozym, Elastase, Kathepsin B u. a.) Mechanismen abtöten (vgl. Abb. 1, zur Übersicht siehe [220]).

Als eine eher indirekte, aber nicht minder effektive Strategie zur Elimination von Pathogenen wird die PMN-eigene Produktion diverser Chemokine oder Zytokine zur Anlockung und Aktivierung weiterer Immunzellen angesehen. Über die Synthese von IL-12 und TNF-α [27, 32, 89, 56] nehmen PMN z. B. Einfluss auf die Ausprägung sich entwickelnder adaptativer Immunreaktionen. Auch die Befunde, dass PMN Antigen-tragend sein [215] und in direkte Interaktion mit dentritischen Zellen treten können [26, 370] sprechen für eine wichtige immunoregulatorische Funktion dieser Immunzellen.

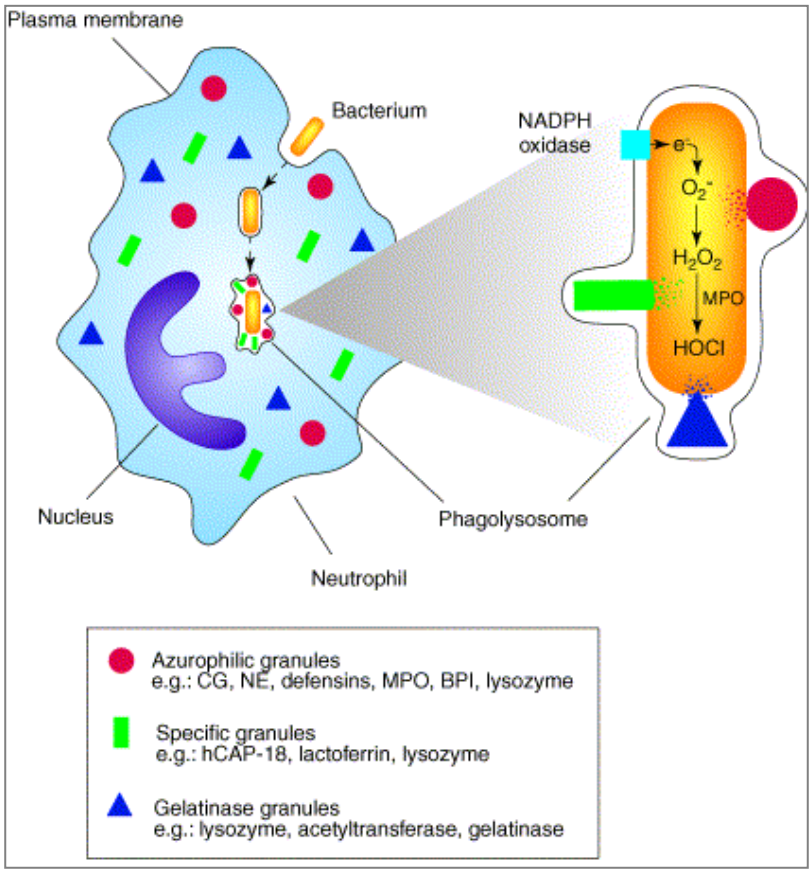

Abbildung aus: Mayer-Scholl et al., 2004

Abb. 1: Sauerstoff-abhängige- und -unabhängige Mechanismen während der Phagozytose
Der Sauerstoff-unabhängige Weg beinhaltet die Freisetzung der Inhalte dreier Granula-Typen: azurophilischer, PMN-spezifischer und Gelatinase-enthaltender Granula, welche insgesamt Proteasen, antimikrobielle Proteine und Peptide und Enzyme enthalten. Eines dieser Enzyme, Lysozym, zerstört z. B. die anionischen Oberflächen von Bakterien und verursacht eine erhöhte Permeabilität der Bakterien. Die neutrophile Elastase, degradiert dagegen Virulenzfaktoren.
Der Sauerstoff-abhängige Mechanismus beruht auf dem NADPH-Oxidase-Komplex, der sich an der Membran des Phagolysosoms zusammenfügt und die Produktion von O_2^- beinhaltet. Dieses wird schnell in Hydogenperoxid überführt, welches seinerseits unter Einfluss der aus den azurophilen Granula stammenden Myeloperoxidase (MPO) zur Bildung von HOCl dient.

In 2004 wurde ein zusätzlicher Effektormechanismus für humane PMN beschrieben [43], welcher die Bildung sog. „Neutrophil Extracellular Traps“ (NETs) beinhaltet und zur extrazellulären Immobilisierung, Entwaffnung und/oder Tötung von Pathogenen führt (zur Übersicht siehe [220, 223, 384, 264]). Die *in vivo*-Relevanz dieses Mechanismus wurde inzwischen in unterschiedlichen Untersuchungen bestätigt [23, 43, 48].

Die Ausbildung solcher NETs wird v. a. hinsichtlich der Bekämpfung großer Pathogene diskutiert [368], ist mittlerweile speziesübergreifend auch für PMN von Maus [48, 103], Kaninchen [43], Pferd [7], Rind [202] und Fisch [263, 262] beschrieben und ist zudem nicht einzigartig für PMN. Vergleichbare antimikrobielle Aktivitäten besitzen so z. B. auch Mastzellen [376, 385], eosinophile Granulozyten [377, 394] und aviäre heterophile Zellen [64].

NETs sind extrazelluläre Strukturen aus nukleärem Material (DNA und Chromatin) mit eingelagerten Inhalten der cytoplasmatischen Granula [43, 119], die im Rahmen eines programmierten Zelltods von PMN im Moment des Absterbens freigesetzt werden (Abb. 2), wobei diese Freisetzung von funktionellen Tubulin- und Aktinfilamenten abhängt [247].

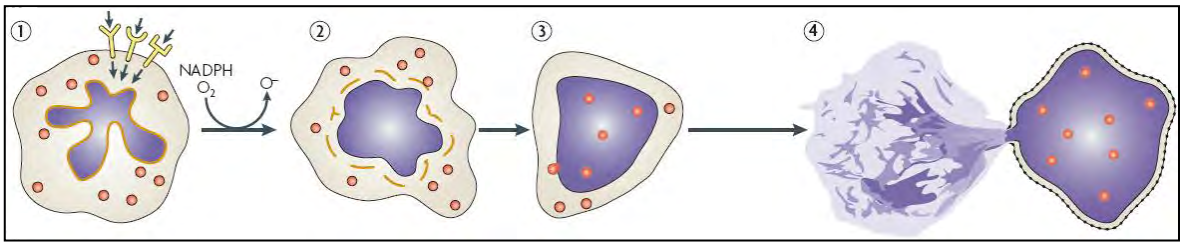

Abb. 2: Bildung von „Neutrophil Extracellular Traps“ (NETs)
(1) Die Aktivierung der PMN führt zur Bildung von ROS
(2) Die Zellkernmembran löst sich auf, die Integrität der Granula geht fortschreitend verloren
(3) Die Kerne verlieren ihre lobuläre Struktur, das Zellkernmaterial vermischt sich mit Inhalten der Granula
(4) Die Zellen runden sich ab, kontrahieren sich und entlassen NETs

Abbildung aus: Brinkmann und Zychlinsky, 2007

Der über die Bildung von NETs implementierte Zelltod von PMN unterscheidet sich von Apoptose oder Nekrose und unterliegt einer NADPH-Oxidase-abhängigen Synthese von ROS (reactive oxygen species) [43, 119]. Pathogene, wie Bakterien (z. B. *Staphylococcus aureus, Shigella flexneri*), Pilze (z. B. *Candida albicans*) oder auch *Leishmania amazonensis*-Stadien [129] werden immobilisiert und z. T. über eine hohe lokale Konzentration antimikrobieller Proteine abgetötet [43, 44, 368]. Im

Falle einiger Bakterien (z. B. Gruppe A *Streptococcus* oder *Pneumococcus*) kommt es jedoch über die Produktion pathogeneigener, DNA-degradierender Moleküle [23, 48] oder des M1-Proteins [192] als Kontramechanismen nur zum Abfangen, nicht jedoch zur Abtötung der Mikroorganismen.

Über die Beteiligung von PMN bei *E. bovis*-Infektionen liegen bisher kaum Daten vor, obwohl Untersuchungen bei anderen Eimerienarten über die vermehrte Präsenz von PMN in der Darmmukosa *in vivo* [31, 229, 325] auf die Relevanz dieser Zellen deuten. Einen Hinweis zu *E. bovis* liefern ältere histologische Untersuchungen von Friend und Stockdale [118], die von PMN, eosinophilen Granulozyten und Makrophagen infiltrierte reife Meronten (16-18 Tage p. i.) beschreiben. Auch die bei *E. bovis*-infizierten Endothelzellen *in vitro* beobachtete, gesteigerte Adhäsion von PMN weist auf eine Beteiligung dieses Zelltyps am Immungeschehen hin [147].

Im Falle von *E. maxima* in Hühnern und *E. nieschulzi* in Ratten wurde im Verlauf der Primärinfektion ein biphasischer Anstieg der PMN im peripheren Blut gemessen [289]. Bei *E. nieschulzi*-Infektionen wurde zudem eine positive Korrelation zwischen der Anzahl der PMN im Darm und der induzierten Pathologie der Erkrankung beobachtet [222]. Auch deutet die erhöhte Oozystenausscheidung in PMN-depletierten, *E. papillata*-infizierten Mäusen auf eine Rolle von PMN im Immungeschehen hin [316]. Ebenso zeigten Mäusestämme mit defekten PMN eine erhöhte Empfänglichkeit gegenüber Primärinfektionen mit *E. vermiformis* [298]. *In vitro*-Untersuchungen zu *E. falciformis* zeigten, dass PMN zwar nicht in der Lage waren, Sporozoiten zu phagozytieren, aber bei Zusatz von Serum zur Lyse der Parasiten beitrugen [24].

Auch bei *T. gondii* wurde über PMN-depletierte Mäuse eine Beteiligung dieser Immunzellen am Immungeschehen belegt, da so behandelte Mäuse u. a. empfänglicher für die akute *Toxoplasma*-Infektion sind [33, 310, 315]. Untersuchungen von MacLaren et al. [213, 212] stellen in eindrucksvollen Bildern dar, dass *T. gondii*-Tachyzoiten nicht nur in der Lage sind, PMN zu infizieren, sondern, dass umgekehrt PMN offensichtlich auch zur Phagozytose und Abtötung dieser Parasiten befähigt sind. Zudem reagieren PMN bei *in vitro*-Stimulation mit *T. gondii*-Antigen mit der Hochregulation verschiedener Mediatoren sowohl auf Protein-Ebene (IL-12, TNFα, MIP-1α, MIP-1β) als auch auf transkriptioneller Ebene (IL-12, MIP-1α, MIP-1β, MIP-3α, CCL5, CCL2) [26, 34, 33, 87, 88] und scheinen über

Kommunikationen mit dentritischen Zellen auch indirekt an der T-Zell-Aktivierung und Generierung von Th1-Zellen beteiligt zu sein [26, 88, 88].

2.2.2 Makrophagen und ihre Vorläuferzellen

Makrophagen sind in der vorderen Linie der Abwehr bei jedweder Infektion zu sehen und treffen i. d. R. kurz nach PMN am Infektionsort ein. Dennoch ist im Zusammenhang mit Eimerien beim Rind relativ wenig zu Interaktionen mit diesen Zellen bekannt und die bisher vorliegenden Untersuchungen beziehen sich in erster Linie auf Nagetiermodelle oder aviäre *Eimeria*-Infektionen.

Rose et al. [289] beobachteten einen biphasischen Anstieg der Anzahl großer mononukleärer Zellen im peripheren Blut von *E. nieschulzi*-infizierten Ratten und *E. maxima*-infizierten Hühnern. Histopathologische Untersuchungen zu *E. bovis*-infizierten Kälbern weisen Makrophagen, wenn auch nur gelegentlich, in Makromeronten nach [118]. Infiltrationen von Makrophagen wurden in der Darmmukosa von *E. tenella* oder *E. acervulina*-infizierten Hühnern sowie in *E. separata*-infizierten Ratten beobachtet [327, 365, 372], wobei in ersteren Fällen auch intrazelluläre Sporozoiten beschrieben werden. Interessanterweise wird für aviäre *Eimeria*-Arten ein Makrophagen-inhibitierender Faktor (MIF) beschrieben, der stadienspezifisch in Merozoiten, nicht oder allenfalls kaum jedoch in Sporozoiten oder Oozysten produziert wird [234].

In vitro-Untersuchungen zu *E. falciformis* belegen eine, wenn auch relativ geringe Aufnahme von Sporozoiten über Makrophagen aus naiven und immunen Mäusen, die unter Einfluss von hitzeinaktiviertem Immunserum erheblich gesteigert ist [25]. Eine anschließende Lyse der Sporozoiten fanden die Autoren nur bei Makrophagen aus immunen Tieren unter Zusatz von Immunserum und Komplement. Eine von Makrophagen-vermittelte Phagozytose von Sporozoiten bei immunen Tieren wird auch für *E. tenella* und *E. maxima* beschrieben, wobei die Morphologie intrazellulärer Sporozoiten nicht angegriffen schien und eine peakförmig gesteigerte Phagozytose-Aktivität im Verlauf der Infektion gemessen wurde [283, 295].

Die Rolle von Makrophagen-produzierten ROS oder NO als Abwehrreaktion gegen Eimerien ist bisher nicht vollständig geklärt. Während für andere Parasiten, wie z. B.

Leishmania spp., die Produktion von NO als Makrophagen-vermittelter Tötungsmechanismus nachgewiesen ist [259, 197], konnte bisher kein direkter Beweis zur NO-vermittelten Abtötung von *Eimeria*-Stadien geführt werden. Allerdings zeigen Lillehoj und Li [201] sowie Dalloul et al. [72], dass Makrophagen NO bzw. iNOS-Gentranskripte als Antwort auf *E. tenella*-Sporozoiten bilden. Auch wurden in der Darmschleimhaut *Eimeria*-infizierter Ratten und Hühner vermehrt iNOS-Transkripte nachgewiesen, wobei die entsprechende Quelle jedoch unklar bleibt [191, 326].
Zudem produzieren Leukozyten *Eimeria*-infizierter Wirte vermehrt freie Sauerstoffmetaboliten [260, 270], die ihrerseits wirksam gegen *Eimeria*-Stadien zu sein scheinen [154, 231].

Neben den bisher genannten Effektormechanismen stellt die Fähigkeit zur Zytokin- und Chemokin-Synthese eine entscheidende Eigenschaft von Makrophagen dar, mittels derer sie aktiv eine sich entwickelnde Immunantwort beeinflussen können. Aviäre Makrophagen, die während und nach *E. tenella*- und *E. maxima*-Infektionen isoliert wurden, produzieren *in vitro* mehr IL-1 und TNF-α als solche von Kontrolltieren [52]. Eine verstärkte TNF-α-Synthese wurde auch in aviären Makrophagenzelllinien, die mit *E. tenella*-Sporozoiten und -Merozoiten ko-kultiviert wurden, beobachtet [396].

Neuere Untersuchungen zu aviären Makrophagen belegen auf transkriptioneller Ebene, dass diverse Zytokine und Chemokine nach Ko-Kultur mit *E.tenella*-, *E. acervulina*- und *E. maxima*-Sporozoiten induziert werden [72]. So wurden z. B. für alle Arten gleichermaßen eine vermehrte Transkription IL-1β-, IL-6-, CXCL8- und MIP-1α-kodierender Gene und eine verminderte Synthese von IL-16- und dem „quiescence-specific protein“-mRNAs nachgewiesen. Insgesamt unterschieden sich die über die verschiedenen *Eimeria* spp. modulierten Transkriptionsprofile aber auch deutlich voneinander, was spezies-spezifische Reaktionen nahelegt. Interessanterweise war insbesondere die Induktion von IFN-γ-mRNAs spezies-abhängig und wurde ausschließlich bei der pathogenen Art *E. tenella* beobachtet [72].

2.2.3 Natürliche Killerzellen

Die Effektormechanismen von NK-Zellen umfassen mindestens drei Kategorien [257]: (i) die über die Freisetzung von Perforin/Granzym oder über die FAS-vermittelte Induktion der Apoptose verursachte Zytotoxizität gegen erkannte Zellen, (ii) die über die Freisetzung von Zytokinen (v. a. IFN-γ und TNF-α) und Chemokinen getragene indirekte Aktivierung des Immunsystems und (iii) eine Kontakt-abhängige Ko-Stimulation von T- und B-Zellen.
Lange Zeit wurde angenommen, dass NK-Zellen nicht direkt mit Pathogenen interagieren können, sondern ausschließlich veränderte Wirtszellen attackieren. In letzter Zeit mehren sich jedoch die Hinweise über eine wirtszellunabhängige, direkte Aktivierung von NK-Zellen, so z. B. über extrazelluläre *Leishmania*- oder *N. caninum*-Stadien [36, 253].

Untersuchungen zu NK-Zellen im bovinen System sind erst seit wenigen Jahren mit der Beschreibung eines bovinen, NK-Zell-spezifischen Rezeptors (NKp46, [348] und der Charakterisierung entsprechender Zellen im Rind [37, 184] möglich geworden. So konnte z. B. die lange Zeit unverstandene Jungtierresistenz bei *Babesia*-Infektionen des Rindes zumindest teilweise über eine vermehrte Aktivität der NK-Zellen in der Milz von Jungtieren erklärt werden [124].

Zur Rolle von NK-Zellen bei der Abwehr von *Eimeria*-Infektionen liegen wenige Befunde vor. Bei *E. papillata*-infizierten Mäusen wurde gezeigt, dass NK-Zellen eine IFN-γ-vermittelte Reduktion der Oozystenausscheidung herbeiführen [316]. Dagegen wurde eine Bedeutung von NK-Zellen in *E. vermiformis*-infizierten Tieren über Untersuchungen NK-Zell-defekter und Antikörper-depletierter Mäuse sowie über ausbleibende IFN-γ-Synthese verneint [292, 337]. In SCID-Mäusen bestätigte sich ein Effekt der NK-Zellen im Falle der *E. papillata*- und *E. ferrisi*-, nicht jedoch bei *E. falciformis*- oder *E. vermiformis*-Infektionen [317]. Bei aviären *Eimeria*-Infektionen konnte eine gesteigerte zytotoxische Aktivität von NK-Zellen aus Darm und Milz v. a. nach Reinfektion gemessen werden, während früh nach Primärinfektion dieser Mechanismus eher gehemmt erschien [198]. Aviäres NK-Lysin zeigte zudem zytotoxische Wirkung auf *E. acervulina*- und *E. maxima*-Sporozoiten [153].

Auch bei anderen Apikomplexa sind die Befunde zu NK-Zellen z. T. kontrovers, so z. B. für *T. gondii* (zur Übersicht siehe [180, 313]). Sie sprechen einerseits für eine

entscheidende Rolle von NK-Zellen während der akuten Infektion. So wurde gezeigt, dass NK-Zellen in der Lage sind, infizierte Zellen sowie extrazelluläre Tachyzoiten über zytotoxische Mechanismen zu lysieren [138, 136, 137, 164, 349]. Andererseits schien dieser Effektormechanismus nur von untergeordneter Bedeutung für die Infektionsabwehr zu sein, da Mäuse mit einen Defekt der NK-Zell-Zytotoxizität eine *T. gondii*-Infektion gleichermaßen überlebten wie Kontrolltiere [163, 155]. Dagegen legen sowohl der schwere Verlauf der *T. gondii*-Infektion bei NK-depletierten Mäusen [159] als auch Befunde, die zeigen, dass NK-Zellen in der Lage sind, das zum Überleben der Infektion essentielle IFN-γ nach Kontakt mit Tachyzoiten oder Parasitenextrakten zu generieren [90, 322, 314] eher eine protektive Rolle dieser Immunzellen nahe.

Bei *N. caninum*-Infektionen des Rindes wurde die Infiltration von NK-Zellen im materno-fetalen Grenzgewebe nachgewiesen [216]. Diese Zellen waren zudem in der Lage, sowohl extrazelluläre Tachyzoiten als auch *N. caninum*-infizierte Wirtszellen abzutöten und sowohl früh im Verlauf der Infektion als auch *in vitro* nach Kontakt mit Tachyzoiten IFN-γ zu produzieren [36, 174].

2.2.4 Apikomplexa und ihre Interaktionen mit Toll-like Rezeptoren

Zellen des nicht-adaptativen Immunsystems müssen in der Lage sein, direkt mit invadierenden Pathogenen zu interagieren. Dabei spielen ihre sog. Toll-like Rezeptoren (TLR), die konservierte molekulare Produkte auf der Oberfläche der Pathogene erkennen, eine Schlüsselrolle. TLR-vermittelte Signalwege in der Zelle sind meist an die Aktivierung des Myeloid-Differenzierungsfaktors 88 (MyD88), eines Adaptorproteins für die meisten der TLRs, gekoppelt [254, 167, 179]. Somit spielt MyD88 eine essentielle Rolle in der über TLRs vermittelten Signaltransduktion.

TLRs interagieren mit den sog. “pathogen associated molecular patterns“ (PAMPs, [224, 269]), deren chemische Zusammensetzung je nach interagierendem TLR variieren kann. So erkennt z. B. TLR2 Lipoproteine/Lipopeptide von $Gram^+$-Bakterien; TLR3 und TLR7 binden dagegen an doppel- und einsträngige RNA und TLR9 erkennt CpG-basierte DNA-Motive [352, 141, 1]. Einige PAMPs und ihre interagierenden TLRs wurden mittlerweile für einige Protozoen (zur Übersicht siehe [122]) wie Leishmanien [78, 221], Trypanosomen [16, 54, 97, 214, 256], *Plasmodium*

falciparum [182, 162, 66] oder *Toxoplasma gondii* [79, 392], nicht jedoch für Eimerien identifiziert.

Innerhalb der *E. bovis* nahe verwandten Arten liegen Daten zu Interaktionen mit TLRs vornehmlich zu *T. gondii* vor. Hier scheinen insbesondere TLR2, TLR4, TLR9 und TLR11 eine Rolle zu spielen, wobei die Bedeutung der einzelnen Rezeptoren z. T. kontrovers diskutiert wird (zur Übersicht siehe [169, 391, 390]). TLR11 wird v. a. eine Rolle bei der parasitenspezifischen, MyD88-kontrollierten IL12-Produktion von Makrophagen und PMN zugewiesen [392], während die Bedeutung von TLR2 und TLR4 eher im Rahmen der Chemokin/Zytokin-Synthese der Makrophagen zu sehen ist [79, 83]. Auch bezüglich *Cryptosporidium parvum* scheinen diese zwei TLRs bedeutend zu sein [61, 62]. Die zudem bei Babesien und Kryprosporidien beschriebene Interaktion mit TLR9 [330, 330, 18] wird einerseits für *T. gondii* bestätigt [169, 233], andererseits auch in Frage gestellt [390].

2.3 Adaptative Immunreaktionen gegen Eimerien

Über die Mechanismen, die bei der Entwicklung der Immunität gegen Reinfektionen zum Tragen kommen, ist beim Kalb nur wenig bekannt. Aus Ergebnissen bei anderen Infektionen, vorwiegend in Nagermodellen, kann geschlossen werden, dass sowohl die immunologisch induzierte Termination der erstmaligen Eimerien-Infektion als auch die schützende Immunität gegen nachfolgende Infektionen T-Zell-abhängig sind (zusammenfassend bei [395, 381]). Die meist im Maussystem generierten Daten mögen jedoch nur von beschränktem Wert sein, da einerseits das Rind in seiner Immunologie von der Maus abweicht und so z. B. nicht einer strikten Th1/Th2-Dichotomie folgt [45]. Weiterhin unterscheiden sich die meisten der beim Rind oder anderen Wiederkäuern vorkommenden, pathogenen *Eimeria*-Arten (z. B. *E. bovis, E. bakuensis, E. arloingi, E. ninakohlyakimovae*) deutlich von denen der Nagetiere bezüglich ihrer primären Wirtszellen, der Ausbildung von Makromeronten und der Dauer der Replikation, Fakten, die sicherlich die Entwicklung einer Immunantwort beeinflussen. Zusätzlich ist von Bedeutung, dass die über Eimerien induzierte Immunität weitestgehend speziesspezifisch ist und Kreuzimmunitäten zwischen *Eimeria*-Arten eines Wirtes i. d. R. keine ausreichende Protektion erwirken [284]. Auch neuere Untersuchungen im aviären System sprechen eindeutig dafür, dass

speziesspezifische Reaktionen z. B. auf Ebene der Makrophagen-vermittelten Zytokinsynthese induziert werden [72]. Diese Fakten vermitteln insgesamt den dringenden Bedarf an immunologischen Untersuchungen zu pathogenen *Eimeria* spp. im Nutztiersektor, dessen Gesundheit und Wirtschaftlichkeit im Gegensatz zu den Labortieren tatsächlich von diesen Infektionen beeinflusst wird.

2.3.1 Humorale Immunreaktionen bei *Eimeria*-Infektionen

Bezüglich *E. bovis*-Infektionen bestehen in Übereinstimmung mit anderen *Eimeria*-Infektionen bei Säugern grundsätzlich keine Zweifel, dass zirkulierende Antikörper induziert werden [14, 110, 309]. Außer im Serum sind spezifische Antikörper bei *Eimeria*-infizierten Tieren auch in Galle, Darm, Milch sowie im Eidotter nachweisbar [110, 299, 383]. Bei *E. bovis*-infizierten Kälbern stiegen sowohl gegen Merozoiten I-Antigene gerichtete IgG1- als auch IgG2-Spiegel während der Primär- und Belastungsinfektion deutlich an, dagegen wurde jedoch nur ein geringes Maß an spezifischem IgM nachgewiesen [110]. Für aviäre *Eimeria*-Infektionen konnten parasitenspezifische IgM-, IgG- und IgA-Antikörper sowohl in Serum als auch im Darm nachgewiesen werden [123, 359]. Bei *E. falciformis*-Infektionen trat die höchste Zahl IgA^+- und IgG^+-B-Zellen in der Darmmukosa und in den Mesenterial-lymphknoten ca. 11 Tage p. i. auf [248]. Bei *E. nieschulzi*-infizierten Ratten wurden maximale Serumkonzentrationen an spezifischem IgM und IgG (IgG1, IgG2a, IgG2b, IgG2c) 8-12, bzw. 20-30 Tage p. i. gemessen, spezifisches IgA wurde dagegen nicht im Serum, sondern nur in Darm und Galle gefunden [299, 338].

Die Bedeutung von Antikörpern im Immungeschehen von *Eimeria*-Infektionen wird im Vergleich mit zellvermittelten Reaktionen generell als gering eingestuft [173, 301], auch wenn in dieser Hinsicht z. T. widersprüchliche Befunde vorliegen. So scheint zwischen dem Antikörperprofil *E. bovis*-infizierter Kälber und einer schützenden Immunität kein klarer Zusammenhang zu bestehen, auch wenn die Antikörperspiegel mit der Schwere einer Infektion korreliert sind [110, 115, 320]. Eine kolostrale Übertragung von Antikörpern oder die mehrfache intravenöse oder intraperitoneale Verabreichung von Seren immuner Rinder erbrachte keine nachweisbare Protektion für die Kälber [110, 115, 320], dagegen konnte über maternale Antikörper bei Hühnern und Lämmern zumindest ein Teilschutz gegen *Eimeria*-Infektionen erzielt werden [284, 57]. Auch Befunde, die verdeutlichen, dass sekretorisches IgA und IgM

bei *E. tenella*-Belastungsinfektionen zur Elimination von Sporozoiten aus dem Darmlumen führen können [286], sowie dass schädigende *in vitro*-Effekte von Hyperimmunseren *E. bovis*-infizierter Tiere auf Merozoiten I ausgeübt werden [14], sprechen zumindest für teilprotektive Funktionen der Antikörper. Allerdings zeigen Untersuchungen zu *E. vermiformis*-Infektionen bei mit B-Zell-defizienten Mäusen oder solchen mit Defekten in der Antikörperbildung, dass sich der Antikörpermangel nur in der Primärinfektion, nicht jedoch nach Belastungsinfektion auswirkt [298].
Unstrittig erscheint dagegen, dass Immunseren zumindest in Kombination mit Immunzellen wie PMN oder Makrophagen im Rahmen Antikörper-vermittelter zytotoxischer (ADCC) Reaktionen eine wichtige Rolle bei der Elimination der Parasiten spielen [25, 24, 286].

2.3.2 Zellulär-adaptative Immunreaktionen bei Primärinfektionen mit *Eimeria* spp.

Infektionen mit Eimerien werden bereits während der Primärinfektion immunologisch über T-Zell-abhängige Reaktionen terminiert [395, 381]. So zeigen athymische, *Eimeria*-infizierte Ratten und Mäuse nach Primärinfektion eine deutlich höhere Oozystenproduktion als Kontrolltiere [228, 297]. Bei T-Zell-depletierten, *E. falciformis*-infizierten Mäusen ergab sich zudem eine Verlängerung der Patenz [344]. Einschränkend muss jedoch erwähnt werden, dass bei Primärinfektionen mit *E. pragensis* in immunosupprimierten Mäusen die Oozystenausscheidung nur geringgradig erhöht und die Dauer der Patenz nicht beeinflusst war [287]. Ebenso hatte in B- und T-Zell-defizienten SCID-Mäusen dieser Immundefekt weder Einfluss auf die Höhe der Oozystenausscheidung noch auf die Dauer der Patenz bei *E. papillata*-Infektionen [317].
Zellulär-adaptative Immunreaktionen des Rindes auf *E. bovis*-Infektionen sind bisher nur in geringem Umfang untersucht worden. Lymphozytäre Ansammlungen um Makromeronten, wie sie für *E. zuernii*-Infektionen des Rindes beschrieben wurden [347], konnten bei *E. bovis*-infizierten Tieren nicht beobachtet werden [118].
Eine Studie von Hermosilla et al. [144] charakterisiert lymphozytäre Reaktionen nach einer relativ schweren Erstinfektion. Die Daten widersprechen teils älteren Befunden von Hughes et al. [158], sind aber wegen der größeren Tierzahl [n=6, Hughes et al. arbeiteten mit 2 Tieren] verlässlicher. Zusammengefasst zeigten Hermosilla et al. [144] über T-Zell-Proliferationen und durchflusszytometrische Analysen mit

Blutlymphozyten eine auf die Präpatenz beschränkte Proliferationssteigerung als Reaktion auf *E. bovis*-Antigen und eine Vermehrung des Anteils der $CD4^+$- und $CD8^+$-T-Zellen in der Primärinfektion. Der Anteil CD2-exprimierender („aktivierter“) T-Zellen blieb dagegen über die Patenz hinaus erhöht. $\gamma\delta$-TCR^+-Zellen waren in die Prozesse offensichtlich nicht involviert. Die spezifische Reaktivität von T-Zellen aus lymphatischen Organen war gleichfalls über die Patenz hinaus erhalten, vor allem in den Lymphknoten, die die betroffenen Gebiete drainieren.

Aus der Mehrzahl der Untersuchungen bei anderen *Eimeria*-Infektionen lässt sich annehmen, dass die Termination von Primärinfektionen mit Eimerien v. a. über αβ-TCR^+-Zellen vermittelt wird (zusammenfassend unter [333]). So zeigten TCR-α- und TCR-β-defiziente Mäuse eine deulich verstärkte Suszeptibilität gegenüber *E. vermiformis*-Infektionen [280, 334], während γδ-TCR-defiziente oder γδ-TCR^+-T-Zell-depletierte Tiere in ihren Reaktionen unbeeinflusst waren [280, 290]. Jedoch wiesen γδ-TCR-defiziente Tiere erheblich stärkere pathologische Darmveränderungen auf als Kontrolltiere [280]. Aleksandersen et al. [6] beschrieben bei *E. ovinoidalis*-infizierten Schafen eine Verringerung oder sogar ein völliges Fehlen von γδ-TCR^+-IEL am Infektionsort. Die Infiltration von αβ-TCR^+-Zellen in die Darmmukosa wurde nach Primärinfektionen mit *E. acervulina, E. tenella* und *E. maxima* bei Hühnern [199, 307, 372] sowie für *E. vermiformis* bei Mäusen [111] berichtet. Eine gesteigerte Anzahl von γδ-TCR^+-T-Zellen wurde dagegen in der Darmschleimhaut bei *Eimeria*-Infektionen von Hühnern und Mäusen beobachtet [199, 307, 111]. Smith und Hayday [335] zeigten zudem, dass Mäuse mit einem αβ-TCR^+-Defekt, die zusätzlich keine γδ-TCR^+-T-Zellen besaßen, empfänglicher für *E. vermiformis*-Infektionen waren als solche ohne letzteren Defekt. Dabei ist jedoch nicht klar, ob dieser Effekt über direkte Interaktionen oder über indirekte Einflüsse zustande kam. Insgesamt wird γδ-TCR^+-T-Zellen, im Gegensatz zur protektiven Funktion der αβ-TCR^+-T-Zellen, jedoch eher eine immunregulative Rolle bei der Kontrolle von Primärinfektionen mit Eimerien zugesprochen [280].

Innerhalb der Population der αβ-TCR^+-Zellen scheinen $CD4^+$-Zellen bei Primärinfektionen mit Eimerien eine wichtigere Rolle zu spielen als $CD8^+$-Zellen (zusammenfassend siehe unter [333]), obwohl auch in dieser Hinsicht die Daten z. T. widersprüchlich sind. CD4-depletierte Mäuse wiesen bei *E. vermiformis*- und *E. pragensis*-Infektionen eine erhöhte Empfänglichkeit auf [293, 291]. Mäuse, die

über genetische Defekte in der MHC-II-Expression nicht mit $CD4^+$-T-Zellen interagieren können, zeigten wesentlich höhere Oozystenausscheidungen nach Primärinfektion mit *E. vermiformis* als intakte Kontrolltiere, während bezüglich der $CD8^+$-T-Zellen bei MHC-I-defekten Tieren kein Einfluss zu erkennen war [333]. Auch bei über anti-CD4-Antikörper depletierten Hühnern wurde eine erhöhte Oozystenausscheidung nach *E. tenella*-Infektion gemessen [367]. Zudem weist die Proliferation von $CD4^+$-T-Zellen in der Darmmukosa von Hühnern nach Primärinfektion mit *E. maxima* und *E. tenella* [307, 372], bei *E. separata*-infizierten Ratten [325] und bei mit *E. papillata*-infizierten Mäusen, sowie im peripheren Blut *E. bovis*-infizierter Kälber [144] auf die Wichtigkeit dieser Subpopulation hin.
Im Gegensatz dazu hatte eine Antikörper-vermittelte Depletion von $CD4^+$-T-Zellen bei Hühnern und ein CD4-Gendefekt bei Mäusen keinerlei Einfluss auf die Primärinfektion mit *E. acervulina* bzw. *E. papillata* [318, 367]. Auch blieb bei *E. acervulina*-infizierten Hühnern der Anteil $CD4^+$-IEL im Verlauf der Infektion im Gegensatz zu ansteigenden $CD8^+$-IEL unbeeinflusst [200]. *E. intestinalis*-infizierte Kaninchen zeigten zunächst früh nach Primärinfektion einen gleichermaßen Anstieg von $CD4^+$- und $CD8^+$-T-Zellen; im späteren Verlauf der Infektion dominierten jedoch $CD8^+$-T-Zellen, die vornehmlich die Darmmukosa infiltrierten [278].

Bezüglich $CD4^+$-T-Helferzellen gilt als gesichert, dass vornehmlich Th1-Zellen an der immunologischen Termination der Primärinfektion beteiligt sind. Beim gegen *E. vermiformis* relativ resistenten BALB/c-Mäusestamm wurden nach Erstinfektion v. a. Th1-Zellen aktiviert, während bei den empfänglicheren C57BL/6-Mäusen auch eine signifikante Th2-Aktivität zu beobachten war [382].
IFN-γ als Th1-assoziiertem Zytokin wird grundsätzlich bei *Eimeria*-Infektionen eine Schüsselrolle bezüglich der Kontrolle von Primärinfektionen zugewiesen. Eine Depletion von IFN-γ verursachte bei *E. vermiformis*-infizierten Mäusen sowohl eine erhöhte Oozystenausscheidung als auch die Verlängerung der Patenz [303, 304]. Auch IFN-γ-knock-out-Mäuse schieden signifikant mehr Oozysten nach Infektionen mit *E. papillata* oder *E. vermiformis* aus [316, 333]. Im Gegensatz dazu schien die Patenz und Oozystenproduktion nach *E. pragensis*-Infektionen von IFN-γ-depletieren Mäusestämmen unbeeinflusst [304].
Eine gesteigerte Antigen-induzierte Produktion von IFN-γ wurde in diversen *Eimeria*-assoziierten Infektionssystemen beschrieben, z. B. bei T-Zellen der Zäkumschleimhaut, bei peripheren Blutlymphozyten und Makrophagen *E. tenella*-infizierter

Hühner [39, 72, 271], bei Lymphknotenzellen *E. vermiformis*-infizierter Mäuse [382] und in IEL und Lymphknotenzellen *E. separata*-infizierter Ratten [326].

Der Wirkungsmechanismus von IFN-γ bei der Kontrolle der Erstinfektion ist bisher nicht vollständig geklärt. IFN-γ selbst wirkt nicht toxisch auf Eimerien [178, 300] und müsste demnach seinen Einfluss über indirekte Effekte, die z. B. eine Zellaktivierung oder Immunregulation bewirken, ausüben. Neben allgemeinen Effekten wie die Hemmung einer Th2-orientierten Immunantwort wirkt IFN-γ auch direkt auf Wirtszellen ein. Durch eine Vorbehandlung von MDBK-Zellen (eine bovine Nierenzelllinie) mit rekombinantem IFN-γ wurde sowohl das Eindringen von *E. tenella*-Sporozoiten als auch deren intrazelluläre Weiterentwicklung gehemmt [177, 178]. Vergleichbare Befunde wurden bei *E. vermiformis*-Sporozoiten und murinen Fibroblastenzellen oder Rattenepithelzellen erabeitet [300]. IFN-γ beeinträchtigt nach Studien von Hughes et al. [156] auch die intrazelluläre Etablierung von *E. bovis*-Sporozoiten *in vitro*. Ovington und Smith [260] vermuten als Ursache des anti-replikativen Effektes die IFN-γ-stimulierte Synthese von Stickoxiden (NO), welche als Effektormoleküle von einer Reihe von Zellen wie z. B. Endothelzellen, Fibroblasten, Enterozyten, Muskelzellen, PMN und Makrophagen gebildet werden [161, 275]. Shi et al. [326] fanden eine erhöhte Gentranskription der induzierbaren NO-Synthase (iNOS) in der Zäkumschleimhaut *E. separata*-infizierter Ratten. Auch in iNOS-defekten Mäusen konnte die Relevanz dieses Enzyms während der Primärinfektion mit *E. vermiformis* dargelegt werden [334]. Die im Plasma von primär mit *E. tenella*- und *E. maxima*-infizierten Hühnern beobachteten, erhöhten NO-Konzentrationen [9, 10, 11] könnten ebenfalls für einen solchen Effektormechanismus sprechen.

Neben NO stimuliert IFN-γ auch die Synthese von reaktiven Sauerstoffmetaboliten (ROS), welche ihrerseits abträgliche Effekte auf Sporozoiten von *E. bovis* [154] und *E. tenella* [231] *in vitro* ausüben. Darüber hinaus war die Produktion von ROS über aktivierte Zellen in *E. vermiformis*-resistenten BALB/c-Mäusen signifikant höher als bei den empfänglichen C57BL/6-Mäusen [261]. Vergleichbare Befunde wurden für Zellen *E. tenella*-infizierter Hühnern erabeitet [270].

Zur Bedeutung Lymphozyten-assoziierter Zytokine außer IFN-γ liegen relativ wenige Befunde vor. Eine gesteigerte Transkription IL-2-kodierender Gene wurde in Lymphknoten *E. bovis*-infizierter Kälber [144] und *E. separata*-infizierter Ratten [326]

beschrieben, während mRNAs für das Th2-assoziierte IL-4 unbeeinflusst blieben. Zusätzlich zeigten Shi et al. [326] eine gesteigerte Transkription des IL-10-Gens bei gleichzeitiger Abwesenheit von IL-5-mRNAs in der Zäkumschleimhaut infizierter Tiere. Bei *E. vermiformis*-infizierten Mäusen wurden neben dem Th1-assoziierten IFN-γ auch die Th2-orientierten Zytokine IL-5 und IL-10 von Lymphknotenzellen nach Stimulation mit Parasiten-Antigen vermehrt produziert [382]. Bei *E. papillata*-Infektionen zeigte sich eine zeitliche Abfolge von frühen Th2-orientierten Reaktionen über vermehrte Produktion von IL-4 und IL-10 und nachfolgenden Th1-assoziierten Reaktionen in der Patenz mit erhöhten Werten für IL-2 und IFN-γ [319]. Bei *E. vermiformis*-infizierten, IL-4-knock-out-Mäusen blieb die Patenz unverändert, so dass die Autoren IL-4 keinerlei Bedeutung bei der Terminierung einer Primärinfektion beimessen [333].

IL-6, dessen Einfluss auf die Entwicklung sowohl Th1- als auch Th2-dominierter Immunreaktionen belegt ist [282, 279, 73], wurde eine Rolle bei der Initiierung der Immunantwort bei Primärinfektionen mit *E. vermiformis* zugesprochen. Entsprechend zeigten IL-6-knock-out-Mäuse eine zwei- bis dreifach erhöhte Oozysten-ausscheidung, wenn auch die Dauer der Patenz unverändert blieb. Zudem wurden erhöhte IL-6-Konzentrationen früh nach Infektion im Plasma *E. vermiformis*-infizierter Mäuse und *E. tenella*-infizierter Hühner gemessen [333].

2.3.3 Zellulär-adaptative Immunreaktionen bei Belastungsinfektionen mit *Eimeria* spp.

Infektionen mit *Eimeria*-Arten induzieren in kompetenten Wirten in aller Regel einen Immunschutz gegen Reinfektionen, der in erster Linie über T-Zellen vermittelt wird. Diese Immunität ist jedoch weitestgehend artspezifisch [284, 285] und z. T. sogar nur stammspezifisch ausgeprägt, wie beispielsweise für *E. maxima* beim Huhn belegt [114, 219, 252, 329, 336]. Dementsprechend werden Kreuzimmunitäten zwischen Arten selten beschrieben.

Zu immunologischen Reaktionen nach Belastungsinfektion mit *E. bovis* liegen kaum Daten vor. Dabei ist für *E. bovis* zweifelsfrei erwiesen, dass Primärinfektionen einen effizienten Immunschutz herbeiführen können (u. a. [76, 110]). Nach Feldstudien bei neonatalen Kälbern kommt dieser nicht über die maternalen Antikörper zustande

[106], d. h. wahrscheinlich spielen zelluläre Mechanismen wie bei *Eimeria*-Infektionen anderer Wirte (vgl. [395]) die entscheidende Rolle.

Über eine ausbleibende protektive Immunität in *Eimeria* spp.-infizierten T-Zell-defizienten Mäusen konnte insgesamt die Schlüsselrolle der T-Zellen bei Reinfektionen klar belegt werden [172, 228, 298, 302, 287, 346]. Zudem zeigten immune Mäuse nach der Antikörper-vermittelten Depletion von T-Zellen bei Belastungsinfektion mit *E. falciformis* eine erhöhte Oozystenausscheidung und verlängerte Patenz [344]. Auch die über Transfer von Lymphozyten immuner Hühner und Nager auf naive Tiere vermittelte, partiell schützende Immunität gegen *E. maxima* bzw. *E. vermiformis* spricht für T-Zell-vermittelte Immunreaktionen [288, 293, 305, 381].

Die Ausbildung der Immunität nach Belastungsinfektionen mit *Eimeria* spp. scheint maßgeblich von αβ-TCR$^+$-T-Zellen getragen zu werden. Entsprechend waren αβ-TCR-defiziente Tiere im Gegensatz zu γδ-TCR-defizienten Mäusen nicht in der Lage, eine Immunität gegen Reinfektionen mit *E. vermiformis* auszubilden [280, 334] . Auch über Depletionsstudien zeigte sich dieser Effekt im Falle von *E. tenella* und *E. acervulina* primär und reinfizierter Hühner [367]. Außerdem kann die bei immunen Tieren gemessene Proliferation von αβ-TCR$^+$-Zellen nach Belastungsinfektion mit *E. tenella* [372], *E. acervulina* [199] und *E. papillata* [319] in dieser Hinsicht interpretiert werden.

Als entscheidende Subpopulation der αβ-TCR$^+$-T-Zellen werden in der Mehrzahl der Untersuchungen CD8$^+$-T-Zellen gewertet. Entsprechend zeigten Studien von Rose et al. [291] und Trout und Lillehoy [367], dass mit der Depletion von CD8$^+$-T-Zellen bei immunen Tieren stärkere Oozystenausscheidungen nach homologen Belastungs-infektionen mit *E. vermiformis* und *E. pragensis* bei der Maus sowie *E. tenella* und *E. acervulina* beim Huhn einhergingen. Die Depletion der CD4$^+$-T-Zellen hatte dagegen keinen Einfluss auf die Immunitätsbildung [291, 367].

Als indirekte Bestätigung für eine protektive Rolle der CD8$^+$-Zellen kann auch angesehen werden, dass bei gegen *E. separata* immunen Ratten [327] und gegen *E. acervulina* immunen Hühnern [366] CD8$^+$-T-Zellen nach Reinfektionen im parasitierten Darmbereich akkumulieren. Allerdings liegen hinsichtlich der Bedeutung von CD8$^+$-T-Zellen für die protektive Immunität auch anderslautende Befunde vor, so bei *E. vermiformis*- [333] und *E. papillata*-Infektionen der Maus [318], bei denen der Schutz durch CD4$^+$-T-Zellen vermittelt werden soll.

Die im Rahmen der protektiven Immunität greifenden Effektormechanismen sind nicht vollständig geklärt. Wie über Depletionsstudien sowie über Verwendung von IFN-γ-knock-out-Mäusen ersichtlich, scheint IFN-γ zumindest im murinen System bei homologen Reinfektionen mit *E. vermiformis* und *E. papillata*, im Gegensatz zur Primärinfektion, keine entscheidende Rolle zu spielen [304, 319, 333, 334, 382].
Ebenso hängt die Immunität nach Belastungsinfektionen mit *E. vermiformis* weder von IL-4 und IL-6 noch von FasL/Fas-vermittelter Apoptose ab [333]. Auch bei gegen *E. papillata*-Infektionen immune Mäusen konnte weder IFN-γ, IL-4 noch IL-10 in Lymphknotenzellen nachgewiesen werden [319]. Die Autoren schlagen dagegen von $CD8^+$-T-Zellen getragene, IL-2-abhängige Effektormechanismen vor.
Bezüglich $CD8^+$-spezifischer, zytotoxischer Wege sprechen Daten aus knock-out-Mäusen zumindest für eine teilweise Beteiligung von Perforin-abhängigen Mechanismen bei *E. papillata* [316], während Ergebnisse anderer Untersuchungen diesem Effektormechanismus bei *E. vermiformis* eine Bedeutung absprechen [333].

2.4 Reaktionen *Eimeria*-infizierter Wirtszellen

Die Entwicklung von *E. bovis* findet streng intrazellulär statt. Entsprechend muss der Parasit dafür sorgen, dass er nicht über wirtszelleigene Abwehrreaktionen eliminiert wird. Dies gilt v. a. für endotheliale Wirtszellen der ersten Merogonie, da *E. bovis* hier seine >15 Tage dauernde Replikation zu Merozoiten I absichern muss. Insbesondere Endothelzellen sind jedoch nicht als wehrloses Ziel einer Infektion anzusehen, sondern agieren als potente Partner im Verlauf von Entzündungsreaktionen. Sie sind in der Lage, ein breites Spektrum von Molekülen (z. B. Adhäsionsmoleküle, Chemokine oder Zytokine) zur Anlockung und Adhäsion von Immunzellen zu exprimieren und damit inflammatorische Reaktionen zu initiieren und zu dirigieren (zur Übersicht siehe [100, 380]).
Insgesamt liegen im Bezug auf Protozoen zu Adhäsionsmolekülen nur wenige Untersuchungen vor. So wurden im Fall der *T. gondii*-Infektion eine vermehrte Produktion von ICAM-1 in Epithelzellen der Retina, Fibroblasten, Endothelzellen des Gehirns oder Mikroglia sowie von VCAM-1 in Gehirngefäßen infizierter Mäuse beschrieben [80, 81, 246]. In einer weiteren Studie wurden erhöhte Konzentrationen löslichen ICAMs oder E-Selektins in Sera *T. gondii*-infizierter Menschen [101]

nachgewiesen. Hermosilla et al. [147] zeigten, dass *in vitro* Infektionen von bovinen Endothelzellen mit *E. bovis*-Sporozoiten zwar grundsätzlich zu einer gesteigerten Adhäsion von PMN führten, diese jedoch im Detail besehen vornehmlich an nicht-infizierten Zellen innerhalb des infizierten Zellrasens erfolgte. Im Vergleich zu anderen, über Tachyzoiten vermittelten Infektionen apikomplexer Erreger wie *T. gondii* und *N. caninum,* war die Adhäsion von PMN zudem nach *E. bovis*-Infektion vergleichsweise niedrig [354]. Die Vermutung einer *E. bovis*-induzierten Hemmung der PMN-Adhäsion konnte in Versuchen mit zuvor über TNFα stimulierten Endothelzellen bestätigt werden [147].
Auch die Chemo- oder Zytokinsynthese infizierter Endothelzellen wurde bisher wenig untersucht. *T. gondii*-Tachyzoiten führen in HeLa-Zellen oder Fibroblasten zu einer vermehrten Bildung von CXCL8, CCL2 und CXCL1 [92, 42]. Wie Hemmungsstudien in einem *T. gondii*-Mausmodell zu entnehmen, scheint die Produktion von CXCL10 ein kritischer Faktor bei der Ausbildung einer spezifischen Immunantwort zu sein [171]. Im Falle von *C. parvum* reagierten infizierte Kolon-Epithelzellen mit einer vermehrten Produktion von CXCL8 und CXCL1 [190]. Weitere Befunde zur Chemokinsynthese bei Infektionen mit intrazellulär lebenden Protozoen beziehen sich auf *Trypanosoma cruzi* [8], Leishmanien [236] oder Plasmodien [51].

Endothelzellen besitzen die Fähigkeit fremde Antigene zu präsentieren und können direkte immunregulatorische Funktionen der adaptativen Immunantwort übernehmen [351, 230]. Aktivierte Endothelzellen können z. B. $CD4^+$- und $CD8^+$-T-Zellpopulationen aktivieren und zur Proliferation anregen [351]. Bisher liegen kaum Daten in diesem Zusammenhang zu Apikomplexa-infizierten Endothelzellen vor, obwohl unterschiedliche Vertreter dieser Protozoen, wie *T. gondii*, *Sarcocystis* spp. oder *Eimeria* spp. Endothelzellen *in vivo* befallen. Eine Beeinflussung der an der Antigenpräsentation beteiligten MHC-Moleküle infizierter Zellen wurde für *T. gondii* [208, 210, 209] und *Leishmania amazonensis* [69] in dem Sinne nachgewiesen, dass der Parasit eine gezielte Verminderung der MHCII-Moleküle als Evasionsmechanismus nutzt. Im Gegensatz dazu wurde bei *E. bovis*-infizierten Zellen eine, wenn auch geringe Steigerung dieser Moleküle nachgewiesen [146], was alternative Evasionsstrategien für diesen Parasiten nahelegt.

Die Entwicklung intrazellulärer Stadien bei Apikomplexa setzt insbesondere bei solchen Arten, die zur Bildung großer Stadien wie Makromeronten führen, eine

Reorganisation von Zellkomponenten voraus. Um v. a. dem resultierenden mechanischen Stress bei maximaler Ausdehnung bzw. Vergrößerung der Wirtszelle standhalten zu können, müssen Elemente des Zytoskeletts maßgeblich verändert werden. Insgesamt spielen drei Typen von Zytoskelett-Elementen sowie assoziierte Moleküle in Zellen eine Rolle: intermediäre Filamente, Mikrotubuli und Aktinfilamente. Bei *T. gondii*-infizierten Zellen wurde gezeigt, dass das zu den Intermediärfilamenten gehörende Vimentin über die Expression unterschiedlicher Isoformen moduliert wird [248] und eine Reorganisierung sowohl dieses Moleküls [130, 131] als auch von Mikrotubuli [225] um die parasitophore Vakuole stattfindet. Zudem benutzt *T. gondii* die Mikrotubuli der Wirtszelle zur parasiteneigenen Versorgung mit endolysosomalen Produkten [67]. Nach Invasion von *C. parvum*-Sporozoiten kommt es dagegen zur Anreicherung von wirtszelleigenen Aktinfilamenten am Ort der Infektion sowie zur Vermehrung Aktin-bindender Moleküle [116, 85]. Im Verlauf der *in vitro*-Entwicklung von *E. bovis*-Makromeronten erfolgt ebenfalls eine zunehmende Vermehrung und Umorganisierung von Aktinfilamenten und Mikrotubuli um die parasitophore Vakuole; Vimentin als intermediäres Filament bleibt dabei jedoch unverändert [145].

Die Invasion endothelialer Wirtszellen als auch die enorme Größen- und Volumenzunahme jenseits physiologischer Grenzen bei der Entstehung intrazellulärer Makromeronten führt zudem zu Zellstress [112, 117]. Dieser dokumentiert sich im Allgemeinen im Zusammenhang mit intrazellulären Parasiten einerseits in der Induktion bestimmter Stressfaktoren, wie z. B. von Hitzeschockproteinen (HSPs, [255, 248]), zum anderen besteht die physiologische Reaktion der Wirtszelle in der Einleitung der zellstressbedingten Apoptose [128, 127]. So müssen apikomplexe Parasiten gewährleisten, dass befallene Wirtszellen den Zeitraum intrazellulären Wachstums überleben; d. h. sie dürfen nicht apoptotisch werden. Die Apoptose wird als programmierter Zelltod vornehmlich über drei Hauptwege initiiert: über Granzym/Perforin aus $CD8^+$-T-Zellen (*i*), über die Bindung sog. Tod-Liganden an ihre spezifischen Zelloberflächenrezeptoren wie Fas/APO-1 oder TNF-Rezeptor I (*ii*) und über einen "internen" Weg mit der Freisetzung von Cytochrom c aus den Mitochondrien in das Zytosol der Zelle (*iii*). Stimuli sind oben benannter Zellstress, das Fehlen von Wachstumsfaktoren u. a. In allen Fällen werden als zentrale Komponenten der Apoptose-Maschinerie Cysteinproteasen mit einer Spezifität für Asparaginsäure-Reste - sog. Caspasen - aktiviert. Die Abläufe können in verschiedenen Phasen in spezifischer Weise von HSPs [151] und

Vertretern aus einer Reihe von Molekülfamilien, u. a. der BCL-2-Familie und den IAPs (**i**nhibitor of **a**poptosis **p**rotein) gehemmt werden. Die meisten dieser Proteine unterliegen der Kontrolle des Transkriptionsfaktors NF-$_K$B (zusammenfassend unter [148, 206]).

Generell ist die Hemmung der Wirtszellapoptose eine häufig genutzte Evasionsstrategie von apikomplexen Parasiten und wurde z. B. bei *C. parvum* [60], *T. parva* [149, 148], *T. gondii* [95, 53, 207, 55, 168] und *N. caninum* [250, 249, 142] nachgewiesen (zur Übersicht siehe [126]). Bei mit aviären Eimerien infizierten Wirtszellen erfolgt die Hemmung der Apoptose über vermehrte Produktion anti-apoptotisch wirkender Faktoren sowie über Aktivierung von NF-$_K$B [82]. Lang et al. [188] zeigten kürzlich, dass auch *E. bovis* in der Lage ist, über die vermehrte Expression der Apoptoseinhibitoren c-IAP und c-FLIP das Überleben der Wirtszelle zu sichern. Über diese Moleküle beeinflusst der Parasit sowohl den internen [99, 203, 94, 160, 38] als auch den Rezeptor-vermittelten [232, 312, 125] Signalweg der Apoptose. Untersuchungen zu *E. bovis*-infizierten Epithelzellen, d. h. zu Zellen, die eine Weiterentwicklung zum Meronten I nicht erlauben, lassen zudem auf eine Beteiligung von NF-$_K$B schließen [4].

Globale Analysen zur parasitenvermittelten Beeinflussung der Wirtszelle auf Ebene des Transkriptoms oder Proteoms liegen zurzeit nur für einige apikomplexe Parasiten vor. Neben Untersuchungen auf transkriptioneller Ebene zu *T. gondii* [30, 255, 175, 120], *T. cruzi* [369] oder *Plasmodium* spp. [321] wurden auch Microarrays zu einer mit aviären *Eimeria* spp. infizierten Makrophagen-Zelllinie durchgeführt [248], wobei dieser Zelltyp jedoch nicht permissiv für eine Weiterentwicklung der Sporozoiten ist. Umfassende Untersuchungen zur parasiten-induzierten Modulation des Wirtszellproteoms wurden bisher ausschließlich für *T. gondii*-infizierte Wirtszellen durchgeführt [248].

3 METHODIK

3.1 In den vorgelegten Veröffentlichungen verwendete Methoden

Für die vorliegende Arbeit sind die in den entsprechenden Veröffentlichungen (siehe Abschnitt 7) beschriebenen Methoden verwendet worden.

- *In vitro*-Kultur von *E. bovis, T. gondii* und *N. caninum*
- Präparation und Kultivierung primärer boviner Endothelzellen aus Nabelschnurvenen
- Präparation und Kultur boviner mononukleärer Zellen aus dem peripheren Blut
- Quantifizierung der Proliferationsleistung boviner PBMC
- Phänotypisierung von T-Zell-Subpopulationen über Durchflusszytometrie und Immunhistologie
- Isolation von $CD4^+$-, $CD8^+$- und $\gamma\delta TCR^+$-T Zellen mittels magnetgekoppelter Antikörper
- Quantifizierung der Adhäsion von PBMC an Endothel unter physiologischen Fließbedingungen (laminar flow plate chamber)
- Quantifizierung von bovinem IFN-γ in Zellkulturüberständen
- Quantifizierung der IFN-γ- und IL-4-Expression in PBMC mit Hilfe der Durchflusszytometrie
- Präparation und Kultur von bovinen neutrophilen Granulozyten (PMN)
- Präparation und Kultur von bovinen Monozyten
- *In vitro*-Transformation von Monozyten zu Makrophagen
- Präparation und Kultur von bovinen NK-Zellen
- Quantifizierung der Gentranskription von bovinen Zytokinen, Chemokinen und Adhäsionsmolekülen über Realtime RT-PCR
- Quantifizierung der Phagozytoseleistung und der „Oxidative Burst"-Aktivität von bovinen PMN und Monozyten mit Hilfe der Durchflusszytometrie
- Raster- und Transmissionselektronenmikroskopie
- Genomweite Transkriptom-Analysen (bovine Microarrays inkl. Auswertung über Ingenuity® Pathways Analysis)
- Proteom-Analysen über ein- und zweidimensionale Gelelektrophorese und MALDI-TOF-MS-Analysen

Die Ergebnisse zu BoMAC (siehe 4.1.2.3., 4.1.2.4.) und NK-Zellen (siehe 4.1.3.), sowie einige andere Resultate enthalten bisher unveröffentlichte Originaldaten. Die diesen Arbeiten zugrunde liegenden und von bereits veröffentlichten Untersuchungen abweichenden Methoden sind im Folgenden dargestellt:

3.2 Untersuchungen zur Interaktion primärer Makrophagen oder BoMac mit *E. bovis*

3.2.1 Ko-Kultur von BoMac und *Eimeria bovis*-Sporozoiten/Merozoiten I-Antigen

Die SV40-transformierte bovine Makrophagenzelllinie (BoMac, [341]), die adhärent als Zellrasen wächst, wurde bis zur Konfluenz kultiviert (37°C, 5 % CO_2, RPMI mit 10 % FKS, 1 % Penicillin/Streptomycin)

Zur Bestimmung der BoMac-vermittelten Elimination von Sporozoiten wurden BoMac abgelöst und mit vitalen oder Hitze-inaktivierten (HI, 60°C, 30 min, serumfrei) *E. bovis*-Sporozoiten in Ab- oder Anwesenheit von Immunserum im Verhältnis 2:1 für 4 h ko-kultiviert. Anschließend wurde die Anzahl verbleibender Sporozoiten bestimmt.

Zur Untersuchung der Gentranskription wurden konfluente BoMac mit *E. bovis*-Sporozoiten (5 x 10^5/25 cm^2) ko-kultiviert oder mit Merozoiten-Antigen (10 µg/ml) stimuliert. Die nachfolgende Isolation von Total-RNA, Reverse Transkription in cDNA und die Realtime PCR zum Nachweis von IL-6-, CXCL8-, CCL2-, CXCL1-, CCL5- und COX-2-cDNA erfolgte wie unter **7.1** und **7.6** beschrieben.

3.2.2 Semiquantitative Erfassung der TLR2-, TLR4- und TLR9- Gentranskripte in bovinen Makrophagen

Die Isolation und Kultur boviner Makrophagen, die Gewinnung der Total-RNA als auch die Synthese der cDNA nach Konfrontation mit *E. bovis* Sporozoiten bzw. nach Stimulation mit Merozoiten I-Antigen erfolgte wie unter **7.3** beschrieben. Die RT-PCR zur Erfassung der TLR2-, TLR4- und TLR9-Gentranskripte wurde mittels bereits für die semiquantitative Analyse etablierter Primersysteme durchgeführt, deren Sequenzen (siehe Tab. 1) von Prof. Werling (Royal Veterinary College, London)

freundlicherweise zur Verfügung gestellt wurden. Die Amplifikate der auf konventionelle Weise durchgeführten PCR (initiale Denaturierung: 94°C, 2 min, dann 29 Zyklen à 40 s Denaturierung bei 94°C, 1 min Annealing bei 55°C und 1 min Elongation bei 72°C, abschließende Elongation für 7 min bei 72°C) wurden in 1%igen Aragosegelen aufgetrennt und mit Ethidiumbromid gefärbt. Die sich darin wiedergespiegelnde DNA-Menge wurde densitometrisch bestimmt (Programm: ImageJ) und über die gleichermaßen gemessene Aktin-cDNA als „Housekeeper" validiert.

Tab. 1: Sequenzen der zur Erfassung boviner TLR2-, TLR4- und TLR9-cDNA verwendeten Primersysteme
(freundlicherweise von Prof. Werling zur Verfügung gestellt)

Gen	Sense Primer	Anti-Sense Primer
Aktin	CCA GAC AGC ACT GTG TTG GC	GAG GAA GCT GTG CTA CGT CGC
TLR2	CAG CAA CTG AAG ACG TTG GA	CAC CAC TCG CTC TTC ACA AA
TLR4	TGC TGG CTG CAA AAA GTA TG	TCT GCA GGA CGA TGA AGA TG
TLR9	CAA GTG CTC GAC CTG AGT GA	CCA TGG TAC AGG TCC AGC TT

3.3 Untersuchungen zur Interaktion von NK-Zellen mit *E. bovis*

3.3.1 Isolation und Kultivierung von NK-Zellen

Die Isolation boviner NK-Zellen wurde nach Boysen et al. [36] durchgeführt. Dazu wurden PBMC isoliert (siehe **7.4**) und zweimal mit PBS/2 mM EDTA (PBS/EDTA) gewaschen. Die in der Gesamtfraktion der PBMC enthaltenden NK-Zellen wurden unter Zugabe eines gegen bovine NK-Zellen gerichteten Antikörpers (3 µl/5 x 10^7 Zellen in 5 ml, mouse anti bovine CD335, MCA 2365, 1 mg/ml, Serotec, 30 min auf Eis) erfasst. Nach zweimaliger Waschung der Zellen in PBS/EDTA/0,5% BSA (372 x *g*, 4°C) wurden sie mit zuvor in PBS/EDTA gewaschenen, magnetgekoppelten anti-Maus-IgG Antikörpern (Dynabeads® Pan mouse IgG, Invitrogen) in NK-Zellmedium (RPMI Medium 1640 mit 60 µg/ml Penicillin, Sigma, 100 µg/ml /Streptomycin, Sigma, 1 mM Natriumpyruvat, Sigma, 1 ml/500 ml nicht-essentielle Aminosäuren, Invitrogen, 50 µM 2-Mercaptoethanol, Invitrogen, 10 % fetales Kälberserum, Biowest) inkubiert (4 x 10^6 Beads/ml Zellen, 5 x 10^6 Zellen in 5 ml NK-Zellmedium, langsame Rotation im MACS-Mix™ Tube Rotator, Miltenyi Biotec,

30 min, 4°C). Die so markierten NK-Zellen wurden über einen Magneten (Dynal MPC-L®, Invitrogen) von den übrigen PBMC getrennt und dreimal mit Hilfe des Magneten mit je 4,5 ml PBS/EDTA/0,5 % BSA gewaschen. Die Zellen wurden in 4 ml NK-Zellmedium, supplementiert mit bovinem rekombinanten IL-2 (boIL-2, 10.000 U/ml, 1:50 in NK-Zellmedium, freundlicherweise überlassen von Prof. Storset, Oslo) aufgenommen, in die Vertiefung einer 6-Well-Platte (Greiner) gegeben, unter kreisenden Bewegungen zentriert und bei 37°C, 5 % CO_2 kultiviert. Nach 48 h wurden die mittlerweile abgelösten Magnetkügelchen im Magneten abgetrennt und die NK-Zellen unter Zugabe frischen NK-Zellmediums (1:1, supplementiert mit boIL-2, 1:100) auf zwei Vertiefungen einer 6-well-Platte aufgeteilt. Zur weiteren Vermehrung wurden die NK-Zellen unter Zusatz von boIL-2 (1:100-1:200) alle 2-3 Tage - je nach individueller Proliferationsleistung - gesplittet. 8-10 Tage nach Primärisolation wurden die IL-2-aktivierten NK-Zellen geerntet und weiter verwendet.

3.3.2 Quantifizierung der NK-Zelldichte im Blut *E. bovis*-infizierter Kälber

Schwankungen der NK-Zelldichte im peripheren Blut experimentell *E. bovis*-infizierter Kälber wurden mittels durchflusszytometrischer Analysen unter Verwendung von PBMC bestimmt. Die Tiere wurden wie unter **7.4** und **7.5** beschrieben infiziert und PBMC zu verschiedenen Zeitpunkten der Infektion isoliert (siehe **7.4**). Triplikate von PBMC (1 x 10^5/well) wurden in eine 96er V-Boden-Zellkulturplatte (Greiner) verbracht und pelletiert (10 min, 4°C, 150 x *g*). Der Überstand wurde durch vorsichtiges Ausschlagen der Platte entfernt. Das Zellpellet wurde in 50 µl Gebrauchslösung des gegen bovine NK-Zellen gerichteten Primärantikörpers (Anti-bovine CD335, Serotec, 1:400 in PBS) resuspendiert und 30 min auf Eis inkubiert. Nach Zentrifugation (10 min, 4°C, 150 x *g*) wurden die Zellen zweimal mit 150 µl PBS gewaschen und dann in 50 µl Gebrauchslösung des FITC-markierten Sekundärantikörpers gegen Maus-Immunoglobuline (Ziege-anti-Maus IgG+L, 1:200, Dianova) inkubiert (30 min, auf Eis). Nach zwei erneuten Waschungen mit je 150 µl PBS wurde das Zellpellet in 50 µl PBS aufgenommen, mit je 1 µl Propidiumjodid (2 µg/ml, Sigma, in PBS verdünnt) versetzt und 1 min inkubiert. Diese Suspension wurde in 400 µl PBS enthaltende Teströhrchen für die Durchflusszytometrie überführt. Die Proben wurden mittels eines FACSCalibur™ Durchflusszytometer ausgewertet. Pro Probe wurden 5 x 10^3 Zellen durchflusszytometrisch bestimmt und der Anteil markierter Zellen als Prozentsatz ausgedrückt.

3.3.3 Quantifizierung der NK-Zell-vermittelten Elimination von *E. bovis*-Sporozoiten *in vitro*

IL-2-aktivierte NK-Zellen wurden mit oder ohne Zusatz von boIL-2 (1:100) mit frisch isolierten *E. bovis*-Sporozoiten im Verhältnis 10:1 und 1:1 über 4 h ko-kultiviert (37°C, 5 % CO_2). Anschließend wurden sowohl die Anzahl verbliebener Sporozoiten als auch der Anteil toter Stadien (Vitalfärbung mit Trypanblau, siehe **7.1**) bestimmt. Um den Perforin-vermittelten Tötungsmechanismus zu überprüfen, wurden in einem Teil der Ansätze NK-Zellen mit Concanamycin A (Sigma, 10 mM, 2 h, 37°C) vorbehandelt.

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4 ERGEBNISSE UND DISKUSSION

4.1 Reaktionen von Zellen des angeborenen Immunsystems auf *E. bovis*

4.1.1 Neutrophile Granulozyten (PMN)

4.1.1.1 Phagozytose-Aktivität von PMN ex vivo aus E. bovis-infizierten Kälbern und in vitro nach Konfrontation mit E. bovis-Sporozoiten

Über *ex vivo*-Analysen, bei denen PMN aus dem Blut *E. bovis*-infizierter Kälber auf ihre allgemeine Phagozytoseleistung überprüft wurden, konnte eine Induktion dieses Effektormechanismus über *E. bovis* gezeigt werden. Bei diesen Untersuchungen wurden PMN ohne weitere Aufarbeitungsschritte unmittelbar aus Blut analysiert und somit arbeitsaufwendige Isolierungsmethoden vermieden, die einen Einfluss auf den Aktivierungsstatus der PMN haben könnten [108, 139]. Diese Methode wurde bereits bei *Babesia bovis*-infizierten Rindern erfolgreich eingesetzt [70] und die Ergebnisse sollten die *in vivo*-Situation direkt widerspiegeln.

Im Verlauf der Infektion kam es zu einer biphasischen Hochregulation der Phagozytose-Aktivität von PMN (vgl. **7.1**). Die Aktivitätsmaxima traten mit einem Tag p. i. und 13-18 Tagen p. i. zu Zeitpunkten auf, zu denen unterschiedliche *E. bovis*-Stadien außerhalb der Wirtszelle verfügbar und damit angreifbar sein sollten. Während zum Zeitpunkt des frühen Anstiegs der Phagozytose-Aktivität kurz nach der Infektion Sporozoiten auf der Suche nach geeigneten Endothelzellen der Lymphkapillaren sowohl im Interstitium als auch in der Lymphe zu erwarten sind, werden zum zweiten Anstieg Merozoiten I und II freigesetzt. Das zuerst im Infektionsverlauf auftretende Aktivitätsmaximum fiel dabei deutlich niedriger aus als das spätere, was indirekt die Anzahl momentan vorhandener Parasitenstadien widerspiegeln könnte, da initial deutlich weniger Sporozoiten im Tier vorhanden sind verglichen mit den später nach erfolgten Merogonien amplifizierten Merozoiten I oder II.

Indirekt vergleichbare Daten zur biphasischen Hochregulation der PMN-getragenen Phagozytoseaktivität wurden von Rose et al. [289] bezüglich der Anzahl von PMN im peripheren Blut *Eimeria maxima*-infizierter Hühner und *Eimeria nieschulzi*-infizierter Ratten beschrieben. Somit könnte es sich um einen allgemein gültigen Mechanismus

bei *Eimeria*-Infektionen handeln. Einschränkend muss jedoch erwähnt werden, dass die Zeitpunkte der Aktivitätsmaxima im Vergleich zu *E. bovis* insofern verschoben waren, als das zeitlich später auftretende Maximum bei Rose et al. [289] erst nach der Oozystenausscheidung erfolgte.

Während das spätere Aktivitätsmaximum bei *E. bovis*-infizierten Tieren zeitlich mit Zellzerstörungen über freiwerdende Merozoiten und Oozysten korreliert und in Folge die verstärkte Phagozytose-Aktivität sowohl mit der Entfernung des Debris als auch mit Interaktionen zwischen Merozoiten und PMN zusammenhängen könnte, findet der erste Anstieg der Phagozytose-Aktivität in einem Zeitraum statt, in dem über eindringende Sporozoiten keine allzu großen Läsionen zu erwarten sind. *In vitro* zeigen *E. bovis*-Sporozoiten, wie auch Sporozoiten anderer Eimerien [74, 75], ein wiederholtes Eindringen und Auswandern aus Wirtszellen [21], so dass sie *in vivo* über einen längeren Zeitraum verfügbar und damit angreifbar für PMN sein sollten. Um solche Interaktionen zu verifizieren, wurden *in vitro*-Untersuchungen mit Ko-Kulturen von bovinen PMN und *E. bovis*-Sporozoiten durchgeführt. Es zeigte sich, dass ca. ein Drittel der Sporozoiten von PMN in Antikörper-unabhängiger Weise eliminiert wird. Der Zusatz von Immunserum verstärkte diese Reaktionen signifikant, was klassische ADCC-Reaktionen vermuten lässt (siehe **7.1**). Zusatz von Neonatenserum, d. h. von Serum, das von neugeborenen Kälbern vor der ersten Kolostrumaufnahme gewonnen wurde und somit keine spezifischen Antikörper enthält, führte entsprechend nicht zu einer Steigerung der Parasiteneliminierung. Bereits die Tatsache, dass ca. ein Drittel der Sporozoiten über Antikörper-unabhängige und damit über rein nicht-adaptative Reaktionen eliminiert werden, spricht für PMN als wichtige Effektorzellen früh nach der Infektion.

Über die bisherigen Analysen konnte nicht ausgeschlossen werden, dass die Reduktion der extrazellulären Sporozoiten auch über aktive Invasion der PMN erfolgt war. PMN stellen zwar keine adäquate Wirtszelle für die Weiterentwicklung von *E. bovis*-Sporozoiten dar, trotzdem könnten Sporozoiten befähigt sein, in diese Zellen einzudringen, wie es bereits für andere nicht-permissive Zellen gezeigt wurde [143]. Im Falle von *T. gondii*-Tachyzoiten, die jedoch im Gegensatz zu *E. bovis* Sporozoiten ein extrem weites Spektrum permissiver Wirtszellen aufweisen, konnte eine aktive Invasion der PMN als auch die Phagozytose der Tachyzoiten gleichermaßen belegt werden [212]. Zur Abklärung wurden mittels durchfluss-zytometrischer Analysen die Aufnahme CFSE-gefärbter (diese Färbung beeinflusst

weder die Vitalität noch die Infektiosität der Sporozoiten, vgl. [146]) Sporozoiten, die entweder vital oder über Hitzeinaktivierung abgetötet waren, überprüft. Es zeigte sich, dass grundsätzlich sowohl aktive Invasion als auch passive Aufnahme der Sporozoiten über Phagozytose vorkommen, wobei ersteres jedoch eine untergeordnete Rolle spielt (siehe **7.1**).

Direkte Interaktionen zwischen PMN und Sporozoiten wurden über rasterelektronenmikroskopische (REM) Analysen illustriert. Die Interaktionen erstrecken sich von eher minimalen Kontakten über PMN-induzierte Filopodien und breitflächigere Vorwölbungen der Zelloberfläche über finger- und armartige Auswüchse der PMN, mit denen Sporozoiten „umarmt" wurden, bis hin zum Umfließen der Sporozoiten durch einen oder mehrere PMN. Dabei schien es keine bevorzugte Stelle für PMN-Parasit-Kontakte zu geben. Auch die Phagozytose des Parasitens wurde dokumentiert (sieh **7.1**).

Für Sporozoiten aviärer *Eimeria*-Arten fungieren Leukozyten auch als Transportmedien [2, 109, 193]. Der Frage, ob PMN nicht nur in der Lage sind, Sporozoiten aufzunehmen, sondern sie auch nach Phagozytose abzutöten, wurde über Kinetiken mittels transmissionselektronenmikroskopischer (TEM) Analysen nachgegangen (vgl. **7.1**). Zwei Stunden nach Inkubation erschienen die Membranen der intrazellulär gelegenen Sporozoiten noch unbeeinflusst. Erste Anzeichen einer Degradation intrazellulärer Sporozoiten, sichtbar als vermehrt auftretende Aufwerfungen der äußeren Membranen, waren ab der 8. Stunde der Inkubation zu vermerken. Nach 12 Stunden waren diese Reaktionen deutlich verstärkt. Die Sporozoiten zeigten klare Anzeichen einer Degradation und einige Parasiten waren bereits stark in ihrer Morphologie verändert im Gegensatz zu extrazellulär liegenden Parasiten (siehe **7.1**). Die zunehmende Schädigung der Sporozoiten ging dabei zeitlich einher mit der Abnahme von PMN-Granula, so dass der letale Effekt über Einwirkung verschiedener Granulainhalte zu vermuten ist. Insgesamt muss davon ausgegangen werden, das PMN im Falle von *E. bovis* nicht als Transportvehikel, sondern als effektiv agierende Abwehrzellen zu beurteilen sind.

4.1.1.2 Oxidative Burst-Aktivität von PMN ex vivo aus E. bovis-infizierten Kälbern und in vitro nach Konfrontation mit E. bovis-Sporozoiten

Um einen weiteren Effektormechanismus von PMN in Relation zu *E. bovis* zu überprüfen, wurden Untersuchungen zur oxidativen Burst-Aktivität durchgeführt.

Vergleichbar mit den Daten zur Phagozytose-Aktivität (vgl. 4.1.1.1) zeigten die *ex vivo*-Messungen einen biphasischen Anstieg der oxidativen Burst-Aktivität von PMN aus *E. bovis*-infizierten Kälbern (siehe **7.1**). Die Aktivitätsmaxima traten gleichfalls früh nach der Infektion sowie kurz vor bzw. zeitgleich mit der Oozystenausscheidung auf. Um zu überprüfen, ob sich diese Effekte auch in erhöhten Spiegeln von Sauerstoffradikalen im Blut niederschlugen, wurden Peroxidmessungen im Plasma infizierter Tiere vorgenommen. Zumindest in der späten Phase der Infektion wurden ab dem 20. Tag p. i. vermehrt freie Peroxide im Blut infizierter Tiere nachgewiesen (Abb. 3).

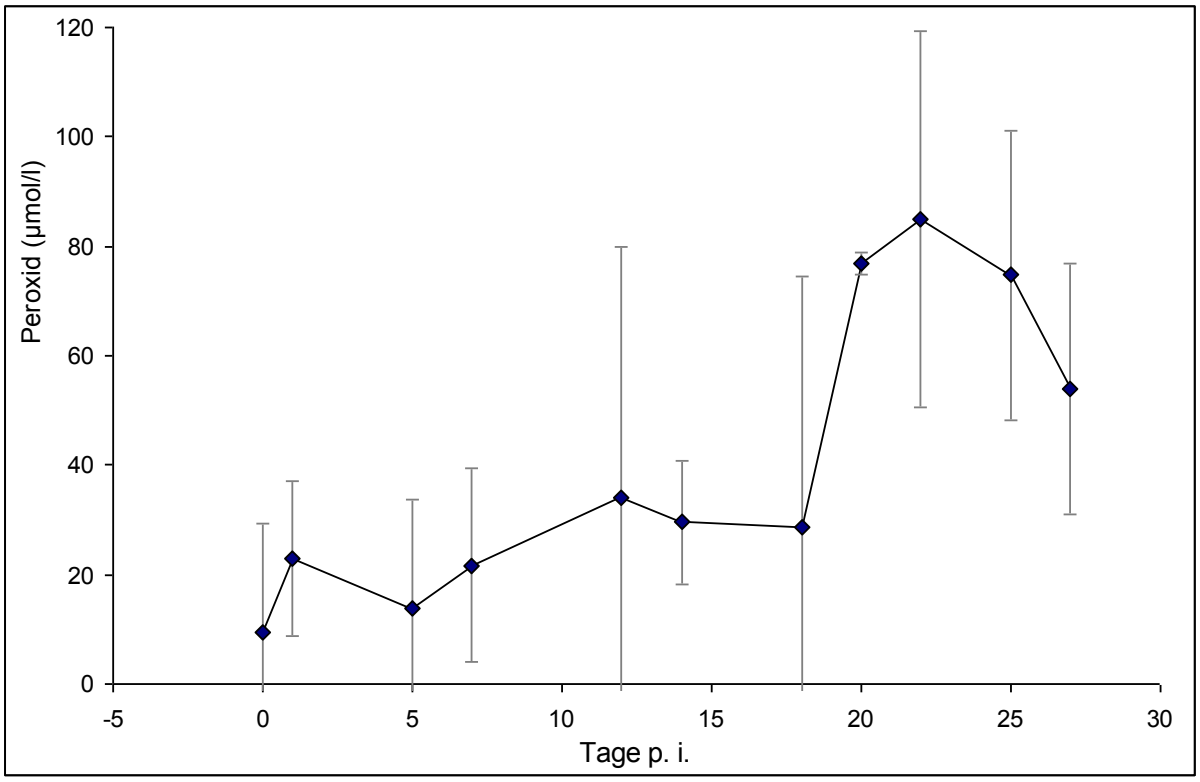


Abb. 3: Peroxide im Plasma *E. bovis*-infizierter Kälber
Plasmaproben wurden von experimentell *E. bovis*-infizierten Kälbern (n = 3) gewonnen und freie Peroxide im Verlauf der Infektion mittels des Oxistat®-Testkits nachgewiesen.

In vitro-Untersuchungen bestätigten, dass PMN nach Konfrontation mit *E. bovis*-Stadien mit einer gesteigerten oxidativen Burst-Aktivität reagierten. Dabei wurde dieser Effektormechanismus signifikant und in Dosis-abhängiger Form induziert (siehe **7.1**). Dass dieser Mechanismus bei der Abtötung zumindest eines Teils der intrazellulären Sporozoiten beteiligt ist, kann anhand der unter 4.1.1.1 dargelegten TEM-Daten angenommen werden. Ob auch extrazellulär freigesetzt Sauerstoffradikale *E. bovis* Sporozoiten nachhaltig schädigen, ist nicht abschließend geklärt. Ältere Untersuchungen von Hughes et al. [154] zur Empfindlichkeit von

E. bovis-Sporozoiten gegen Sauerstoff-Zwischenprodukte lassen dies jedoch vermuten.

4.1.1.3 Transkription immunmodulatorischer Gene nach Konfrontation mit E. bovis-Sporozoiten in vitro

Untersuchungen zu unterschiedlichen Chemo- und Zytokinen sowie zu iNOS zeigten auf transkriptioneller Ebene, dass PMN auch in dieser Hinsicht auf *E. bovis*-Sporozoiten bzw. -Merozoiten I-Antigen reagieren. Konfrontation mit lebenden Sporozoiten führte zur Induktion der Gentranskription von CCL2, CXCL1 und IL-6, d. h. von solchen Molekülen, die in erster Linie in das Anlocken nicht-adaptativ agierender Immunzellen involviert sind. Stimulation mit Merozoiten I-Antigen führte zusätzlich zur Hochregulation von CXCL10 und IL-12, welche wiederum in erster Linie auf Lymphozyten wirken [121, 353, 363] und somit zur Initiierung und Entwicklung adaptativer Immunreaktionen beitragen können. Insbesondere über die nachgewiesene Generierung von IL-12 könnten PMN die Charakteristik der sich *in vivo* entwickelnden T-zellulären Reaktionen früh nach Infektion in Richtung Th1-Dominanz beeinflussen, wie es beispielsweise auch für *T. gondii* diskutiert wird [215, 350].

Merozoiten I-Antigen, induzierte grundsätzlich stärkere Reaktionen bei PMN als vitale Sporozoiten (mit Ausnahme von iNOS). Dies könnte in der effektiveren Aufnahme von löslichen Antigenen oder in der evtl. besseren Verfügbarkeit von PAMP-Motiven hinsichtlich der Aktivierung von PMN begründet sein.

Gentranskripte von iNOS wurden in PMN als einziges untersuchtes Molekül von vitalen Sporozoiten und von löslichem Antigen gleichermaßen induziert (siehe **7.1**). Über iNOS werden NO-Radikale generiert, denen grundsätzlich eine wichtige Rolle bei der Tötung intrazellulärer, apikomplexen Parasiten zugesprochen wird [260, 259, 315], so dass dieser Mechanismus auch im Falle von *E. bovis* eine Rolle spielen sollte.

4.1.1.4 Induktion von „Neutrophil Extracellular Traps“ (NETs) über E. bovis-*Sporozoiten* in vitro

Im Rahmen der REM-Analysen fanden wir wiederholt Vernetzungen zwischen degradierten PMN und Sporozoiten, wobei die Parasiten in einer Art „Faserstraße“ gefangen schienen. Diese sowohl aus sehr feinen als auch aus dickeren filamentartigen Strukturen bestehende Fasernetze schienen z. T. regelrecht auf den Sporozoiten zu kleben und diese somit zu immobilisieren (**vgl. 7.2**). Die nachgewiesenen Strukturen ähnelten in ihrer Morphologie stark denen von NETs [43, 368]. Um die DNA-Natur der Fasern zu bestätigen wurde mit dem Fluoreszenz-Farbstoff Sytox® Orange gearbeitet, der extrazelluläre DNA anfärbt, nicht jedoch vitale Zellen. Bei allen Untersuchungen wurden immer parallel quantitative Messungen der Fluoreszenz (Fluoroscan®) als auch fluoreszenzmikroskopische Kontrollen durchgeführt.

Die Stimulation von PMN mit PMA, die allgemein als Positivkontrolle verwendet wird [119], führte zu einer deutlichen Induktion von NETs. Auch nach Konfrontation der PMN mit *E. bovis* Sporozoiten konnten wir die Bildung von NETs, in denen z. T. Sporozoiten gefangen sind, nachweisen (vgl. **7.2**). Über Kinetik-Analysen wurde deutlich, dass Sporozoiten – im Vergleich zu PMA – NETs viel schneller und stärker induzieren. Während PMN mit PMA mindestens für drei Stunden stimuliert werden mussten, war eine messbare Induktion von NETs nach Ko-Kultur mit *E. bovis*-Sporozoiten bereits nach 30 Minuten nachzuweisen (dies war der früheste, untersuchte Zeitpunkt, vermutlich läuft diese Reaktion noch schneller an). Vergleichbare Beobachtungen machten auch Fuchs et al. [119], die ebenfalls schnellere und stärkere Reaktionen für *Staphylococcus aureus*-induzierte NETs im Vergleich zu PMA messen konnten.

Um die DNA-Natur der Sporozoiten-induzierten NETs weiterhin zu verifizieren, wurden Experimente unter Zusatz von DNase durchgeführt. Es zeigte sich eine signifikante Reduktion sowohl der Sporozoiten-vermittelten als auch der PMA-induzierten Reaktionen in Anwesenheit von DNase (vgl. **7.2** und Abb. 4). Die auch bei nicht-stimulierten PMN auftretende Verminderung der Fluoreszenz lässt sich über grundsätzlich in PMN-Präparationen enthaltene, tote PMN erklären, deren freigesetzte DNA als Hintergrund zum Sytox® Orange-Signal beiträgt (siehe auch Abb. 4-5).

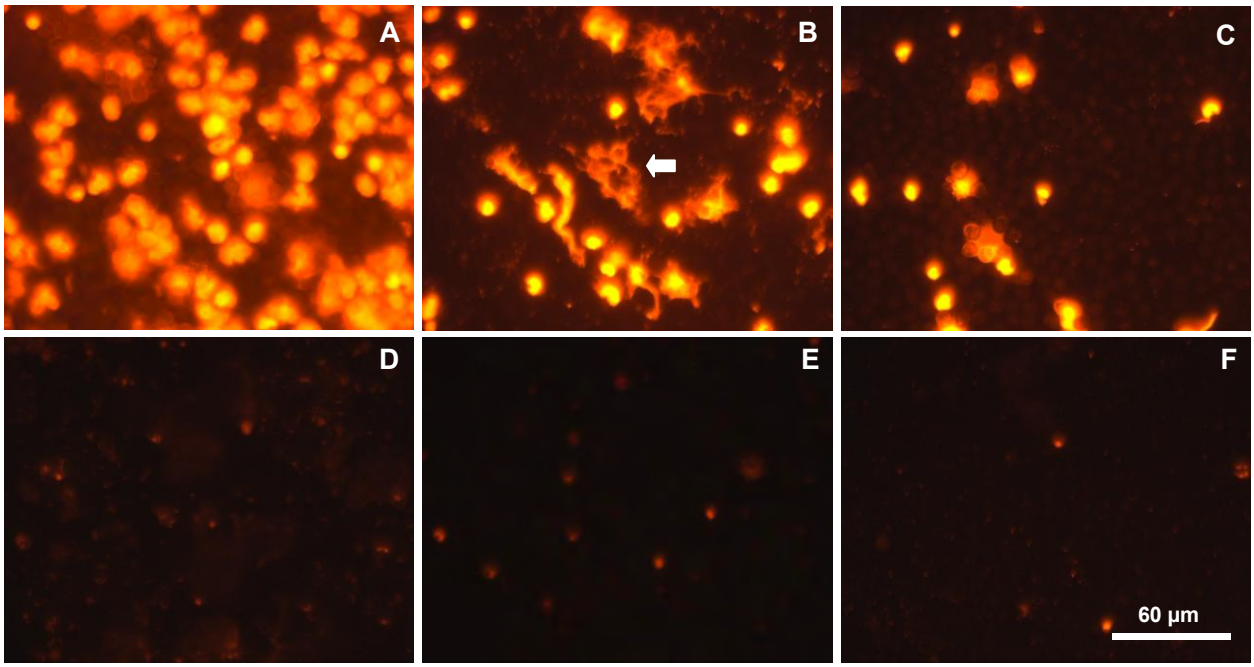


Abb 4: Einfluss der DNase-Behandlung auf die Ausbildung Sporozoiten-induzierter NETs. Bovine PMN wurden mit *E. bovis*-Sporozoiten, PMA oder Medium in Ab- (A-C) oder Anwesenheit (D-F) von DNase I für 3 Stunden ko-kultiviert. DNA wurde über Sytox® Orange Färbung sichtbar gemacht.

Untersuchungen unter simultanen Ausschluss der Phagozytoseaktivität der PMN über Cytochalasin D-Behandlung bestätigten zudem, dass die Induktion von NETs nach Kontakt mit lebenden Sporozoiten dosisabhängig und über DNase-Behandlung hemmbar ist. Die bei diesen Experimenten parallel durchgeführte Lyse der PMN über TritonX-100, die die maximal erreichbare Freisetzung von DNA aus PMN widerspiegeln sollte, erlaubte eine etwaige Quantifizierung der beteiligten PMN. Je nach eingesetzter Sporozoitenzahl wurde bis zu 30,6 ± 4,4 % der maximal erreichbaren DNA extrazellulär freigesetzt, was für eine Beteiligung von bis zu einem Drittel der PMN spricht.

Die Bildung von NETs unterliegt der NADPH-Oxidase-vermittelten Synthese von ROS [43, 119]. Um den hier gezeigten, über Kontakt mit Sporozoiten induzierten Zelltod der PMN von Apoptose oder Nekrose abzugrenzen und NET-spezifische Eigenschaften aufzuzeigen, wurden Hemmungsversuche unter Zusatz von Diphenyleniodonium (DPI), das als potenter Inhibitor der NADPH-Oxidase gilt [71], durchgeführt. Zusatz von DPI reduzierte die Sporozoiten-induzierte Fluoreszenz-intensität signifikant. Auch bei Stimulation mit PMA kam es zu einer Verminderung der NET-Bildung, während der gleiche Effekt im Falle der Negativkontrolle nicht zu beobachten war (vgl. **7.2** und Abb. 5).

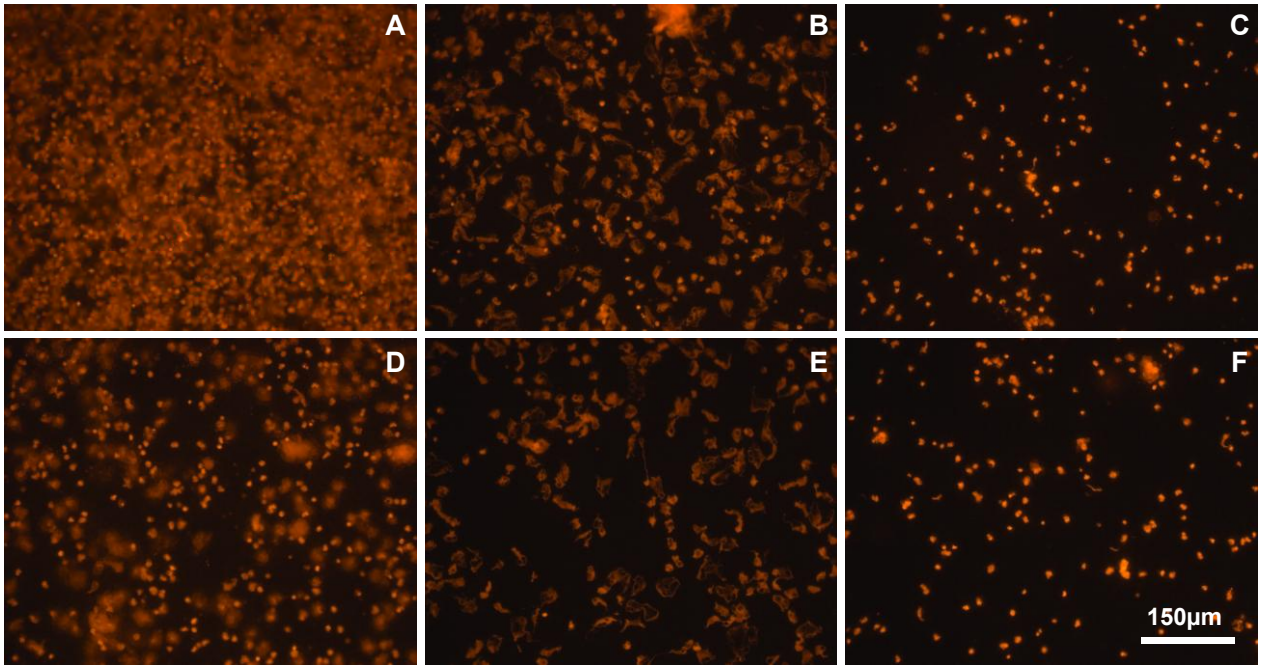


Abb 5: Einfluss der DPI-Behandlung auf die Ausbildung Sporozoiten-induzierter NETs. Bovine PMN wurden mit *E. bovis*-Sporozoiten, PMA oder Medium in Ab- (A-C) oder Anwesenheit (D-F) von DPI I für 3 Stunden ko-kultiviert. DNA wurde über Sytox® Orange Färbung sichtbar gemacht.

Die in der Literatur beschriebene Hemmung von NETs über Serumzusatz [119] wurde ebenfalls für Sporozoiten bestätigt. Wir verwendeten dabei neonatales Kälberserum, da dieses frei von Antikörpern gegen *E. bovis* ist und somit ein antikörpervermittelter Effekt auf Sporozoiten (siehe 4.2.1) ausgeschlossen werden konnte. Allerdings führte der generell hohe DNA-Gehalt des Serums zu einer hohen Hintergrundfluoreszenz, so dass eine fluorometrische Quantifizierung in diesem Fall keine verlässlichen Ergebnisse brachte. Die simultan durchgeführten fluoreszenzmikroskopischen Untersuchungen der Proben legen jedoch einen inhibitorischen Effekt nahe (Abb. 6). Eine hemmende Wirkung von Serum erscheint insofern sinnvoll, als die Ausbildung von NETs in Blutgefäßen schwerwiegende Folgen für den Wirt, wie Thrombenbildung, Verstopfung kleinerer Gefäße etc., nach sich ziehen könnte. In Übereinstimmung beschreiben von Köckritz-Blickwede et al. [375] NET-degradierende, hitze-stabile Nucleasen in fetalem Kälberserum. Die Ergebnisse zur inhibitorischen Wirkung von Serum stehen allerdings im Gegensatz zu Daten zu NETs in Blutproben von *Plasmodium falciparum*-infizierten Patienten [17] oder von Patienten mit multiplen Traumen [218].

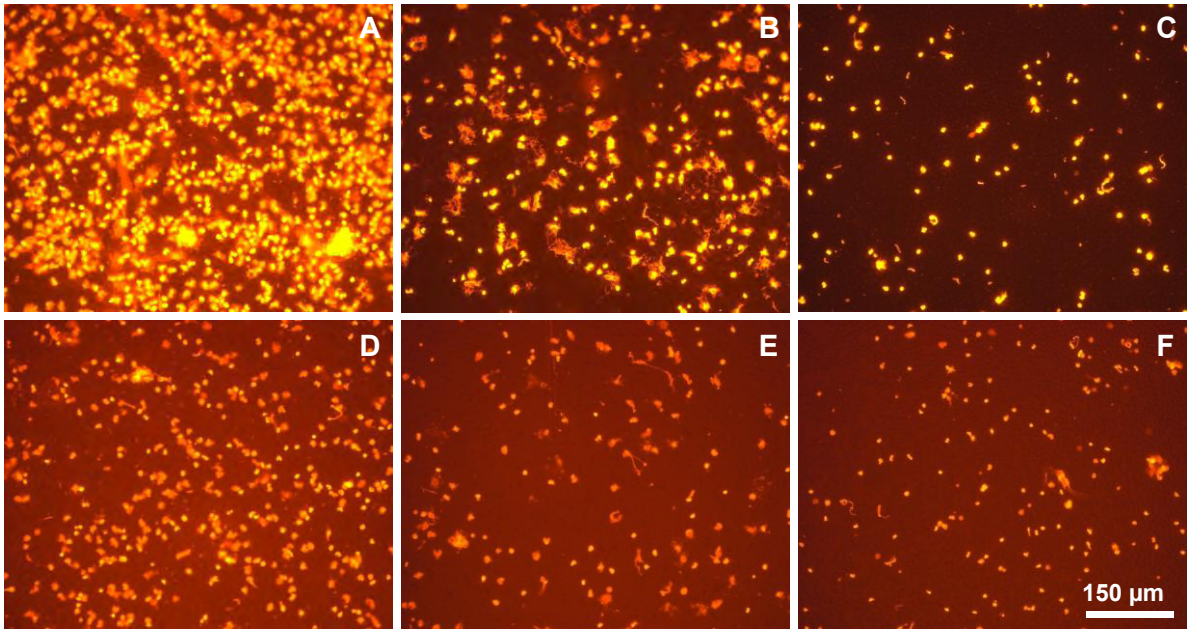


Abb 6: Einfluss von neonatalem Kälberserum auf die Ausbildung Sporozoiten-induzierter NETs. Bovine PMN wurden mit *E. bovis*-Sporozoiten, PMA oder Medium in Ab- (A-C) oder Anwesenheit (D-F) von 10 % neonatalem Kälberserum für 3 Stunden ko-kultiviert. DNA wurde über Sytox® Orange Färbung sichtbar gemacht.

In weitergehenden Untersuchungen wurde geklärt, ob die Induktion von NETs in Abhängigkeit zur Vitalität oder Integrität der Sporozoiten steht. Dazu wurden vitale, über Hitzeinaktivierung abgetötete sowie homogenisierte Sporozoiten verwendet. Es zeigte sich, dass intakte, vitale Sporozoiten die Bildung von NETs am effektivsten auslösten. Sowohl die Hitzeinaktivierung als auch die Homogenisierung der Parasiten reduzierte die Ausbildung der NETs erheblich; die Werte lagen jedoch noch signifikant über denen der Negativkontrolle (vgl. **7.2**). Bei beiden Behandlungen muss jedoch in Betracht gezogen werden, dass die von toten Sporozoiten stammende DNA in diesem Messsystem miterfasst wird. Die fluoreszenz-mikroskopischen Beobachtungen wiesen jedoch trotzdem auf eine, wenn auch geringe, Induktion von NETs bei beiden Behandlungen hin (Abb. 7).

Die stark verminderte Induktion von NETs bei inaktivierten oder homogenisierten Sporozoiten könnte einerseits in dem entsprechend fehlenden mechanischen Einfluss über die Parasitenmotilität begründet sein. Andererseits könnten die Behandlungen zur Zerstörung multipler Liganden geführt haben, deren simultane Bindung als Voraussetzung für eine effektive NET-Induktion postuliert wird (vgl. [44]).

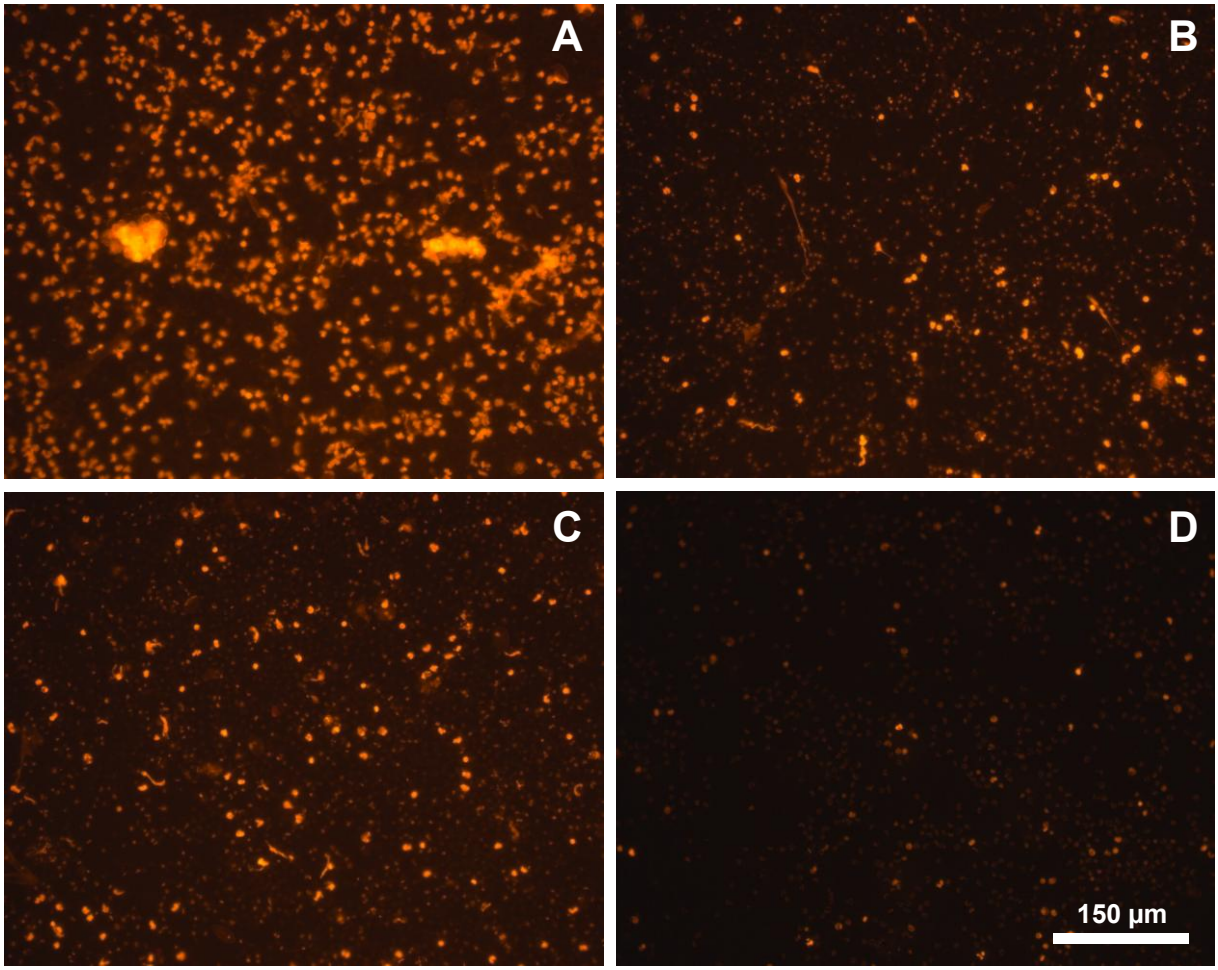


Abb 7: Einfluss der Vitalität und Integrität von Sporozoiten auf die Ausbildung von NETs. Bovine PMN wurden mit vitalen (A), Hitze-inaktivierten (B) und homogenisierten (C) *E. bovis*-Sporozoiten oder Medium (D) für 3 Stunden ko-kultiviert. DNA wurde über Sytox® Orange Färbung sichtbar gemacht.

Bei den Untersuchungen zu NETs fiel immer wieder auf, dass deren Freisetzung offensichtlich über mechanische Einflüsse verstärkt wird. Dies erscheint insofern logisch, als es bisher kaum Hinweise auf eine aktive Extrusion der NETs seitens der absterbenden Zellen gibt. Sollte jedoch Bewegung im umgebenden Milieu sein - und *in vivo* sollte diese omnipräsent sein - könnten die NETs deutlich effizienter freigesetzt werden. Bei Ko-Kulturen mit lebenden Sporozoiten sorgen die motilen Parasiten für ein entsprechend bewegtes Milieu. Andererseits vermuten Brinkmann und Zychlinsky [44], dass eine simultane Stimulation multipler Rezeptoren von Nöten ist, um eine effiziente Ausbildung von NETs zu erreichen. Dies könnte bei so komplexen Organismen wie Sporozoiten im Gegensatz zu PMA gegeben sein.

Bisher sind die molekularen Grundlagen der Pathogen-PMN-Interaktion bei der NET-Induktion unbekannt. Diskutiert werden sowohl auf elektrostatischen Interaktionen beruhende Mechanismen [223] als auch über Rezeptor/Liganden funktionierende

Systeme. Bei der Induktion von NETs über promastigote Formen von *L. amazonensis* scheint ein parasitäres, oberflächengebundenes Lipophoshoglykan causal beteiligt zu sein [129]. Im Falle nicht-opsonierter Mikroorganismen wird die Beteiligung von sog. „pattern recognition receptors“ (PRR), wie z. B. Toll-like Rezeptoren (TLRs) oder Dectin [368] diskutiert. Über einen indirekten Mechanismus scheint z. B. TLR4 in der Thrombozyten-vermittelten Induktion von NETs zum Abfangen von Bakterien eine Rolle zu spielen [65]. Direkt beteiligte Rezeptoren von PMN sind jedoch bisher nicht identifiziert.

Für intrazelluläre Parasiten würden sich TLRs anbieten, da bei diversen parasitischen Protozoen über solche Interaktionen berichtet wurde (zur Übersicht siehe [122]). Im Falle von *T. gondii* werden z. B. TLR2 und TLR4 über parasitenspezifische Gykosylphosphatidylinositole (GPIs) aktiviert [79]. Über REM-Analysen wurden im Rahmen dieser Arbeit erste Hinweise zu über *T. gondii*-Tachyzoiten induzierte, NET-ähnliche Strukturen nach Ko-Kultur mit bovinen PMN gewonnen (Abb. 8). Weitere Analysen zur Bestätigung von NETs im Zusammenhang mit *T. gondii*-Stadien stehen jedoch noch aus.

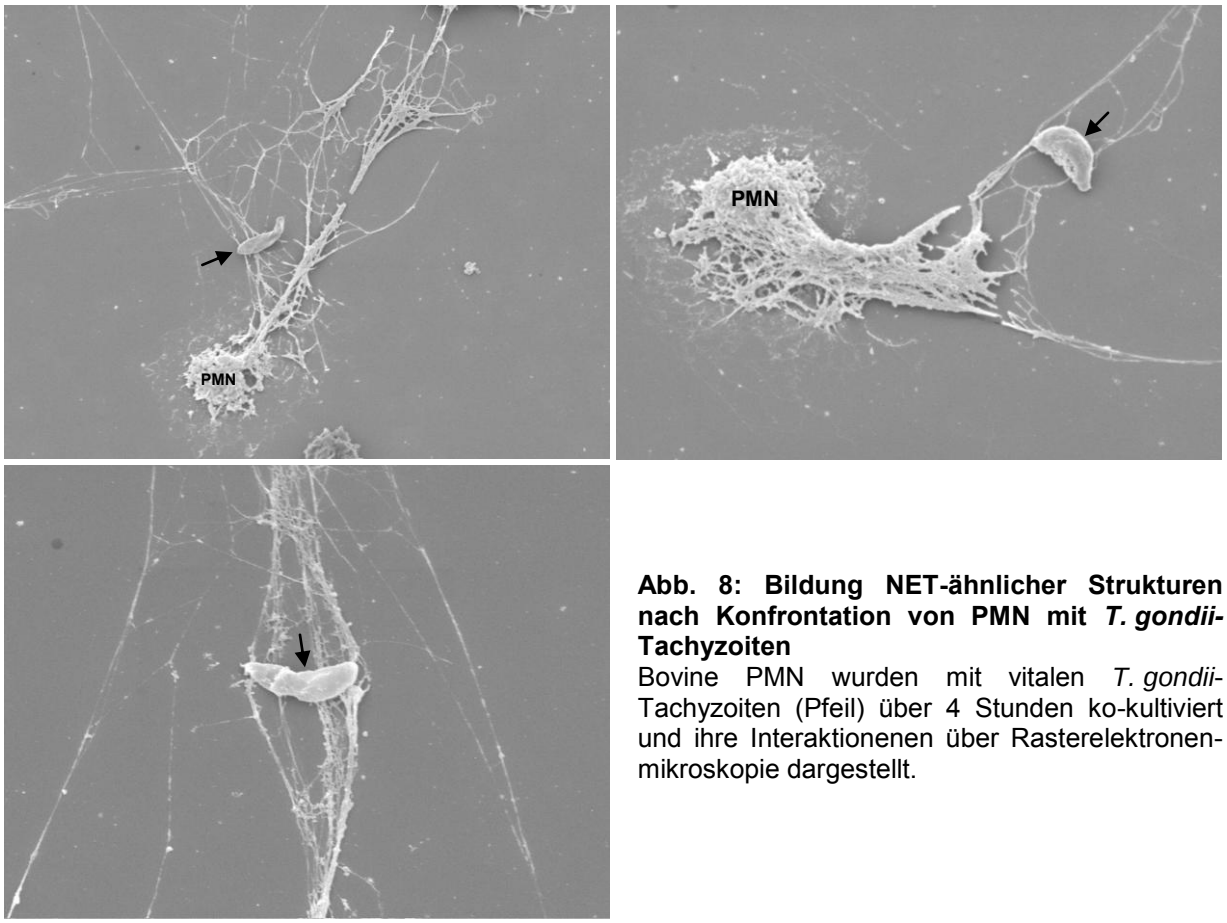


Abb. 8: Bildung NET-ähnlicher Strukturen nach Konfrontation von PMN mit *T. gondii*-Tachyzoiten
Bovine PMN wurden mit vitalen *T. gondii*-Tachyzoiten (Pfeil) über 4 Stunden ko-kultiviert und ihre Interaktionenen über Rasterelektronen-mikroskopie dargestellt.

Aus biochemischen Analysen zu ^{3}H-Glucosamin-markierten Glykolipiden von *E. bovis* (in Zusammenarbeit mit Prof. Schwarz, Klinikum der Philipps-Universität Marburg, unveröffentlichte Ergebnisse) haben wir zumindest erste Hinweise auf GPIs in *E. bovis*-Merozoiten I (mit Sporozoiten können diese Untersuchungen wegen unzureichender Verfügbarkeit nicht durchgeführt werden), so dass Interaktionen mit TLRs durchaus denkbar wären.

Laut Literatur haben NETs eine abtötende Wirkung auf verschiedene Bakterien, Pilze (zur Übersicht: [377]) und promastigote Formen von *L. amazonensis* [129]. Im Gegensatz dazu wurden in unseren Untersuchungen bisher keine letalen Effekte auf Sporozoiten *in vitro* festgestellt. Die über REM dargestellten Interaktionen wiesen eher auf ein Abfangen der Parasiten im Netzwerk der NETs über fest angeheftete Fasern hin. Insofern sehen wir den Wirkungsmechanismus von NETs in der Immobilisierung der *E. bovis*-Sporozoiten, denen damit nachfolgend die Invasion von Wirtszellen verwehrt wird, die ihrerseits – im Gegensatz zu den sich meist extrazellulär vermehrenden Bakterien oder Pilzen – eine unabdingbare Vorraussetzung für die effektive, ausschließlich intrazellulär stattfindende Replikation des Parasiten darstellt. Allein über die Hemmung der Wirtszellinvasion könnte der gesamte Lebenszyklus des Parasitens abgebrochen und seine Pathogenität, die in erster Linie auf der von späteren Stadien vermittelte Zellzerstörung beruht, erheblich abgemildert werden. Diese Hypothese wurde *in vitro* über funktionelle Untersuchungen bestätigt, da die Infektiosität von Sporozoiten, die zuvor mit PMN ko-kultiviert wurden, deutlich herabgesetzt war (vgl. **7.2**). Verglichen mit Sporozoiten, die vorab keinen Kontakt mit PMN hatten, waren die Infektionsraten bei BUVEC um ca. 65% reduziert. Da bei DNase-behandelten diese Verminderung der Infektionsrate nahezu volllständig ausblieb (vgl. **7.2**), muss der abträgliche Effekt auf die Wirkung der NETs zurückgeführt werden.

Ein ebenfalls nicht mit der Abtötung einhergehendes Abfangen und Immobilisieren von Pathogenen über NETs wurde auch für Gruppe A-Streptokokken und Pneumokokken beschrieben [23, 48], wobei hier pathogeneigene, DNA-abbauende Moleküle zugrunde zu liegen scheinen. Die oben benannte Hemmung der Wirtszellinfektion über NETs spricht nicht für die Produktion ähnlicher Moleküle über *E. bovis*-Sporozoiten.

4.1.2 Monozyten und Makrophagen

4.1.2.1 Dichte von Makrophagen in der Darmmukosa E. bovis-infizierter Kälber

Immunhistologische Untersuchungen zur Infiltration von Makrophagen in die Darmschleimhaut *E. bovis*-infizierter Kälber belegen die Relevanz dieser Immunzellen, da sie in der Schleimhaut von Jejunum, Ileum, Zäkum und Kolon bei primär- und reinfizierten Kälbern vermehrt nachzuweisen waren (siehe **7.3**).

In aller Regel erreichen PMN als erste Zellen den Ort einer Irritation, gefolgt von Monozyten bzw. Makrophagen und γδ-TCR$^+$-T-Zellen. Für PMN konnten wir zeigen, dass eine Konfrontation mit Sporozoiten zur gesteigerten Synthese von z. B. TNF-α und CCL2 führt (vgl. **7.3**), beides Moleküle, die auf Makrophagen wirken respektive sie anlocken. Die in der Präpatenz der *E. bovis*-Infektion gemessene, deutlich gesteigerte Transkription IFN-γ-kodierender Gene in Lymphozyten drainierender Lymphknoten als auch im peripheren Blut könnte ebenfalls im Zusammenhang mit der Infiltration bzw. der Aktivierung der Makrophagen stehen (vgl. **7.4**). Infiltrationen von Makrophagen wurden in der Darmmukosa von *E. tenella*- und *E. acervulina*-infizierten Hühnern sowie in *E. separata*-infizierten Ratten beobachtet [327, 365, 372]. Auch ältere histopathologische Untersuchungen zu *E. bovis* von Friend und Stockdale [118] beschreiben eine, wenn auch geringe Infiltration mit Makrophagen und anderen Zellen in reife Makromeronten. Dabei waren solche Zellen insbesondere in bereits degenerierten Makromeronten anzutreffen. Allerdings ist aus diesen Untersuchungen nicht ersichtlich, ob die Zellen vor oder nach dem Absterben der Parasitenstadien einwanderten.

Über die Reinfektion der Kälber kam es abermals zur Infiltration aller untersuchten Darmabschnitten mit Makrophagen. Dies spricht für eine effektive Beteiligung dieser Zellen an der Elimination der Parasiten. Da die untersuchten Proben 12 Tage nach Reinfektion gewonnen wurden, deuten die Daten auf einen frühen Abbruch der Infektion hin, d. h. dass vermutlich bereits Sporozoiten- oder Trophozoiten-Stadien eliminiert werden. Für Makrophagen wäre der Zugang auf extrazelluläre Stadien beschränkt und somit kämen lediglich Sporozoiten in Frage. In den nachfolgenden *in vitro*-Untersuchungen haben wir uns entsprechend auf Interaktionen mit Sporozoiten als vitale Stadien beschränkt.

4.1.2.2 Phagozytische und Oxidative Burst-Aktivität von Monozyten E. bovis-infizierter Kälber ex vivo

In primär experimentell *E. bovis*-infizierten Kälbern wurden die oxidative Burst- und phagozytischen Aktivitäten von peripheren Monozyten als Vorläuferzellen von Makrophagen im Infektionsverlauf überprüft. Beide Effektormechanismen wurden in Monozyten zu ähnlichen Zeitpunkten wie in PMN (siehe 4.1.1.1., 4.1.1.2.) verstärkt induziert. Entsprechend waren zwei Aktivitätsmaxima jeweils kurz nach der Infektion und im Zeitraum der Merogonien I-II erkennbar (vgl. **7.3**). Insgesamt fiel das Ausmaß der Aktivierung bei Monozyten deutlich geringer verglichen mit PMN aus (52 % vs. 97 % bei oxidativem Burst und 38 % vs. 64 % bei Phagozytose). Einen vergleichbaren biphasischen Verlauf beschrieben auch Rose et al. [289] bezüglich der Anzahl großer mononukleärer Zellen im peripheren Blut von *E. nieschulzi*-infizierten Ratten und *E. maxima*-infizierten Hühnern. Ob diese Aktivitäten jedoch in direktem Zusammenhang mit einer Interaktion mit *E. bovis*-Stadien stehen oder über absterbende Zellen und resultierende Zelltrümmer induziert wurden, kann aus diesen Analysen nicht geklärt werden.

4.1.2.3 Elimination von E. bovis-Sporozoiten in vitro

Um die bezüglich **Monozyten** *ex vivo* gemessene Situation *in vitro* nachzuvollziehen und somit auszuschließen, dass die Aktivierung der Monozyten evtl. nicht parasitenspezifisch, sondern z. B. über Zelldetritus zustande kommt, wurden bovine Monozyten isoliert und *in vitro* mit Sporozoiten konfrontiert. Zwar verschwanden nach Ko-Kultur ca. ein Drittel der Sporozoiten, dies konnte jedoch unter Zugabe von Immunserum nur geringfügig gesteigert werden. Die Verwendung von Hitze-inaktivierten Sporozoiten erwies zudem, dass diese Reaktionen in erster Linie auf aktiver Invasion seitens der Parasiten beruhen, so dass Monozyten bei der phagozytosevermittelten Elimination von Sporozoiten insgesamt eher eine untergeordnete Rolle spielen (vgl. **7.3**).

Mit **BoMac** stand uns eine permanente bovine Makrophagenzelllinie zur Verfügung, die bereits in anderen Untersuchungen, z. B. zu *Theileria* sp. [258, 328] oder *Mycobacterium* sp. [358, 339, 389, 189], erfolgreich verwendet wurde.
Nach Ko-Kultur von BoMac mit vitalen *E. bovis*-Sporozoiten zeigte sich im mikroskopischen Bild, dass viele Parasiten bereits nach kurzer Zeit intrazellulär zu

finden waren. Dabei ähnelte die Kultur sowohl in ihrer Kinetik als auch morphologisch (kernnahe Lage der i. d. R. einzeln in der Zelle liegenden Sporozoiten, siehe Abb. 9b) sehr dem Bild infizierter BUVEC oder anderer Wirtszellen, so dass Zweifel aufkamen, ob BoMac in der Lage sind, Sporozoiten zu phagozytieren. Über den Einsatz Hitze-inaktivierter Sporozoiten wurde bereits in die Kultur ersichtlich, dass die meisten Sporozoiten aktiv invadieren und folgerichtig nur sehr wenige Parasiten tatsächlich phagozytiert werden (Abb. 9c).
Dementsprechend zeigten quantitative Experimente, dass bei Verwendung von Hitze-inaktivierten Sporozoiten kein signifikanter Unterschied in der Anzahl verbleibender Parasiten im Vergleich zur zellfreien Kontrolle vorlag, während bei Verwendung vitaler Sporozoiten auch ohne Zusatz von Immunserum nahezu alle aus dem Medium verschwunden waren (Abb. 9a). Da es sich bei BoMac um eine bereits über Jahre verwendete, permanente Zelllinie handelt, steht zu vermuten, dass sich einige ihrer ursprünglichen Eigenschaften wie ihre phagozytischen Fähigkeiten, über die Zeit dahingehend verändert haben, dass sie kaum noch in der Lage waren, *E. bovis*-Sporozoiten zu phagozytieren.

Verglichen mit BoMac zeigte die Ko-Kultur von primären bovinen **Makrophagen** und Sporozoiten ein anderes Bild, da bereits lichtmikroskopisch z. T. prall mit Sporozoiten angefüllte Makrophagen erkennbar waren. Extrazellulär verbliebene Parasiten blieben vital und beweglich und schienen nicht über sezernierte Moleküle angegriffen. Die Elimination von Sporozoiten aus dem extrazellulären Milieu war zudem titrierbar (vgl. **7.3**). Bei einem Verhältnis von Sporozoiten zu Makrophagen von 1:2 wurden 70-90 % der Sporozoiten aufgenommen. Bei steigender Anzahl von Sporozoiten war die Aufnahmekapazität der Makrophagen zunehmend erschöpft. So verblieben bei einem Verhältnis von 2:1 50-70% der Parasiten extrazellulär.

Auch hier stellte sich die Frage nach der aktiven Invasion der Makrophagen über Sporozoiten. Vergleichende Untersuchungen mit vitalen und Hitze-inaktivierten Sporozoiten zeigten, dass Sporozoiten von Makrophagen im Gegensatz zu Monozyten oder BoMac fast ausschließlich über Phagozytose aufgenommen wurden, da die Hitze-Inaktivierung der Sporozoiten das Ausmaß der Reaktion nicht signifikant beeinflusste. In Übereinstimmung wurden vergleichbare Daten auch für Ko-Kulturen von *E. acervulina* und aviären Makrophagen generiert [365].

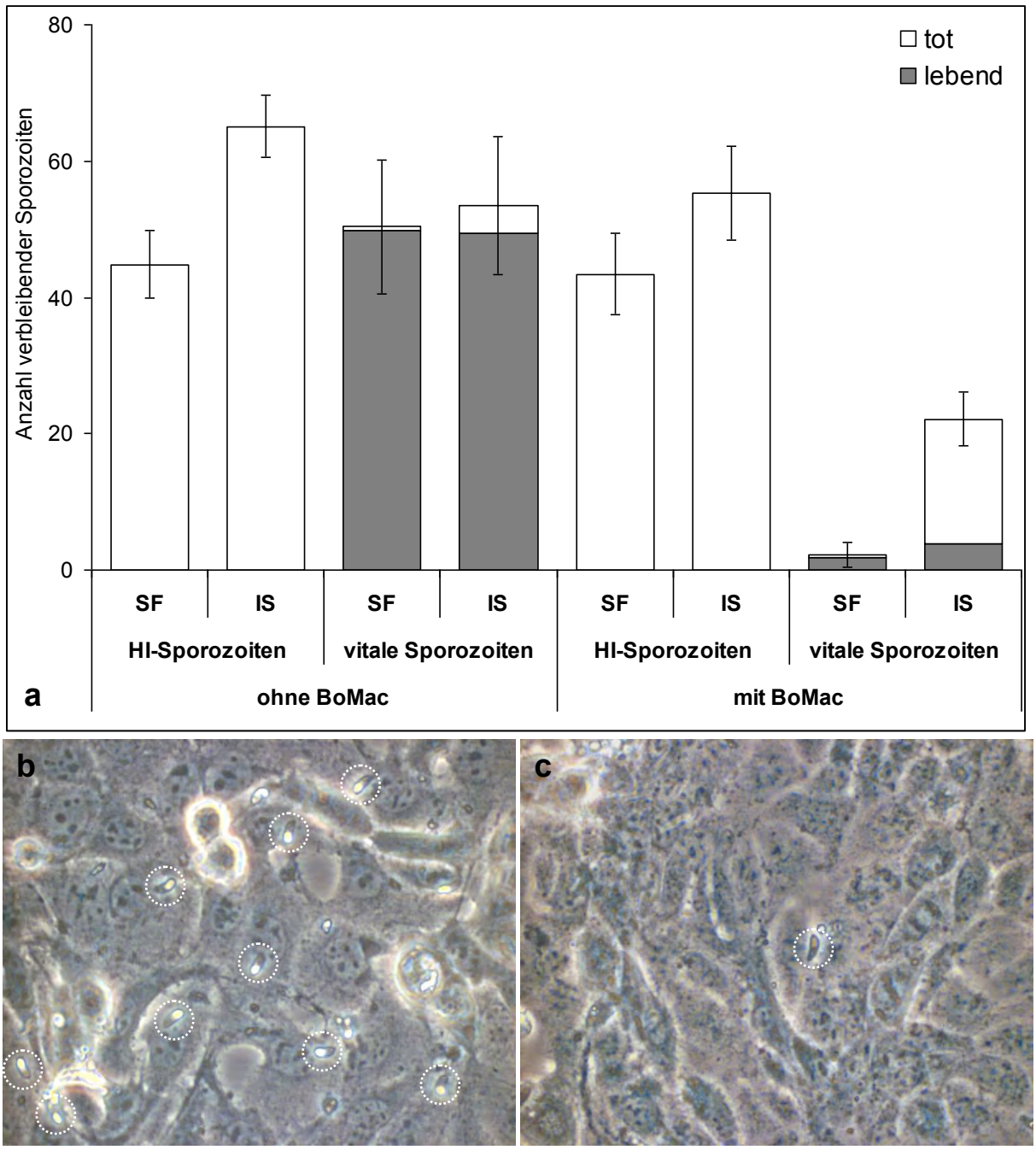


Abb. 9: BoMac-vermittelte Elimination von *Eimeria bovis*-Sporozoiten
(a) BoMac wurden mit vitalen oder Hitze-inaktivierten (HI, 60°C, 30 min, serumfrei) *E. bovis*-Sporozoiten in Ab- (SF = serumfrei) oder Anwesenheit von Immunserum (IS) im Verhältnis 2:1 für 4 h ko-kultiviert. Anschließend wurde die Anzahl extrazellulär verbleibender Sporozoiten bestimmt. **(b-c)** zeigt beispielhaft das Bild nach Ko-Kultur mit vitalen (b) oder Hitze-inaktivierten (c) Sporozoiten. Eingekreist sind intrazellulär gelegene Parasiten.

Im *E. bovis*-System wurden bereits mehr als ein Drittel der vitalen Sporozoiten als „echte“ nicht-adaptative Reaktion, d. h. ohne Serumzusatz von Makrophagen phagozytiert. Diese Reaktion war über Immunserum zu verstärken, was eine Effizienzsteigerung über ADCC-Reaktionen nahe legt (vgl. **7.3**). Da spezifische Antikörper gegen *E. bovis* erst im Verlauf der Infektion entwickelt werden und

kolostrale Immunglobuline evtl. bereits abgebaut sind [110] spiegeln erstere Ergebnisse wahrscheinlicher die Reaktionen der Makrophagen früh nach der Infektion *in vivo* wieder. Über eine Elimination von ca. einem Drittel der Sporozoiten kann die Infektion zwar nicht verhindert werden, da jedoch nachfolgend die Amplifikation des Parasitens über Merogonien ausbleibt, ist über diese Elimination trotzdem eine erhebliche Reduktion der Parasitenlast zu erwarten.

Um die Ergebnisse in einem anderen Messsystem zu verifizieren, wurden vitale und über Hitze inaktivierte Sporozoiten mit dem Fluoreszenzfarbstoff CFSE gefärbt [146] und nach Ko-Kultur der Anteil intrazelluläre Sporozoiten enthaltende Makrophagen mittels FACS-Analysen bestimmt. Es bestätigte sich, dass die Verwendung toter Sporozoiten keinen Einfluss auf den Anteil intrazellulärer Sporozoiten hat (vgl. **7.3**). Insgesamt kann die aktive Invasion von Makrophagen als gelegentlich vorkommendes Ereignis zwar nicht ausgeschlossen werden; das Gros der Sporozoiten wird jedoch über Phagozytose aufgenommen.

Über die bisherigen Untersuchungen kann nicht geschlossen werden, ob Makrophagen tatsächlich in der Lage sind, aufgenommene Sporozoiten intrazellulär abzutöten. Mikroskopische Untersuchungen verlängerter Kulturen gaben darüber optisch keinen Aufschluss. Zukünftig müsste geklärt werden, ob bovine Makrophagen zur effizienten Tötung von *E. bovis*-Sporozoiten nur in Anwesenheit spezifischer Antikörper und Komplement in der Lage sind, wie im Maussystem bezüglich *E. falciformis*-Sporozoiten berichtet wurde [25], oder ob sie als reine nicht-adaptative Reaktion auch ohne Zusatz von Immunserum direkt die Parasiten intrazellulär lysieren können. Ältere Untersuchungen [156, 340] beschreiben eine „Phagozytose-aktive, bovine Monozyten-Zelllinie“ (genauere Angaben werden nicht gemacht, diese Zellen werden aber als Makrophagen diskutiert) als permissive Wirtszelle für die Entwicklung von *E. bovis*-Meronten I, die jedoch nach Stimulation mit Zellüberstand Con A-aktivierter T-Zellen eine Weiterentwicklung der Sporozoiten unterbinden können [340]. Eine Weiterentwicklung der Sporozoiten konnte bisher weder in der bovinen Makrophagenzelllinie (BoMac) noch in primären bovinen Makrophagen nachvollzogen werden, allerdings waren zumindest letztere nicht über einen entsprechend langen Zeitraum kultivierbar. Die Daten zu Hitze-inaktivierten Sporozoiten sprechen jedoch dafür, dass die aktive Infektion der Makrophagen insgesamt vernachlässigbar ist.

4.1.2.4 Transkription immunmodulatorischer Gene nach Konfrontation mit **E. bovis-*Sporozoiten* in vitro**

Auch wenn Monozyten oder BoMAC kaum in der Lage waren, so große Pathogene wie Sporozoiten zu phagozytieren und somit direkt zu eliminieren, so können sie trotzdem eine Rolle in der Anlockung anderer Immunzellen und in der Präsentation von Antigenen zur Einleitung adaptativer Immunreaktionen spielen. Deshalb wurden ihre Reaktionen, ebenso wie die primärer Makrophagen, auf Ebene der Gentranskription verschiedener immunmodulatorisch wirkender Moleküle überprüft.

Monozyten zeigten sich insgesamt auch in diesem Zusammenhang als relativ unbeeindruckt sowohl von vitalen Sporozoiten als auch von Merozoiten-Antigen. Lediglich die Transkription IFN-γ- und CXCL10-kodierender Gene wurden deutlich induziert, während die Gentranskription anderer Moleküle eher moderat beeinflusst war (TNF-α, IL-6, CCL2, CXCL8, iNOS) oder ausblieb (IL-12, CXCL1, vgl. **7.3**). Die Steigerung von IFN-γ-spezifischen mRNAs nach Ko-Kultur mit lebenden Sporozoiten und nach Stimulation mit Merozoiten I-Antigen gleichermaßen spricht durchaus für eine wichtige Rolle der Monozyten sowohl bei der Aktivierung von Makrophagen als auch bei der Initiierung der adaptativen Immunantwort, die nach unseren Untersuchungen (vgl. **7.4**) in der Präpatenz Th1-dominiert ist. Auch die verstärkte Transkription CXCL10-kodierender Gene legt die Rekrutierung von T-Zellen nahe, welche dann eine entsprechend T-Zell-getragene Immunität gewährleisten können. Insofern erscheinen Monozyten zumindest für früh sich entwickelnde, zelluläre Immunreaktionen bei der *E. bovis*-Infektion von Bedeutung zu sein.

Um zunächst die grundsätzliche Reaktivität von **BoMac** zu prüfen, wurden sie mit TNF-α und IFN-γ stimuliert und nachfolgend die Gentranskription einiger Moleküle untersucht. BoMac reagierten insgesamt relativ schwach auf beide Stimulanzien; einige Moleküle wie CXCL10 oder IL-6 wurden jedoch induziert (Abb. 10). Wir konnten jedoch mit keiner Behandlung die Transkription TNF-α- oder iNOS-kodierender Gene steigern (Daten nicht gezeigt).

Im Gegensatz zu Monozyten reagierten BoMac relativ deutlich auf Sporozoiten und Merozoiten I-Antigen hinsichtlich der Produktion immunmodulatorisch wirkender Moleküle. Die Stimulation mit Merozoiten-Antigen I induzierte die Transkription IL-6-, CCL2-, CXCL8-, CXCL1- und COX-2-kodierender Gene (in absteigender

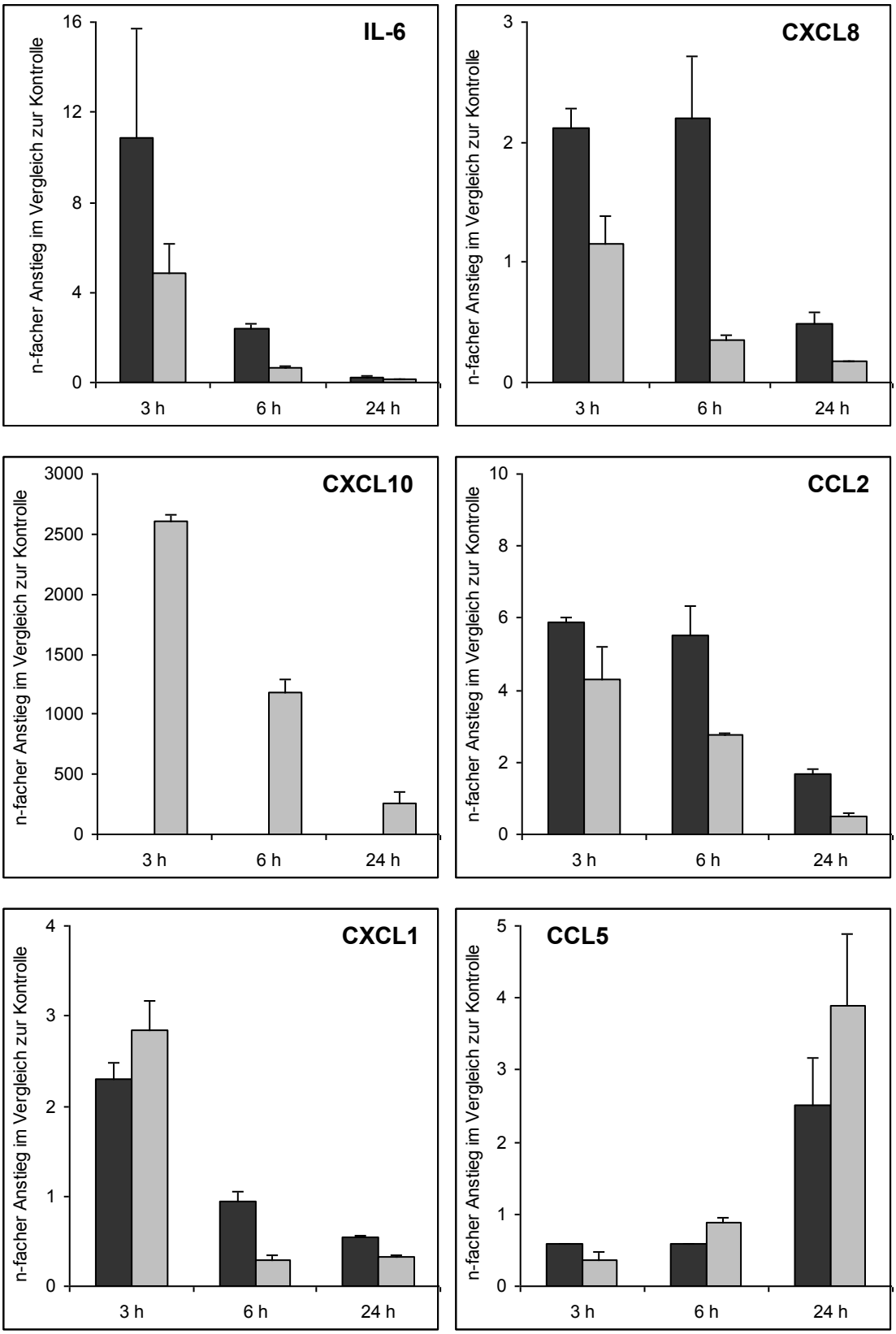


Abb. 10: Transkription IL-6-, CXCL8-, CXCL10-, CCL2-, CXCL1- und CCL5-kodierender Gene in BoMac nach Stimulation mit TNF-α und IFN-γ
BoMac wurden mit humanem rekombinanten TNF-α (schwarz) oder IFN-γ (grau) über verschiedene Zeiträume stimuliert. Total-RNA wurde gewonnen, in cDNA umgeschrieben und mittels Realtime PCR unter Verwendung IL-6-, CXCL8-, CXCL10-, CCL2-, CXCL1- und COX-2-spezifischer Systeme analysiert.

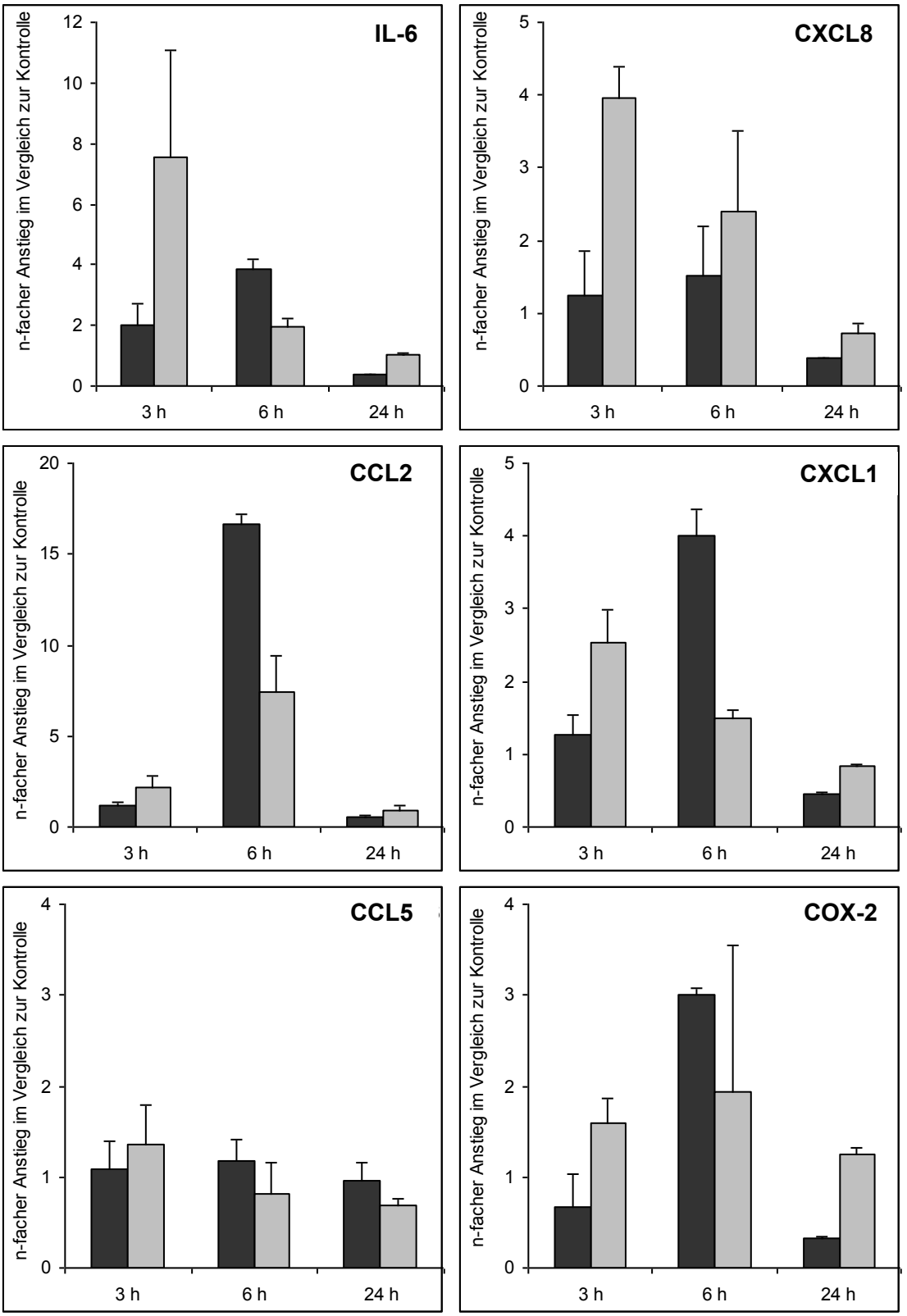


Abb. 11: Transkription IL-6-, CXCL8-, CCL2-, CXCL1-, CCL5- und COX-2-kodierender Gene in BoMac nach Exposition mit *E. bovis*-Sporozoiten und Merozoiten-Antigen
BoMac wurden mit *E. bovis*-Sporozoiten (5 x 10^5/25 cm^2, schwarze Balken) ko-kultiviert oder mit Merozoiten-Antigen (10 µg/ml, graue Balken) stimuliert. Total-RNA wurde gewonnen, in cDNA umgeschrieben und mittels Realtime PCR unter Verwendung IL-6-, CXCL8-, CCL2-, CXCL1-, CCL5- und COX-2-spezifischer Systeme analysiert.

Reihenfolge); nach Konfrontation mit Sporozoiten waren Gentranskripte für CCL2, CXCL1, IL-6 und COX-2 (in absteigender Reihenfolge) vermehrt nachzuweisen (Abb. 11). Die Gentranskription von CCL5, CXCL10, TNFα und iNOS wurde nicht beeinflusst.

Die von beiden Zelltypen gleichermaßen induzierten mRNA-Synthesen von IL-6, CCL2 und CXCL1 sprechen für eine präferentielle Anlockung von Zellen des nicht-adaptativen Immunsystems. Interessanterweise wurden dieselben Moleküle auch in PMN induziert (siehe 4.1.1.3.). Chemo- und Zytokine mit bevorzugter chemotaktischer bzw. aktivierender Wirkung auf Lymphozyten wie CCL5 oder CXCL10 wurden in BoMac dagegen nicht vermehrt nachgewiesen. Verglichen mit Monozyten zeigten BoMac eine gesteigerte Reaktivität sowohl gegenüber Sporozoiten als auch Merozoiten-I-Antigen. Eine generelle Tendenz zur verstärkten Reaktionen gegen Antigen verglichen mit Sporozoiten, wie sie für PMN belegt ist, war hier nicht zu erkennen.

Insgesamt gesehen erschienen BoMac somit zwar als reaktiv gegen *E. bovis*, jedoch zumindest in ihren phagozytischen Eigenschaften relativ verändert gegenüber primären Makrophagen, so dass die Ergebnisse nur bedingt als die *in vivo*-Situation wiederspiegelnd angesehen werden konnten.

Bovine **Makrophagen** reagierten auch auf Ebene der Gentranskription immunmodulatorisch agierender Moleküle differenzierter als Monozyten oder BoMac. Im Gegensatz zu PMN zeichnete sich eine generelle Tendenz dahingehend ab, dass die Ko-Kultur mit vitalen Sporozoiten i. d. R. (mit Ausnahme von IL-12) stärkere Reaktionen hervorrief als die Stimulation mit Merozoiten I-Antigen (vgl. **7.3**).
In Makrophagen waren nach Konfrontation mit Sporozoiten sowohl mRNAs von Zytokinen als auch von Chemokinen und COX-2 erhöht. Insbesondere IL-6 und IFN-γ wurden stark induziert, aber auch die Gene von IL-12 und TNF-α wurden vermehrt transkribiert, während die Stimulation mit Merozoiten I-Antigen nur IL-6- und IL-12-spezifische mRNAs induzierte. Zusätzlich wurden über Sporozoiten auch die Transkriptionen CXCL8-, CXCL1-, CXCL10- und COX-2-kodierender Gene stimuliert (vgl. **7.3**), was insgesamt für eine Makrophagen-vermittelte Anlockung sowohl von Zellen des nicht-adaptativen als auch des adaptativen Immunsystems spricht. Die Transkription IL-10-, CCL2- und CCL5-kodierender Gene blieb bei beiden Behandlungen unbeeinflusst. Insbesondere die Induktion IFN-γ- und IL-12-

kodierender Gene spricht für eine immunmodulierende Rolle der Makrophagen bezüglich nicht-adaptativer Reaktionen, wie z. B. der Aktivierung von NK-Zellen, als auch bezüglich der Induktion einer Th1-orientierten, adaptativen Immunreaktion (vgl. 4.2.4), der bei *Eimeria*-Infektionen grundsätzlich eine Schlüsselrolle zukommt. Zudem können über die vermehrte Synthese von TNF-α dendritische Zellen aktiviert und in Konsequenz eine effektivere Immunreaktion initiiert werden. Eine vermehrte Produktion von TNF-α wurde auch bei aviären Makrophagen beschrieben, die während und nach einer Primärinfektion mit *E. tenella* oder *E. maxima* isoliert wurden [52]. Auch Makrophagenzelllinien aus Hühnern reagierten nach Stimulation mit *E. tenella*-Sporozoiten und -Merozoiten mit einer vermehrten Produktion von TNF [396].

Im Gegensatz zu PMN konnte weder nach Stimulationen mit Parasitenmaterial noch über Behandlungen mit IFN-γ, TNF-α oder LPS iNOS-mRNAs in bovinen Makrophagen nachgewiesen werden. Auch eine Voraktivierung der Makrophagen mit IFN-γ, wie z. B. in Untersuchungen zu *B. bovis* beschrieben [343], führte nicht zur Induktion von iNOS-Transkripten (Daten nicht gezeigt), so dass NO bei der Makrophagen-vermittelten Tötung von *E. bovis*-Sporozoiten eher eine untergeordete Rolle spielen sollte. Im Gegensatz dazu stehen Befunde im aviären System. Die Ko-Kultur von *E. tenella*-, *E. maxima*- und *E. acervulina*-Sporozoiten mit einer aviären Makrophagenzelllinie resultierte hier in einer Steigerung der iNOS-Gentranskription unterschiedlichen Ausmaßes [72]. Zudem waren die Reaktionen der Makrophagen-zelllinie auf Sporozoiten zum Teil speziesspezifisch. So wurde z. B. die Gen-transkription von IFN-γ nur nach Konfrontation mit Sporozoiten der weniger immunogenen, aber pathogeneren Art *E. tenella* induziert; Ko-Kulturen mit *E. maxima*- und *E. acervulina*-Sporozoiten führten dagegen nicht zu einer Steigerung dieser mRNA [72]. Da IFN-γ eine Schlüsselrolle bei der Immunabwehr von Säugetier-Eimerien spielt [259, 303, 304, 326, 334] könnten über die frühe Synthese dieses Zytokins eine Verstärkung der Makrophagenreaktionen über Rückkopplungseffekte bzw. die Aktivierung anderer Makrophagen oder NK-Zellen induziert werden. Zudem könnte die sich entwickelnde adaptative Immunantwort früh in Richtung Th1 dirigiert werden. Der abträgliche Effekt von IFN-γ auf die Replikation von *Eimeria* spp. ist zudem gut belegt [177, 178, 260, 300].

4.1.2.5 Transkription TLR2-, TLR4- und TLR9-kodierender Gene in Makrophagen nach Konfrontation mit E. bovis-Sporozoiten

Die molekulare Natur der Interaktionen zwischen *Eimeria*-Sporozoiten und Makrophagen ist nicht geklärt. Um zu überprüfen, ob TLRs beteiligt sind, wurde auf transkriptioneller Basis die parasiteninduzierte Regulation solcher Moleküle überprüft. Allerdings muss einschränkend erwähnt werden, dass diese Ergebnisse vorläufigen Charakters sind, da ausreichende Tierzahlen bisher nicht untersucht werden konnten.

Eine Beeinflussung von TLR2-mRNAs nach Konfrontation mit Sporozoiten oder Stimulation mit Merozoiten I-Antigen war nicht gegeben (Abb. 12). Bei anderen Apikomplexa wie *T. gondii* oder *C. parvum* wird der Interaktion der Parasiten mit diesem TLR eine Bedeutung zugemessen [61, 79, 83, 244], wobei die Untersuchungen allerdings nur z. T. auf transkriptioneller Ebene durchgeführt wurden und nur im Falle von *T. gondii* Makrophagen als Wirtszellen verwendet wurden. Bei *T. gondii*-infizierten PMN wird über TLR2-Vermittlung die MyD88-abhängige Synthese von CCL2 beeinflusst [83], d. h. eines Chemokins, dessen Gentranskription auch nach Konfrontation mit *E. bovis* Sporozoiten induziert wurde (vgl. **7.1**).

Die Gentranskripte von TLR4 wurden dagegen nach Ko-Kultur mit Sporozoiten, nicht jedoch über Merozoiten I-Antigen gesteigert (Abb. 12). Dies weist auf eine stadienspezifische Reaktion hin, wobei jedoch einschränkend erwähnt werden muss, dass über die Homogenisierung der Merozoiten I entsprechende Liganden evtl. zerstört wurden. Bei *C. parvum*-infizierten humanen Cholangiozyten wurden nach Infektion vermehrt TLR2- und TLR4-Moleküle an die Parasit-Wirtszell-Grenzfläche verbracht, was letztendlich in einer über Aktivierung von NF-κB bewerkstelligten Zyto- und Chemokinsynthese der Wirtszelle resultierte [61]. Allerdings waren weder die Anheftung noch die Invasion der Wirtszellen dabei abhängig von TLR2 oder TLR4.

Im Falle von *T. gondii* wird die Rolle von TLR4 z. T. kontrovers diskutiert. Während die nahezu unveränderte Empfänglichkeit von TLR4-knock-out-Mäusen gegenüber *T. gondii* nicht auf eine bedeutene Rolle dieses Rezeptors bei der *T. gondii*-Erkennung schließen ließ [244, 311], wiesen andere Untersuchungen durchaus auf eine Beteiligung von TLR4 in der Generierung nicht-adaptativer Immunreaktionen gegen *T. gondii* hin [79, 140].

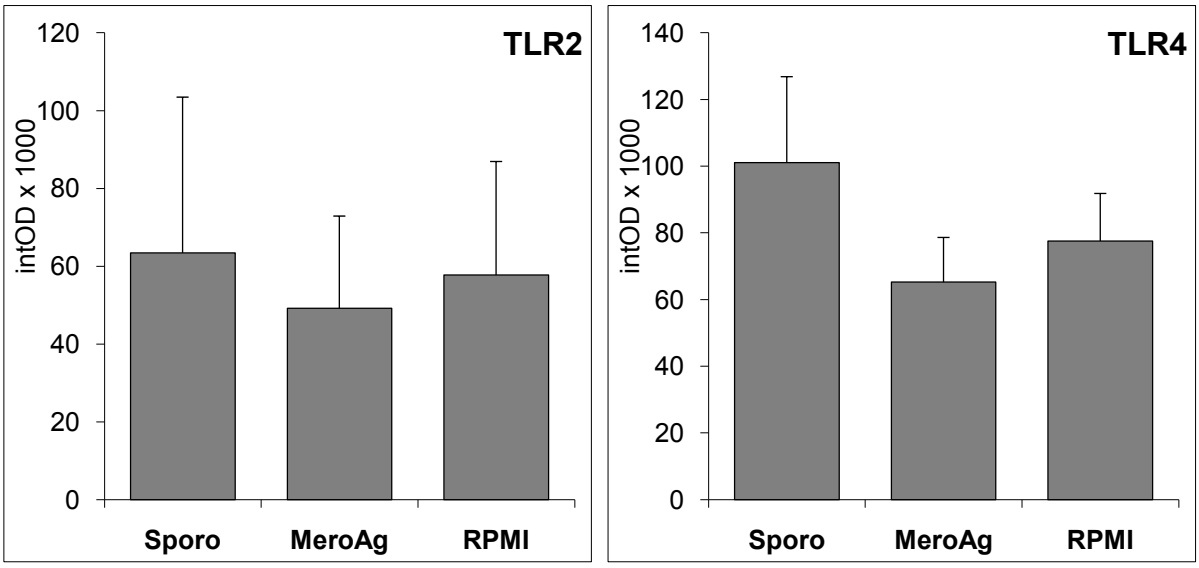


Abb. 12: Transkription TLR2- und TLR4-kodierender Gene in Makrophagen nach Konfrontation mit *E. bovis* Sporozoiten oder Merozoiten-Antigen
Bovine Makrophagen wurden mit *E. bovis*-Sporozoiten (= Sporo) ko-kultiviert oder mit Merozoiten I-Antigen (MeroAg, 10 µg/ml) stimuliert. Total-RNA wurde gewonnen, in cDNA umgeschrieben und mittels konventioneller, semiquantitativer RT-PCR unter Verwendung TLR2- und TLR4-spezifischer Primersysteme analysiert.
(intOD = integrierte optische Dichte)

Unter keiner der gewählten Bedingungen wurde cDNA für TLR9 nachgewiesen. Während gemäß Yarovinsky [390] *T. gondii* keine Aktivierung von TLR9 induziert, weisen andere Untersuchungen mit TLR9-knock-out-Mäusen auf eine direkte Beteiligung dieses Rezeptors bei der *T. gondii*-Erkennung hin [169, 233], wie es auch für *B. bovis* [330] oder *P. falciparum* [66] beschrieben ist.

Sequenzen zur Erfassung des bei *T. gondii*-Infektionen eine wichtige Rolle spielenden TLR11 standen leider nicht zur Verfügung. TLR11 kontrolliert IL-12-vermittelte Abwehrreaktionen von dentritischen Zellen [392]. Dies erscheint insofern interessant, als Makrophagen mit der vermehrten Gentranskription dieses Zytokins sowohl nach Ko-Kultur mit Sporozoiten als auch nach Stimulation mit Merozoiten I-Antigen reagierten (vgl. **7.3**), was auf vergleichbare Mechanismen hinweisen könnte.

4.1.3 Natürliche Killerzellen

4.1.3.1 NK-Zelldichte im peripheren Blut E. bovis-infizierter Kälber

Um eine infektionsbedingte Beeinflussung der NK-Zelldichte im peripheren Blut zu überprüfen, wurden von experimentell *E. bovis*-infizierten Tieren Blutproben zu unterschiedlichen Zeitpunkten nach Infektion auf NK-Zelldichten überprüft.

Interessanterweise kam es jeweils zu den Zeitpunkten, zu denen unterschiedliche *Eimeria*-Stadien extrazellulär anzutreffen sind (Tag 2: Sporozoiten, Tag 15: Merozoiten I, Tag 23: Oozysten), zu einem mehr oder weniger stark ausgeprägten Absinken der NK-Zelldichte im peripheren Blut (Abb. 13). Diese Beobachtung spricht für eine Wanderung dieser Zellpopulation aus dem peripheren Blut in das infizierte Gewebe, wie es auch für *N. caninum*-infizierte Rinder aufgrund ähnlicher Beobachtungen früh nach Infektion diskutiert wurde [174]. Bei *Eimeria*-infizierten Hühnern wurde ebenfalls kurz nach Infektion ein Abfall der NK-Zell-Aktivität GM1-positiver Zellen aus intraepithelialen Lymphozyten (IEL) beobachtet [198]. Die Autoren sehen die Ursache dafür entweder in suppressiven Effekten seitens der Eimerien oder in der hemmenden Wirkung von IEL auf NK-Zellen, wie es z. B. im murinen System beschrieben wurde [242].

Die Anlockung und Aktivierung der NK-Zellen wird über Chemokine und Zytokine bewirkt [174, 28, 205, 13]. Die in Makrophagen vermehrt nachgewiesene Gentranskription von CXCL10 und IL-12 (siehe **7.3**) nach Konfrontation mit Sporozoiten ist auch in diesem Zusammenhang zu bewerten, da CXCL-10 chemotaktisch [243, 186] und IL-12 aktivierend auf NK-Zellen wirken [28, 349, 364, 362, 361, 360].

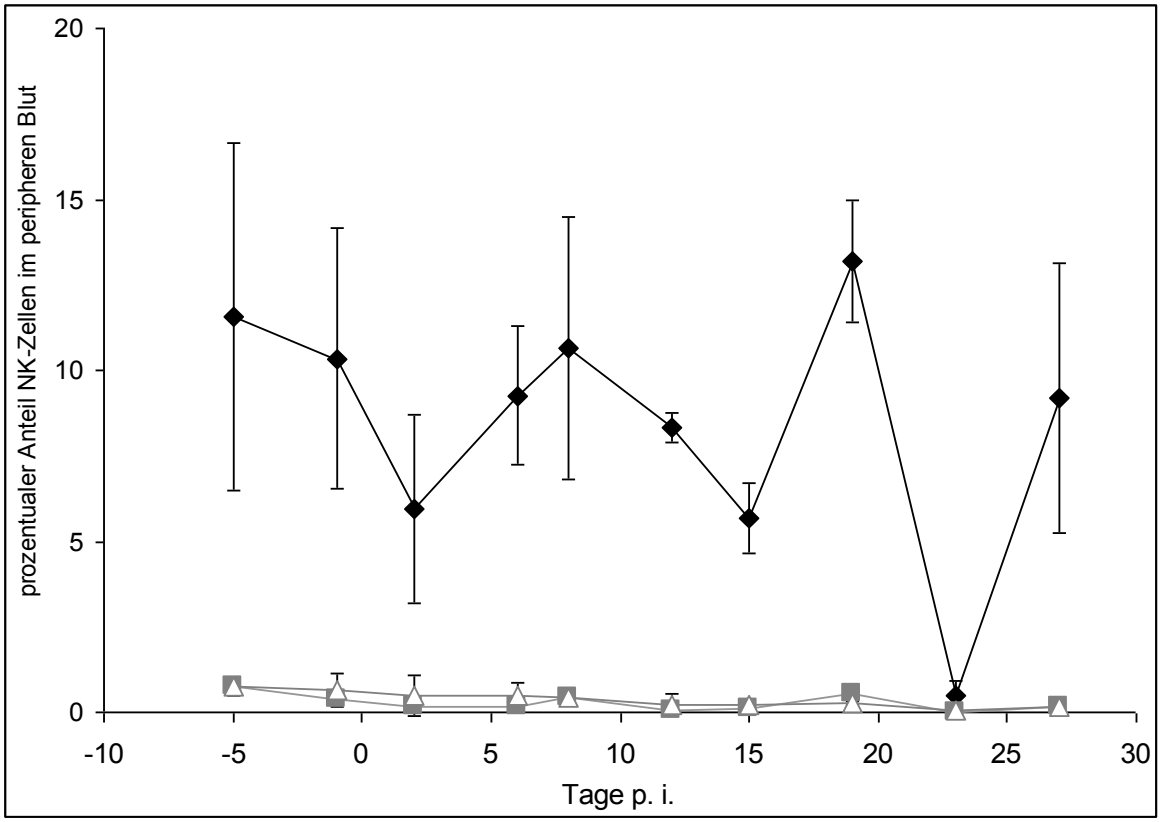


Abb. 13: NK-Zellen im peripheren Blut *E. bovis*-infizierter Kälber. Blutproben *E. bovis*-infizierter Kälber (n = 3) wurden in regelmäßigen Abständen auf die Dichte von NK-Zellen durchflusszytometrisch überprüft. NK-Zellen (schwarze Rauten), Erstantikörperkontrolle (graue Quarder), Zweitantikörperkontrolle (offene Dreiecke)

4.1.3.2 NK-Zell-vermittelte Elimination von E. bovis-Sporozoiten in vitro

Die *in vitro*-Konfrontation IL-2-aktivierter NK-Zellen mit vitalen *E. bovis*-Sporozoiten führte zu einer deutlichen Reduktion der extrazellulären Sporozoiten (Abb. 14), was eine direkte, wirtszellunabhängige Aktivierung der NK-Zellen nahelegt. Dabei reagierten die NK-Zellen der Einzeltiere relativ gleichförmig und es kam zu einer mittleren Sporozoitenreduktion von 50,6 ± 4,6%. Da ein simultaner Anstieg toter extrazellulärer Sporozoiten nicht zu beobachten war, muss eine vollkommene Lyse der Parasiten vermutet werden.

Die Vorbehandlung der NK-Zellen mit Concanamycin A zur Hemmung des perforinabhängigen Effektorwegs beeinflusste die Reaktionen jedoch nicht (Daten nicht gezeigt). Damit bleibt unklar, über welchen Mechanismus die Parasiten eliminiert wurden. Direkte Interaktionen zwischen NK-Zellen und Stadien apikomplexer Parasiten sind auch für *T. gondii*- und *N. caninum*-Tachyzoiten belegt

[36, 138]. Allerdings konnte hier zumindest im letzteren Falle eine Beteiligung der perforinvermittelte Abtötung gezeigt werden [36].

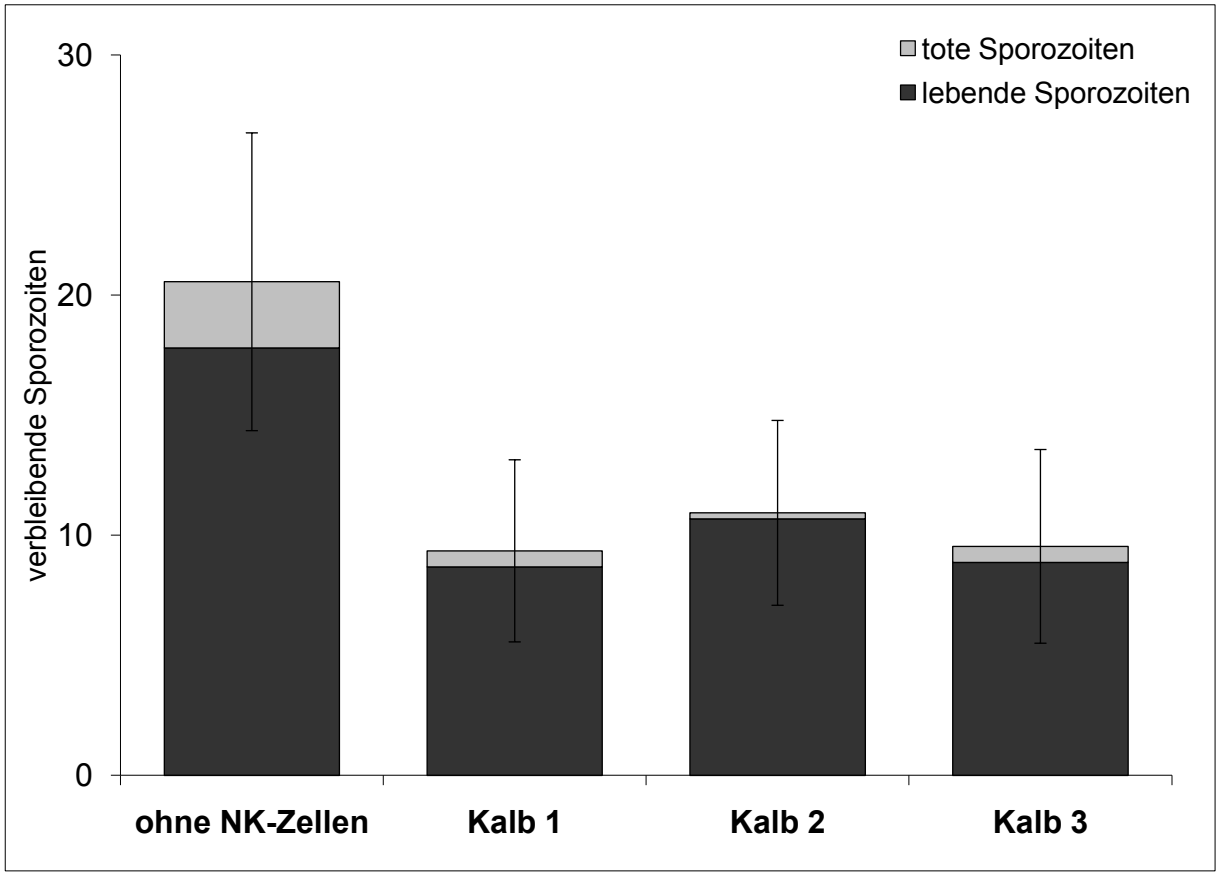


Abb. 14: NK-Zell-vermittelte *in vitro* Elimination von Sporozoiten. IL-2-aktivierte, bovine NK-Zellen wurden mit vitalen *E. bovis*-Sporozoiten *in vitro* konfrontiert. Nach 4 h wurde die Anzahl extrazellulär verbleibender lebender (dunkelgrauer Anteil der Balken) und toter (hellgrauer Anteil der Balken) Sporozoiten bestimmt.

Im Gegensatz zu Befunden bei *N. caninum*, die eine IL-12-unabhängige Steigerung der IFN-γ-Synthese in IL-2-aktivierten NK-Zellen nach direktem Kontakt mit Tachyzoiten zeigten [36], konnten wir in Abwesenheit von IL-12 keinerlei Induktion dieses Zytokins nach Ko-Kultur mit *E. bovis*-Sporozoiten nachweisen (bovines IL-12 stand uns leider nicht zur Verfügung). Im Gegensatz dazu erschien sowohl die Transkription des IFN-γ-Gens als auch die Freisetzung des Proteins gehemmt (Abb. 15). Ob Sporozoiten dabei einen direkten suppressiven Einfluss auf NK-Zellen ausüben, wie es in ähnlicher Weise für andere Parasiten beschrieben wird [194] oder ob die Anwesenheit von IL-12 essentiell ist, konnte in diesen Versuchen nicht geklärt werden.

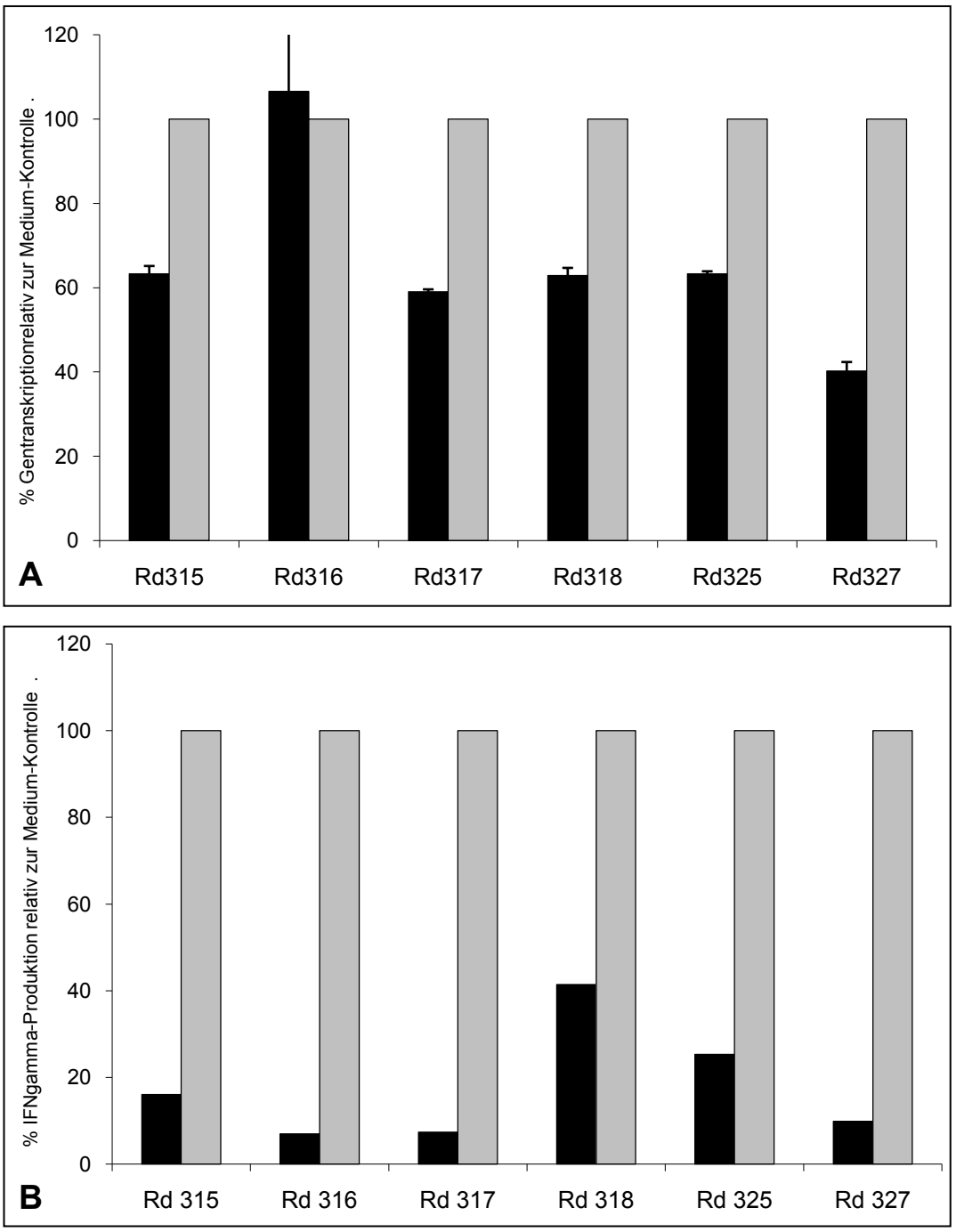


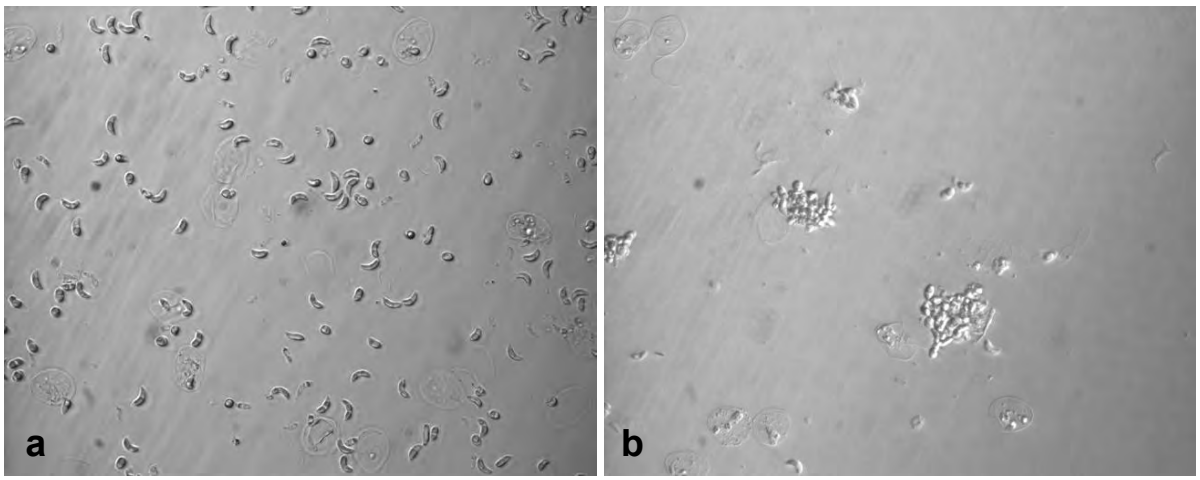
Abb. 15: IFN-γ-Gentranskription (A) in NK-Zellen und Sekretion von IFN-γ (B) nach Ko-Kultur mit *E. bovis*-Sporozoiten. Bovine, IL-2-aktivierte NK-Zellen wurden mit vitalen Sporozoiten (schwarze Balken) oder Medium (= 100%, graue Balken) in Anwesenheit von IL-2 ko-kultiviert. Nach Inkubation (3 h für Gentranskription, 24 h für Zellkultur-Überstände) wurde Total-RNA gewonnen, in cDNA umgeschrieben und mittels IFN-γ-spezifischer Realtime PCR analysiert (A), sowie IFN-γ in Zellkulturüberständen mittels eines kommerziell erhältlichen Tests (bovine IFN-γ EASIA, Biosource) bestimmt (B).

4.2 Adaptative Immunreaktionen bei Primär- und Belastungsinfektionen mit *E. bovis*

4.2.1 *Einfluss von Immunserum auf E. bovis-Sporozoiten in vitro*

Wie bereits bezüglich PMN und Makrophagen gezeigt, spielt Immunserum bei über *E. bovis*-Sporozoiten induzierten ADCC-Reaktionen eine wichtige Rolle hinsichtlich der effizienten Elimination des Parasiten (siehe **7.1**, **7.3**).

Immunserum war zudem im immunzellfreien System in der Lage, Sporozoiten zu agglutinieren als auch zu einem gewissen Grade zu lysieren (siehe Abb. 16 und Tabelle 2). Dabei war die Agglutination von Sporozoiten zwar unabhängig von Komplementfaktoren, aber abhängig von spezifischen IgG-Molekülen (Tab. 2). Entsprechend waren solche Reaktionen nach Zusatz von Kolostrum, nicht jedoch nach Inkubation in Neonatenserum zu messen (Tab. 2).



	Agglutinat.
serumfrei	-
Immunserum[a]	+
Immunserum, Hitze-inaktiviert[b]	++
Immunserum, IgG-depletiert[c]	-
Neonatenserum[d]	-
Kolostrum	++

[a] Serumentnahme: 3 Wochen nach Reinfektion von Kälbern
[b] Hitzeinaktivierung: 30 min, 56°C
[c] Depletion der IgG-Moleküle über Protein G-Säulen
[d] die Blutentnahme erfolgte vor der ersten Kolostrumaufnahme

Abb. 16 /Tab. 2: Agglutination von *E. bovis*- Sporozoiten über Seren
Vitale *E. bovis*-Sporozoiten wurden mit verschiedenen Seren (Tab. 2, 1:200) über drei Stunden inkubiert und anschließend die Agglutination der Parasiten mikroskopisch beurteilt.
(a) serumfrei
(b) Immunserum

Im Gegensatz dazu war die Serum-bedingte Lyse von Sporozoiten sowohl abhängig von Komplement als auch von spezifischen IgG-Antikörpern (Abb. 17), so dass hier eine klassische Aktivierung des Komplementsystems mit nachfolgender Lyse der Parasiten über die Ausbildung eines „membrane attacking complex“ (MAC) nahe liegt. In Übereinstimmung induzieren weder Hitze-inaktiviertes oder IgG-depletiertes Immunserum noch Neonatenserum eine vermehrte Lyse der Parasiten (Abb. 17). Der erhöhte Anteil toter Sporozoiten in Proben unter Zusatz von Immunserum mag über bereits abgetötete, aber noch nicht komplett lysierte Sporozoiten zustande kommen.

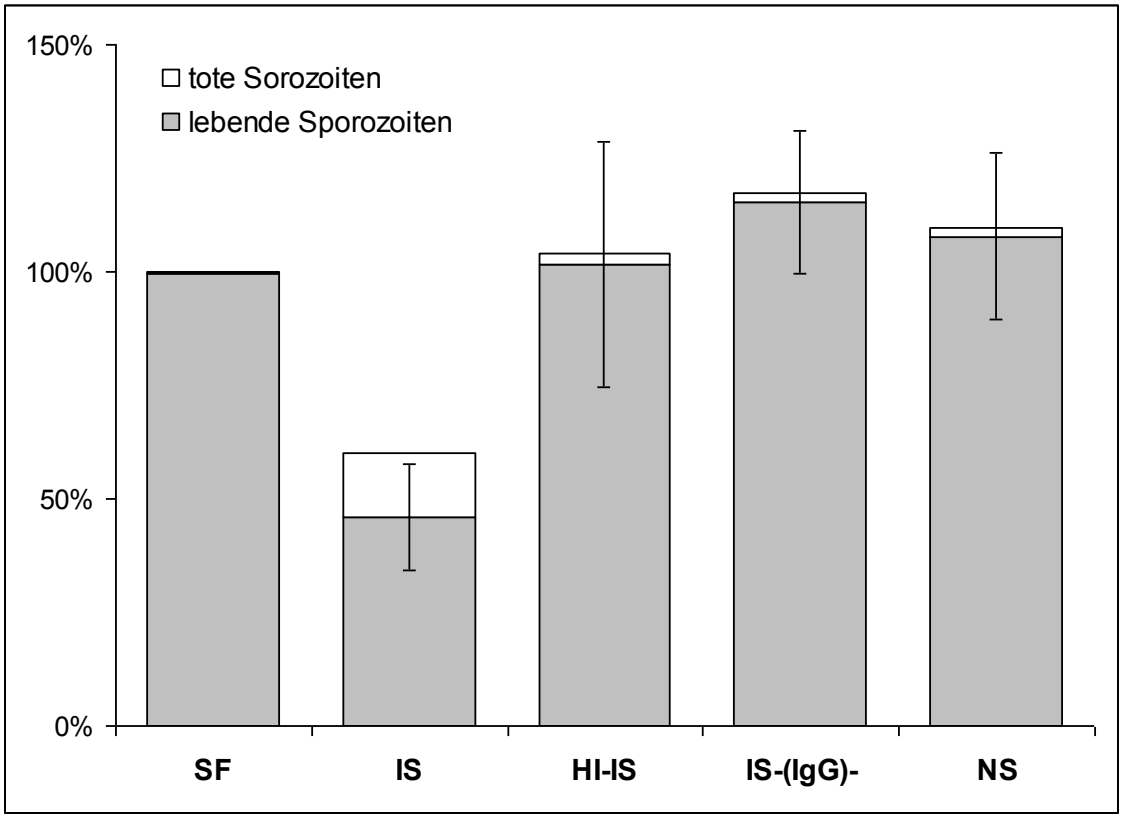


Abb. 17: Effekte von Serumkomponenten auf *E. bovis*-Sporozoiten
Vitale *E. bovis*-Sporozoiten wurden in Abwesenheit von Serum (serumfrei = SF) oder unter Zusatz von Immunserum (IS), Hitze-inaktiviertem Immunserum (HI-IS), IgG-depletiertem Immunserum (IS-(IgG)-) oder Neonatenserum (NS) über 3 h inkubiert und anschließend die Anzahl verbliebener Parasiten bestimmt.

Der Einfluss der humoralen Immunantwort auf den Ausgang einer *E. bovis*-Infektion wird z. T. kontrovers diskutiert. Es steht außer Frage, dass spezifische Antikörper im Verlauf der Infektion gebildet werden, diese scheinen jedoch nicht protektiv zu sein [110]. Nach unseren Untersuchungen könnten sie jedoch zumindest einen Beitrag zur effizienten Elimination von Sporozoiten leisten. Diese Stadien wären entweder über kolostrale Antikörper bei Infektionen sehr junger Kälber angreifbar oder im späteren Verlauf der Infektion nach erneutem Verlassen der Wirtszellen (wie wir es in

der Kultur bis zu 3 Wochen nach Infektion beobachten) über kälbergenerierte Antikörper erfassbar. Insbesondere nach Reinfektionen sollten spezifische Antikörper für effiziente ADCC-Reaktionen zur Verfügung stehen [110]. Dass spezifische Antikörper grundsätzlich direkt mit *Eimeria*-Stadien interagieren können und im Zusammenhang mit Immunzellen und/oder Komplement eine Elimination der Parasiten verstärken, steht außer Frage und wurde mehrfach belegt [14, 25, 24, 388].

4.2.2 Antigenspezifische T-Zellproliferation von PBMC und Lymphknoten-zellen

Die Primärinfektion mit *E. bovis* war in Übereinstimmung mit Hermosilla et al. [144] über eine kurzzeitig erhöhte antigenspezifische Proliferationsleistung von PBMC in der Präpatenz charakterisiert (vgl. **7.5**). Kurzfristige proliferative Aktivitäten von Lymphozyten wurden auch im Zusammenhang mit *E. vermiformis*-Infektionen der BALB/c-Maus [382] und *E. tenella*-Infektionen des Huhnes [40] beschrieben, wobei diese jedoch nur im ersteren Falle während der Präpatenz zu messen waren.

Die antigenspezifische Proliferation der PBMC war insgesamt innerhalb der Präpatenz auf die Tage 8-15 p. i. beschränkt mit maximaler Aktivität am 8. Tag p. i. und anschließend stark abfallender Reaktivität (vgl. **7.5**). Diese Reaktionen liefen offensichtlich zeitlich synchron mit dem Beginn der ersten Merogonie des Parasiten ab, d. h. es bestand ein enger Zusammenhang mit der frühen Proliferation des Erregers. Interessanterweise treten ebenfalls ab diesem Zeitpunkt erstmalig parasitenspezifische Antigene auf der Oberfläche *E. bovis*-infizierter Endothelzellen auf [15]. Unter Berücksichtigung der Tatsache, dass Endothelzellen über Antigen-präsentierende Eigenschaften verfügen [20, 35, 176, 379] und T-Zellen antigenabhängig aktivieren können [102, 268, 281], lässt sich eine Meront I-vermittelte Induktion von T-Zellaktivitäten vermuten.

Die in der Peripherie erhöhte Proliferationsleistung von Lymphozyten an Tag 8 p. i. konnte auch lokal über Untersuchungen von aus drainierenden Lymphknoten stammenden Zellen bestätigt werden (vgl. **7.6**) und überschnitt sich zudem zeitlich mit einer gesteigerten, antigenspezifischen Synthese von IFN-γ (vgl. 4.2.4) so dass die lymphozytären Reaktionsmuster insgesamt für eine Th1-dominierte Immunreaktion während der Präpatenz sprechen.

Der sich an Tag 8 p. i. anschließende, fast vollständige Rückgang der Zellreaktivität kann auch als parasiteninduzierter Mechanismus, der die lange, über 15 Tage dauernde Entwicklung von *E. bovis* in der Wirtszelle ermöglicht, interpretiert werden. Bei *E. intestinalis*-Infektionen des Kaninchens wird ebenfalls eine zeitgleich beginnende, antigenspezifische Lymphozytenproliferation und Merogonie beschrieben, allerdings war bei dieser sich durch eine kurze Merogonie I auszeichnenden Infektion die Proliferationsaktivität im weiteren Verlauf noch anhaltend gesteigert [278].

Die unspezifische Reaktivität der PBMC auf Concanavalin A variierte unregelmäßig im Verlauf der Infektion, schien insgesamt aber nicht über *E. bovis* beeinflusst zu sein (Daten nicht gezeigt). Dies entsprach Beobachtungen von Hermosilla et al. [144] und Hughes et al. [158] im Rahmen von *E. bovis*-Infektionen sowie von Renaux et al. [278] bei *E. intestinals*-infizierten Kaninchen. Somit sprechen die vorliegenden Ergebnisse nach Con A-Stimulation gegen eine unspezifische Immunsuppression als Ursache des Rückgangs der Proliferationsleistung in der Präpatenz.

Nach Belastungsinfektion blieben proliferative Aktivitäten sowohl peripherer als auch lokal in Lymphknoten befindlicher Lymphozyten aus (vgl. **7.5**). Diese Befunde entsprechen solchen zur fehlenden oder verminderten Lymphozytenproliferation bei Reinfektionen mit *E. papillata* [319] oder *E. vermiformis* [382] in der Maus. Im Gegensatz dazu zeigten immune *E. intestinalis*-infizierte Kaninchen eine signifikant gesteigerte Proliferationsleistung von Lymphozyten nach Stimulation mit Parasitenantigen [278]. Zu berücksichtigen ist hierbei insgesamt, dass nach den meisten bisherigen Untersuchungen bei *Eimeria* spp. bereits die frühen Stadien vor bzw. nach Befall der Wirtszelle mit Sporozoiten die Zielstadien protektiver Immunreaktionen sind [296, 325]. Mit deren zeitiger Elimination könnte die Entwicklung der nach unseren Befunden die T-Zell-Reaktionen vermittelnden frühen Meronten I verhindert worden und in Folge eine T-Zell-Proliferation ausgeblieben sein.

4.2.3 Phänotypisierung peripher zirkulierender und lokal in der Darmmukosa befindlicher T-Zellen

Die Primärinfektion mit *E. bovis* zeichnete sich in Übereinstimmung mit Hermosilla et al. [144] durch eine Expansion des Anteils $CD4^+$-T-Zellen im peripheren Blut aus (vgl.

7.5). Diese gegen Ende der Präpatenz einsetzende Vermehrung der $CD4^+$-T-Zellen blieb über den Verlauf der Primärinfektion erhalten, wurde jedoch mit erfolgter Belastungsinfektion nicht weiter beeinflusst. Entsprechend und in Übereinstimmung mit Daten bei anderen *Eimeria*-Infektionen [307, 372, 325] kam es auch zu einer Infiltration mit $CD4^+$-T-Zellen sowohl früh nach Infektion mit Tag 8 p. i. in der Ileum- als auch nach Abschluss der Primärinfektion in der Kolonschleimhaut.

Im Gegensatz zu Hermosilla et al. [144] wurde während der Primärinfektion keine Expansion des Anteils $CD8^+$-T-Zellen im Blut gemessen. Stattdessen erschienen $CD8^+$-T-Zellen während der Präpatenz im Vergleich zu nicht-infizierten Kontrolltieren im peripheren Blut vermindert (vgl. **7.5**). Auch in der Ileummukosa kam es während der Präpatenz nicht zu einer signifikanten Steigerung der Anzahl $CD8^+$-T-Zellen. Allerdings akkumulierte dieser Zelltyp in der späten Postpatenz (40 Tage p. i.) in der Kolonschleimhaut, so dass die Primärinfektion insgesamt von der lokal früh einsetzenden Dominanz von $CD4^+$-T-Zellen sowie der erst nach Ablauf der Infektion einsetzenden Reaktivität $CD8^+$-T-Zellen gekennzeichnet ist.

Die Belastungsinfektion mit *E. bovis* führte nicht zu einer Verschiebung der Anteile unterschiedlicher T-Zell-Phänotypen ($CD4^+$-, $CD8^+$-, $\gamma\delta TCR^+$-T-Zellen) im peripheren Blut (vgl. **7.5**). Der Einfluss der Belastungsinfektion manifestierte sich stattdessen über die gesteigerte Infiltration parasitierter Darmabschnitte sowohl mit $CD4^+$- als auch mit $CD8^+$-T-Zellen (vgl. **7.5**). Auch hier erfolgte die Akkumulation von $CD4^+$-T-Zellen zeitlich etwas eher als die von $CD8^+$-T-Zellen. Bei anderen *Eimeria*-Infektionen deuten nur wenige Befunde auf eine protektive Rolle von $CD4^+$-T-Zellen bei Reinfektionen hin. Während Depletionsstudien bei aviären und murinen *Eimeria*-Infektionen keinen Einfluss der $CD4^+$-T-Zellen auf die Immunitätsbildung aufzeigten [291, 367], wiesen Daten sowohl von Smith und Hayday [333] als auch von Schito et al. [318] diesem Zelltyp eine protektive Funktion zu. Zu bedenken ist in dieser Hinsicht, dass $CD4^+$-T-Zellen ebenfalls über zytotoxische Effektormechanismen verfügen, denen z. B. bei Infektionen mit anderen Apikomplexa wie *N. caninum* eine größere Bedeutung zugeschrieben wird als denen von $CD8^+$-T-Zellen [342].

Die Infiltration der Darmschleimhaut mit $CD8^+$-T-Zellen nach erfolgter Belastungsinfektion entspricht Befunden bei *E. separata*-infizierten Ratten [325, 327] oder *E. acervulina*-infizierten Hühnern [366] und dürfte im Zusammenhang mit der Immunelimination der Parasiten zu sehen sein. Entsprechend konnte für andere

Eimeria spp.-Infektionen über Depletionsstudien zumindest teilprotektive Funktionen der $CD8^+$-T-Zellen nach Belastungsinfektionen gezeigt werden [291, 367]. Der tatsächliche Nachweis der in diversen Arbeiten diskutierten und gegen *Eimeria*-infizierte Wirtszellen gerichteten, zytotoxischen Funktionsweise von $CD8^+$ T-Zellen wurde jedoch bisher - im Gegensatz zu z. B. *T. gondii* [165, 91, 170] - bei *Eimeria*-Infektionen noch nicht geführt.

Neben der oben erwähnten Infiltration parasitierter Darmabschnitte mit $CD4^+$-T-Zellen kam es während der Primärinfektion, im Gegensatz zur Belastungsinfektion, auch zu einer Einwanderung von $\gamma\delta TCR^+$-Zellen in die Ileum- und Kolonmukosa (vgl. **7.5**), was Beobachtungen bei aviären und murinen *Eimeria*-Infektionen entspricht [199, 307, 111]. Dabei muss die Akkumulation der $\gamma\delta TCR^+$-Zellen nicht unbedingt allein mit Schutzeffekten in Zusammenhang gebracht werden. Mit ihrer bevorzugten Lokalisation in Schleimhäuten werden sie zwar grundsätzlich als erste Abwehrkräfte in der Infektabwehr ("sentinel function", vgl. [77]) gesehen, doch verneinten Untersuchungen unter Verwendung $\gamma\delta TCR^+$-zelldepletierter oder -defizienter Tiere eine protektive Rolle dieser Immunzellen bezüglich parasitologischer Parameter [280, 290]. Vielmehr zeigten sich dort protektive Funktionen in Bezug auf die klinischen Erscheinungen, so dass Roberts et al. [280] von einer regulatorischen Funktion im Ablauf immunpathologischer Vorgänge ausgehen.

4.2.4 Antigeninduzierte Synthese Th1- und Th2-assoziierter Zytokine

Die Primärinfektion mit *E. bovis* zeichnet sich durch eine Abfolge Th1- und Th2-orientierter Immunreaktionen aus. Während die Präpatenz sowohl über einen starken Anstieg der IFN-γ-Gentranskription als auch über die moderat erhöhten Werte für IL-2 einer Th1-orientierten Immunreaktion zuzuordnen ist, folgen mit verstärkter Transkription des IL-4-kodierenden Gens zu Beginn der Patenz Th2-assoziierte Reaktionen (vgl. **7.4**).

Die Steigerung von IFN-γ in der Präpatenz konnte gleichermaßen in Antigen-stimulierten PBMC, über Biopsien gewonnenen Lymphknotenzellen (*Lnn. ileocaecales*) und PBMC-Überständen gemessen werden. Dabei zeigten an Tag 8 p. i. sowohl $CD4^+$- als auch $CD8^+$-T-Zellen eine verstärkte Transkription IFN-γ-kodierender Gene, während IL-2 maßgeblich von $CD4^+$-T-Zellen produziert wurde (vgl. **7.4**). Auch die unter 4.2.2 und von Hermosilla et al. [144] beschriebene T-

Zellproliferation fand zeitgleich statt, so dass typische Kriterien eines Th1-assoziierten Reaktionsmusters erfüllt sind. Die von Fiege et al. [110] beschriebene Induktion parasitenspezifischer IgG2-Antikörper passt ebenfalls zu den Daten, da dieser Isotyp im bovinen System über IFN-γ positiv reguliert wird [104].

Die gesteigerte IFN-γ-Synthese während der Primärinfektionen stimmt mit diversen Studien zu anderen Kokzidiosen überein [326, 334, 303, 304] und scheint eine grundsätzliche Schlüsselrolle bei der Kontrolle der Primärinfektion inne zu haben (zur Übersicht, siehe [260]). Dabei wirkt IFN-γ auf unterschiedliche Weisen. Als ein wichtiger Effektormechanismus ist die Aktivierung von Makrophagen anzusehen. Dieser Zelltyp akkumuliert nach *E. bovis*-Infektionen in parasitierter Darmschleimhaut und ist befähigt Sporozoiten zu eliminieren (vgl. **7.3**). Makrophagen ihrerseits könnten neben phagozytischen Aktivitäten mit der Generation von Sauerstoffradikalen zusätzlich abtötende Effekte auf Eimerien ausüben [154, 231]. Neben diesen Mechanismen ist die abträgliche Wirkung von IFN-γ auf die intrazellulär ablaufende Replikation von *Eimeria* spp. gut belegt [154, 177, 178, 300, 260], wobei die Synthese von intrazellulärem NO als wichtigster Effektormechanismus angesehen wird [260]. Sowohl die unter 4.2.5 gezeigte Aktivierung von endothelialen (Wirts)Zellen als auch die negative Regulation Th2-assoziierter Reaktionen über IFN-γ spielen vermutlich zudem eine Rolle bei den sich nachfolgend entwickelnden Immunreaktionen.

Neben antigenspezifischen Effekten auf die IFN-γ-Synthese kommt es auch zur unspezifischen, aber infektionsbedingten Beeinflussung dieses Zytokins. Beginnend und maximal ausgeprägt in der frühen Präpatenz, aber auch darüber hinaus anhaltend, wurden über *E. bovis*-Infektionen bei mit Mitogen stimulierten (PMA + Ionomycin) PBMC sowohl der Anteil IFN-γ-positiver, als auch deren Gehalt an IFN-γ gesteigert (vgl. **7.4**). Eine alleinige oder zusätzliche Stimulation mit Parasitenantigen beeinflusste diese Reaktion nicht. Diese Beobachtungen wurden auch über einen höheren Gehalt an IFN-γ in Überständen von Mediumkontrollen an den Tage 8 und 12 p. i. bestätigt (vgl. **7.4**).

Die Daten weisen insgesamt auf einen infektionsbedingten, aber antigen-unspezifischen Mechanismus hin, der vermutlich in einer grundsätzlich gesteigerten T-Zellreaktivität begründet ist, da ähnliche Effekte auch für IL-4-positive Zellen zu beobachten waren. Die Beeinflussung der Reaktivität scheint jedoch insgesamt auf

die Zytokinsynthese beschränkt zu sein, da die unspezifisch über Concanavalin A stimulierte T-Zellproliferation in Übereinstimmung mit Hermosilla et al. [144] und Hughes et al. [158] nicht verändert war. Bisher sind ähnliche Effekte bei anderen *Eimeria*-Infektionen nicht beschrieben worden. Bei Berücksichtigung der vielfältigen Wirkungen von IFN-γ, insbesondere jedoch seiner anti-replikativen Effekte, liegt eine maßgebliche Beeinflussung des Ausmaßes der Infektion über diesen unspezifischen Mechanismus nahe.

Th2-assoziierte Zytokine wurden mit IL-4 im Gegensatz zu Daten von Hermosilla et al. [144] ebenfalls über *E. bovis*-Primärinfektionen in antigenabhängiger Weise beeinflusst. Übereinstimmend wird eine z. T. zeitversetzte Induktion sowohl Th1- als auch Th2-spezifischer Zytokine auch bei anderen *Eimeria*-Infektionen beschrieben [319, 326, 333, 382]. Verglichen mit der Synthese Th1-typischer Zytokine erfolgte die Induktion von IL-4 nach Infektion mit *E. bovis* zeitlich verzögert und fand erst in der frühen Patenz seinen Höhepunkt (vgl. **7.4**). Die kurzfristige Überlappung Th1- und Th2-spezifischer Reaktionen könnte die Koexistenz von IgG1- und IgG2-Isotypen in *E. bovis*-infizierten Tieren erklären. Da sich zudem ein infektionsbedingter, antigenunabhängiger Effekt auf Anzahl und Gehalt IL-4-positiver PBMC ergab, erklären sich die bei Fiege et al. [110] beschriebenen, parasitenspezifischen, über IL-4 im bovinen System positiv regulierten IgG2-Spiegel (vgl. [104]), die offensichtlich aber nicht zur Protektion der Tiere beitragen. Als ebenfalls nicht relevant bei der Terminierung der Primärinfektion erwies sich IL-4 in Versuchen mit *E. vermiformis*-infizierten knock-out-Mäusen, da die Patenz in diesen Tieren nicht beeinflusst war [333].

Die im Verlauf der Infektion ausbleibende Steigerung der Gentranskription von IL-10, das im wesentlichen Maß als Th2-assoziiert angesehen wird (u. a. [239]) sprechen gegen eine entscheidende, immunregulatorische Rolle dieses Zytokins. Unabhängig davon kann nicht ausgeschlossen werden dass die IL-10-Synthese über die dominanten Th1-Reaktionen gehemmt wird. In diesem Zusammenhang erscheint interessant, dass Merozoitenantigen auch unspezifisch in PBMC nicht-infizierter Tiere eine, wenn auch moderate IFN-γ-Gentranskription induziert (Taubert, unveröffentlichte Ergebnisse), die ihrerseits für eine Herunterregulierung Th2-assoziierter Zytokine wie IL-10 sorgen könnte.

Da neben IL-10 auch TGFβ zu keinem Zeitpunkt der Infektion gesteigert war (Taubert, unveröffentlichte Ergebnisse), sprechen die Ergebnisse zudem nicht für eine Beteiligung sog. regulativer T-Zellen am Immungeschehen, wie sie im Zusammenhang mit anderen Darm-assoziierten Entzündungsreaktionen beschrieben wurde (u. a. [12, 217, 237]).

Belastungsinfektionen mit *E. bovis* führten weder im peripheren Blut noch in Lymphknotenzellen zu einer Beeinflussung Th1-assoziierter Zytokine. In Übereinstimmung wurde auch bei Hughes et al. [157] keine erhöhte IL-2-Synthese in PBMC immuner Tieren nachgewiesen. Da in unseren Untersuchungen ebenso wie bei anderen *Eimeria*-Reinfektionen [382, 334, 319] keine gesteigerte Synthese von IFN-γ gemessen wurde, scheint dieses Zytokin - im Gegensatz zur Primärinfektion - keine essentielle Rolle bei der Kontrolle von Reinfektionen zu spielen. Ähnliche Schlussfolgerungen ergeben sich aus Depletionsstudien und Untersuchungen mit IFN-γ-knock-out-Mäusen bei gegen *E. vermiformis* und *E. papillata* immunen Tieren [304, 319, 333, 334, 382].
Im Gegensatz dazu wurde die Transkription des IL-4-Gens über die Belastungsinfektion hinaus zunehmend in Lymphozyten aus Lymphknoten gesteigert, was Th2-dominierte Immunreaktionen nahelegt. Ob diesem Zytokin neben antikörperinduzierender Wirkung auch protektive Eigenschaften zukommen, ist nicht geklärt. Zumindest bei *E. vermiformis*-Reinfektionen der Maus scheint dies nicht der Fall zu sein [333].

4.2.5 Einfluss von IFN-γ auf BUVEC als potenzielle Wirtszellen

Wir konnten in den bisherigen Untersuchungen zum einen zeigen, dass die Frühphase der Primärinfektion mit *E. bovis* von einer parasitenspezifischen, IFN-γ-geprägten Th1-Antwort dominiert ist und zum anderen, dass PBMC im späteren Verlauf der Infektion zu einer verstärkten unspezifischen Produktion dieses Zytokins neigen (vgl. 4.2.4). Die verstärkte Adhäsion von PBMC an *E. bovis*-infizierte Endothelien (vgl. 4.2.6) lässt eine infektionsbedingte, unmittelbare Nähe von Lymphozyten und Wirtszellen und damit eine mögliche Wirkung von IFN-γ auf Endothelzellen annehmen. Aus diesem Grund wurde zudem untersucht, wie bovine Endothelzellen (BUVEC) als adäquate Wirtszellen unter Einfluss von IFN-γ reagieren.

BUVEC wurden grundsätzlich über IFN-γ aktiviert, wie anhand unterschiedlicher Aktivierungsindices, wie gesteigerte endotheliale Gentranskription verschiedener Chemokine/Adhäsionsmoleküle und vermehrte PMN-/PBMC-Adhäsion erkennbar war (vgl. **7.6**). Dabei waren einige Reaktionen spezifisch für IFN-γ, andere entsprachen den nach TNF-α-Stimulation ermittelten Daten.

Ähnlich humanen Endothelzellen [19, 276] führte die Stimulation mit IFN-γ zur selektiven Induktion von CXCL10 und CCL2 in BUVEC (vgl. **7.6**) und sollte somit an der präferentiellen Anlockung von Lymphozyten und Monozyten zum Infektionsort beteiligt sein. Insbesondere über CC-Chemokine, aber auch über das zusätzlich unter IFN-γ-Einfluss stimulierte COX-2 (vgl. humanes System, [397, 204]) könnte so über endothelvermittelte Reaktionen der Übergang von nicht-angeborenen in adaptative Immunreaktionen induziert werden.

Neben pro-inflammatorischen werden IFN-γ auch anti-inflamammatorische Eigenschaften zugeschrieben. So hemmt es z. B. die Rekrutierung von PMN im humanen System. In Übereinstimmung mit Beobachtungen bei humanen Endothelzellen [19, 150] resultierte eine Behandlung von BUVEC mit IFN-γ, im Gegensatz zu TNF-α, entsprechend nicht in der Hochregulation von GM-CSF und beeinflusste die Gentranskription von CXCL1 und CXCL8, Mediatoren der PMN-Anlockung, allenfalls marginal (vgl. **7.6**).

Vergleichbar mit Reaktionen im humanen System [357, 96, 226] führte die Stimulation mit IFN-γ zudem zu einer präferentiellen Induktion der Adhäsionsmoleküle VCAM-1 und ICAM-1, d. h. von Molekülen, die die feste Adhäsion von Immunzellen an aktiviertes Endothel vermitteln (zur Übersicht siehe [100, 380]). Die das reversible Rollen („rolling“) von Leukozyten vermittelnden Selektine P- und E-Selektin, die im bovinen System im Gegensatz zu humanen Zellen beide über eine Behandlung mit einem einzelnen Zytokin grundsätzlich induzierbar sind [387, 371, 29], wurden dagegen, analog zum humanen System [245, 273], nicht oder allenfalls moderat beeinflusst (vgl. **7.6**). In Folge zeigte sich in Untersuchungen zur Adhäsion von PMN und PBMC unter Verwendung von Durchflusskammersystemen eine signifikant gesteigerte Adhäsion beider Leukozytenpopulationen (vgl. **7.6**).

Die Adhäsion von PMN und PBMC wird im humanen System über Interaktionen mit unterschiedlichen Adhäsionsmolekülen vermittelt. Während PMN in erster Linie über ICAM-1 gebunden werden, interagieren PBMC v. a. mit VCAM-1 (zur Übersicht siehe [100, 380]). Da beide Moleküle gleichermaßen in IFN-γ-stimulierten BUVEC induziert wurden, war die ermittelte Adhäsion beider Leukozytentypen in ihrer Kinetik vergleichbar. Allerdings bedeutete dies Unterschiede zum humanen System, da hier IFN-γ, im Gegensatz zur PBMC-Adhäsion [357], nicht als Stimulator der PMN-Adhäsion angesehen wird [226, 357].
Zu der relativ starken PBMC-Adhäsion an IFN-γ-stimulierte BUVEC könnte indirekt endotheliales, über IFN-γ induziertes CXCL10 beigetragen haben, da es auch im humanen System solche Adhäsionsreaktionen potenziert [353]. Bezüglich PBMC zeigen immunhistologische Analysen, dass bei der IFN-γ-vermittelten Adhäsion an BUVEC $CD4^+$-und $CD8^+$-T-Zellen sowie γδ-TCR^+-T-Zellen gleichermaßen beteiligt sind (vgl. **7.6**), so dass IFN-γ insgesamt als Quelle einer sehr breit angelegten, endothelialen Immunreaktion anzusehen ist.

Neben den oben genannten Effekten wurde über IFN-γ auch die Gentranskription von MHC I- und II-Molekülen induziert (Taubert und Hermosilla, unveröffentlichte Daten), was bei *E. bovis*-infizierten BUVEC bezüglich möglicher Interaktionen insbesondere mit $CD4^+$- oder $CD8^+$-T-Zellen von Bedeutung sein könnte.

Die gezeigte, wenn auch moderate Steigerung von iNOS-Transkripten unter IFN-γ-Einfluss (vgl. **7.6**) könnte sich zudem über NO-Synthese hemmend auf die Replikation des Parasiten auswirken.

4.2.6 Adhäsion von PBMC an *E. bovis*-infizierte Endothelzellen *in vitro*

Der erfolgreiche Ablauf der mehrstufigen Kaskade der Leukozytenextravasation stellt die Voraussetzung für inflammatorische Prozesse im Bereich des Endothels bzw. im tieferen Gewebe dar. Hierbei müssen frühe Interaktionen zwischen Leukozyten und Endothelzellen gegeben sein, die einerseits MHC-unabhängig sind und somit antigenunspezifisch ablaufen und deren Qualität andererseits vom Expressionsmuster endothelialer bzw. leukozytärer Oberflächenmoleküle abhängt. So wird beispielsweise die Adhäsion von PMN und PBMC über unterschiedliche, präferentiell genutzte endotheliale Adhäsionsmoleküle gesteuert [100, 380, 374,

105]. Die Unabhängigkeit vom MHC-System ermöglicht Untersuchungen im allogenen System, so dass die vorliegenden Studien mit Endothelzellen und PBMC unterschiedlicher Spendertiere durchgeführt werden konnten. Da das Expressionsmuster der Rezeptoren bzw. Liganden auf PBMC jedoch im Zusammenhang mit ihrem Differenzierungs- bzw. Aktivierungszustand variieren kann [274, 374] und solche Einflüsse ausgeschaltet werden sollten, wurde mit PBMC aus experimentell *E. bovis*-infizierten Kälbern in Kombination mit zeitgleich *in vitro*-infizierten BUVEC gearbeitet.
Die Untersuchungen zur Adhäsion von PBMC wurden unter Verwendung der "parallel plate flow chamber" durchgeführt, einem Durchflusskammersystem, das den Leukozytenfluss in kleineren Gefäßen simuliert und somit die *in vivo*-Situation relativ realistisch widerspiegeln sollte.

Die Adhäsion von PBMC an infizierte BUVEC war früh nach der Infektion gesteigert, was sich in Maximalwerten bereits einen Tag p. i. niederschlug (vgl. **7.7**) und auf die Invasion der Sporozoiten als auslösendes Moment hinweist. Ob noch frühere Reaktionen stattfinden, konnte wegen des Designs der Studie nicht geklärt werden. Anschließend kam es zu einer graduell abfallenden PBMC-Adhäsion, so dass mit Tag 8 p. i. keine signifikanten Effekte mehr zu messen waren. Damit erfolgten offensichtlich keine Interaktionen mit sich entwickelnden Meronten I, sondern die Adhäsionsreaktionen waren mit der beginnenden Replikation (ab Tag 7-8 p. i.) des Parasitens kaum noch nachzuweisen. Die in ähnlicher Weise für die PMN-Adhäsion an *E. bovis*-infizierte BUVEC beschriebenen Daten werden von den Autoren als Evasionsstrategie des Parasitens beschrieben [147]. Tatsächlich konnte in dieser Arbeit eine *E. bovis*-vermittelte Hemmung der TNF-α-induzierten PMN-Adhäsion gezeigt werden. Ähnliche Effekte wären auch bezüglich der PBMC-Adhäsion denkbar. Zudem zeigen die Daten, dass sich die über *E. bovis*-Infektionen der Kälber verursachte Aktivierung der PBMC nicht auf Adhäsionsreaktionen auswirkt, da PBMC um Tag 8-12 p. i. zwar hochreaktiv gegen spezifische Antigene sind (vgl. 4.2.2, 4.2.4), aber nicht vermehrt adhärieren.

Im Prinzip sind die Daten zur PBMC-Adhäsion mit solchen zur PMN-Adhäsion an *E. bovis*-infizierte BUVEC [147] vergleichbar, da sie auf eine grundsätzliche Aktivierung der Wirtszellen hindeuten, jedoch weisen Unterschiede in der Kinetik (die PMN-Adhäsion war am stärksten an Tag 2 p. i. und verblieb über einen längeren

Zeitraum auf erhöhtem Niveau) auf einen schneller ablaufenden und kürzer andauernden Prozess hin. Diese differenzierte Reaktivität ist über die unterschiedliche Nutzung endothelialer Adhäsionsmoleküle wie ICAM-1 und VCAM-1 seitens PMN und PBMC erklärbar. Dass *E. bovis*-Infektionen bei Endothelzellen grundsätzlich eine, wenn auch eher moderate, Hochregulation der für das Rollen und die Adhäsion von Leukozyten essentiellen Adhäsionsmoleküle bewirkt, konnten Hermosilla et al. [147] zeigen.

Immunhistologische Untersuchungen adhärierter PBMC belegen, dass sowohl $CD4^+$- und $CD8^+$-T-Zellen als auch γδ-TCR^+-T-Zellen an diesen Reaktionen beteiligt waren (vgl. **7.7**). Somit interagieren insgesamt solche T-Zellpopulationen mit infiziertem Endothel, die alle grundsätzlich zu zytotoxischen Reaktionen befähigt sind. Interessant erscheint insbesondere die relativ hohe Beteiligung von $CD8^+$-T-Zellen (41,78 % der adhärierten PBMC waren $CD8^+$, vgl. **7.7**), welche bei anderen *Eimeria*-Infektionen als Effektorzellen vermutet werden. Zytotoxische $CD4^+$-T-Zellen spielen dagegen beispielsweise bei der Termination von *N. caninum*-Infektionen eine essentielle Rolle [342]. Jedoch sind auch γδ-TCR^+-T-Zellen in der Lage, *T. gondii*-infizierte Makrophagen zu lysieren [166]. Allerdings finden die Interaktionen der PBMC nach Erstinfektion mit einem Tag p. i. sehr früh statt, so dass effektive zytolytische Reaktionen zumindest über erstere Subpopulationen kaum zu vermuten sind. Nichtsdestotrotz erscheint in diesem Zusammenhang erwähnenswert, dass Endothelzellen Antigen-präsentierende Eigenschaften haben und $CD4^+$- und $CD8^+$-T-Zellen entsprechend antigenabhängig aktivieren können [211, 268, 102, 281]. Ähnlich wie bei Shi et al. [325] diskutiert, wäre eine Elimination *E. bovis*-infizierter Endothelzellen über protektive zytotoxische Reaktionen gegen frühe intrazelluläre Stadien nach Reinfektionen über diesen Mechanismus durchaus denkbar.

Die Adhäsion von $CD4^+$-, $CD8^+$- und γδ-TCR^+-T-Zellen an *E. bovis*-infizierte endotheliale Zellrasen erfolgte erstaunlicherweise zu nahezu gleichen Teilen sowohl an infizierte als auch an nicht-infizierte Zellen innerhalb eines Monolayers. Ähnliche Befunde liegen zur PMN-Adhäsion vor [147], jedoch war hier das Verhältnis deutlich zugunsten nicht-infizierter Zellen verschoben (90 % der PMN adhärierten an nicht-infizierte Zellen). Es ist bisher nicht klar, warum PBMC oder PMN an nicht-infizierte Zellen innerhalb eines infizierten Zellrasens adhärieren. Zu bedenken ist die Hypersensibilität von Endothelzellen gegenüber mechanischen Einflüssen. Eventuell

reicht bereits das Gleiten der Parasiten über die Zelloberfläche bei der Suche nach geeigneten Wirtszellen zur Aktivierung der Endothelzellen aus. Diese motilen Aktionen werden über komplexe Interaktionen zwischen Parasit und Wirtszelle vermittelt und resultieren z. B. in einer nachweisbaren Proteinspur auf Wirtszellen [49], so dass eine Registrierung seitens der Wirtszelle durchaus denkbar erscheint. Auch ein wiederholtes Eindringen und Verlassen scheinbar nicht geeigneter Wirtszellen seitens der Sporozoiten [21, 74, 75] könnte ursächlich zugrunde liegen. Zusätzlich könnten diese Effekte über eine parasitenvermittelte, parakrine Aktivierung benachbarter Zellen erfolgt sein, wie es z. B. für Zytomegalievirus-infizierte HUVEC über IL-1β-Synthese belegt wurde [86].

4.3 Reaktionen *E. bovis*-infizierter Wirtszellen

4.3.1 Beeinflussung des Wirtszelltranskriptoms über *E. bovis*-Infektion

4.3.1.1 Einfluss von E. bovis auf die Gentranskription immunmodulatorischer Moleküle in Wirtszellen

Untersuchungen von Hermosilla et al. [147] zur *E. bovis*-vermittelte Beeinflussung von Adhäsionsmolekülen in Endothelzellen legen eine infektionsbedingte Verminderung dieser Moleküle nahe. Um ähnliche Reaktionen bezüglich immunmodulatorisch agierender Moleküle erfassen zu können, wurden Infektionen mit Tachyzoiten von *T. gondii* und *N. caninum* zu vergleichenden Untersuchungen herangezogen. Da beide Parasiten im Gegensatz zu *E. bovis* mit max. drei Tagen eine schnelle Replikation in der Zelle durchlaufen und zudem Untersuchungen mit Stimulatoren wie TNF-α und IFN-γ (vgl. **7.6**) auf eine zügige Reaktivität der Endothelzellen innerhalb der ersten 48 h schließen lassen, wurde der Untersuchungszeitraum auf 72 Stunden beschränkt. Untersuchungsgegenstand waren bevorzugt auf PMN wirkende CXC-Chemokine (CXCL1, CXCL8, CXCL10), v. a. Monozyten und Lymphozyten anlockende CC-Chemokine (CCL2, CCL5), der PMN- und Makrophagen-stimulierende Faktor GM-CSF, das als Marker der Prostaglandinsynthese dienende COX-2 sowie iNOS als Indikator der induzierbaren NO-Synthese.

Insgesamt zeigte sich, dass die unterschiedlichen Infektionen grundsätzlich zu einer Aktivierung der Wirtszellen führten, allerdings von unterschiedlichem Ausmaß und Reaktionsmuster (vgl. **7.8**). Analog zu Untersuchungen zu Adhäsionsmolekülen [355] kam es über *T. gondii*- oder *N. caninum*-Infektionen früh nach Infektion zu einer Steigerung aller untersuchten Gentranskripte, während diese im Falle der *E. bovis*-Infektionen entweder nur moderat (CCL2, CCL5, GM-CSF) induziert wurden oder mehr oder weniger unbeeinflusst (CXCL1, CXCL8, CXCL10, COX-2, iNOS) blieben, obwohl bei Verwendung gleicher Infektionsdosen die resultierenden Infektionsraten bei *E. bovis*-infizierten BUVEC ungleich höher lagen (25,1 % bei *E. bovis*, 8,5 % bei *T. gondii* und 7,9 % bei *N. caninum*, vgl. **7.8**). Die Ergebnisse entsprechen bezüglich *T. gondii* Untersuchungen von Knight et al. [175] zu infizierten Endothelzellen aus Gefäßen der Retina der Ratte, die eine früh nach der Infektion auftretende, vermehrte Gentranskription und Proteinexpression von CCL2, CCL5 und CXCL1 nachwiesen. Auch in *T. gondii*-infizierten Fibroblasten wurde eine Hochregulation der Transkription der CXCL1-, CCL2-, oder CXCL8-Gene [30, 92, 120] beschrieben, während Brenier-Pinchart et al. [41] zwar eine Steigerung der Transkription und Expression von CCL2, nicht jedoch von CXCL8-, CCL5- und CXCL10-mRNAs nachweisen konnten. Interessanterweise scheint der CCL2-assoziierte Effekt stadienabhängig zu sein, da eine Induktion dieses Chemokins nur über die sich schnell entwickelnde Tachyzoiten, nicht jedoch über Bradyzoiten erreicht wurde [42]. Da es sich bei *E. bovis*-Sporozoiten ebenfalls um sich langsam entwickelnde Stadien handelt, die eine deutlich geringere Steigerung der CCL2-Gentranskription als *T. gondii*-Tachyzoiten bewirken, könnten hier die Ergebnisse ihre Entsprechung finden. Insgesamt könnte die Induktion von CCL2 auf bei *Eimeria* spp. konserviertes Antigen (18 kDa Protein) zurückgeführt werden, wie von Rosenberg et al. [306] in murinen dendritischen Zellen beschrieben. Eine auf die Induktion von CC-Chemokinen beschränkte Reaktivität der Wirtszelle findet sein Pendant auch in Proben *E. tenella*- und *E. maxima*-infizierter Hühner [191].

Der zusätzlich auftretende Effekt von *T. gondii*-Infektionen auf GM-CSF (vgl. **7.8**), eines Moleküls mit bekannter Wirkung auf diesen Parasiten [246, 113, 84], bestätigt Befunde in *T. gondii*-infizierten Fibroblasten [59]. Auch die vermehrte Synthese von COX-2 entspricht im Prinzip den Ergebnissen anderer Gruppen [209, 356, 58, 393] und kann im Sinne der bei Zerstörung von Zellen beschriebenen Hochregulation dieses Moleküls [196, 251] interpretiert werden.

Die im Rahmen aller drei Infektionen moderat induzierte Gentranskription von iNOS sollte eine vermehrte Synthese von NO nach sich ziehen. NO ist ein wichtiges Effektormolekül gegen intrazelluläre Pathogene in Phagozyten [259, 47] und könnte auch bei Endothelzellen zur Wirkung kommen.

Während in der Frühphase der *E. bovis*-Infektion kaum pro-inflammatorische Reaktionen seitens der Wirtszelle gemessen wurden, was auch über Transkriptomanalysen 4 Stunden und 4 Tage p. i. bestätigt wurde (vgl. **7.9.**), zeigten an Tag 8 p. i. durchgeführte Microarray-Analysen (vgl. **7.9**) ein anderes Bild. Die signifikante Beeinflussung zellulärer Funktionen bezüglich des „trafficking“ von Immunzellen und der inflammatorischen Antwort der Wirtszelle als auch die Induktion eines immunassoziierten Netzwerks unter Beteiligung diverser, zuvor im Verlauf der Infektion in der Mehrzahl nicht vermehrt transkribierter, immun-modulatorisch wirkender Moleküle belegen hier die grundsätzliche Potenz der endothelialen Wirtszelle (vgl. **7.9**). Interessanterweise waren hier im Gegensatz zu oben beschriebenen Befunden früh nach Infektion ausschließlich CXC-Chemokine (CXCL1, CXCL3, CXCL6, CXCL8) hochreguliert (vgl. **7.9**), was eine chemotaktische Einwirkung v. a. auf PMN, aber auch auf Lymphozyten nahelegt [204, 397], wobei für beide Zelltypen Befunde zur Interaktion mit *E. bovis*-infizierten Endothelien vorliegen (vgl. 4.2.6 und [147]). In einem direkten Zusammenhang dazu steht die Hochregulation der Adhäsionsmoleküle P-Selektin, VCAM1 und ICAM1 (vgl. **7.9**), da diese Moleküle in Übereinstimmung mit Hermosilla et al. [147] die Adhäsion von PMN an *E. bovis*-infizierte BUVEC vermitteln.

Zusätzlich waren solche Moleküle in ihrer Abundanz vermehrt, die in den Komplex der Blutgerinnung eingebunden sind (vgl. **7.9**). Welche Rolle eine vermehrte Gentranskription von PLAU (plasminogen activator, urokinase), PLAUR (plasminogen activator, urokinase receptor), PLAT (plasminogen activator, tissue) und SERPINE1 (serpin peptidase inhibitor, clade E) im Rahmen der *E. bovis*-Infektion spielt, ist unklar. Es erscheint unwahrscheinlich, dass sie mit den über tiefe mukosale Defekte verursachten hämorrhagischen Durchfällen im Zusammenhang steht, da diese klinischen Erscheinungen erst später im Verlauf der Infektion im Zuge der Patenz zu erwarten sind. Eher ist sie als Ausdruck der parasiteninduzierten Aktivierung der Wirtszellen zu verstehen, da inflammatorische Reaktionen des Endothels generell von einer erhöhten Permeabilität des endothelialen Zellverbands

begleitet werden. Gelockerte Zellverbände wurden auch bei *C. parvum*-infizierten Epithelzellen beobachtet [5] und könnten bei *E. bovis* in Verbindung mit der Runterregulation des tight junction-assoziierten Claudin 1 (CLD1, vgl. **7.9**) in Verbindung stehen.
Auch die gesteigerte Transkription von in der Komplement-Kaskade eingebundenen Molekülen wie Komplementfaktor B und H, C8, C1S und C3 in der späteren Phase der *E. bovis*-Infektion spricht für den Aktivierungszustand der Endothelzellen.

Die oben besprochene Modulation immunassoziierter Moleküle an Tag 8 p. i. schlug sich auch in der Regulation bestimmter Signalwege nieder. So waren in *E. bovis*-infizierten BUVEC Moleküle der Koagulations-, IL-8- und Leukozytenextravasations-Signalwege signifikant überrepräsentiert (vgl. **7.9**). Zusätzlich wurde der im Rahmen der Freisetzung pro-inflammatorischer Moleküle betroffene HMBG1-Signalweg an den Tagen 4, 8 und 14 p. i. induziert.

Die immunologischen Konsequenzen einer restriktiven Synthese inflammatorischer Moleküle zu bestimmten Zeitpunkten nach der Infektion sind offensichtlich. Die Unterdrückung diverser Chemotaxis-relevanter und anderer Moleküle zu einem frühen Zeitpunkt der Infektion, in dem *E. bovis* sich noch nicht stabil in der Zelle etabliert hat, erhöht die Überlebenschancen des Parasiten. Die später auftretende Induktion diverser pro-inflammatorisch agierender Moleküle am Tag 8 p. i. spricht für eine pro-Wirtszell ausgerichtete Reaktion, passt zeitlich z. B. zu den unter 4.2.2. und 4.2.4. beschriebenen T-Zell-assoziierten Reaktionen und verdeutlicht die immunologische Potenz der endothelialen Wirtszelle. Inwiefern die zu diesem Zeitpunkt induzierten, pro-inflammatorischen Reaktionen tatsächlich zu einer Begrenzung der Infektion führen, bleibt zu klären.

4.3.1.2 Transkriptionelles Profil E. bovis-infizierter Endothelzellen

Um globale Einsichten in die über *E. bovis* vermittelte Modulation der Wirtszelle zu erhalten, wurden bovine Microarrays an *E. bovis*-infizierten BUVEC durchgeführt. Dabei wurden folgende Termine berücksichtigt: 4 Stunden p. i. (= kurz nach der Invasion der Parasiten), 4 Tage p. i. (= Umwandlung zum Trophozoiten), 8 Tage p. i. (= beginnende Proliferation des Parasiten) und 14 Tage p. i. (= Ausreifung des Makromeronten) (vgl. **7.9**).

Die vergleichende Analyse der Datensätze zeigt einen gesteigerten Einfluss des Parasiten auf die Transkriptionsmaschinerie der Wirtszelle mit zunehmender Infektionsdauer (vgl. **7.9**). Bei Berücksichtigung sowohl der Anzahl modulierter Gene/Termin als auch der innerhalb der Termine überlappenden Datensätze verdeutlicht sich eine Zweiteilung der *in vitro*-Entwicklung dahingehend, dass in der Frühphase - d. h. vor Beginn der Proliferation des Parasiten mit 4 Stunden und 4 Tagen p. i. - allenfalls eine schwache Modulation der Wirtszelltranskription auszumachen war, die tendenziell eher durch inhibitorische Effekte auf die Endothelzellaktivierung gekennzeichnet war. Mit beginnender Proliferation des Parasitens erfolgte dann eine massive, im Zuge der Weiterentwicklung bzw. Ausreifung der Makromeronten zunehmende Beeinflussung des Wirtszelltranskriptoms. So wurden beispielsweise > 60 % der am 8. Tag p. i. modulierten Gene in derselben Weise auch 6 Tage später beeinflusst, jedoch i. d. R. in einem noch stärkerem Ausmaß (vgl. **7.9**).

Manipulationen an *E. bovis*-infizierten Wirtszellen in den ersten zwei Tagen p. i., wie z. B. über Zugabe von Apoptoseinduktoren [187] oder Kalzium-Ionophoren [22] zeigen, dass das Parasit-Wirtszellverhältnis innerhalb dieses Zeitraums noch relativ labil ist und in der Folge Sporozoiten die Wirtszellen verlassen, um neue aufzusuchen. Offensichtlich hat sich der Parasit innerhalb dieses Zeitraums noch nicht irreversibel in der Wirtszelle etabliert und übernimmt erst später die vollständige Kontrolle, was sich z. B. dahingehend äußert, dass über Apoptoseinduktoren die Wirtszelle dann nicht mehr in den programmierten Zelltod getrieben werden kann [188]. Prinzipiell ist dieses Phänomen indirekt auch an den Daten zur Transkriptomanalyse *E. bovis*-infizierter BUVEC ablesbar. Der Zeitraum unmittelbar nach der Invasion der Wirtszelle 4 Stunden p. i. bewirkte nur eine minimale Reaktion der Wirtszelle auf transkriptioneller Ebene. Dies erscheint zunächst zwar verwunderlich, da der Prozess der Invasion mit mechanischem Stress einhergeht und insbesondere Endothelzellen empfindlich auf solche Manipulationen reagieren sollten. Wir haben uns bezüglich dieses verwendeten Zeitpunkts an Daten zur optimalen Reaktionszeit von BUVEC auf lösliche Stimulatoren, wie z. B. TNF-α oder IFN-γ orientiert. Folgerichtig ist zu vermuten, dass entweder die Invasion nahezu unbemerkt von der betroffenen Wirtszelle erfolgt oder dass die initiale Modulation der betroffenen Wirtszelle seitens des Parasiten auf Proteinebene oder über epigenetische Mechanismen erfolgt und somit nicht über Transkriptomanalysen

erfasst werden kann. Die Abgabe präformierter, blockierend eingreifender Proteine erscheint zumindest als rasch greifende, wenn auch nicht dauerhaft wirkende Strategie einleuchtend; unterstützende Daten zu dieser Hypothese liegen jedoch bisher nicht vor.

Gemäß der Transkriptomdaten nimmt *E. bovis* Einfluss auf unterschiedlichste Aspekte der Wirtszelle. Auffallend war eine i. d. R. bei zunehmender Infektionsdauer vermehrte, parasiteninduzierte Regulation von allgemeinen Zellfunktionen (zelluläre Entwicklung, Zell-zu-Zell-Signaling, Zellzyklus, Zellmorphologie, Zelltod, Zellbewegung, zelluläres Wachstum und Proliferation), von Wirtszellmetabolismus (v.a. Kohlenhydrat-, Lipidmetabolismus als auch Energiegewinnung) und von der wirtszelleigenen Immunantwort (vgl. **7.9**). Zudem regulierte *E. bovis* diverse molekulare Netzwerke und zelluläre Signalwege.

Die mit einer enormen Größenzunahme verbundene intrazelluläre Makromerontenentwicklung zieht eine strukturelle Reorganisation der Wirtszelle über Beeinflussung unterschiedlicher Zytoskelettelemente nach sich, wie im Prinzip auch für andere Apikomplexa beschrieben wurde [85, 116, 130, 131, 225, 248]. Bei *E. bovis*-infizierten Zellen berichten Hermosilla et al. [145] über die Zunahme der wirtszelleigenen Aktinfilamente und Mikrotubuli um die parasitophore Vakuole, was insgesamt zu einer Stabilisierung der Wirtszelle im Verlauf der *in vitro*-Infektion führt. Die Transkriptomanalysen offenbaren zusätzlich beteiligte Moleküle v. a. in der späten Phase der Infektion (vgl. **7.9**). So waren unterschiedliche Tubuline (TUBB, TUBB4, TUBB6) und Mikrotubuli-assoziierte Moleküle [“tubulin polymerization promoting protein“ (TPPP), „dedicator of cytokinesis 7“ (DOCK7), “cytoskeleton-associated protein 4“ (CKAP4), “dynactin 3”, (DCT3)] in ihrer Abundanz vermehrt. Zusätzlich induzierte die Infektion Moleküle, die mit Aktin-Signalwegen interagieren oder auf anderem Weg Aktin-assoziiert sind , wie „capping protein gelsolin-like“ (CAPG), “calponin” (CNN2), “transgelin” (TAGLN) oder “paladin” (PALLD).

Die im Zuge der Makromerontenreifung einhergehenden Vergrößerung der Wirtszelle sollte nicht unbeträchtlichen Zellstress bewirken. In Analogie zu anderen Apikomplexa-infizierten Wirtszellen [255, 248], wurden charakteristische Stressfaktoren, wie z. B. unterschiedlicher Hitzeschockproteine (HSP90AA1, HSP90B1, HSP70, HSP70-3, HSP70-5, HSP27, HSPB6, vgl. **7.9**) induziert. Auch andere stressassoziierte Moleküle wie SERP1 („stress-associated endoplasmic

reticulum protein 1“) und STIP1 („stress-induced-phosphoprotein 1“) wurden an Tag 14 p. i. vermehrt transkribiert, wobei letztgenanntes wiederum als Koordinator von Molekülen der HSP70- und HSP90-Familie fungiert.

HSPs sind zudem in die Regulation der Apoptose involviert, wobei bestimmte Mitglieder der HSP27-, HSP70- und HSP90-Familien inhibitorisch einwirken können [151]. Dass die Wirtszellapoptose *E. bovis*-infizierter Zellen grundsätzlich gehemmt wird, wurde kürzlich von Lang et al. [188] belegt. Als vermittelnde Faktoren wurden die inhibitorisch agierenden Moleküle c-IAP (syn. BIRC2) und c-FLIP benannt, wobei ersteres auch in den Transkriptomanalysen von Tag 8. p. i. als gesteigert vorliegend aufgezeigt wurde. Zusätzlich wurden neue, bisher im Zusammenhang mit der *E. bovis*-vermittelten Hemmung der Apoptose unbekannte Faktoren identifiziert, die entweder über ihre Hochregulation [z. B. “DNA-damage-inducible transcript 4“ (DDIT4), „BCL2-related protein A1“ (BCL2A1)] oder über eine verminderte Abundanz [z. B. „BCL2-like 14“ (BOK)] inhibitorisch einwirken (vgl. **7.9**). Allerdings darf nicht übersehen werden, dass auch eine Reihe pro-apoptotisch wirkender Moleküle, wie „BCL2-associated athanogene 5“ (BAG5), „BCL2-antagonist/killer 1“ (BAK1), „BCL2-like 14“ (BCL2L14), „cytochrome c“ (CYCS), „cytochrome c oxidase subunit Va“ (COX5A) und „death associated protein 3“ (DAP3) induziert wurde (vgl. **7.9**). Es ist schwierig zu beurteilen, welche der Komponenten den Ausschlag geben werden. Grundsätzlich sind auch pro-apoptotische Reaktionen bei *Eimeria* spp.-infizierten Wirtszellen in ähnlicher Situation gegen Ende der intrazellulären Entwicklung zur Unterstützung der Lyse der Wirtszelle beschrieben [82].

Ein Charakteristikum der Makromerontenentwicklung ist die mit beginnender Proliferation andauernde Veränderung des Zellkerns infizierter Wirtszellen, ein Phänomen, das auch in der Kultur von *E. ninakohlyakimovae*-Meronten I beobachtet wurde (vgl. **7.11**) Die veränderte Zellkernmorphologie, die auf eine vermehrte Abundanz des transkriptionsaktiven Euchromatin hinweist, findet ihre Entsprechung in der steigenden Anzahl regulierter Gene ab Tag 8 p. i. Auch die in diesen Datensätzen in ihrer Abundanz vermehrt identifizierten Schlüsselfaktoren der Transkription wie FOS, MYC oder STAT1 als auch anderer Transkriptionsfaktoren (z. B. ANKRD1, ANKRD52, BHLHB2, GTF2E2, GTF2H3, EZH2, KLF4, KLF5) bestätigen die erhöhte Transkriptionsaktivität infizierter Zellen (vgl. **7.9**). Ein am Tag 14 p. i. hochsignifikant über *E. bovis* beeinflusstes molekulares Netzwerk mit MYC

als zentralem Element weist auf die Schlüsselrolle dieses Transkriptionsfaktors hin. Die Aktivierung von MYC trägt z. B. bei *Theileria* spp.-infizierten B-Lymphozyten zum Überleben der Wirtszellen bei [93]. Ähnliche Phänomene wären auch bei *E. bovis*-infizierten Wirtszellen denkbar.

Neben der veränderten Abundanz des Euchromatins ist auch das Konfluieren und Wachsen der Nukleoli charakteristisch für die „Spiegelei"-Form der Zellkerne makromerontentragender Wirtszellen. Dieses Phänomen gilt als Indikator einer stark proteinsynthetisch aktiven Zelle [3]. In Übereinstimmung wurden typische Translations-assoziierte Moleküle, wie eukaryontische Translations-initiierende Faktoren (EIF2B1, EIF4A1, EIF4E2, EIF4E3) oder eukaryontische Translations-elongierende Faktoren (EEF1A1, EEF1E1) in *E. bovis*-infizierten BUVEC induziert (vgl. **7.9**).

Die oben beschriebene Veränderung der Zellkernmorphologie entspricht grundsätzlich der eines in der Interphase befindlichen Zellkerns [3]. Während die Kerne umgebender, nicht-infizierter Zellen i. d. R. eine andere, mehr gepunktete Morphologie aufwiesen, verblieben die der infizierten bis zum Ende der Merogonie I in dem „Spiegelei"-Zustand. Dies legt die Vermutung einer *E. bovis*-induzierten Arretierung des Zellzyklus nahe. Bei *T. gondii*-infizierten Wirtszellen wird eine Arretierung in der G_2-Phase [235, 46] zum Benefit der parasitären Proliferation beschrieben, die u. a. über eine Verminderung des Zyklins B1 erklärt wird [46]. Auch *Leishmania* spp. verzögern die Wirtszellproliferation über die Induktion der den G1/S-Übergang hemmenden Zyklinkinase-Inhibitoren p21 und p27 [185].

Die bei *E. bovis*-makromerontentragenden Zellen beobachtete Hochregulation verschiedener Zykline (CCNB2, CCND2, CCNE1, CCNE2) spricht grundsätzlich gegen ein Verbleiben der infizierten Zellen in der G_0-Phase und eher für den Eintritt der makromerontentragenden Zelle in den Zellzyklus (vgl. **7.9**), was durchaus als sinnvoll erscheint, da die Zelle in der G_1-Phase wächst und sich auf eine Replikation vorbereitet. Der signifikante Eingriff in die Signalwege sowohl zum G_1/S- als auch zum G_2/M-Kontrollpunkt spricht zumindest grundsätzlich für eine *E. bovis*-bedingte Modulation des Zellzyklus (vgl. **7.9**). Anhand der regulierten Gene ist jedoch nicht klar zu entscheiden, zu welchem Resultat dieser Eingriff führt. Neben den oben benannten hochregulierten Zyklinen und -assoziierten Molekülen (CDK2AP1, GADD45A) spricht auch die Hemmung des Zyklin-abhängigen Kinase Inhibitors 1C

(CDKN1C) eher für die Progression des Zellzyklus in die Synthesephase [324, 272, 323, 345]. Allerdings könnte die gemessene Steigerung der Abundanz des Zyklin-abhängigen Kinase Inhibitor 1A (CDKN1A) auch das Gegenteil bewirken (vgl. **7.9**). Zudem spricht die Hochregulation des inhibitorisch wirkenden BRCA1 für eine Arretierung in der G_2-Phase.

Sobald *E. bovis* mit seinem Wachstum bzw. seiner Proliferation innerhalb der Wirtszelle beginnt, ist er in zunehmendem Maß auf wirtszelleigene Nährstoffe angewiesen. In Übereinstimmung wurden die meisten Metabolismus-assoziierten Moleküle in der späten Phase der Infektion induziert. Dabei waren verschiedene metabolische Kategorien, wie Kohlenhydrat-, Nukleinsäure- und Lipidmetabolismus als auch die Generierung von Energieträgern betroffen. Entsprechend wurden verschiedene Wege der Kohlenhydratbiosynthese bzw. deren Metabolismus über *E. bovis* moduliert, wie der Aminozucker-, Fruktose/Galaktose-, Nucleotidzucker- oder Pyrovat-Metabolismus als auch die Glykolyse/Glukoneogenese und der Pentose-Phosphat-Weg (vgl. **7.9**), was insgesamt dafür spricht, dass die transkriptionelle Beeinflussung des Grundmetabolismus der Wirtszelle essentiell für das intrazelluläre Wachstum von *E. bovis* ist.

Über den tatsächlichen Nährstoffbedarf seitens *E. bovis* ist jedoch - im Gegensatz zu *T. gondii*, dessen essentieller Bedarf an Purin [183], Hypoxanthin, Xanthin und andere Faktoren dokumentiert ist [331] - nichts bekannt. *T. gondii* hängt zudem von der wirtszelleigenen, über den Melanovat-Weg ablaufenden Synthese des Cholesterols ab [68, 332]. Interessanterweise wurden in *E. bovis*-infizierten Zellen 16 Moleküle hochreguliert, die in Biosynthese und Metabolismus von Cholesterol eingebunden sind (vgl. **7.9**), was für einen ähnlichen diesbezüglichen Nährstoffbedarf von *E. bovis* und *T. gondii* spricht. Insbesondere die Hochregulation des Enzyms Squalenepoxidase (SQLE), über die in unterschiedlichen *T. gondii*-infizierten Zelltypen berichtet wird [30, 255] und die als eine wichtige Komponente des Melavonat-Weges angesehen wird, spricht für die oben genannte Hypothese.
Die an Tag 14 p. i. auftretende Hochregulation der im Metabolismus der Apfelsäure eingebundenen Enzyme ME1, MDH1 und MDH2 spricht zusätzlich für einen erhöhten Energiebedarf von *E. bovis*-Makromeronten gegen Ende der Reifung (vgl. **7.9**).

Die in gesteigertem Maß an Tag 8 p. i. auftretenden Immunreaktionen seitens der Wirtszelle werden unter 4.3.1.1 besprochen.

4.3.2 Beeinflussung des Wirtszellproteoms über *E. bovis*-Infektion

Um den globalen Einfluss von *E. bovis* auf die Wirtszelle auch auf Ebene der Proteinsynthese zu überprüfen, wurden Proteomanalysen an Tag 14 p. i. durchgeführt. Leider standen BUVEC nicht in ausreichender Menge zu Verfügung, so dass auf BFGC ausgewichen werden musste. Allerdings verläuft die Merogonie I von *E. bovis* in diesem Zelltyp ca. 3-4 Tage schneller, so dass die Vergleichbarkeit mit den ebenfalls an Tag 14 p. i. in BUVEC durchgeführten Transkriptomanalysen eingeschränkt ist.

Bereits am Tag der Gewinnung der Zellproben wurden erste Merozoiten I freigesetzt, d. h. die Daten spiegelten die Situation unmittelbar gegen Ende der Merogonie I wieder. Vergleichende 2D-Analysen von *E. bovis*-infizierten und nicht-infizierten BFGC zeigten, dass insgesamt 211 Proteine in ihrer Abundanz verändert waren (vgl. **7.10**). 21 neu aufgetauchte Proteine waren parasitären Ursprungs, 23 Moleküle konnten nicht zugeordnet werden. Der Hauptanteil der modulierten Wirtszellproteine war in seiner Abundanz vermindert (137 Proteine). 111 Proteine wurden einer Analyse über MALDI-TOF-MS unterzogen, auch hier waren mit 84 Proteinen das Gros runterreguliert (vgl. **7.10**).

Die weitergehende Analyse der regulierten Proteine zeigte eine *E. bovis*-vermittelte Beeinflussung unterschiedlicher funktioneller Bereiche der Wirtszelle. Der Hauptanteil der hochregulierten Proteine war im Zellmetabolismus involviert (35 %), ein geringerer Anteil in Proteinprozessierung (19 %) und Zellstruktur (15 %). Die in ihrer Abundanz verminderten Proteine wurden ebenfalls in Zellmetabolismus (20 %) und Zellstruktur (23 %), aber auch in „Core“-Proteine und Transkriptions-assoziierte Moleküle (beide 17 %) neben anderen Bereichen gruppiert (vgl. **7.10**).

Innerhalb der Metabolismus-assoziierten Moleküle waren mit 16 Vertretern die meisten runterreguliert. Viele dieser Proteine sind in Glykolyse und Zitratzyklus eingebunden, was nahelegt, dass diese Hauptwege der Energiegewinnung entweder grundsätzlich nicht, oder, was wahrscheinlicher erscheint, gegen Ende der Merogonie nicht mehr benötigt werden (vgl. **7.10**). Im Gegensatz dazu stehen Daten

von *T. gondii*-infizierten Wirtszellen, bei denen v. a. die in die Gykolyse involvierten Proteine hochreguliert waren [248]. Die Probengewinnung erfolgte hier jedoch in der proliferativen Phase des Parasiten und die Daten spiegeln somit Vorgänge eines anderen Stadiums wider.

In Untersuchungen zur Modulation wirtszelleigener Zytoskelettelemente wurde gezeigt, dass die fortschreitende Merogonie I von *E. bovis* mit einer Vermehrung von Aktinfilamenten und Mikrotubuli verbunden ist [145]. Die Proteomanalysen gegen Ende der Merogonie zeigten dagegen, dass sowohl Aktin- und Myosin- als auch Tubulin-assoziierte Proteine in ihrer Abundanz vermindert waren (vgl. **7.10**). Insgesamt deuten diese Daten darauf hin, dass unmittelbar vor Freisetzung der Merozoiten I insbesondere die Hauptelemente des den Meronten I umgebenden Zytoskeletts vermindert werden und somit evtl. die Lyse der Zelle erleichtert wird.

Auch die Transkriptions- und Translationsmaschinerie der Wirtszelle erschien zum Zeitpunkt der vorliegenden Untersuchung eher gehemmt zu werden. Entsprechend wurden 7 Vertreter der „heterogeneous nuclear ribonuclear protein"-Familie, die eine Rolle in der Prozessierung der prä-mRNA innerhalb des Kerns [98] sowie in deren Transport [267] spielen, in ihrer Abundanz vermindert, ebenso wie Histon-assoziierte Proteine, wichtige Transkriptionfaktoren wie „Ras" und das Protein „Ran", das einen Schlüsselfaktor beim nukleären Ex- und Import von Molekülen darstellt [238, 266] (vgl. **7.10**). Auch der Prozess der Translation wurde eher gehemmt als gefördert, da wichtige Teilnehmer wie der "elongation factor 2" runterreguliert waren und der Parasit somit bestenfalls den *Status quo* der Zelle bis zur letztendlichen Auflösung erhalten konnte.

Nichtsdestotrotz scheint *E. bovis* zumindest seinen inhibitorischen Einfluss auf die Apoptosefähigkeit der Wirtszellen zu erhalten, da - in Analogie zu *T. gondii*-infizierten Zellen [378] - Kaspase 8 in einer deutlich verminderten Abundanz vorlag. Auch die z. T. hochregulierten Vertreter der Heatshockproteine (z. B. HSP90, HSP70-9B) könnten für eine Hemmung der Apoptose sprechen.

Bei Vergleich der an Tag 14 p. i. generierten Proteom- und Transkiptomanalysen muss die oben benannte Problematik der unterschiedlichen Zelltypen in Betracht gezogen werden. Zwar trat nach funktioneller Analyse grundsätzlich eine signifikante Modulation ähnlicher Funktionsbereiche zutage (Abb. 18), jedoch wurden 18 der 26

gleichermaßen in BUVEC und BFGC modulierten Moleküle in gegensätzlicher Weise reguliert (Tab. 3). Gegensätzliche Befunde von Proteom- und Transkriptomanalysen sind zwar grundsätzlich nicht ungewöhnlich und wurden so auch für andere Apikomplexa-infizierte Zellen berichtet [248, 386], dennoch sprechen die vorliegenden Daten insgesamt für eine unterschiedliche entwicklungsbiologische Situation in beiden Zelltypen, insbesondere da von infizierten BUVEC erst frühestens 3 Tage später erste Merozoiten I freigesetzt wurden und sich dieser Zelltyp somit im Gegensatz zu BFGC noch in der Proliferationsphase befand.

Tab 3: Gleichermaßen im Transkriptom und Proteom *E. bovis*-infizierter Wirtszellen regulierte Moleküle (14 Tage p. i.)

Molekül	Gentranskript (n-fache Modulation)	Protein (n-fache Modulation)
ACO2	1,71	-2,93
APOA1	-1,51	-2,19
CAPG	2,39	-3,68
CNN2	2,23	2,39
ECHS1	1,66	2,68
ENO1	1,66	-2,65
HSP90AA1	1,95	2,69
HSPA8	1,52	4,01
IDH3A	2,30	-2,38
ITGAW	1,54	-5,02
MDH1	2,53	-2,25
MDH2	1,59	3,84
PDLIM1	-1,69	-4,19
PGAM1	2,05	-2,15
PGK1	1,61	-3,77
PHB	1,93	-2,11
PRDX2	1,61	-2,19
PSMD14	1,50	-2,25
RUVBL1	2,56	-3,38
SLC25A4	3,33	3,61
STIP	1,60	-2,22
TAGLN	1,60	-13,5
TPI1	1,77	-3,69
TUBB6	1,80	-2,27
VIL2	1,87	-3,42
YARS	1,52	-2,01

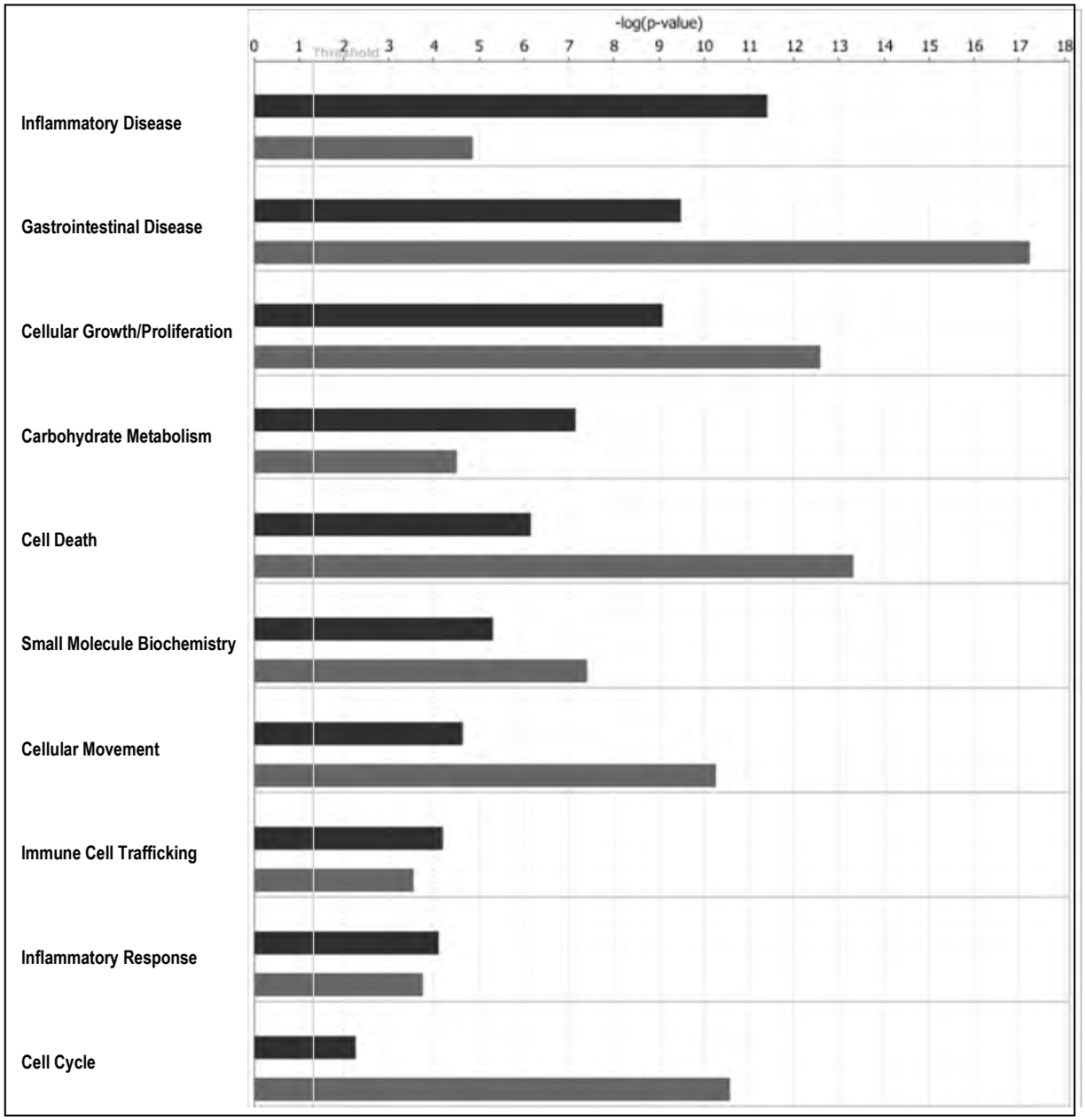


Abb. 18: Einordnung *E. bovis*-modulierter Proteine und Gentranskripte in funktionelle Bereiche der Wirtszelle. Vergleichende Analysen der über Proteom- (schwarze Balken) und Transkriptom- (graue Balken) Analysen generierten Datensätze vom 14. Tag p. i. (Ingenuity Pathway Analyses). Der Schwellenwert bezieht sich auf $p \leq 0{,}05$.

4.4 Etablierung eines *Eimeria ninakohlyakimovae*-Zellkultur-systems für vergleichende Untersuchungen

Alle in der Literatur zu Vergleichszwecken heranziehbare, *Eimeria*-bezogene Untersuchungen zur zellulären Immunantwort als auch zur Modulation der Wirtszelle beziehen sich auf solche Arten, die entwicklungsbiologisch mit *E. bovis* nicht vergleichbar sind, da z. B. Epithelzellen als Wirtszellen fungieren oder keine Makromeronten ausgebildet werden und damit die Verweildauer des Parasiten innerhalb der Wirtszelle deutlich verkürzt ist. Um aus den bisher beschriebenen Untersuchungen allgemeine, für Makromeronten-produzierende *Eimeria* spp. gültige Aussagen ableiten zu können, wurde eine *in vitro* Kultur für *Eimeria ninakohlyakimovae* für vergleichende Untersuchungen etabliert. *E. ninakohlyakimovae* ist ein weltweit verbreitetes Kokzid von Ziegen, das sowohl klinisch (schwere hämorrhagische Typhlocolitis bei Ziegenlämmern, [181]) als auch entwicklungbiologisch (Ausbildung von Makromeronten in Endothelzellen [373]) mit *E. bovis* vergleichbar ist und ebenfalls die Profitabilität der Tierhaltung beeinträchtigt [308].

Für die *in vitro* Kultur wurden primäre und permanente Zelllinien unterschiedlichen Ursprungs herangezogen: primäre caprine, bovine und humane Endothelzellen aus der Nabelschnurvene (CUVEC, BUVEC, HUVEC), permanente bovine Zelllinien von fötalen Gastrointestinalzellen (BFGC) und Kolonepithelzellen (BCEC) sowie eine permanente Nierenepithelzelllinie vom Affen (VERO-Zellen, African green monkey kidney cells) (vgl. **7.11**).

Die Invasion der *E. ninakohlyakimovae*-Sporozoiten erfolgte innerhalb eines Zeitrahmens von bis zu einer Stunde unabhängig vom verwendeteten Zelltypus. Dieses Wirtszell-unspezifische Invasionsverhalten entspricht dem bei anderen *Eimeria* spp. beschriebenen [21, 143, 107]. Die höchsten initialen Infektionsraten am ersten Tag p. i. wurden in absteigender Reihenfolge in BFGC, BCEC, HUVEC, CUVEC, BUVEC und VERO gemessen. Es wurden immer wieder Sporozoiten beobachtet, die eine zuvor invadierte Wirtszelle verließen, um neue zu infizieren (vgl. **7.11**). Ein solches Verhalten ist auch für Sporozoiten anderer *Eimeria* spp. dokumentiert [21, 22, 75, 143] und spiegelt wahrscheinlich die Fähigkeit mancher Sporozoiten wider, Zellverbände zu durchwandern um tiefere Schichten zu erreichen [21, 22]. Dieses Durchwandern kommt vermutlich über einen alternativen

Invasionsprozess zustande, der analog zu *E. bovis* [21] und *P. falciparum* [240, 241] mit dem Aufbrechen der Plasmamembran der Wirtzelle und deren schneller Reparatur verbunden ist.

Im Gegensatz zum unspezifischen Invasionsprozess erfolgte die intrazelluläre Weiterentwicklung auf Wirtszell-spezifischer Ebene, da sie ausschließlich in Zellen ruminanten Ursprungs stattfand. Dementsprechend kam es in VERO-Zellen analog zu *E. bovis* [143] nicht zur Weiterentwicklung der Sporozoiten, die jedoch vital innerhalb einer sehr großen parasitophoren Vakuole verblieben (vgl. **7.11**). HUVEC erlaubten zwar eine gewisse Weiterentwicklung, es kam jedoch nicht zur Ausbildung maturer Makromeronten. Erstaunlicherweise war die Bildung reifer Makromeronten nicht auf endotheliale Zellen beschränkt (CUVEC, BUVEC, anteilig in BFGC), sondern erfolgte ebenso in Zellen epithelialen Ursprungs (BCEC). Diese ursprünglich aus bovine Kolonkrypten isolierte Zelllinie zeigte jedoch nach der Immortalisierung eine Veränderung ihrer ursprünglichen Eigenschaften und wurde entsprechend ihrer Charakteristik eher Makrophagen-ähnlichen Zellen zugeordnet (C. Menge, persönliche Mitteilung). Es erscheint als wahrscheinlich, dass sich zudem solche Eigenschaften geändert haben, die letztendlich eine Reifung von Makromeronten unterstützen. Nichtsdestotrotz bleibt es erstaunlich, dass BCEC nicht nur grundsätzlich eine Makromerontenentwicklung erlauben, sondern sogar den Zelltyp mit der höchsten Ausbeute an Merozoiten I repräsentieren.

Die in der *in vitro* Kultur entwickelten *E. ninakohlyakimovae*-Makromeronten variierten bezüglich ihrer Größe und Morphologie sowohl innerhalb eines Zellrasens als auch zwischen den verwendeten Zelltypen. Die im Mittel bei weitem größten Makromeronten wurden in CUVEC nachgewiesen. Unterschiedliche Morphologie-typen, wie gekammerte/ungekammerte Typen, unstrukturierte Formen, rosettenförmige Anteile enthaltende Typen oder solche, die Merozoiten in der Peripherie ansammelten, wurden in allen permissiven Zellen gleichermaßen gefunden. Die zelluläre Grundlage oder Bedeutung dieser Morphologietypen ist bisher nicht geklärt.

Mit der Entwicklung der *E. ninakohlyakimovae*-Makromeronten stellten sich analog zur *E. bovis*-Kultur (vgl. **7.9** und 4.3.1.2) Veränderungen der Zellkernmorphologie infizierter Zellen ein (vgl. **7.11**). Neben der bei jedoch nicht allen infizierten Zellen gleichermaßen beobachteten Vergrößerung des Zellkerns kam es mikroskopisch

sichtbar frühestens ab Tag 7 p. i. zu einer Zunahme des sich hell darstellenden Euchromatins zulasten des dunkel erscheinenden Heterochromatins, was auf eine replikations- oder transkriptionsaktive Zelle schließen lässt [3]. Zudem konfluierten und wuchsen die Nukleoli zu einem oder mehreren großen Nukleoli, so dass sich der Zellkern der infizierten Zellen dauerhaft ab Tag 7 p. i., also mit dem vermuteten Beginn der Parasitenproliferation, als sog. „Spiegelei“-Form darstellte. Da der Nukleolus den Ort der rRNA-Prozessierung sowie der ribosomalen Untereinheiten darstellt, indizieren die geschilderten Veränderungen der Nukleoli einen Zelltyp, der große Mengen an Protein produziert [3]. Sobald der Morphologiewechsel eingetreten war, verblieben die Zellkerne der infizierten Wirtszellen in diesem Zustand bis zur Ruptur der Zelle mit anschließender Freisetzung der Merozoiten I (vgl. **7.11**).

Mit der hier entwickelten Zellkultur für *E. ninakohlyakimovae* steht uns nun ein wichtiges Instrument zur Verfügung, das einerseits für zukünftige vergleichende Untersuchungen zu *E. bovis* herangezogen werden kann, andererseits aber natürlich auch dem Fernziel der Vakzinierung gegen *E. ninakohlyakimovae* bei Ziegen dienen wird.

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5 ZUSAMMENFASSUNG

Die individuell variierende Empfänglichkeit von Kälbern für eine *E. bovis*-Erstinfektion könnte mit unterschiedlich stark ausgeprägten, nicht-adaptativen Immunreaktionen zusammenhängen. Um deren Natur zu überprüfen, wurden Analysen zu Interaktionen von Monozyten, Makrophagen, NK-Zellen und PMN mit *E. bovis*-Sporozoiten durchgeführt. Während Monozyten nur eine geringe Reaktivität nach Konfrontation mit *E. bovis*-Sporozoiten *in vitro* zeigten, waren alle anderen Zelltypen hochreaktiv, führten zur m. o. w. stark ausgeprägten Elimination der Parasiten und produzierten (mit Ausnahme der NK-Zellen) immunmodulatorisch wirkende Mediatoren. Immunhistologische Untersuchungen zeigten, dass der Makrophagenbesatz der infizierten Darmschleimhaut nach Primär- und Reinfektion zunehmend gesteigert ist. Sowohl PMN als auch Monozyten reagierten *ex vivo* im Infektionsverlauf mit gesteigerten Phagozytose- und Oxidative Burst-Aktivitäten. PMN-vermittelte Reaktionen führten zudem zu der hier erstmalig für die Abwehr von *Eimeria*-Stadien beschriebenen Bildung sog. „Neutrophil extracellular traps“, mit der Sporozoiten zwar nicht abgetötet wurden, jedoch über Vernetzung immobilisiert und an der Invasion von Wirtszellen gehindert wurden. Insgesamt ließen die Ergebnisse auf eine wichtige Beteiligung der oben genannten Zellen an der initialen Abwehr der *E. bovis*-Infektion schließen.

Adaptative Immunreaktionen spielen generell eine wichtige Rolle bei der Abwehr von *Eimeria*-Infektionen, sowohl bezüglich der Kontrolle von Erstinfektionen als auch der Entwicklung der Immunität gegen Reinfektionen. Die Überprüfung solcher Reaktionen bei PBMC *E. bovis* experimentell primär- und reinfizierter Kälber verdeutlichte eine anhand antigenspezifischer T-Zellproliferation und zeitgleicher Synthese von IFN-γ und IL-2 als Th1-dominiert einzustufende Immunreaktion in der Präpatenz der Erstinfektion. Anschließend waren mit beginnender Patenz die Immunreaktionen über IL-4 eher Th2-orientiert und die PBMC proliferierten nicht mehr. Sowohl $CD4^+$- als auch $CD8^+$-T-Zellen wurden als Quelle der IFN-γ-Synthese in der Primärinfektion identifiziert, während IL-2 und IL-4 in erster Linie von $CD4^+$ T-Zellen gebildet wurden. Lymphknotenlymphozyten reagierten insgesamt ähnlich wie PBMC, wobei es unerheblich war, aus welchem Lymphknoten sie stammten. PBMC primärinfizierter Tiere reagierten zusätzlich im Verlauf der Infektion in Antigen-unabhängiger Weise mit einer vermehrten Produktion von IFN-γ und IL-4, was einen bisher nicht beschriebenen Mechanismus bei *Eimeria*-Infektionen darstellt. Anhand von FACS-Analysen ließ sich nach Erstinfektion im peripheren Blut eine Expansion der $CD4^+$-T-Zellen beobachten. Auf den Anteil der $CD8^+$- und γδ-TCR^+-positiven Zellen hatten die Infektionen kaum Einfluss. Nach Reinfektion blieben sowohl antigenspezifische proliferative Aktivitäten und Expansion der T-Zellsubpopulationen als auch Zytokinsynthese mit Ausnahme von IL-4 völlig aus, was einen frühen Abbruch der Reinfektion noch vor Entwicklung derjenigen Stadien, die während der

Primärinfektion Reaktionen hervorgerufen hatten, vermuten lässt. Die Infiltration der infizierten Darmschleimhaut sowohl während der Primär- als auch der Reinfektion mit $CD4^+$- und $CD8^+$-T-Zellen lässt insgesamt auf protektive Funktionen beider Zelltypen schließen.

E. bovis-Sporozoiten infizieren mit Endothelzellen hochreaktive Zellen, die ein breites Spektrum an pro-inflammatorischen und immunmodulatorischen Molekülen produzieren können und zudem sehr empfindlich auf widrige Bedingungen reagieren. Die im Rahmen dieser Arbeit durchgeführten Untersuchungen zeigten, dass Infektionen mit *E. bovis*-Sporozoiten im Gegensatz zu solchen mit Tachyzoiten der nahe verwandten Arten *T. gondii* und *N. caninum* innerhalb von 3 Tagen p. i. zur vergleichsweisen schwachen Induktion immunmodulatorischer Mediatoren führt. Analysen zur Modulation des Wirtszelltranskriptoms 4 Stunden und 4 Tage nach der Infektion bestätigen dies. Dagegen erfolgte am Tag 8 der Merogonie I eine pro-inflammatorisch ausgerichtete Immunreaktion der Wirtszellen.

Das transkriptionelle Profil von mit *E. bovis*-Sporozoiten infizierten bovinen Endothelzellen ließ insgesamt auf eine Zweiteilung der parasiten-induzierten Modulation der Wirtszelle schließen. Während in der Frühphase mit 4 Stunden und 4 Tage p. i. der Einfluss des Parasitens auf das Wirtszelltranskriptom gemessen an der Zahl regulierter Gene schwach war und eher eine hemmende Wirkung auf die Aktivierung der Endothelzellen vermuten ließ, kam es ab beginnender Proliferation des Parasitens 8 und 14 Tage p. i. zum massiven Eingriff in die wirtszelleigene Transkriptionsmaschinerie. Dabei waren diverse funktionelle Bereiche der Wirtszelle betroffen. Mit zunehmender Infektionsdauer nahm *E. bovis* vermehrt Einfluss auf Zellwachstum und -Proliferation, den Zell-Zyklus, den Zell-Tod sowie den wirtszelleigenen Metabolismus. Die Qualität der Regulation lassen auf eine parasiten-induzierte Hemmung der Apoptose und auf eine Arretierung des Zellzyklus als auch auf eine zunehmende Ausbeutung der wirtszelleigenen Nährstoffe zur Erleichterung der Parasitenproliferation schließen. Während die Nährstoffbedürfnisse von *E. bovis* bisher völlig unbekannt waren, weist die Induktion charakteristischer metabolischer Moleküle erstmalig auf eine Abhängigkeit des Parasitens von der zellulären Cholesterolsynthese während der proliferativen Phase hin.

Gegen Ende der Merogonie I durchgeführte Proteomanalysen verdeutlichten dagegen, dass der Parasit zum Zeitpunkt der Merozoiten I-Freisetzung in erster Linie den *Status quo* der Wirtszelle erhält indem er z. B: weiterhin ihre Apoptose verhindert. Ansonsten wurde das Gros der nachgewiesenen Moleküle in seiner Abundanz vermindert, was mit auch mit einem Erschöpfungszustand der Wirszelle zusammenhängen könnte.

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6 SUMMARY

Primary-infected calves show individually varying susceptibilities for *E. bovis* which may be based on differentially displayed innate immune reactions. To characterize such reactions we analysed interactions of PMN, monocytes, macrophages and NK-cells with *E. bovis* sporozoites. Besides monocytes, all other cell types showed high reactivity *in vitro* leading to sporozoite elimination and to the production of various immunomodulatory molecules (except for NK cells). Immunohistology revealed enhanced infiltration of macrophages in gut mucosa of primary and challenge infected animals. Both PMN and monocytes displayed increased phagocytic and oxidative burst activities *ex vivo* during *E. bovis* infection. Moreover, upon exposure to sporozoites, PMN reacted by the formation of neutrophil extracellular traps (NETs) which is a newly described effector mechanism against *Eimeria* stages. NET formation did not directly promote parasite death but led to sporozoite immobilisation and subsequent inhibition of host cell infection. Overall, the data indicate an important role of innate immune reactions in *E. bovis* defense.

Adaptive immune reactions against *Eimeria* species generally lead to control of primary infection and promote immunity against reinfections. We here analyse adaptive immune reactions of *E. bovis* primary and challenge infected calves. Primary infection was characterised by Th1-dominated immune responses in prepatency as measured by enhanced antigen-specific cell proliferation and IFN-γ/IL-2 gene transcription in PBMC. Thereafter, the augmented production of IL-4 in PBMC with beginning patency indicated a switch to Th2-associated immune reactions. Overall, lymphocytes isolated from lymph nodes reacted equally as PBMC. Besides antigen-specific reactions we also found an infection-triggered induction of the non-specific activation state of PBMC in the course of primary infection as detected by enhanced intracellular IFN-γ and IL-4 content of phorbol-12-myristate-13-acetate/ionomycin-stimulated PBMC. This may represent a new mechanism of immune cells of *E. bovis*-infected calves contributing to ongoing immune reactions. FACS analyses revealed an expansion of $CD4^+$ T cells during primary infection, whilst $CD8^+$ and γδ-TCR^+ T cell were hardly affected. After homologous reinfection PBMC neither proliferated nor produced cytokines, except for IL-4, suggesting an early abrogation of parasite development. Both *E. bovis* primary and challenge infections had an impact on local tissue T cell distribution. Primary infection was characterised by a $CD4^+$ T cell infiltration early in prepatency in ileum and later in colon mucosa, whereas $CD8^+$ T cells were only found accumulating in the latter gut segment.

Challenge infection led to infiltration of both $CD4^+$ and $CD8^+$ T cells in small intestine and large intestine segments indicating protective functions of both cell types.

In vivo E. bovis sporozoites must invade endothelial cells, which are highly immuno-reactive cells being able to produce a broad range of pro-inflammatory and immunomodulatory molecules. However, infections of BUVEC with *E. bovis* sporozoites led to a much weaker induction of host cell-derived immunomodulatory molecules within 3 days after infection than infections with tachyzoites of the closely related *T. gondii* and *N. caninum*. Analyses on the *E. bovis*-mediated modulation of the host cell transcriptome 4 hours and 4 days p. i. confirmed these results. In contrast, 8 days p. i. host cell response was characterised by a strong pro-inflammatory reaction indicating host cell activation.

Considering the numbers of *E. bovis*-regulated genes, the results of microarray-based transcriptional profiling suggested that merogony I was dissected in an early low-responsive phase and a late, highly reactive one beginning with the onset of parasite proliferation. Thus, 4 hours and 4 days p. i. host cells hardly reacted on the transcriptional level and *E. bovis* infection rather led to inhibition of host cell activation. Beginning with parasite proliferation at day 8 p. i. *E. bovis* increasingly regulated the transcriptional machinery influencing various functional categories of host cell transcriptome. With increasing merogony I duration *E. bovis* escalated its influence on molecules involved in cell growth/proliferation, cell cycle, cell death and host cell metabolism. The nature of regulation suggested parasite-induced inhibition of apoptosis and an arrest of cell cycle both allowing for parasite proliferation. Moreover, *E. bovis* increasingly exploited host cell nutritients during merogony I. So far, nothing was known on the precise nutritional needs of *E. bovis*. The induction of characteristic metabolic molecules measured in this work indicates the dependency of this parasite on host cell cholesterol synthesis during its proliferative phase.

Analyses of host cell proteome modulation at the very end of *E. bovis* merogony I suggest that the parasite sustains the *status quo* of the host cell to support its merozoite I formation and release. Parasite-induced differences in protein abundance concerned distinct functional categories, with most proteins being involved in host cell metabolism, cell structure, protein fate and gene transcription. The majority of modulated molecules were down-regulated and some regulated molecules indicated inhibition of host cell apoptosis allowing the parasite to complete its development.

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7 VORGELEGTE VERÖFFENTLICHUNGEN

7.1 "PMN-mediated immune reactions against *Eimeria bovis*"

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PMN-mediated immune reactions against *Eimeria bovis*

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Abstract

For successful *in vivo* infection, *Eimeria bovis* sporozoites have to traverse the mucosal layer of the ileum to infect lymphatic endothelial cells and may, thereby, be exposed to the interstitial fluid and to the lymph representing potential targets for leukocytes. To mimic this situation *in vitro*, we exposed *E. bovis* sporozoites to bovine PMN and found enhanced elimination of the parasites. Addition of immune serum clearly increased these reactions, whereas neonatal calf serum had no effect, thus proposing a PMN-derived antibody-dependent cytotoxicity. Scanning and transmission electron microscopy showed PMN engulfing sporozoites or extending filopodia towards them and occasionally incorporating the parasites. PMN reacted with enhanced transcription of IL-6, MCP-1, GROα, TNF-α, and iNOS genes after exposure to sporozoites while stimulation with merozoite-antigen, in addition, upregulated IL-8, IP-10 and IL-12 gene transcription. Furthermore, enhanced *in vitro* oxidative burst and phagocytic activities were observed after contact of PMN with viable sporozoites. To verify the potential role of PMN in the *in vivo* situation, we analysed the general phagocytic and oxidative burst activities of PMN obtained *ex vivo* from *E. bovis* experimentally infected calves. Enhanced levels of both activities were found early p.i. (1–5 days) and towards the end of the first schizogony (days 13–22 p.i.) underlining the *in vitro* data. Our results suggest that PMN-mediated, innate immune reactions play an important role in the early immune response to *E. bovis* infections in calves.

Keywords: *Eimeria bovis*; Polymorphonuclear neutrophils; Phagocytosis; Oxidative burst; Chemokines; iNOS

1. Introduction

Eimeria bovis infection in cattle is an important protozoan parasitosis, causing economic losses and severe clinical disease in calves (Fitzgerald, 1980; Daugschies et al., 1998). *Eimeria* infections, in general, are under immunological control and a variety of studies performed on cell-mediated, adaptive immune responses emphasized the crucial role of lymphocytes in *Eimeria* infections in mice and rats (Rose and Hesketh, 1982; Rose et al., 1988a,b, 1990, 1992; Shi et al., 2001) and in *E. bovis*-infected calves (Speer et al., 1985; Hughes et al., 1988, 1989; Fiege et al., 1992; Hermosilla et al., 1999). Relatively little is known, however, so far on innate immune responses to *Eimeria* infections. The first-line defence against invading pathogens, in general, is represented by professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear neutrophils (PMN). Main effector functions utilised by PMN during infection are phagocytosis of foreign material, the release of oxidative radicals as a result of the oxidative

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burst reaction and the production of immunomodulatory molecules, such as cytokines or chemokines, to attract immunopotent cells to the site of infection, thereby, initiating acquired immune responses. As additional effector mechanism, PMN-mediated killing of pathogens by forming neutrophil extracellular traps has recently been described (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). Accordingly, PMN show distinct infiltration of parasitised tissues and accumulate very early at the site of schizont formation in *Eimeria*-infected rodents (Mesfin et al., 1978; Blagburn and Todd, 1984; Schito and Barta, 1997). For successful infection, *E. bovis* sporozoites have to traverse the mucosal layer of the ileum to reach the lymphatic capillary for infection of the adequate host cells, lymphatic endothelial cells. In addition, these stages are able to egress from once-invaded cells (Behrendt et al., 2004), a phenomenon that we frequently observe in cell culture. In consequence, sporozoites should be exposed to the interstitial fluid and to the lymph representing potential targets for PMN. The importance of PMN in *Eimeria* infections is further underlined by the observation, that PMN-depleted mice produce significantly more *E. papillata* oocysts than control mice (Schito and Barta, 1997). There is also some evidence that PMN can directly interact with apicomplexan parasites. Thus, they are able to actively lyse *E. falciformis* sporozoites in the presence of antibodies and complement (Bekhti et al., 1992). In the case of *Toxoplasma gondii*, PMN have been shown to kill tachyzoites *in vitro* (Wilson and Remington, 1979; MacLaren and de Souza, 2002; MacLaren et al., 2004). Furthermore, PMN generate proinflammatory cytokines (e.g. IL-1β, IL-10, IL-12 and TNF-α) and chemokines (e.g. IL-8, MCP-1, MIP-1α and RANTES) (Bliss et al., 1999a,b, 2001; Denkers, 2003) when stimulated *in vitro* with *T. gondii* antigens, thereby, most probably attracting other immunocompetent cells to the site of infection and indirectly promoting adaptive cell-mediated immune responses. In addition, several studies dealing with PMN-depleted mice suggest the immunoregulatory role of PMN during *Toxoplasma* infection (Sayles and Johnson, 1996; Scharton-Kersten et al., 1997; Bliss et al., 1999b, 2001).

In order to characterise early PMN-mediated, innate immune reactions to *E. bovis*, we analysed *in vitro* interactions between PMN and sporozoites. We show that PMN eliminate sporozoites *in vitro* in an improved way in the presence of immune serum and identify PMN as an *in vitro*-source of several cytokines, chemokines and iNOS upon exposure to sporozoites and parasite antigens. Furthermore, the potential role of PMN in parasite control was underlined by *in vitro* and *ex vivo* data showing enhanced phagocytic and oxidative burst activities of PMN either exposed to sporozoites *in vitro* or derived from *E. bovis*-infected calves throughout infection.

2. Materials and methods

2.1. Parasite and infection

The *E. bovis* strain H used in the present study was maintained by passages in Holstein-Frisian calves for oocyst production. Calves were infected orally with 5×10^5 sporulated *E. bovis* oocysts. For *in vitro* experiments sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al., 2002). Free sporozoites were collected and suspended in medium (RPMI 1640, GIBCO, 1% penicillin/streptomycin, v/v).

2.2. Sera and E. bovis merozoite I antigen

Immune serum was obtained from a calf after primary (4×10^4 *E. bovis* oocysts) and challenge (3×10^4 *E. bovis* oocysts) infections. Neonatal serum was derived from a calf before the first-time uptake of colostrum.

E. bovis merozoites I, collected from culture as described previously (Hermosilla et al., 2002), were homogenised by repeated freezing and thawing followed by sonication (20 kHz, 5× 15 s pulses) on ice. After centrifugation (11,000 × *g*, 4 °C, 20 min) the supernatants were passed through 0.2 μm sterile filters (Renner). Protein concentration was determined using the Bradford method (Bradford, 1976). The antigen (EbAg) was stored at −80 °C until further use.

2.3. Isolation of bovine PMN

Calves were bled by puncture of the jugular vein and blood was collected in 50 ml plastic tubes (Nunc) containing 0.1 ml heparin (Sigma) as anticoagulant. Heparinised blood was centrifuged in a discontinuous Percoll (Amersham) gradient according to Hjorth et al. (1981) to yield a PMN fraction of >97% purity. PMN were washed twice with medium (RPMI 1640) to remove Percoll and resuspended in medium (RPMI 1640).

2.4. Detection of phagocytic and oxidative burst activities of PMN

Phagocytic and oxidative burst activities of PMN were determined by using Phagotest® and Phagoburst®

kits (ORPEGEN-Pharma, Heidelberg, Germany), according to Moussay et al. (2006). All tests were performed in duplicates. Blood samples were drawn from *E. bovis* experimentally infected calves on days −1, 1, 5, 7, 13, 15, 18, 20, 22 and 25 p.i. by puncture of the jugular vein. In addition, we tested PMN from three non-infected calves on days corresponding to days 1, 7, 15, 22 p.i. Four milliliters of heparinised blood were mixed with 36 ml distilled water (40 s, shaking) to lyse erythrocytes, supplemented with 10× Hank's buffer (GIBCO) and pelleted (10 min, 400 × *g*). After washing (10 ml PBS/EDTA, 10 min, 400 × *g*) cells were transferred to V-shaped microtitre plates (Nunc, 2×10^5 cells/well) and centrifuged (4 °C, 200 × *g*, 7 min).

For *ex vivo* quantification of phagocytic activity cells were suspended in 100 μl ice-cold, autologous plasma. After addition of 10 μl FITC-labelled *E. coli* pre-opsonised with human serum (provided with the kit), cells were incubated for 10 min at 37 °C (shaking waterbath) or on ice (=negative control). After transferring the plates on ice, the quenching of surface-bound bacteria, fixation and permeabilisation of cells was performed according to the manufacturer's instructions.

For *ex vivo* quantification of the inducible oxidative burst activity, cells were suspended in 100 μl ice-cold PBS, supplemented with either 10 μl non-labelled *E. coli*, phorbol-12-myristate 13 acetate solution (PMA, 8.1 μM, ORPEGEN-Pharma; =positive control) or PBS (=negative control) and incubated at 37 °C (shaking waterbath). After 10 min, 10 μl dihydrorhodamine 123 substrate solution was added and cells were incubated for a further 20 min (37 °C, shaking waterbath). After transferring the plates on ice, cells were fixed and permeabilised according to the manufacturer's instructions. In both assays PBS–EDTA were then added to the wells (4 °C, 5 min) to recover plastic-adherent cells. Cells were counterstained with "DNA-staining solution" (provided with the kits) and analysed by flow cytometry (FCM; FACScalibur, BD Biosciences) gating for PMN corresponding to their light-scattering properties.

In order to test PMN for *in vitro* oxidative burst activity in response to *E. bovis* sporozoites, we isolated cells from six cattle as described above. PMN were incubated with viable *E. bovis* sporozoites at a ratio of 10:1, 1:1 or in medium alone for 18 h (37 °C, 5% CO_2 atmosphere). For positive control, PMA was added to the cultures 10 min before the end of incubation. Cells were collected and processed as described above to estimate oxidative burst activity.

In vitro phagocytosis assays for sporozoite phagocytosis used stained viable or heat-inactivated parasites. Freshly isolated viable *E. bovis* sporozoites (1×10^6/ml PBS) were pulsed with the fluorescent dye 5(6)-carboxyfluorescein diacetate succimidyl ester (CFSE, Invitrogen, 2.5 μM, 10 min, 37 °C) and washed thereafter four times in PBS. CFSE neither affects the viability nor the infectivity of sporozoites (Hermosilla, unpublished). For heat inactivation, sporozoites were incubated for 30 min at 60 °C in a waterbath. Viable and heat-inactivated, CFSE-stained sporozoites were then exposed to bovine PMN (3 h, 37 °C) at a ratio of 1:1. Phagotest® was performed as described above (but without adding bacteria to the test samples).

In a corresponding series of experiments we controlled the elimination of the parasites from the incubation medium in the presence of PMN. Freshly isolated PMN of five donor calves were incubated with freshly isolated *E. bovis* sporozoites at a ratio of 10:1 and either supplemented with medium RPMI 1640 alone, immune serum (1:30) or neonatal serum (1:30). After a 3.5 h incubation the total number of remaining sporozoites was counted in a Neubauer chamber. Sporozoite viability was estimated by trypan blue exclusion (trypan blue, Sigma, 5 min, 1:2 dilution).

2.5. Scanning electron microscopy

Bovine PMN were incubated with freshly isolated *E. bovis* sporozoites at a ratio of 10:1 for 4, 8 and 12 h in the presence of immune serum (1:30) on poly-L-lysine-coated coverslips. Subsequently, cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 15 min, washed with 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, washed three times in distilled water, dehydrated in an ascending ethanol sequence, critical point-dried with CO_2 and sputtered with gold according to standard procedures. Specimens were scanned using a Philips PSEM 500 or Philips XL20.

2.6. Transmission electron microscopy

PMN were fixed after 2, 8 and 12 h of incubation with *E. bovis* sporozoites in 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed and postfixed in 1% osmium tetroxide in buffer. After thoroughly washing in distilled water they were incubated overnight in 2% aqueous uranyl acetate at 4 °C, dehydrated in ethanol and embedded in Epon. Ultrathin sections of the cured blocks were mounted on formvar-coated grids and stained with uranyl acetate and Reynolds lead citrate. The sections were inspected in a ZEISS EM912 AB (Oberkochem, Germany).

2.7. Gene transcription in PMN after exposure to E. bovis sporozoites and to merozoite I antigen

For RT-PCR analyses, freshly isolated PMN from six donors were either exposed to freshly isolated *E. bovis* sporozoites at a ratio of 10:1 or incubated in *E. bovis* merozoite-antigen (10 μg/ml final concentration) for 6 h (37 °C, 5% CO_2). PMN incubated in medium alone served as negative control.

2.8. Isolation and reverse transcription of total RNA

Isolation of total RNA from 1×10^6 PMN after cultivation was performed according to the manufacturer's instructions using TRIZOL® reagent (Invitrogen). RNA was resuspended in 20 μl RNase-free, double distilled water and stored at −80 °C until further use. For cDNA synthesis total RNA was treated with RNase-free DNase I (1 U/μg RNA, Roche; 30 min, 37 °C). After inactivation of DNase I (75 °C, 6 min) cDNA synthesis was performed using M-MLV-reverse-transcriptase (Invitrogen). Briefly, 14 μl of DNase I-treated total RNA isolated from 1×10^6 PMN was mixed with 5 μl 5× RT-buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCL, 15 mM $MgCl_2$, Roche), 2 μl DTT (0.1 M, Roche), 2 μl hexanucleotides (62.5 A_{260}/ml; Roche), 1 μl dNTPs (10 mM, MBI Fermentas), and 1 μl M-MLV-reverse transcriptase (200 U/μl) and incubated at 37 °C for 60 min. The synthesized cDNA was diluted with 25 μl TE buffer (10 mM Tris–HCl, pH 8.1 mM EDTA) and stored at −20 °C until further use.

2.9. Relative quantification of the IL-8, IP-10, MCP-1, GROα, IL-6, TNF-α, IL-12, iNOS and GAPDH gene transcripts by real-time PCR

Sequences of primers (MWG Biotech) and probes (Eurogentec) are depicted in Table 1. Primers and probes for IL-6 and IL-12p40 were designed using the Primer Express software (Version 2.0; Applied Biosystems). Probes were labelled at the 5′-end with the reporter dye

Table 1
Sequences of bovine primers and probes used for real-time RT-PCR

Specificity	Primers and probes (5′–3′)	Reference
IL-8-forw	CAC TGT GAA AAA TTC AGA AAT CAT TGT TA	Leutenegger et al. (2000)
IL-8-rev	CTT CAC CAA ATA CCT GCA CAA CCT TC	
IL-8-probe	AAT GGA AAC GAG GTC TGC TTA AAC CCC AAG	
IP-10-forw	AAG TCA TTC CTG CAA GTC AAT CCT	Taubert et al. (2006)
IP-10-rev	TTG ATG GTC TTA GAT TCT GGA TTC AG	
IP-10-probe	CCA CGT GTC GAG ATT ATT GCC ACA ATG A	
MCP-1-forw	CGC TCA GCC AGA TGC AAT TA	Taubert et al. (2006)
MCP-1-rev	GCC TCT GCA TGG AGA TCT TCT T	
MCP-1-probe	CCC AAG TCG CCT GCT GCT ATA CAT TCA A	
GROα-forw	CGC CTG TGG TCA ACG AAC T	Taubert et al. (2006)
GROα-rev	CAC CTT CAC GCT CTG GAT GTT	
GROα-probe	CCA GTG CCT GCA GAC CTT GCA GG	
IL-6-forw	CTG AAG CAA AAG ATC GCA GAT CTA	This study
IL-6-rev	CTC GTT TGA AGA CTG CAT CTT CTC	
IL-6-probe	CAC TCC AGC CAC AAA CAC TGA CCT GCT	
TNFα-forw	TCT TCT CAA GCC TCA AGT AAC AAG T	Leutenegger et al. (2000)
TNFα-rev	CCA TGA GGG CAT TGG CAT AC	
TNFα-probe	AGC CCA CGT TGT AGC CGA CAT CAA CTC C	
IL-12-forw	GCA GCT TCT TCA TCA GGG ACA T	This study
IL-12-rev	CCT CCA CCT GCC GAG AAT T	
IL-12-probe	CAC CCA AGA ACC TGC AAC TGA GAC CAT TAA	
iNOS-forw	GGC CCA GGA AAT GTT CGA A	Taubert et al. (2006)
iNOS-rev	ACA GTG ATG GCC GAC CTG AT	
iNOS-probe	AGA CAC GTG CGT TAT GCC ACC AAC AA	
GAPDH-forw	GCG ATA CTC ACT CTT CTA CCT TCG A	Taubert et al. (2006)
GAPDH-rev	TCG TAC CAG GAA ATG AGC TTG AC	
GAPDH-probe	CTG GCA TTG CCC TCA ACG ACC ACT T	

FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). PCR amplification was performed employing an automated fluorometer (ABI PRISM™ 5700 Sequence Detection System, Applied Biosystems) using 96-well optical plates. Samples were analysed in duplicate. For PCR 5 μl cDNA (corresponding to 25 ng total RNA) were used in a 25 μl PCR reaction mixture containing 12.5 μl TaqMan® PCR Master Mix (Eurogentec), 300 nM of each primer and 200 nM probe. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s and at 60 °C for 60 s.

Semi-quantitative analyses used the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the ABI PRISM™ 5700 Sequence Detector manufacturer and reported as *n*-fold differences in comparison to the respective medium control (after normalising the samples referring to their corresponding housekeeping gene GAPDH).

To determine the linear range and amplification efficiencies of the GAPDH versus IL-6 and IL-12p40 systems, six 4-fold dilution steps were amplified from two different cDNAs derived from Con A-stimulated PBMC in duplicate to obtain standard curves. The differences of the slopes between standard curves obtained from GAPDH and the genes of interest (which should be <0.1 for reliable quantification) were plotted against the logarithm of input total RNA and a regression line was calculated.

2.10. Statistical analyses

Statistical analyses used the programme package BMDP for XP, Release 8.1 (Dixon, 1993). For the description of the data arithmetic means were calculated. To describe the variability of the data, for very low sample sizes ($n = 3$) minima and maxima were given, for all other cases standard deviation was used. As some statistical distributions of the original data were skewed to the right, if necessary, logarithmic (for gene transcription data) or arc-sine (for percentages data) transformations were performed to obtain an approximately normal distribution of the values. In accordance to the design of the experiments, co-culture/stimulation conditions were either compared by *t*-test for dependent samples (BMDP3D, for gene transcription data) or by one/two-factorial analysis of variance (ANOVA) with repeated measures (BMDP2V, for all other data) followed by the Student–Newman–Keuls test for pairwise treatment comparison. Differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. Phagocytic and oxidative burst activities of PMN are enhanced after E. bovis infection

We directly analysed PMN *ex vivo* from whole blood samples, i.e., without performing any further isolation techniques or stimulation; consequently, these results should reflect the *in vivo* situation. Data generated on day −1 p.i., which reflect the situation of non-infected animals, revealed low phagocytic and oxidative burst activities. The same held true for the non-infected calves (data not shown), whilst a biphasic upregulation of the general phagocytic and oxidative burst activities of PMN was observed in *E. bovis*-infected calves (Fig. 1). Phagocytic activity was enhanced early after infection at day 1 p.i. reflecting the phase of sporozoite transmigration through the epithelium of the ileum and invasion of endothelial cells. A second peak was detected at days 13–18 p.i., corresponding to the phase of first meront maturation and the release of first generation merozoites. Highest values occurred on day 18 p.i. when, by means, more than 60% of PMN showed increased phagocytic activity. A similar time course dynamics was detected in the case of oxidative burst activity of PMN with increased values at day 5 p.i. and at days 15 and 22 p.i. At 15 days p.i. almost all (97.4 ± 3.2%) PMN displayed oxidative burst activity. For both activities ANOVA showed significant differences when calculating dynamics of time (for burst activity $p = 0.013$, for phagocytic activity $p = 0.0002$) and when comparing test with control samples represented by the interaction (for burst activity $p \leq 0.0001$, for phagocytic activity $p = 0.0001$). Stimulation of PMN with PMA, serving as positive control, led to consistently high values of oxidative burst activity (94.5 ± 6.8%) throughout *E. bovis* infection (data not shown).

To mimic the *in vivo* situation and to exclude non-parasite-induced reactions driven by, e.g., host cell degradation, we directly exposed PMN to *E. bovis* sporozoites *in vitro* and measured the intracellular oxidative burst after 18 h. In comparison to the PBS control, addition of sporozoites significantly enhanced (for both doses $p < 0.01$) burst activity of PMN (Fig. 2). In addition, these reactions were dose-dependent (Fig. 2: differences between the two doses were significant with $p < 0.05$).

3.2. PMN eliminate E. bovis sporozoites

Global comparison of the different culture conditions revealed significant differences ($p < 0.0001$).

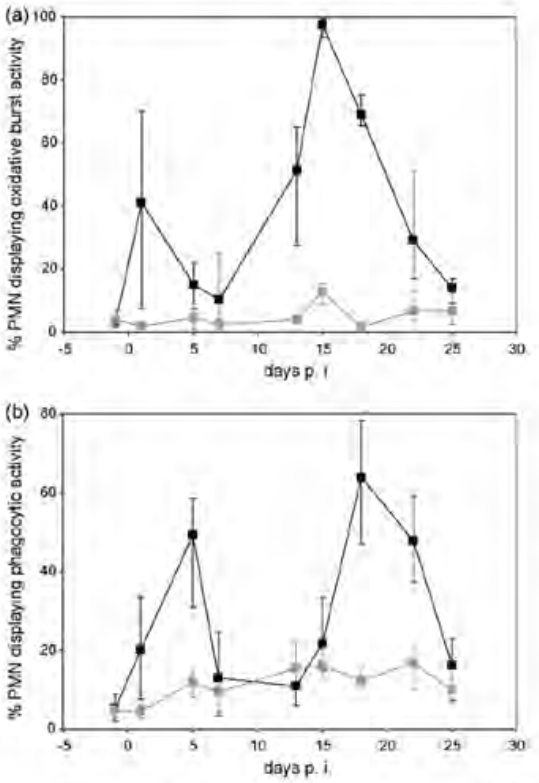

Fig. 1. Oxidative burst (a) and phagocytosis (b) activity of PMN isolated *ex vivo* from *Eimeria bovis* infected calves. Calves ($n = 3$) were orally infected with *E. bovis* oocysts (3×10^4 oocysts/animal). Bovine PMN were isolated from infected calves on various days after infection. Oxidative burst activity (a) was investigated by flow cytometry estimating the oxidation of dihydrorhodamine 123 fluorogen stimulated by *Escherichia coli* phagocytosis. The general phagocytic activity (b) was estimated by flow cytometry determining the uptake of FITC-labelled *E. coli*. Incubations were performed at 37 °C (black symbols) or on ice (controls, grey symbols). Arithmetic means of three PMN donors, minimum, maximum.

Exposure of viable *E. bovis* sporozoites to PMN in the absence of serum significantly reduced ($p < 0.01$) the number of parasites by approximately 30%, suggesting the direct elimination of parasites (Fig. 3). The percentage of remaining, dead parasites was not affected. Incubation of sporozoites and PMN in the presence of immune serum resulted in a significantly enhanced ($p < 0.01$) elimination of parasites (Fig. 3); on average 66% of the sporozoites were eliminated indicating PMN-mediated, antibody-dependent cytotoxicity. In contrast, exposure of PMN to sporozoites in the presence of serum derived from a neonatal calf before colostrum uptake, which should be free of *E. bovis*-specific antibodies, did not increase PMN-mediated elimination of the sporozoites when compared

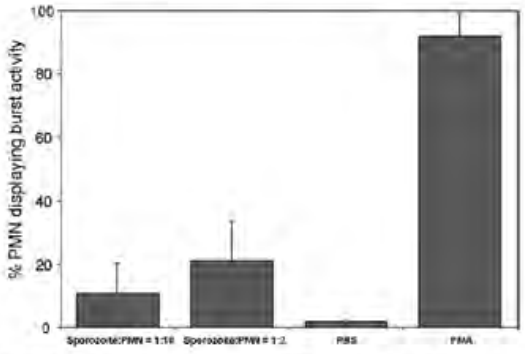

Fig. 2. Oxidative burst activity of PMN exposed to *Eimeria bovis* sporozoites *in vitro*. Bovine PMN were incubated with *E. bovis* sporozoites at ratios of 10:1 and 2:1 for 18 h. Oxidative burst activity was investigated by flow cytometry estimating the oxidation of dihydrorhodamine 123 fluorogen stimulated by sporozoite uptake. Exposure to phorbol-12-myristate 13 acetate (PMA, 8.1 μM) or PBS were used as positive and negative controls, respectively. Arithmetic means of six PMN donors and standard deviations.

to serum-free samples (in consequence, according to ANOVA, differences were not significant).

3.3. *E. bovis* sporozoites both actively invade PMN and are phagocytised by PMN

To exclude, that the reduction in numbers of sporozoites after exposure to PMN *in vitro* was merely owing to active infection of PMN, we tested PMN for parasite uptake using viable and heat-inactivated CFSE-stained sporozoites by FCM analysis (Fig. 4). After exposure to viable sporozoites 6.5% of PMN contained

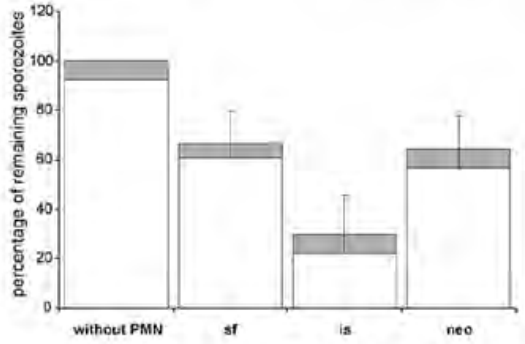

Fig. 3. PMN-mediated elimination of *E. bovis* sporozoites *in vitro*. Bovine PMN were exposed to *E. bovis* sporozoites (10:1) for 3.5 h in the absence (sf = serum free) or presence of immune serum (is) or neonatal serum (neo). The total number of live (white) and dead (grey part of the column) sporozoites were determined. PMN-free controls were set 100%. Columns represent arithmetic means of five PMN donors and standard deviations calculated for the percentage of live sporozoites.

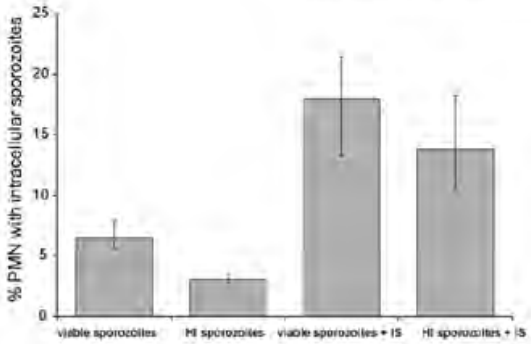

Fig. 4. Intracellular *E. bovis* sporozoites in PMN after *in vitro* exposure. Bovine PMN were incubated (3 h, 37 °C) with CFSE-stained viable or heat-inactivated (HI) *E. bovis* sporozoites (1:1) in the presence or absence of immune serum (IS). Quantification was done by flow cytometry analyses. Arithmetic means of three PMN donors, minimum, maximum.

one or more sporozoites, a situation that may have relied on both, active invasion of sporozoites into PMN or PMN-mediated phagocytosis of sporozoites. When sporozoites were heat-inactivated before exposure and, thereby, no longer able to actively infect PMN, the proportion of cells containing sporozoites decreased to 3.1%, however, ANOVA showed, that differences were barely not significant ($p = 0.069$). Nevertheless, both mechanisms would seem to occur in parallel. Whilst the proportion of PMN phagocytising sporozoites was significantly enhanced by the supplementation of immune serum ($p = 0.044$), the proportion of PMN being actively invaded by sporozoites remained relatively constant independent of the presence or absence of immune serum (Fig. 4). Thus, statistically significant interaction was not detected between IS+/IS− culture conditions.

3.4. PMN attack E. bovis sporozoites by forming filopods, engulfing and incorporation

SEM analyses illustrated early interactions of PMN with *E. bovis* sporozoites (Fig. 5): after first contacts, filamentous structures (a) and protuberances (b) of the PMN surface directed towards the sporozoites were observed. PMN also showed slender and finger-like filopods (c) or more compact protrusions engulfing or partially embracing (d and e) the parasite. Sporozoites were either attacked by single (a–d) or multiple PMN (e). There was no apparent preferential site for cell–parasite contacts. Occasionally, we found a sporozoite stuck with the posterior pole in a neutrophil cell indicating ongoing phagocytosis of the parasite (Fig. 5f). However, during cell culture we also, occasionally, observed sporozoites actively invading PMN, some of them leading to the rupture of the PMN.

TEM analyses showed PMN containing one (Fig. 6a) or more (b and c) sporozoites. Two hours after incubation the membranes surrounding the parasites appeared smooth and intact and PMN-induced degradation did not seem to have taken place. In contrast, after 8 h of incubation the surface of intracellular sporozoites showed multiple swellings, dents and wrinkles (b, arrows) whereas extracellular sporozoites were unchanged in their morphology. After 12 h incubation, these reactions had intensified with the pellicula of the sporozoites showing severe crinkles and foldings (c, arrows). Occasionally, the sporozoite was heavily altered and disrupted in its morphology (c, asterisks). In one case we found a sporozoite sticking with the apical end in a PMN, which may be judged as active invasion of this PMN (d). TEM analyses, furthermore, confirmed observations made by SEM as some PMN were found to embrace and engulf a sporozoite (e).

3.5. Exposure to E. bovis sporozoites or to merozoite I antigen leads to upregulation of immunoregulatory molecule gene transcription in PMN

Exposure of PMN to *E. bovis* sporozoites, in general, and except for iNOS, resulted in a weaker upregulation of gene transcription than addition of merozoite-antigen (Fig. 7). However, ANOVA revealed significant differences merely for IL-12 ($p = 0.0069$). Relative to the medium controls, co-culture of PMN with sporozoites induced an upregulation of the PMN-derived MCP-1 (4.4-fold, n.s.), GRO-α (2.8-fold, n.s.), IL-6 (2.7-fold, $p = 0.0014$) and TNF-α (2.2-fold, $p = 0.032$) genes, whereas the transcriptional levels of the IL-8, IP-10 and IL-12p40 genes were not affected. In the case of iNOS gene transcripts, incubation with sporozoites led to a significant increase compared to the medium control ($p = 0.0032$) and to comparable if not higher values when compared to merozoite-antigen. Exposure to merozoite-antigen induced an upregulation of the transcription of all genes tested. The strongest reactions were found in the case of IL-6 (9.8-fold, $p = 0.0044$) and MCP-1 (7.5-fold, $p = 0.042$), followed by IL-12p40 (6.2-fold, $p = 0.0032$), TNF-α (5.2-fold, n.s.), iNOS (4.5-fold, $p = 0.019$) and GRO-α (4.2-fold, $p = 0.0005$). The gene transcriptions of IP-10 (2.9-fold, $p = 0.017$) and IL-8 (2.2-fold, n.s.) were only moderately influenced.

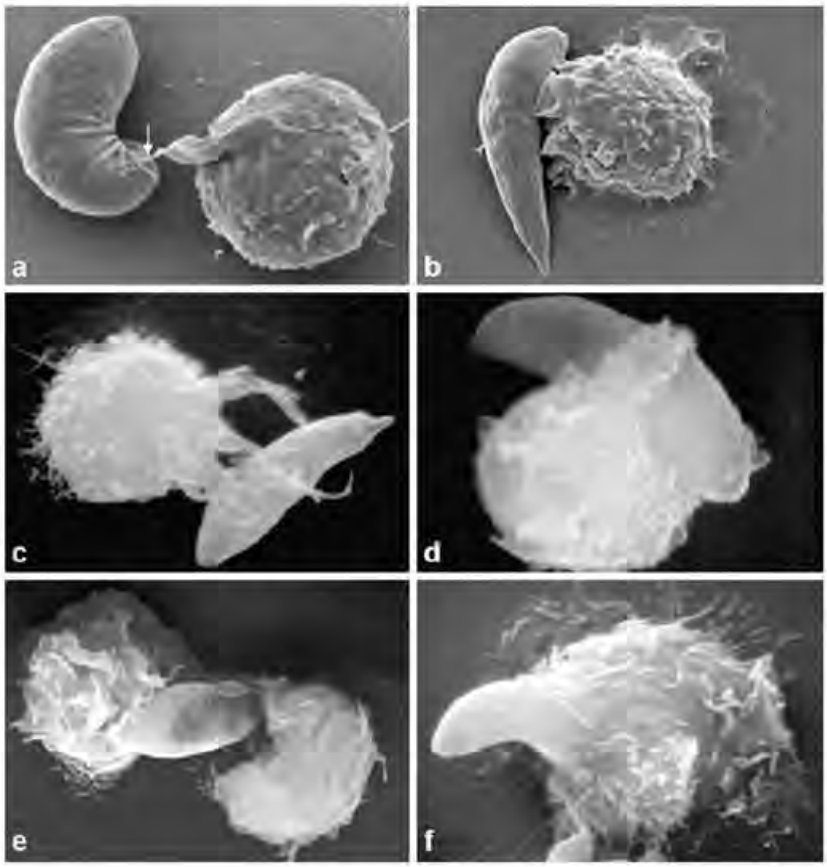

Fig. 5. Interactions of PMN with *E. bovis* sporozoites illustrated by REM analyses. Bovine PMN were exposed to *E. bovis* sporozoites for 4 h in the presence of immune serum. Interactions were illustrated by REM analyses ranging from surface-derived filamentous structures (a) or protuberances (b) directed towards the parasite to finger-like filopods (c) or more compact protrusions (d) engulfing the sporozoite, in some cases from the apical and posterior side (e), or leading to parasite uptake (f).

4. Discussion

Innate immune reactions against *E. bovis* have scarcely been investigated in the past, although, the first encounter between parasite and immune system should be decisive for the subsequent outcome of the infection. In this work we have focused on PMN-mediated immune reactions directed against *E. bovis in vitro* and *ex vivo*. We found enhanced phagocytic and oxidative reactivities of PMN obtained from calves experiencing experimental *E. bovis* infections. Direct exposure of PMN to *E. bovis* sporozoites *in vitro* reflected these reactions and, additionally, led to accelerated elimination of the parasite and to activation of PMN, which were identified as the *in vitro*-source of different immunoregulatory molecules upon exposure to parasite antigens. These results suggest PMN as active mediators of innate immune responses against *E. bovis*.

We could demonstrate that PMN of *E. bovis*-infected calves exhibit enhanced, general phagocytic and oxidative burst activities coinciding with periods of time when *E. bovis* stages are most probably not yet or no longer situated intracellularly and, therefore, are highly vulnerable to PMN. Thus, these activities were increased early after infection, i.e., when sporozoites having excysted in the abomasum are migrating through the epithelial layer of the ileum in search for their primary host cell, and during schizogonies, when merozoites are released in the mucosa. For the sporozoite stage, free access for PMN may even occur at days 1–5 p.i. as these stages are able to egress from once-invaded cells (Behrendt et al., 2004), a phenomenon that we frequently observe *in vitro* and that is also described for other *Eimeria* spp. (Danforth et al., 1984, 1992). Furthermore, PMN adhesion to infected endothelial cell layers as reported for 1–2 days p.i. *in*

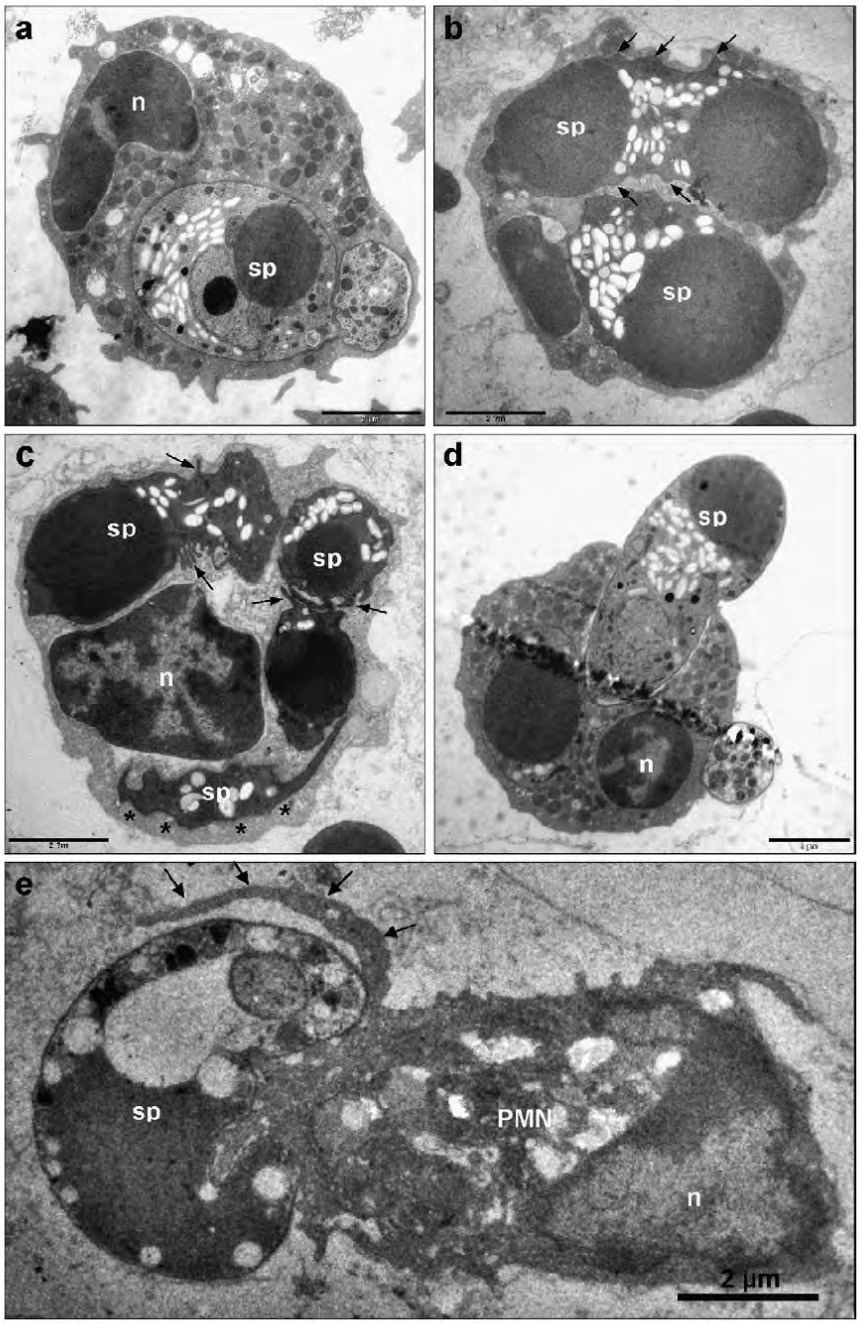

Fig. 6. Interactions of PMN with *E. bovis* sporozoites illustrated by TEM analyses. Bovine PMN were exposed to *E. bovis* sporozoites in the presence of bovine immune serum for different periods of time. Two hours after exposure (a and e) PMN were found engulfing sporozoites (e, arrows); the pellicula of the intracellular sporozoite seemed intact and smooth (a). Six hours later the parasite's surface showed multiple swellings and wrinkles (b, arrows) whilst 12 h after exposure these reactions had intensified (c, arrows) and some sporozoites were heavily altered in their morphology (c, asterisks). Active invasion of PMN was illustrated by a sporozoite sticking with the anterior end in a PMN (d). sp = sporozoite, *n* = nucleus of the PMN.

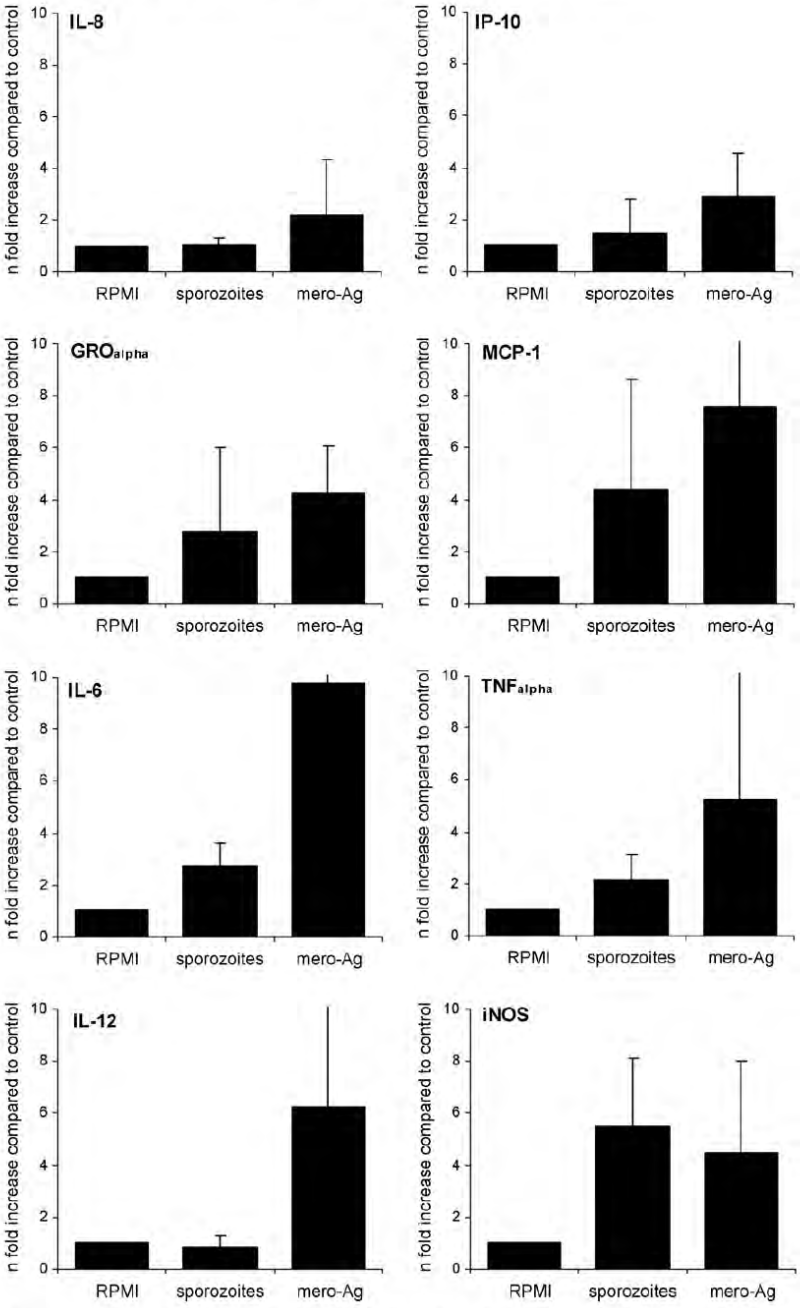

Fig. 7. Transcription of genes encoding for IL-8, IP-10, GRO-α, MCP-1, IL-6, TNF-α, IL-12 and iNOS in PMN after exposure to *E. bovis* sporozoites or merozoite-antigen. Bovine PMN were exposed to *E. bovis* sporozoites (10:1), merozoite-antigen (mero-Ag, 10 μg/ml) or medium alone for 6 h. Total RNA was isolated, reversely transcribed into cDNA and probed with real-time RT-PCR systems for the detection of the respective mRNA equivalents. Arithmetic means of six PMN donors and standard deviations.

vitro (Hermosilla et al., 2006) may also play a role to this respect. In this work, we directly analysed PMN from whole blood samples, thereby, avoiding laborious isolation methods that may alter the activation state of these cells (Fearon and Collins, 1983; Hed et al., 1987), a technique that has been successfully applied for the generation of *ex vivo* data in *Babesia bovis*-infected cattle (Court et al., 2001). We, therefore, consider the reactions as indicating *in vivo*-activity. Since enhanced PMN activities may also have been induced by degrading host cells, we mimicked the *in vivo* situation by co-culturing PMN and sporozoites *in vitro*. The clear induction of oxidative burst and phagocytic activity of PMN underlined the *ex vivo* results.

Mimicking the interaction of PMN and *E. bovis* sporozoites *in vitro*, we could further show that PMN are able to eliminate up to 30% of sporozoites in an antibody-independent manner. Although these reactions are not fully effective, they might contribute to early parasite elimination. The fact that the elimination process took rather a long time (3–4 h) to happen may – besides oxidative burst and phagocytosis activities – suggest the formation of neutrophil extracellular traps (NET), a phenomenon that was recently described to be involved in the capture and killing of relatively large-sized pathogens, such as *Candida albicans* hyphae (Urban et al., 2006; Brinkmann and Zychlinsky, 2007). In REM analyses of long-term incubation samples we observed morphologically likewise phenomena (Behrendt, unpublished data). Ongoing experiments measuring the generation of extracellular DNA after PMN and sporozoite-co-cultures also suggest NET-formation as an additional mechanism in sporozoite killing (Behrendt and Ruiz, unpublished data), but further analyses are needed to confirm this hypothesis.

PMN-mediated killing of sporozoites was also reported for *E. faleiformis*, a coccidian parasite of mice, but was only observed to occur when parasites and PMN were incubated in the presence of immune serum for at least 18 h (Bekhti et al., 1992). Supplementation with immune serum clearly led to reduction in numbers *E. bovis* sporozoites in our assays, although this effect was already observed after 3–4 h of incubation. However, TEM analyses indicated that sporozoites situated intracellularly in PMN were not morphologically affected until 8 h after exposure. Overall, these results suggest PMN-promoted, antibody-dependent cytotoxicity as an additional mechanism of parasite killing, a hypothesis which was further supported by the fact that supplementation with neonatal serum instead of immune serum did not enhance sporozoite elimination. Other *in vitro* studies dealing with PMN-promoted immune reactions showed that contact of these cells with other apicomplexan parasites, i.e., *T. gondii* tachyzoites, resulted in both parasite killing by phagocytosis (MacLaren and de Souza, 2002; MacLaren et al., 2004) and active invasion of PMN. Although PMN neither represent an adequate host cell nor promote *E. bovis* development, *E. bovis* sporozoites, in principle, might be able to invade these immune cells. By using fluorescent viable and heat-inactivated sporozoites in co-cultures, we could clearly show by flow cytometry analyses, that active PMN invasion by sporozoites occurs, but to a minor degree.

We could furthermore show, that PMN reacted upon exposure to viable sporozoites by upregulation of MCP-1, GROα and IL-6 gene transcription, i.e., of molecules involved in attracting cells of the innate immune system. When stimulated with homogenized parasite antigen, PMN reacted more strongly and in a broader range by upregulating additional molecules, such as IP-10 or IL-12, which act predominantly on lymphocytes (see e.g. Taub et al., 1993; Gately et al., 1998; Trinchieri, 1998a,b) and, thereby, probably trigger the development of parasite-specific immune reactions. The fact, that the antigen generally induced stronger reaction than viable sporozoites may be explained by a more effective uptake of soluble antigens or by a better disposability of potential pathogen-associated molecular patterns (PAMP) motives. Interestingly and in contrast to all other molecules under investigation, exposure of PMN to sporozoites induced more iNOS gene transcripts than stimulation with merozoite-antigen. iNOS is essential for the generation of NO radicals, which are known to mediate killing of intracellular parasites (Ovington and Smith, 1992; Ovington et al., 1995; Scharton-Kersten et al., 1997) or to act extracellularly after degranulation. As the proportion of dead sporozoites was not increased in *in vitro* co-cultures of PMN and sporozoites, extracellular PMN-derived NO or other granule-derived molecules seem ineffective in our experiments.

Taken together, the present results suggest PMN as active mediators of innate immune responses against *E. bovis* by orchestrating different effector mechanisms for parasite elimination and as cells involved in the transition from innate to adaptive immune reactions. In addition, some observations, suggest the formation of NET-like structures, probably facilitating the killing of this large-sized pathogen, a hypothesis, which is currently under more detailed investigation.

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7.2 "Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*"

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Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis*

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ABSTRACT

Eimeria bovis infections are under immunological control and recent studies have emphasized the role of early PMN-mediated innate immune responses in infected calves. Neutrophil extracellular traps (NETs) have recently been demonstrated to act as a killing mechanism of PMN against several pathogens. In the present study, the interactions of bovine PMN with sporozoites of *E. bovis* were investigated in this respect *in vitro*. For demonstration and quantification of NET formation, extracellular DNA was stained by Sytox Orange. Fluorescence images after Sytox Orange staining as well as scanning electron microscopy (SEM) showed NET formation to occur upon contact with *E. bovis* sporozoites. Exposure of PMN to viable sporozoites induced stronger NET formation than to dead or homogenized parasites. NET formation was abolished by treatment with DNase and could be reduced by diphenylene iodonium, which is described as a potent inhibitor of NADPH oxidase. After sporozoite and PMN co-culture, extracellular fibres were found attached to sporozoites and seemed to trap them, strongly suggesting that NETs immobilize *E. bovis* sporozoites and thereby prevent them from infecting host cells. Thus, transfer of sporozoites, previously being confronted with PMN, to adequate host cells resulted in clearly reduced infection rates when compared to PMN-free controls. NET formation by PMN may therefore represent an effector mechanism in early innate immune reactions against *E. bovis*. This is the first report indicating *Eimeria*-induced NET formation.

1. Introduction

Eimeria bovis infection in cattle is an important protozoan parasitosis, causing economic losses and severe clinical typhlocolitis in calves (Fitzgerald, 1980; Daugschies et al., 1998).

In general, *Eimeria* infections are under immunological control and a variety of studies performed on cell-mediated, adaptive immune responses emphasized the crucial role of lymphocytes in rodents (Rose et al., 1990, 1992; Shi et al., 2001) and calves (Speer et al., 1985; Hughes et al., 1988, 1989; Fiege et al., 1992; Hermosilla et al., 1999; Taubert et al., 2008). So far, however, relatively little is known on innate immune responses to *Eimeria* infections. The first-line defence against invading pathogens, in general, is represented by professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear neutrophils (PMN). Main effector mechanisms of PMN are the killing of pathogens and the production of immunomodulatory molecules, such as cytokines or chemokines, to attract immunopotent cells to the site of infection, thereby initiating acquired immune responses. Classical PMN-conducted killing involves phagocytosis, the production of reactive oxygen species, and antimicrobial peptides/proteins. In addition, the formation of

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neutrophil extracellular traps (NETs) has been recently identified as a further effector mechanism of PMN-mediated pathogen killing. NETs act effectively against bacteria and fungi (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007) and may represent a common mechanism to eliminate pathogens.

In the case of *Eimeria* infections, PMN show distinct infiltration of parasitized tissue and accumulate very early at the site of meront formation in infected rodents (Mesfin et al., 1978; Blagburn and Todd, 1984) and in *E. bovis*-infected calves (Friend and Stockdale, 1980). The importance of PMN in *Eimeria* infections is further underlined by the observation, that PMN-depleted SCID mice significantly produce more *E. papillata* oocysts after primary infection than control mice (Schito and Barta, 1997).

For successful infection, *E. bovis* sporozoites have to traverse the mucosal layer of the ileum to reach the lymphatic capillaries for infection of the adequate host cells, lymphatic endothelial cells. In consequence, sporozoites of *E. bovis* should be exposed to the interstitial fluid and to the lymph and should be recognized as potential targets by PMN.

There is some evidence that PMN can directly interact with apicomplexan parasites in general. PMN have been shown to actively lyse *E. falciformis* sporozoites in the presence of antibodies and complement (Bekhti et al., 1992). In the case of *Toxoplasma gondii*, PMN have been shown to kill tachyzoites *in vitro* (Wilson and Remington, 1979; MacLaren and de Souza, 2002; MacLaren et al., 2004). We have recently shown that bovine PMN interact directly with *E. bovis* sporozoites (Behrendt et al., 2008) and are able to eliminate sporozoites *in vitro*. Furthermore, bovine PMN were identified as an *in vitro*-source of several pro-inflammatory cytokines (IL-6, IL-12, TNF-α), chemokines (MCP-1, GRO-α, IL-8, IP-10) and iNOS when exposed to *E. bovis* sporozoites or merozoite I antigens (Behrendt et al., 2008). The role of PMN in *E. bovis* control was also underlined by *in vitro* and *ex vivo* data showing enhanced phagocytic and oxidative burst activities of PMN either exposed to sporozoites *in vitro* or derived from *E. bovis*-infected calves (Behrendt et al., 2008).

This study was conducted to characterise early reactions of bovine PMN against *E. bovis* with respect to NET formation. We show here that direct contact of PMN with viable sporozoites triggers the formation of NETs, which, in turn, immobilize these invasive stages of the parasite thus preventing the infection of host cells. As such, sporozoite-induced NET formation was proven as additional effector mechanism directed against *E. bovis* sporozoites *in vitro*.

2. Materials and methods

2.1. Parasite

The *E. bovis* strain H used in the present study was maintained by passages in Holstein–Friesian calves for oocyst production. Calves were infected orally with 5×10^5 sporulated *E. bovis* oocysts. Collection of oocysts, oocyst sporulation and excystation of sporozoites were performed as previously described (Hermosilla et al., 2002). Free sporozoites were collected and suspended in medium free of FCS (RPMI 1640, Gibco, 1% penicillin/streptomycin, v/v).

2.2. Isolation of bovine PMN

Calves were bled by puncture of the jugular vein and blood was collected in 50 ml plastic tubes (Nunc), containing 0.1 ml heparin (Sigma) as anticoagulant. Heparinized blood was diluted in an equal amount of PBS containing 0.02% EDTA (Sigma), layered on Biocoll Separating Solution (Biochrom AG) and centrifuged at 800 × *g* for 45 min. After removal of plasma, lymphocytes and monocytes, the pellet was suspended in distilled water and shaken for 40 s to lyse erythrocytes. Osmolarity was immediately re-adjusted by adding the appropriate amount of Hanks Salt Solution (10×, Biochrom AG). PMN were washed twice with RPMI 1640 medium, resuspended in medium RPMI 1640 (6.6×10^6 cells/ml) and incubated at 37 °C and 5% CO_2 for at least 30 min before use.

2.3. Scanning electron microscopy

Bovine PMN were incubated with freshly isolated *E. bovis* sporozoites at a ratio of 10:1 for 2, 3 and 4 h on poly-L-lysine pre-coated coverslips. After incubation, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 15 min and afterwards washed with 0.1 M cacodylate buffer. The cells were then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point dried with CO_2 and sputtered with gold. Specimens were examined using a Philips XL20 scanning electron microscope.

2.4. Co-culture of bovine PMN and Eimeria bovis sporozoites

PMN were placed in flat bottom 96-well plates at a density of 5×10^5 cells/well. For different experimental conditions, the wells were filled up to a final volume of 200 μl with various incubation mixes and incubated for 3 h at 37 °C in a 5% CO_2 atmosphere. All compounds used were diluted or suspended in medium RPMI 1640 whilst plain medium served as negative control. For positive controls, PMN were stimulated with phorbol 12-myristate 13-acetate (PMA, ORPEGEN-Pharma) at a final concentration of 10 ng/ml. To obtain maximum values for extracellular DNA, PMN were lysed with Triton X100 (0.1%), added 15 min before the end of incubation.

To test for sporozoite-induced NET-formation, 10^5 sporozoites (vital or heat inactivated at 60 °C for 30 min) were added. To test a sporozoite-homogenate for it's ability to induce NET-formation, sporozoites underwent three freeze and thaw cycles (freezing in liquid nitrogen for 1 min and complete thawing at 37 °C) and subsequent sonication (15 min, 50 kHz). The amount of homogenate per well corresponded to 10^5 sporozoites.

For DNase treatment 90 U DNase I (Roche Diagnostics) per well were added at the start of incubation. Inhibition assays were performed using 5 μM diphenylene iodonium (DPI) or 10% neonatal calf serum throughout the incubation period.

2.5. Quantitation of neutrophil extracellular traps (NETs)

NETs were quantified after staining extracellular DNA with Sytox Orange according to others (Martinelli et al., 2004; Lippolis et al., 2006). Samples were stained by Sytox Orange (Invitrogen) at a final concentration of 1 μM for 10 min. They were analyzed by a fluorometric reader (Ascent Fluoroskan, Labsystems) using an excitation wavelength of 530 nm and detecting at 590 nm. Results were always confirmed by microscopical observations.

2.6. Host cells and parasite infection

Bovine umbilical vein endothelial cells (BUVEC) were isolated as previously described (Taubert et al., 2006) and cultured in endothelial cell growth medium (ECGM; PromoCell). Cells were seeded into 25 cm^2 tissue culture flasks and incubated at 37 C and 5% CO_2 atmosphere until confluency.

To test the impact of sporozoite-induced NET formation on the infectivity of the parasites, vital sporozoites (4×10^5/well in duplicates) were incubated with bovine PMN (8×10^5/well) for 3 h (37 C, 5% CO_2) in the presence or absence of DNase (90 U/well). PMN- and DNase-free samples were used as positive controls. After incubation, co-cultures/sporozoites were transferred to confluent BUVEC and incubated for 1 h (37 C, 5% CO_2). Thereafter, cell layers were washed thoroughly with prewarmed ECGM to remove PMN and dead or excrescent sporozoites and infection rates were estimated microscopically.

2.7. Statistical analysis

Co-culture/stimulation conditions were compared by one- or two-factorial analysis of variance (ANOVA) with repeated measures. Differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. Exposure of E. bovis sporozoites to PMN induces NET formation

SEM analyses revealed that co-cultivation of bovine PMN with live sporozoites resulted in the formation of a delicate network of thicker and thinner strands of fibres originating from PMN and being firmly attached to the parasites, seemingly trapping them (Fig. 1a and b). Such structures were exclusively detected in serum-free preparations. PMN involved in this network appeared disrupted and were obviously dead, whilst others, which did not participate in this process, showed normal shapes.

These parasite-induced NET-like structures were proven to contain DNA by Sytox Orange staining. As analysed by simultaneous fluorescence and phase contrast microscopy we found sporozoites in close proximity to NETs and presumably being trapped in these structures (Fig. 2b and c).

Stimulation with PMA, being used as positive control, clearly induced the formation of microscopically detectable, DNA-containing fibres (Fig. 2a), although the appearance of fibres was partially subject to mechanical impact. When conducting the experiments, we noticed that abruptly adding the dye caused a flow of medium, leading to parallel orientation of many fibres as illustrated in Fig. 2a. When avoiding any mechanical impact, stimulation with PMA generally led to a rather cloudy and diffuse fluorescence.

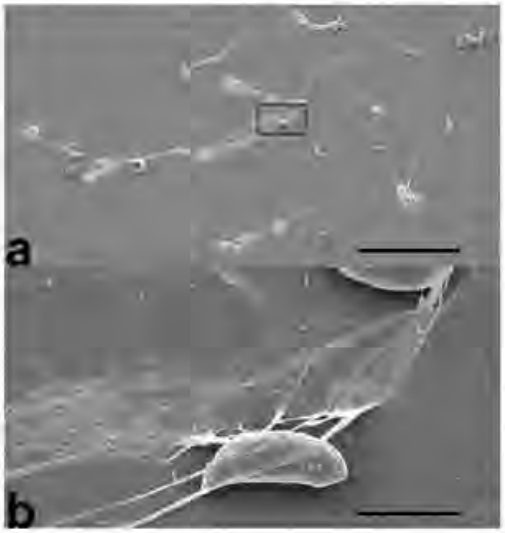

Fig. 1. NETs formed by bovine PMN co-cultured with *E. bovis* sporozoites. Scanning electron microscopy analysis revealed NETs formed by bovine PMN co-cultured with *E. bovis* sporozoites for 4 h in the absence of serum. (a) Several sporozoites can be seen sticking to a network of long drawn-out fibres originating from dead and disrupted PMN. PMN that are not involved in NET formation are not altered in morphology (scale bar 50 μm). (b) The detailed view shows NETs to consist of an outspread filigree meshwork of filaments. A number of strands are firmly attached to an *E. bovis* sporozoite (scale bar 5 μm).

Quantification of fluorescence intensities mirroring NET formation revealed that co-culture of PMN with *E. bovis* sporozoites significantly increased the amount of extracellular DNA when compared to parasite-free (negative) controls (ratio 1:1, $p < 0.01$, Fig. 3). Furthermore, parasite-induced NET formation was dose-dependent, as increasing the amount of vital sporozoites led to enhanced fluorescence intensities (Fig. 3). Given that Triton X100 treatment reflected lysis of all PMN (≈100%), co-cultures of PMN and sporozoites at ratios of 4:1, 2:1 and 1:1 led to 20.7, 22.8 and 30.6% DNA release of the PMN, respectively. In contrast, in parasite-free negative controls 14.8% of the PMN contributed to extracellular DNA content of the samples.

3.2. Sporozoite-induced NET formation follows a rapid kinetic

As illustrated in Fig. 4, different kinetics of DNA release were observed after exposure of PMN to live sporozoites and PMA: the cells responded much faster and stronger to the parasites than to PMA. As such, sporozoites induced high fluorescence intensities already within 30 min of incubation, whilst a clear induction of this feature in PMA-treated samples – compared to the negative control – was soonest achieved after 3 h (Fig. 4).

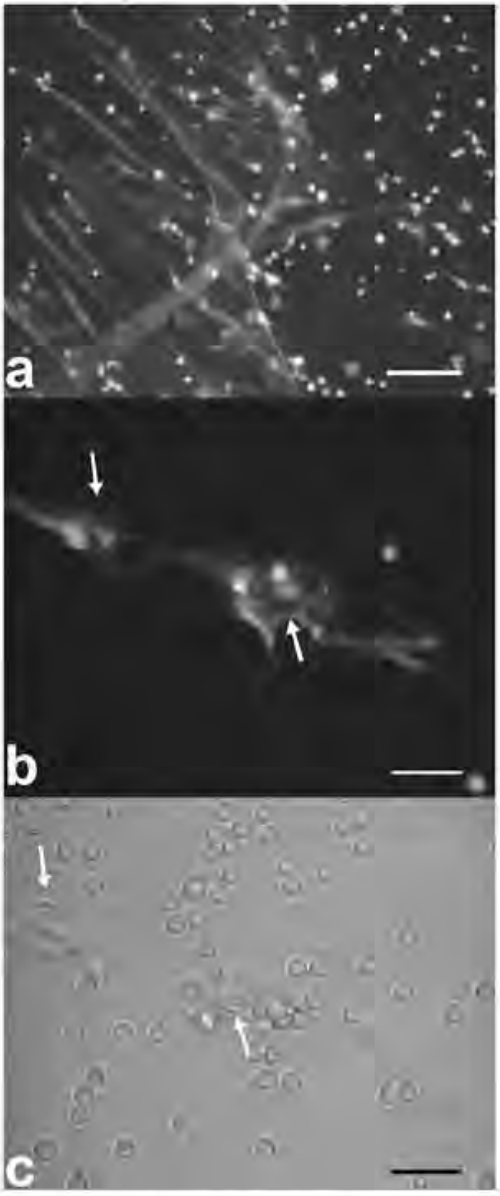

Fig. 2. DNA staining with Sytox Orange confirming NET formation. (a) NET formation after stimulation with PMA (scale bar 100 μm). (b) fluorescence and (c) phase contrast microscopy of NET formation after co-culture of PMN with *E. bovis* sporozoites: several PMN displayed NET formation. Two sporozoites in close proximity to the net and presumably trapped within the NET are indicated by arrows (scale bar 30 μm).

3.3. Effective sporozoite-induced NET formation is dependent on the parasites viability/integrity

Experiments performed with either viable, dead morphologically intact or crushed sporozoites implicated that *E. bovis*-induced NET formation is dependent on the sporozoite's viability and/or integrity (Fig. 5). Thus, homogenized and heat-inactivated sporozoites only slightly, but nevertheless significantly, enhanced the DNA-related fluorescence in comparison to parasite-free controls ($p < 0.01$ and $p < 0.05$, respectively), whilst the use of viable sporozoites led to much stronger signals ($p < 0.01$) (Fig. 5). Stimulation with PMA induced much weaker signals of NET formation than viable sporozoites, but led to stronger fluorescence intensities when compared to dead or homogenized parasites.

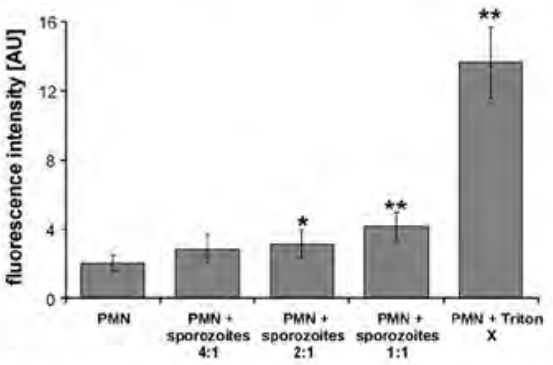

Fig. 3. Quantification of sporozoite-induced NET formation by Sytox Orange. PMN were incubated with *E. bovis* sporozoites at different ratios or in plain medium (negative control) in the presence of cytochalasin D to prevent phagocytosis. To obtain maximum values for extracellular DNA, PMN were lysed by Triton X100. After incubation, the samples were analysed for extracellular DNA by quantifying Sytox Orange-derived fluorescence intensities (displayed in arbitrary units, AU). Samples were compared to the negative control by Student's *t*-test. Significant differences to the negative controls are indicated by asterisks (**$p < 0.01$; *$p < 0.05$). Arithmetic means and standard deviations of three PMN donors.

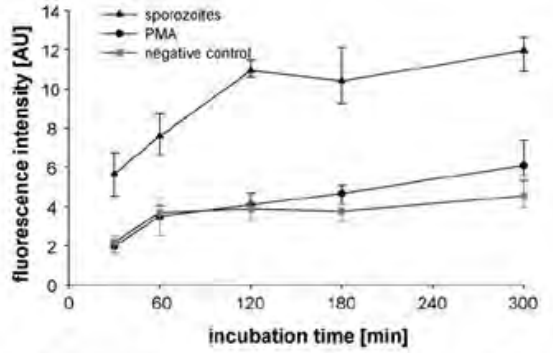

Fig. 4. Kinetics of sporozoite-induced NET formation. PMN were incubated with *E. bovis* sporozoites, PMA or medium (negative control) for different time periods. After incubation, the samples were analysed for extracellular DNA by quantifying Sytox Orange-derived fluorescence intensities (displayed in arbitrary units, AU). Each condition was performed in triplicates for each calf. Arithmetic means of three PMN donors, minimum and maximum.

3.4. Sporozoite-induced NET formation is inhibited by DNase, DPI and serum treatment

The DNA-nature of *E. bovis*-induced NET-like structures was additionally confirmed by DNase treatment (Fig. 6). A significant reduction of Sytox Orange-derived fluorescence

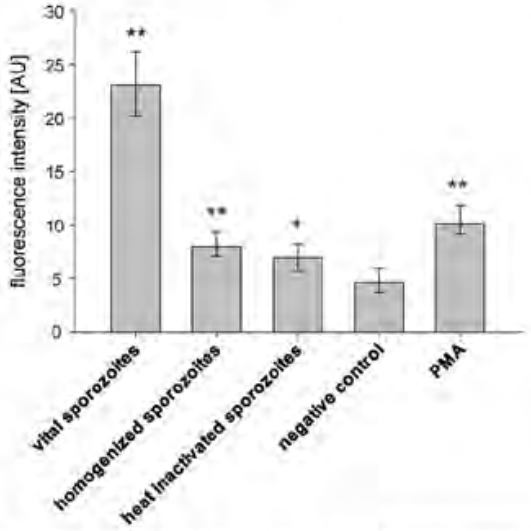

Fig. 5. NET formation induced by vital, homogenized or heat-inactivated sporozoites. Bovine PMN were incubated with vital, homogenized or heat-inactivated sporozoites. Stimulation with PMA was used as positive control; incubation in plain medium served as negative control. Each sample was analysed in triplicates. Fluorescence intensities are displayed in arbitrary units (AU). Different sporozoite preparations were compared to the negative control by the Dunnet's test. Significant differences to the negative controls are indicated by asterisks (**$p < 0.01$; *$p < 0.05$). Arithmetic means of three PMN donors, minimum and maximum.

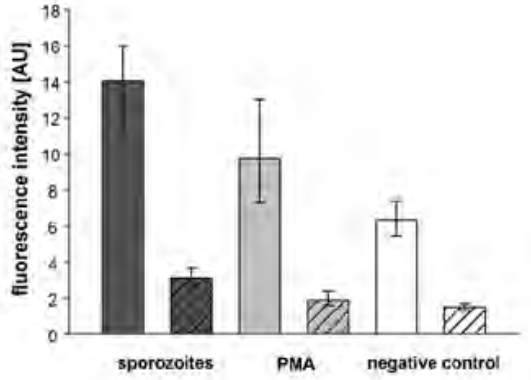

Fig. 6. Sporozoite-induced NET formation after DNase treatment. PMN were incubated for 3 h with *E. bovis* sporozoites, PMA or medium (negative control) in the presence (hatched bars) or absence of DNase. Each condition was performed in triplicates for each calf. Fluorescence intensities are displayed in arbitrary units (AU). Extent of DNA degradation was significantly higher when incubated with sporozoites compared to the negative control ($p < 0.05$). Arithmetic means of three PMN donors, minimum and maximum.

intensities after co-culture with sporozoites was measured in DNase-treated samples ($p < 0.05$). Corresponding effects were observed in the case of PMA-activated PMN ($p < 0.05$). The DNase effect was also visible in PMN lacking any stimulation, but significantly lower compared to PMN co-cultured with sporozoites ($p < 0.05$).

To further confirm the characteristics of NET we performed inhibition assays with DPI, an inhibitor of the

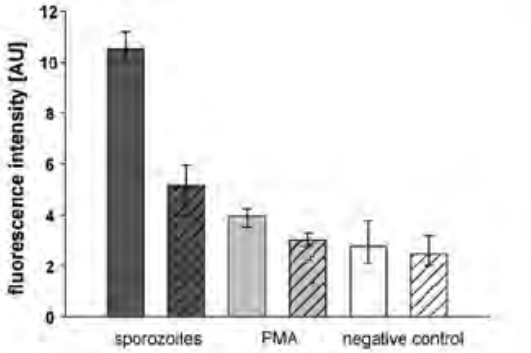

Fig. 7. Sporozoite-induced NET formation after DPI treatment. PMN were incubated for 3 h with *E. bovis* sporozoites, PMA or medium (negative control) in the presence (hatched bars) or absence of diphenylene iodonium (DPI), a potent inhibitor of the NADPH oxidase. Each condition was performed in triplicates for each calf. Fluorescence intensities are displayed in arbitrary units (AU). Supplementation with DPI led to significantly reduced amount of extracellular DNA under either conditions, incubation with sporozoites or PMA, when compared to the negative control ($p < 0.05$ each). Arithmetic means of three PMN donors, minimum and maximum.

NADPH oxidase. Supplementation of DPI throughout the incubation period resulted in significantly reduced parasite- and PMA-induced NET formation (each with $p < 0.05$; Fig. 7), whilst treatment of parasite- and PMA-free samples did not influence the amount of extracellular DNA.

Addition of serum also reduced NET formation as judged by the fluorescence images (not shown in figures). However, an obviously high DNA content of the serum seemed to have masked this inhibition in the fluorescence readings (data not shown).

3.5. Parasite-induced NET formation prevent sporozoites from invading host cells

In all experiments conducted so far, lethal effects of NETs on sporozoites were not observed. SEM analyses rather suggested immobilization of the parasites. As sporozoite motility represents an essential prerequisite for host cell invasion and subsequent multiplication of the parasite, we tested, whether co-culture of sporozoites and PMN influenced the subsequent infectivity of the sporozoites. For these experiments viable sporozoites were first exposed to PMN and transferred to host cell cultures thereafter. As shown in Fig. 8, previous encounter of PMN significantly prevented sporozoites from invading host cells afterwards. Thus, infection rates decreased from 43.9 ± 5.8%, resulting from PMN-free sporozoite samples, to 15.5 ± 2.2% after co-culture. DNase treatment almost completely abolished this effect (40.7 ± 4.5% infection rate) proving NETs as causing agents.

4. Discussion

NETs were first described by Brinkmann et al. (2004) who showed that activated human PMN can form extracellular fibres capable of binding and killing Gram-positive and -negative bacteria. By now, there are several

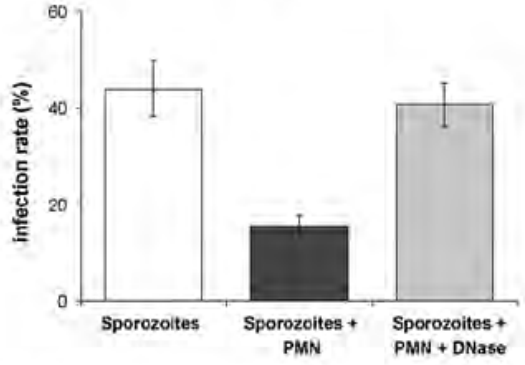

Fig. 8. Infectivity of *E. bovis* sporozoites after exposure to PMN. Vital *E. bovis* sporozoites were co-cultured for 3 h with bovine PMN in the presence (=sporozoites + PMN + DNase) or absence (=sporozoites + PMN) of DNase. Incubation in plain medium served as PMN-free, positive control (=sporozoites). After incubation, the samples were transferred to confluent BUVEC monolayers for 1 h. Thereafter, the cell layers were thoroughly washed and infection rates were estimated. Arithmetic means and standard deviations of three PMN donors.

reports showing NETs to be produced by PMN originating from different vertebrate species like man (Gupta et al., 2005), mouse (Buchanan et al., 2006; Beiter et al., 2006; Wartha et al., 2007), horse (Alghamdi and Foster, 2005), cattle (Lippolis et al., 2006) and fish (Palic et al., 2007). NETs are not only described to be involved in defence against bacteria, but also against fungi (*Candida albicans*, Urban et al., 2006). Furthermore, antimicrobial extracellular trap formation is seemingly not unique for PMN, but is also described as effector mechanism for mast cells (von Köckritz-Blickwede et al., 2008). The formation of extracellular traps in response to parasites has largely not been investigated, although this mechanism was particularly discussed to affect large sized pathogens (Urban et al., 2006). There is one report, however, showing NET formation in blood samples from *Plasmodium falciparum* infected patients. Infected erythrocytes and trophozoites were observed sticking to fibrous structures identified as NET by DNA staining (Baker et al., 2008).

We have recently demonstrated by *in vitro* and *ex vivo* assays that PMN are involved in innate immune reactions against *E. bovis* sporozoites and that they utilize some of their effector mechanisms, such as oxidative burst or phagocytosis to attack these parasitic stages (Behrendt et al., 2008). In this study we now show that the sporozoite stage of *E. bovis* also induces NET formation as additional effector mechanism of PMN.

PMN derived NET-like structures firmly attached to *E. bovis* sporozoites were visualized by scanning electron microscopy. The pictures resembled strikingly those presented by other authors (Brinkmann et al., 2004; Gupta et al., 2005; Urban et al., 2006). Since NETs mainly consist of chromatin (Brinkmann et al., 2004), we proved its DNA-nature by staining with Sytox Orange and double checked it by DNase treatment. Fluorescence images with brightly stained fibres and the complete loss of fluorescence in DNase-treated samples corroborated our assumption that these structures are NETs. The noticeable amount of extracellular DNA in unstimulated controls most likely originated from dead cells which are always present in PMN isolations.

Further evidence for *Eimeria*-induced NETs was obtained by inhibition assays. Assembly and activation of the NADPH oxidase complex, resulting in the formation of reactive oxygen species (ROS), is an essential step in the process of NET formation (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007). Consequently, treatment with DPI as a potent inhibitor of NADPH oxidase (Cross and Jones, 1986) significantly reduced Sytox Orange-derived fluorescence signals. We were not able to quantify NETs in the presence of serum as high amounts of DNA, which are common in serum, obscured the NET derived fluorescence. As judged by microscopic observation, however, serum also clearly inhibited NET formation, which is in agreement with data generated by Fuchs et al. (2007). These findings, however, are somewhat contradictory to reports on NETs detected in blood samples of patients infected with *P. falciparum* (Baker et al., 2008) or multiple trauma patients (Margraf et al., 2008).

There was a great difference in extent and time course of sporozoite- and PMA-induced NET formation, a phenomenon also described for bacterial-induced NETs. As shown for *Staphylococcus aureus* (Fuchs et al., 2007), also *E. bovis* sporozoites induced more NETs and the kinetics were faster than after stimulation with PMA. Brinkmann and Zychlinsky (2007) suggested that the simultaneous stimulation of multiple receptors is required to effectively trigger NET formation. This could explain why intact pathogens induced stronger reactions than PMA-stimulation. To meet the need of simultaneous stimulation, a functional and structurally intact parasite surface might be required, explaining the much lower extent of NET formation induced by heat-inactivated or homogenized parasites. In addition, heat inactivation could cause denaturation of "activation-relevant" molecules on the parasite surface. Not only fluorescence intensities but also the appearance of visible fibres was most prominent after stimulation with vital sporozoites. This may also be attributed to the parasites motility since we observed that mechanical impact, e.g. as caused by incautious pipetting, in general affects NET formation. This appears not too surprising since there is, so far, no indication for an active mechanism of NET extrusion. However, active extrusion may not be needed as, *in vivo*, mechanical influence is omnipresent.

So far, we could not observe lethal effects of NETs on sporozoites, a phenomenon that has also been described for certain bacteria (Buchanan et al., 2006; Beiter et al., 2006). SEM analyses rather suggested immobilization of the parasites, which, in contrast to extracellularly multiplying bacteria and fungi, may have a preventive effect on host cell invasion representing the prerequisite for subsequent replication of the parasite. Thus, we could show that pre-incubation of sporozoites with PMN clearly affects the parasites infectivity causing approximately 65% reduction of infection rates. As supplementation with DNase abolished this effect, it appears convincing that NET formation hampers sporozoites from invasion. Overall, NETs may not kill sporozoites

directly, but might have detrimental effects on successful *E. bovis* establishment by immobilizing the parasite in order to abrogate the parasites development and to facilitate subsequent phagocytosis by other phagocytes. In consequence, sporozoite-induced NET formation should also play an important role in the *in vivo* situation.

Signals and corresponding receptors for NET-activation are still questioned. In case of unopsonized microbes, pattern recognition receptors (PRR), such as Toll-like receptors (TLRs) or dectin are discussed (Urban et al., 2006). Also for intracellular parasites TLRs may be involved since several parasitic protozoans are sensed by TLR molecules (for review see Gazzinelli and Denkers, 2006). However, so far, no data are available concerning *E. bovis* stages.

Taken together, our results suggest PMN as active mediators of innate immune responses against *E. bovis* sporozoites by forming NETs to immobilize these stages and to prevent them from invading host cells.

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7.3 "Monocyte- and macrophage-mediated immune reactions against *Eimeria bovis*"

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Monocyte- and macrophage-mediated immune reactions against *Eimeria bovis*

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ABSTRACT

Innate immune reactions conducted by macrophages may affect the outcome of primary infections and are crucial for the transition to adaptive immune responses. In bovine coccidiosis little is known on early monocyte/macrophage-mediated responses. We therefore investigated *in vivo*, *in vitro* and *ex vivo* reactions of monocytes and macrophages against *Eimeria bovis*, one of the most pathogenic *Eimeria* species in cattle. Macrophages significantly infiltrate the gut mucosa of *E. bovis*-infected calves, particularly after challenge infection. Furthermore, peripheral monocytes of infected animals, as precursor cells of macrophages, exhibited enhanced *ex vivo* phagocytic and oxidative burst activities. Enhanced levels of both activities were found early after infection and towards the end of first merogony. *In vitro* exposure of macrophages to sporozoites led to phagocytosis of the pathogen, whilst monocytes failed to do so. Phagocytosis occurred independently of the viability of the sporozoites, indicating that active invasion by the parasites was negligible. Phagocytosis occurred in the absence of immune serum, but could clearly be enhanced by addition of immune serum, suggesting macrophage-derived antibody-dependent cytotoxicity. Furthermore, co-culture of macrophages with sporozoites and stimulation with merozoite 1 antigen induced distinct levels of cytokine and chemokine gene transcription. Thus, the transcription of genes encoding for IFN-γ, IL-12, TNF-α, IL-6, CXCL1, CXCL8, CXCL10 and COX-2 was upregulated after sporozoite encounter. In contrast, soluble merozoite 1 antigen only induced the gene transcription of IL-6 and IL-12 and failed to upregulate IFN-γ and TNF-α gene transcripts. In monocytes, IFN-γ and CXCL10 were found upregulated, all other immunoregulatory molecules tested were not affected. In summary, our results strongly suggest that macrophage-mediated, innate immune reactions play an important role in the early immune response to *E. bovis* infections in calves.

1. Introduction

Eimeriosis in cattle is an important enteric protozoan parasitosis causing economic losses and severe clinical disease in calves (Fitzgerald, 1980; Daugschies et al., 1998). So far, innate immune responses of ruminant hosts against *Eimeria* spp. have hardly been investigated, although these reactions may be crucial for the outcome of a primary infection with respect to the severity of the disease and an effective induction of adaptive immunity. The first line of defence against invading pathogens is represented by professional phagocytes, such as macrophages, dendritic cells and polymorphonuclear neutrophils (PMNs). PMN have recently been identified as effector cells against one of the most pathogenic *Eimeria* species in cattle, *Eimeria bovis* (Behrendt et al., 2008). Hardly anything, however, is

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known on the early role of monocytes or macrophages against this pathogen. Friend and Stockdale (1980) demonstrated macrophages in degenerating macromeronts of *E. bovis*-infected animals. Hughes et al. (1987) even described macromeront development of *E. bovis* in cultured bovine monocytes. Mucosal macrophage infiltration was reported in *E. tenella*- and *E. acervulina*-infected chickens and in *E. separata*-infected rats (Trout and Lillehoi, 1993; Vervelde et al., 1996; Shi et al., 2000). A biphasic increase of large mononuclear cells was observed in the peripheral blood of *E. nieschulzi*-infected rats and *E. maxima*-infected chickens (Rose et al., 1979). *In vitro* analyses suggested avian and murine macrophages isolated from immune animals as potent phagocytes for *Eimeria* sporozoites (Rose, 1974; Rose and Lee, 1977; Bekhti and Pery, 1989), although elimination of the parasites appeared to depend on the presence of immune serum and complement (Bekhti and Pery, 1989).

Additional effector functions utilised by macrophages are the release of oxidative radicals and the production of immunomodulatory molecules, such as cytokines or chemokines, to attract immunocompetent cells to the site of infection initiating, thereby, acquired immune responses. *In vitro* analyses demonstrate that avian macrophages react upon exposure to sporozoites by upregulating NO production and iNOS gene transcription (Lillehoj and Li, 2004; Dalloul et al., 2007). Enhanced iNOS gene transcription was also found in the mucosa of *Eimeria*-infected chickens and rats (Shi et al., 2001; Laurent et al., 2001), although, the source of this molecule was not defined. In addition, reactive oxygen species (ROS) are reported as enhanced in leukocytes of *Eimeria*-infected hosts (Ovington and Smith, 1992; Prowse et al., 1992) and seem to be effective against *Eimeria* stages (Hughes et al., 1989; Michalski and Prowse, 1991).

Avian macrophages isolated from *E. tenella* and *E. maxima*-infected animals showed enhanced IL-1 and TNF-α production (Byrnes et al., 1993). The latter was also found increased in a macrophage cell line co-cultured with *E. tenella* stages (Zhang et al., 1995). Recent microarray analyses on avian macrophages, which had been exposed to sporozoites of different *Eimeria* subspecies showed parasite-induced effects on the synthesis of various cytokines and chemokines, which were partially subspecies specific (Dalloul et al., 2007).

However, for the bovine system detailed data concerning monocyte/macrophage actions in eimeriosis are still lacking. In order to characterise early macrophage-mediated, innate immune reactions against *E. bovis*, we analysed *in vitro* interactions between macrophages and sporozoites. We show that macrophages phagocytise sporozoites under serum-free conditions and in the presence of immune serum, whilst monocytes fail to do so. In addition, macrophages were identified as an *in vitro*-source of several cytokines and chemokines upon exposure to sporozoites and parasite-antigens. Furthermore, the potential role of monocytes and macrophages in parasite control *in vivo* was underlined by the demonstration of macrophage mucosal infiltration in *E. bovis*-infected animals and by *ex vivo* data showing enhanced phagocytic and oxidative burst activities of monocytes derived from *E. bovis*-infected calves throughout infection.

2. Materials and methods

2.1. Calves

Holstein Friesian calves were purchased from a local farmer at the age of 2 weeks, treated with Baycox® (Bayer) and Halocur® (Intervet) in the second week after birth, assessed for parasitic infections, and when found parasite free, maintained under parasite-free conditions in autoclaved stainless steel cages (Woetho) until experimental *E. bovis* infection. They were fed with milk substitute (Hemo Mischfutterwerke) and commercial concentrates (Raiffeisen). Water and sterilized hay were given *ad libitum*.

2.2. Parasite maintenance

The *E. bovis* strain H used in the present study was maintained by passages in Holstein Friesian calves. For the production of oocysts, calves were infected at the age of 10 weeks with 5×10^4 sporulated oocysts each. Excreted oocysts were isolated from the faeces beginning 18 days p.i. according to the method of Jackson (1964). Sporulation was achieved by incubation in a 2% (w/v) potassium dichromate (Sigma) solution at room temperature. Sporulated oocysts were stored in this solution at 4 °C until further use.

Sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al., 2002). For *in vitro* infections bovine umbilical vein endothelial cells (BUVEC) (Taubert et al., 2006), grown to confluence, were infected with freshly isolated sporozoites (10^6 sporozoites/75 cm^2 culture flask). Culture medium (endothelial cell growth medium, PromoCell) was changed 24 h p.i. and thereafter every second day. From day 18 p.i. onwards, *E. bovis* merozoites I were harvested from culture as previously described (Hermosilla et al., 2002).

2.3. Infections, bleedings and necropsies of experimental animals

Calves ($n = 3$; group 1 = primary infection, group 2 = challenge infection) were infected orally with 5×10^4 sporulated *E. bovis* oocysts. Challenge infection was performed on day 40 after primary infection. Non-infected calves ($n = 3$, group 3) were used as negative controls. Shedding of oocysts was determined from day 18 p.i. onwards by daily faecal examination (McMaster technique). For the determination of oxidative burst and phagocytic activities of monocytes blood samples were drawn from *E. bovis* experimentally infected calves on days −1, 1, 5, 7, 13, 15, 18, 20, 22 and 25 p.i. by puncture of the jugular vein.

Calves were necropsied on days 26 after primary or challenge infection. Tissue samples of the jejunum, ileum, caecum and colon and associated lymph nodes (*Lnn. jejunales*, *Lnn. ileocaecales* and *Lnn. colici*) were excised for immediate fixation (4% formaldehyde in phosphate-buffered saline, 24 h) and embedded in paraffin.

2.4. Immunohistochemistry

5 μm cross-sections of formalin-fixed tissues were deparaffinied according to standard histological procedures. Endogenous peroxidase was inactivated in 0.5% H_2O_2 (30 min, room temperature, Roth). Samples were washed for 5 min in Tris-buffered saline (TBS) and treated with protease (protease type 24, 5 min, 37 °C, Sigma). Protease activity was stopped by dipping the slides in 4 °C TBS. Tissue samples were then probed with monoclonal mouse anti-human monocyte/macrophage-specific antibodies (1:100, 60 min, 37 °C, humidity chamber, MAC387, Serotec), which cross-react with bovine cells (Gutierrez et al., 1999). After rinsing three times in TBS (5 min), samples were incubated in sheep anti-mouse IgG conjugated with peroxidase (1:50, 30 min, 37 °C, humidity chamber, NA 931, Amersham Pharmacia Biotech Europe). After three washings in TBS (5 min), reactions were visualised by adding substrate (0.048 g DAB, Fluka, and 800 μl 3% H_2O_2 in 80 ml imidazole buffer, 3–5 min). After rinsing three times in TBS (5 min) and once in Aqua dest (5 min), the tissue samples were counterstained for 15 s in Papanicolaou solution (1:10, Merck), washed in tap water (5 min), dehydrated according to standard procedures and mounted in Aquatex® (Merck).

Immunostained macrophages present in gut mucosa were counted in 10 randomly chosen vision fields (200× magnification) per sample.

2.5. Detection of the ex vivo phagocytic and oxidative burst activities of monocytes

Phagocytic and oxidative burst activities of monocytes were determined by using Phagotest® and Phagoburst® kits (ORPEGEN-Pharma), according to Moussay et al. (2006). All tests were performed in duplicates. Four ml of heparinised blood were mixed with 36 ml distilled water (40 s, shaking) to lyse erythrocytes, supplemented with 10× Hank's buffer (Gibco) and pelleted (10 min, 400 × g). After washing (10 ml PBS/EDTA, 10 min, 400 × g) cells were transferred to V-shaped microtitre plates (2×10^5 cells/well, Nunc) and centrifuged (4 °C, 200 × g, 7 min).

For *ex vivo* quantification of phagocytic activity cells were suspended in 100 μl ice-cold autologous plasma. After addition of 10 μl FITC-labelled *Escherischia coli* pre-opsonized with human serum (provided with the kit), cells were incubated for 10 min at 37 °C (shaking water bath) or on ice (=negative control). After transferring the plates on ice, the quenching of surface-bound bacteria, fixation and permeabilisation of cells was performed according to the manufacturer's instructions.

For *ex vivo* quantification of the inducible oxidative burst activity, cells were suspended in 100 μl ice-cold PBS, supplemented with either 10 μl non-labelled *E. coli*, phorbol-12-myristate 13 acetate solution (PMA, 8.1 μM, ORPEGEN-Pharma; =positive control) or PBS (=negative control) and incubated at 37 °C (shaking water bath). After 10 min, 10 μl dihydrorhodamine 123 substrate solution were added and cells were incubated for further 20 min (37 °C, shaking water bath). After transferring the plates onto ice, cells were fixed and permeabilised according to the manufacturer's instructions.

In both assays PBS-EDTA were then added to the wells (4 °C, 5 min) to recover plastic-adherent cells. Cells were counterstained with "DNA-staining solution" (provided with the kits) and analysed by flow cytometry (FCM; FACScalibur, BD Biosciences).

2.6. Isolation and cultivation of bovine monocytes and macrophages

For both, monocytes and macrophages, PBMC had to be isolated in advance. Therefore, 18 ml of blood, substituted with 2 ml 3.8% citric acid, were mixed with 17 ml of 0.9% NaCl and applied on the top of 12 ml Ficoll-paque (density = 1.077 g/l, Biochrom) in 50 ml centrifugation tubes (Nunc). After centrifugation (45 min, 400 × g) the lymphocyte/monocyte layer was collected and the cells were washed three times (10 min, 400 × g, 4 °C) in RPMI. Using the trypan blue (Sigma) exclusion test, viable cells were counted in a Neubauer haemocytometer chamber.

Bovine monocytes were isolated as described by Goddeeris et al. (1986). If not stated differently, we used monocytes of infected animals. In brief, 7.5×10^7 PBMC were allowed to adhere (1 h, 37 °C) to 75 cm^2 tissue plastic flasks (Greiner), previously coated with 2% sterile gelatine solution (2 h, 37 °C, thereafter dried) and autologous plasma (1 h, 37 °C, thereafter washed twice with RPMI 1640/1% penicillin/1% streptomycin, Sigma). Non-adherent PBMC were removed and monocytes were washed with prewarmed RPMI 1640/1% penicillin/1% streptomycin. Monocytes were detached (5–10 min in 10 mM EDTA in Mg^{2+}- and Ca^{2+}-free Hank's solution, room temperature), washed (10 min, 400 × g, 4 °C) and resuspended in 4 °C RPMI 1640/1% penicillin/1% streptomycin. The cells were kept on ice until use and counted in a Neubauer haemocytometer chamber.

Bovine macrophages were prepared according to Jungi et al. (1996). If not stated differently, we used macrophages of infected animals. PBMC were sealed in Teflon bags (20 ml, 5×10^6 PBMC/ml) as described by Jungi et al. (1996) and cultured for 7–8 days at 37 °C in a humidified atmosphere of 5% CO_2. The medium was Iscove's modified Dulbecco's Medium (IMDM Glutamax®, Sigma) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 1% (v/v) non-essential amino acids for minimal essential medium (MEM; Gibco), 0.4% (v/v) vitamin solution for MEM (Gibco), 1 mM sodium pyruvate (Gibco), 2.5 μM amphotericin B (Gibco), 50 mM 2-mercaptoethanol (Gibco) and 20% foetal calf serum (FCS; Biowest). From the cell mixture, macrophages were purified by selective adherence to microtitre plate wells for 4 h.

2.7. Determination of monocyte or macrophage-mediated elimination of E. bovis sporozoites in vitro

In a corresponding series of experiments we controlled the elimination of the parasites from the incubation medium in the presence of monocytes or macrophages. The respective cell types were incubated with freshly isolated vital or heat-inactivated (60 °C, 30 min) *E. bovis* sporozoites and either supplemented with plain medium RPMI 1640 or immune serum (1:30). After 4 h incubation

the total number of remaining sporozoites was counted in a Neubauer chamber. Sporozoite viability was estimated by trypan blue exclusion.

For the detection of intracellular sporozoites in macrophages, freshly isolated *E. bovis* sporozoites were stained with the fluorescence dye 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) according to Hermosilla et al. (2008). Sporozoites (10^6/ml) were suspended in the dye (2.5 μM CFSE in PBS) by gently shaking and incubated for 10 min (37 °C, 5% CO_2 atmosphere). In order to stop the labelling process, an equal volume of PBS with 10% FCS was added and CFSE-labelled sporozoites (sporozoites^{CFSE}) were washed four times (400 × g, 10 min) in PBS, resuspended in PBS/10% FCS and protected from light until use. Sporozoites^{CFSE} were co-cultured with macrophages for 4 h. The samples were analysed by flow cytometry (FCM) according to Hermosilla et al. (2008).

2.8. Sera and *E. bovis* merozoite I antigen (EbAg)

Immune serum was obtained from a calf after primary (4×10^4 *E. bovis* oocysts) and challenge (3×10^4 *E. bovis* oocysts) infection.

E. bovis merozoites I, collected from culture as described previously (Hermosilla et al., 2002), were homogenised by repeated freezing and thawing followed by sonication (20 kHz, 5 s × 15 s pulses) on ice. After centrifugation (11,000 × g, 4 °C, 20 min) the supernatants were passed through 0.2 μm sterile filters (Renner). Protein concentration was determined using the Bradford method (Bradford, 1976). The antigen (EbAg) was stored at −80 °C until further use.

2.9. In vitro stimulation of monocytes/macrophages and isolation and reverse transcription of total RNA

For RT-PCR analyses, monocytes/macrophages were either exposed to freshly isolated *E. bovis* sporozoites at a ratio of 1:1 or incubated in EbAg (10 μg/ml final concentration) for 4 h (37 °C, 5% CO_2). Cells incubated in plain medium served as negative control. Stimulation of macrophages with LPS (lipopolysaccharides, *Escherischia coli*, O111:B4, 1 μg/ml, Sigma) and bovine recombinant IFN-γ (100 U/ml, kindly donated by R. Steiger, Novartis Pharma) was used as positive control.

Table 1
Sequences of primers and probes used for Real-time RT-PCR.

Specificity	Primers and probes (5′–3′)	Reference
IFN-γ forw	CAG CTC TGA GAA ACT GGA GGA CTT	Waldvogel et al. (2000)
IFN-γ rev	TGG CTT TGC GCT GGA TCT	
IFN-γ probe	AGC TGA TTC AAA TTC CGG TGG ATG ATC T	
IL-12 forw	GCA GCT TCT TCA TCA GGG ACA T	Behrendt et al. (2008)
IL-12 rev	CCT CCA CCT GCC GAG AAT T	
IL-12 probe	CAC CCA AGA ACC TGC AAC TGA GAC CAT TAA	
IL-6 forw	CTG AAG CAA AAG ATC GCA GAT CTA	Behrendt et al. (2008)
IL-6 rev	CTC GTT TGA AGA CTG CAT CTT CTC	
IL-6 probe	CAC TCC AGC CAC AAA CAC TGA CCT GCT	
TNF-α forw	TCT TCT CAA GCC TCA AGT AAC AAG T	Leutenegger et al. (2000)
TNF-α rev	CCA TGA GGG CAT TGG CAT AC	
TNF-α probe	AGC CCA CGT TGT AGC CGA CAT CAA CTC C	
CCL2 forw	CGC TCA GCC AGA TGC AAT TA	Taubert et al. (2006)
CCL2 rev	GCC TCT GCA TGG AGA TCT TCT T	
CCL2 probe	CCC AAG TCG CCT GCT GCT ATA CAT TCA A	
CXCL1 forw	CGC CTG TGG TCA ACG AAC T	Taubert et al. (2006)
CXCL1 rev	CAC CTT CAC GCT CTG GAT GTT	
CXCL1 probe	CCA GTG CCT GCA GAC CTT GCA GG	
CXCL8 forw	CAC TGT GAA AAA TTC AGA AAT CAT TGT TA	Leutenegger et al. (2000)
CXCL8 rev	CTT CAC CAA ATA CCT GCA CAA CCT TC	
CXCL8 probe	AAT GGA AAC GAG GTC TGC TTA AAC CCC AAG	
CXCL10 forw	AAG TCA TTC CTG CAA GTC AAT CCT	Taubert et al. (2006)
CXCL10 rev	TTG ATG GTC TTA GAT TCT GGA TTC AG	
CXCL10 probe	CCA CGT GTC GAG AIT ATT GCC ACA ATG A	
COX-2 forw	GCA CAA ATC TGA TGT TTG CAT TC	Taubert et al. (2006)
COX-2 rev	AGC TGG TCC TCG TTC AAA ATC T	
COX-2 probe	TTG CCC AGC ACT TCA CCC ATC AAT T	
iNOS forw	GGC CCA GGA AAT GTT CGA A	Taubert et al. (2006)
iNOS rev	ACA GTG ATG GCC GAC CTG AT	
iNOS probe	AGA CAC GTG CGT TAT GCC ACC AAC AA	
GAPDH forw	GCG ATA CTC ACT CTT CTA CCT TCG A	Taubert et al. (2006)
GAPDH rev	TCG TAC CAG GAA ATG AGC TTG AC	
GAPDH probe	CTG GCA TTG CCC TCA ACG ACC ACT T	

Total RNA was isolated from monocytes and macrophages using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. To minimize contamination with genomic DNA and to achieve reliable photometric measurements of the RNA, an on-column-DNase I treatment (Qiagen) was applied during total RNA isolation following the manufacturer's instructions. RNA was controlled for integrity by electrophoresis on a 1% agarose gel. Since on-column-DNase I treatment was not absolutely efficient, the RNA (1 μg) was additionally treated with 1 U RNase-free DNase I (30 min, 37 °C; Roche). DNase I was inactivated afterwards by heating (65 °C, 6 min). Total RNA probes were stored at −80 °C until use. For cDNA synthesis M-MLV-reverse-transcriptase (Gibco) was used. DNase I-treated total RNA was mixed with 5 μl 5× RT-buffer (250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl DTT (0.1 M), 2 μl hexanucleotides (62.5 A_{260}/ml; all Hoffmann La Roche), 1 μl dNTPs (10 mM, MBI Fermentas) and 1 μl M-MLV-reverse transcriptase (200 U/μl). The reaction was carried out in a final volume of 25 μl at 37 °C for 60 min. After addition of 185 μl TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA) the sample was stored at −20 °C until further use.

2.10. Real-time PCR for the relative quantification of IFN-γ, IL-12, IL-6, TNF-α, CXCL1, CXCL8, CXCL10, CCL2, COX-2, iNOS and GAPDH cDNAs

The relative quantification of IFN-γ, IL-12, IL-6, TNF-α, CXCL1, CXCL8, CXCL10, CCL2, COX-2, iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene transcripts was done by real-time PCR applying TaqMan® probes. The sequences of primers (MWG Biotech) and probes (Eurogentec) are depicted in Table 1. Probes were labelled at the 5'-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3'-end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). PCR amplification was performed employing an automated fluorometer (ABI PRISM™ 5700 Sequence Detection System, Applied Biosystems) using 96-well optical plates. Samples were analysed in duplicate. For PCR 5 μl cDNA (corresponding to 25 ng total RNA) were used in a 25 μl PCR reaction mixture containing 12.5 μl TaqMan® PCR Master Mix (Eurogentec), 300 nM of each primer and 200 nM probe. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 s and at 60 °C for 60 s.

Semi-quantitative analyses used the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the ABI PRISM™ 5700 Sequence Detector manufacturer and reported as *n*-fold differences in comparison to the respective medium control (after normalising the samples referring to their corresponding housekeeping gene GAPDH).

2.11. Statistical analyses

Statistical analyses used the programme package BMDP for XP, Release 8.1 (Dixon, 1993). For the description of the

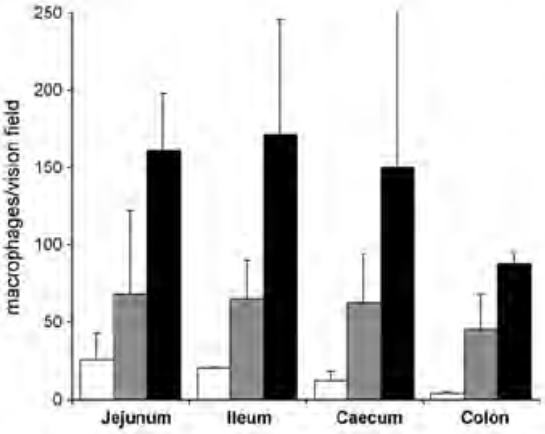


Fig. 1. Macrophages in gut tissues of *Eimeria bovis* primary and challenge infected calves. Tissue samples of the jejunum, ileum, caecum and colon of non-infected (white bars), *E. bovis* primary (26 days p.i., grey bars) and challenge (26 days after challenge, black bars) infected calves were fixed in paraformaldehyde and embedded in paraffin. Cross-sections were probed with anti-macrophage antibodies and macrophages present in the tissue were counted. Arithmetic means of three calves each and standard deviations.

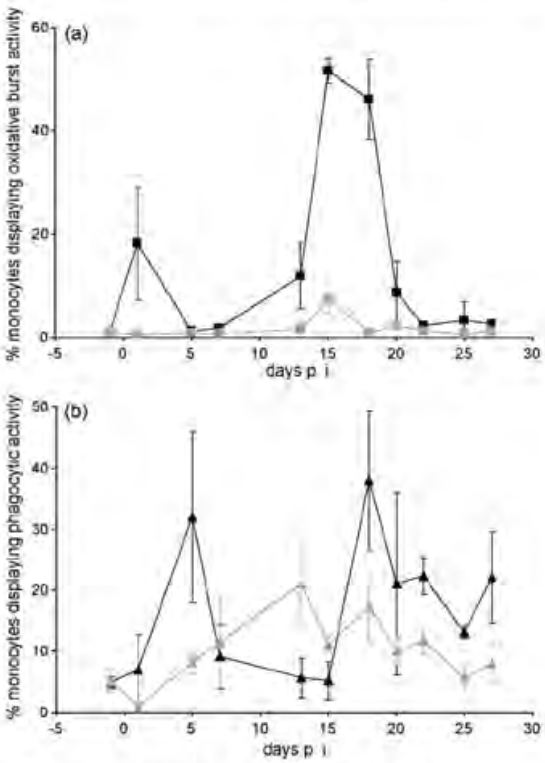


Fig. 2. Oxidative burst and phagocytosis activity of monocytes isolated *ex vivo* from *Eimeria bovis* infected calves. Monocytes were isolated from infected calves ($n = 3$) before and on various days after infection. Oxidative burst activity (a) was determined by flow cytometry estimating the oxidation of dihydrorhodamine 123 fluorogen stimulated by *Escherichia coli* phagocytosis. The general phagocytic activity (b) was estimated by flow cytometry determining the uptake of FITC-labelled *E. coli*. Incubations were performed at 37 °C (black symbols) or on ice (controls, grey symbols). Arithmetic means of three monocyte donors, minimum, maximum.

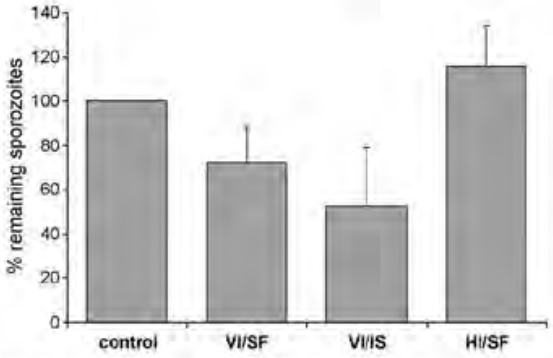


Fig. 3. Monocyte-mediated reactions against *Eimeria bovis* sporozoites *in vitro*. Bovine monocytes were exposed to viable (VI) or heat-inactivated (HI) *E. bovis* sporozoites (10:1) for 4 h in the absence (SF = serum free) or presence of immune serum (IS). Thereafter, the number of free sporozoites was determined. Monocyte-free controls were set 100%. Columns represent arithmetic means of three monocyte donors and standard deviations calculated for the percentage of free sporozoites.

data arithmetic means were calculated. To describe the variability of the data standard deviations were used. As some of the original data were not normally distributed (skewed to the right), if necessary, logarithmic transformations were performed to obtain an approximate normal distribution of the values. Data concerning infiltration of macrophages into parasitized tissue (Fig. 1), *ex vivo* oxidative burst and phagocytosis activities of monocytes (Fig. 2) and macrophage-mediated phagocytosis of CFSE-stained sporozoites (Fig. 6) were analysed by two-factorial analysis of variance (ANOVA) with repeated measures (BMDP2V). For data sets dealing with monocyte and macrophage-mediated elimination of sporozoites (Figs. 3, 5 and 6) statistical analyses were performed by applying one-factorial ANOVA with repeated measures (BMDP2V) followed by the Student–Newman–Keuls test for pairwise treatment comparison. For the gene transcription data sets (Figs. 7 and 8) as well as for the data concerning titrational effects of macrophage-mediated elimination of sporozoites (Fig. 4) co-culture/stimulation conditions were compared by *t*-test for dependent samples (BMDP3D). Differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. Macrophages infiltrate E. bovis infected gut mucosa and accumulate in lymph nodes

E. bovis-infected calves showed significantly more macrophages in the gut wall than naïve animals (Fig. 1). A significant increase in macrophage numbers was apparent in both primary and challenge infected animals (both $p < 0.01$). In challenged calves macrophage counts significantly exceeded those of primary infected animals ($p < 0.01$). Macrophage infiltration occurred in all gut samples tested in comparable proportions (Fig. 1).

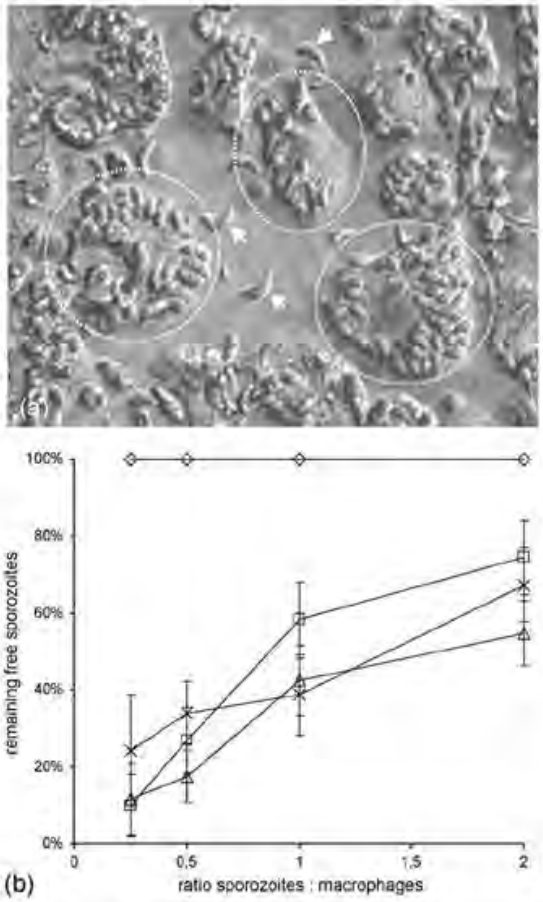


Fig. 4. Macrophage-mediated elimination of *Eimeria bovis* sporozoites *in vitro*: increased elimination of sporozoites with increasing macrophage: sporozoite ratios: (a) sporozoite uptake by macrophages (indicated by circles) and free sporozoites (arrows); (b) bovine macrophages of three animals (cross, quarder, triangle) were exposed to viable *E. bovis* sporozoites at variable ratios for 4 h. Thereafter, the total number of free sporozoites was determined. Macrophage-free samples were used as controls (diamond). All experiments were performed in triplicates. Arithmetic means of triplicates/animal and standard deviations.

In late primary infection (26 days p.i.), enhanced accumulation of macrophages in associated lymph nodes was found only for *Lnn. jejunales* (Table 2). In contrast, challenge infection caused an increase of macrophage numbers in all associated lymph nodes investigated (*Lnn. jejunales*, *Lnn. ileocaecales* and *Lnn. colici*) (Table 2).

Table 2
Immunohistological detection of macrophages in gut-associated lymph nodes of *E. bovis* primary and challenge infected calves.

	Lnn. jejunales	*Lnn. ileocaecales*	*Lnn. colici*
No infection	+	++	++
Primary infection[a]	++	+/++	+/++
Challenge infection[b]	++/+++	+++	+++

[a] 26 days p.i.
[b] 26 days after challenge infection.

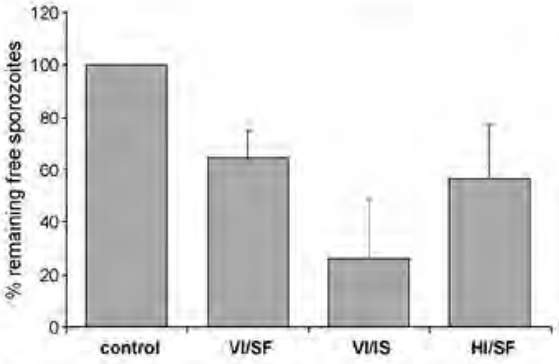


Fig. 5. Influence of immune serum and heat inactivation of the parasites on macrophage-mediated elimination of *Eimeria bovis* sporozoites *in vitro*. Bovine macrophages were exposed to viable (VI) or heat-inactivated (HI) *E. bovis* sporozoites (10:1) for 4 h in the absence (SF = serum free) or presence of immune serum (IS). Thereafter, the total number of free sporozoites was determined. Macrophage-free controls were set 100%. Columns represent arithmetic means of three macrophage donors and standard deviations calculated for the percentage of free sporozoites.

3.2. Monocytes display enhanced oxidative burst and phagocytic activities during *E. bovis* infection

Monocytes were analysed directly *ex vivo* from whole blood samples, i.e., without performing any further isolation techniques or stimulation. Data generated on day −1 p.i., which reflect the situation of non-infected animals, revealed low phagocytic and oxidative burst activities, whilst a biphasic upregulation of the general phagocytic and oxidative burst activities of monocytes was observed when compared to the negative control incubated at 4 °C (Fig. 2). Oxidative burst activity was enhanced already 1 day after infection, most probably reflecting the phase of sporozoite transmigration through the epithelium of the ileum and invasion of lymphatic endothelial cells. A second peak was detected at days 13–18 p.i., corresponding to the phase of macromeront maturation and the release of first generation merozoites. Highest values occurred on day 15 p.i. when, by means, more than 50% of monocytes showed increased oxidative burst activity.

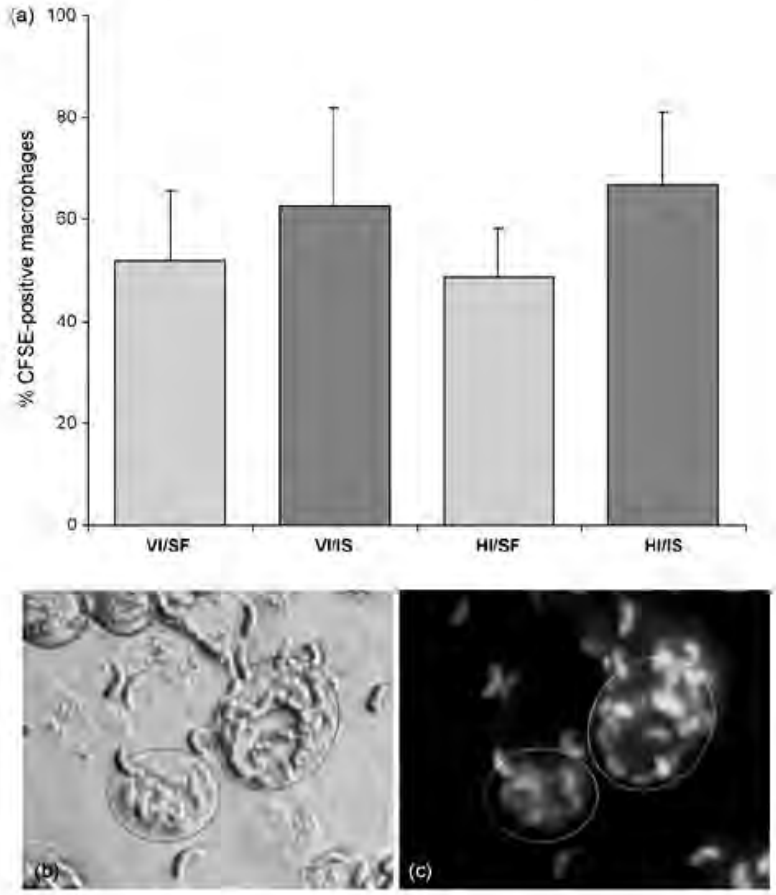


Fig. 6. Macrophage-mediated elimination of CFSE-stained *Eimeria bovis* sporozoites *in vitro*: (a) bovine macrophages were exposed to viable (VI) or heat-inactivated (HI), CFSE-stained *E. bovis* sporozoites (ratio: 10:1) for 4 h in the absence (SF = serum free) or presence of immune serum (IS). The percentages of CFSE-positive macrophages were analysed by flow cytometry. Arithmetic means of three macrophage donors and standard deviations; (b and c) uptake of CFSE-stained sporozoites by macrophages (indicated by circles), illustrated by phase contrast and fluorescence microscopy.

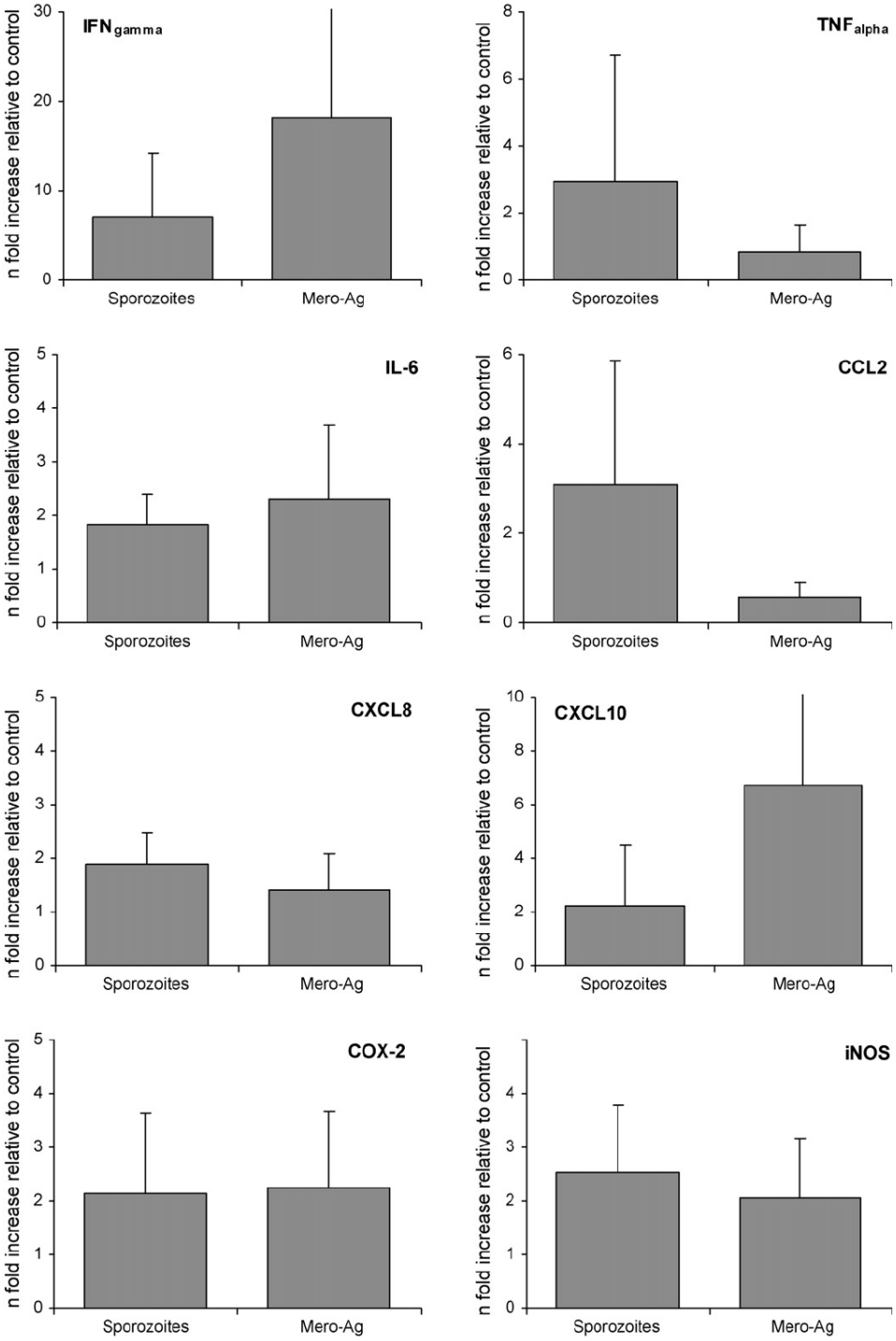
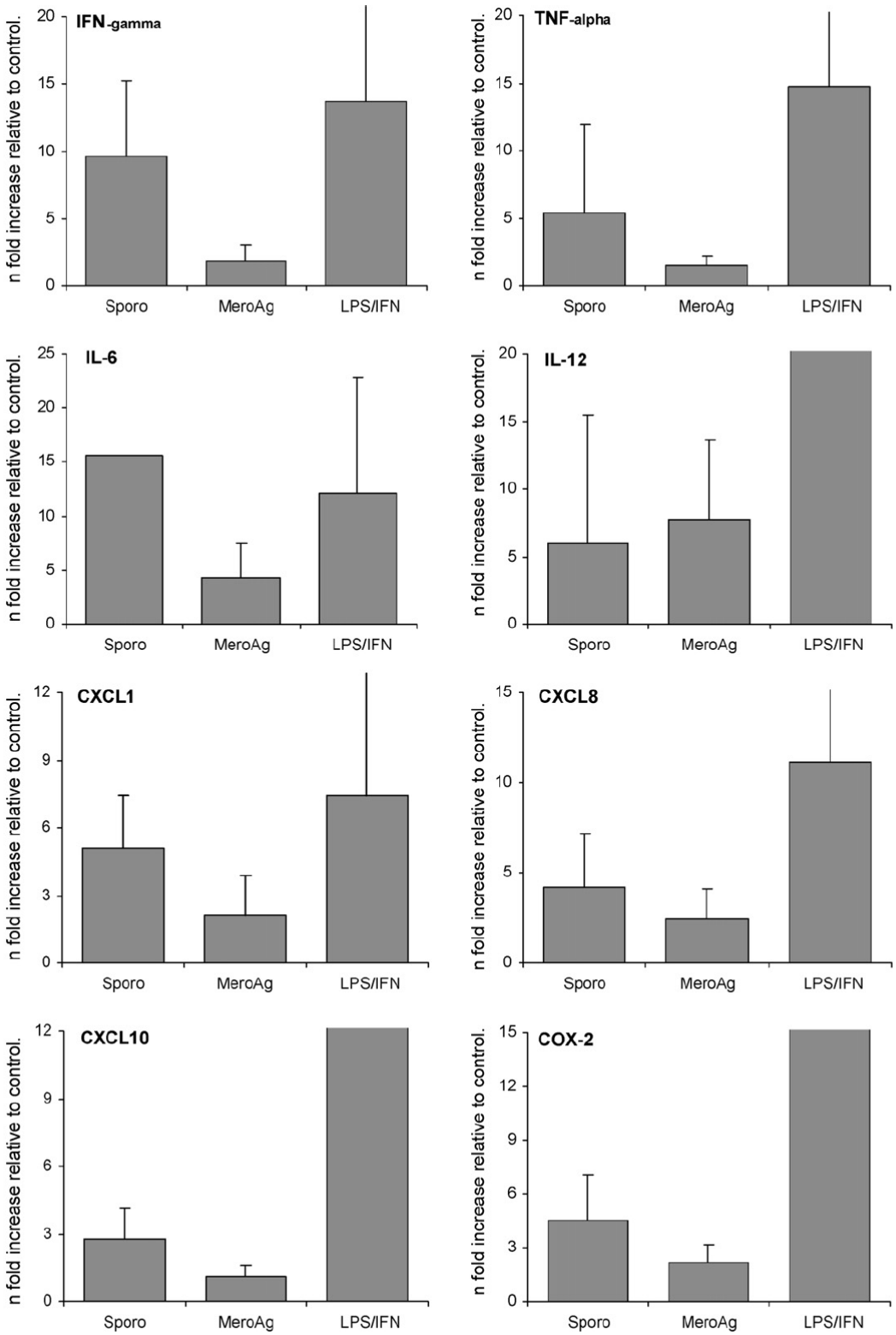


Fig. 7. Transcription of genes encoding for CXCL1, CXCL8, CXCL10, CCL2, IL-6 and iNOS in monocytes after exposure to *Eimeria bovis* sporozoites or merozoite-antigen. Bovine monocytes were exposed to *E. bovis* sporozoites (ratio: 1:1), merozoite-antigen (Mero-Ag, 10 μg/ml) or medium alone for 4 h. Total RNA was isolated, reversely transcribed into cDNA and probed with real-time RT-PCR systems for the detection of the respective mRNA equivalents. Arithmetic means of three monocyte donors and standard deviations.



ranscription of genes encoding for IFN-γ, IL-12, TNF-α, IL-6, CXCL1, CXCL8, CXCL10 and COX-2 in macrophages after exposure to *Eimeria bovis* ites or merozoite-antigen. Bovine macrophages were exposed to *E. bovis* sporozoites (ratio: 1:1), merozoite I antigen (Mero-Ag, 10 μg/ml) or ı alone for 4 h. Stimulation with LPS (1 μg/ml) and bovine recombinant IFN-γ (1000 U/ml) was used as positive control. Total RNA was isolated, y transcribed into cDNA and probed with real-time RT-PCR systems for the detection of the respective mRNA equivalents. Arithmetic means of three hage donors and standard deviations.

Similar time course dynamics were detected in the case of phagocytic activity of monocytes, however, peaks occurred delayed by 5 days p.i. and at days 18–27 p.i. However, the negative controls performed by 4 °C showed increased values at days 12–18 p.i. So far, we have no plausible explanation for this phenomenon.

For both activities ANOVA showed significant differences when calculating dynamics of time (for burst activity $p < 0.0001$, for phagocytic activity $p < 0.001$) and when comparing test with control samples represented by the interaction (for both, burst activity and phagocytic activity $p = 0.0001$). Stimulation of monocytes with PMA, serving as positive control, led to consistently enhanced values of oxidative burst activity throughout *E. bovis* infection, which varied with ongoing infection (data not shown).

3.3. Monocytes fail to phagocytise E. bovis sporozoites in vitro but are invaded by them

In vitro exposure of *E. bovis* sporozoites to monocytes resulted in moderate, but significant reduction of free sporozoites in the medium ($p < 0.05$, Fig. 3). This reaction was slightly enhanced by the supplementation of immune serum (differences compared to negative control were also significant, $p < 0.05$), however, the differences between serum-free conditions and supplementation of immune serum were insignificant. To clarify whether the reduction in numbers of free sporozoites was owing to phagocytosis or to active cell invasion by the parasite, sporozoites killed by heat inactivation were exposed to monocytes. Heat-inactivated sporozoites were not significantly eliminated from plain medium when compared to the cell-free controls (Fig. 3), but the values differed significantly from those of PMN incubated with viable sporozoites in the presence or absence of immune serum (both $p < 0.01$). These data suggest that the parasite had disappeared from the medium because it had actively invaded the monocytes.

3.4. Macrophages phagocytise E. bovis sporozoites in vitro

Macrophages co-incubated with sporozoites were found tightly loaded with parasites after 4 h (Fig. 4a). Extracellular sporozoites appeared fully vital and active. Elimination of sporozoites from the medium increased significantly ($p < 0.05$) with increasing macrophage–sporozoite-ratios (Fig. 4b).

Co-culture of macrophages and sporozoites in the absence of serum resulted in a significant reduction ($p < 0.05$) of the parasites (35.6%) from the medium (Fig. 5). This effect was significantly enhanced to 73.6% ($p < 0.05$ compared to cell-free control, $p < 0.01$ compared to serum-free samples) by immune serum suggesting opsonisation of the parasites. Heat-inactivated sporozoites in a serum-free milieu were eliminated from the medium to a similar degree as viable parasites, indicating that active cell invasion by sporozoites played a negligible role (Fig. 5).

The data were confirmed by flow cytometry analyses using CFSE-stained parasites. This staining does not affect the parasite viability. As observed microscopically (Fig. 6b and c), the sporozoites^{CFSE} accumulated in the macrophages irrespective of heat inactivation of the sporozoites (Fig. 6a). However, in this assay the fraction of CFSE-positive macrophages was only moderately enhanced by supplementation of immune serum and, in consequence, the differences were barely not significant ($p = 0.08$) (Fig. 6a).

3.5. Exposure of monocytes and macrophages to E. bovis sporozoites or to merozoite I antigen (EbAg) leads to differential upregulation of immunoregulatory molecule gene transcription

Monocytes reacted upon exposure to *E. bovis* sporozoites by enhanced IFN-γ gene transcription (Fig. 7). TNF-α, IL-6, CCL2, CXCL8, COX-2 and iNOS gene transcripts were induced rather weakly (Fig. 7). There was no upregulation of IL-12 and CXCL1 mRNAs (data not shown). Transcripts encoding for IFN-γ and CXCL10 were more strongly induced after stimulation with merozoite I antigen (Fig. 7). However, the differences described above were not significant (EbAg vs. sporozoites: CCL2: $p = 0.083$; CXCL8: $p = 0.067$, CXCL10: $p = 0.069$; sporozoites vs. control: IFN-γ: 0.079, IL-6: $p = 0.074$, IL-8: $p = 0.089$).

Co-culture of macrophages with sporozoites and stimulation with EbAg induced distinct levels of cytokine and chemokine gene transcription (Fig. 8). Thus, IFN-γ ($p < 0.01$) and IL-6 ($p < 0.05$) mRNAs were significantly enhanced by viable parasites when compared to medium controls, whilst reactions concerning IL-12 and TNF-α were not significant. Soluble antigen induced the gene transcription of IL-6 ($p = 0.065$) and IL-12 ($p < 0.01$) but failed to upregulate IFN-γ and TNF-α gene transcripts (Fig. 8). In the case of IFN-γ, differences between exposure to sporozoites and stimulation with EbAg were highly significant ($p < 0.001$).

Co-culture with sporozoites significantly enhanced CXCL1 ($p < 0.01$), CXCL8 ($p < 0.05$), CXCL10 ($p < 0.05$) and COX-2 ($p < 0.01$) gene transcription, whilst stimulation with EbAg influenced these mRNAs only moderately (n.s. for CXCL1 and CXCL10, $p < 0.05$ for CXCL8 and $p < 0.05$ for COX-2) (Fig. 8). However, the differences between exposure to sporozoites and stimulation with EbAg were significant only for CXCL10 ($p < 0.001$) and barely not significant for CXCL1 ($p = 0.066$) and COX-2 ($p = 0.083$). The transcription of genes encoding for iNOS or CCL2 was neither induced by sporozoites nor by EbAg (data not shown).

4. Discussion

Macrophage-based, early innate immune reactions against cattle *Eimeria* spp. have scarcely been investigated so far, although, the first encounter between parasite and the innate part of the immune system should be decisive for the subsequent outcome of the infection. In this work we have focused on monocyte- and macrophage-mediated immune reactions against *E. bovis in vivo* and *in vitro*. We found enhanced general phagocytic and oxidative reactivities of monocytes obtained from calves experiencing experimental *E. bovis* infections. Macrophages were shown

to accumulate in the gut mucosa of *E. bovis* infected animals. Direct exposure of macrophages to *E. bovis* sporozoites *in vitro* resulted in elimination of the parasite from the medium and in upregulated transcription of genes encoding for various immunoregulatory molecules. These results suggest macrophages as anti-parasitic effector cells and active mediators of immune responses against *E. bovis*.

The *in vivo* relevance of macrophages was underlined by a significant infiltration of these immune cells into the intestinal mucosa of infected animals, a phenomenon, that is also documented for other *Eimeria* infections (Trout and Lillehoi, 1993; Vervelde et al., 1996; Shi et al., 2000). Also Friend and Stockdale (1980) observed macrophages infiltrating gut tissue of *E. bovis* infected animals, but they found them mainly in degrading macromeronts.

Macrophage infiltration depends on adequate chemotactic signals. PMN, which are generally accepted as the earliest immune cells to be involved in inflammatory processes, have recently been identified as an early source of immunomodulatory molecules upon encounter with *E. bovis* sporozoites (Behrendt et al., 2008), including TNF-α and CCL3, which are of relevance with respect to macrophage infiltration and activation.

The observation of additional macrophage accumulation in the gut tissue of challenged calves in combination with the sporozoite opsonising efficacy of immune serum emphasizes the *in vivo* relevance of these cells in abrogating challenge infections. This is in agreement with reports on avian and murine *Eimeria* infections which also show enhanced *in vitro* anti-sporozoite phagocytic activity of macrophages isolated from immune animals (Rose, 1974; Rose and Lee, 1977; Bekhti and Pery, 1989) and increased macrophage-mediated antibody dependent cytotoxicity (Bekhti and Pery, 1989).

Monocytes of *E. bovis* infected calves exhibited biphasically increased, general phagocytic and oxidative burst activities coinciding with periods of time when *E. bovis* stages most probably are not yet or not longer situated intracellularly and, consequently, should be accessible for immune cells. Comparable time courses have been described for PMN activities in the course of *E. bovis* infection (Behrendt et al., 2008). It is noteworthy, that the proportions of monocytes involved in these reactions are far lower than those of PMN. Interestingly, Rose et al. (1979) reported on a biphasic increase of large mononuclear cells in the peripheral blood of *E. nieschulzi* infected rats and *E. maxima* infected chickens suggesting a common situation for *Eimeria* infections. However, in *in vitro* experiments monocytes failed to effectively phagocytise heat-inactivated sporozoites, although the fact, that sporozoite uptake was enhanced by supplementation of immune serum, argues for the potential ability of monocytes for antibody-dependent phagocytosis.

Furthermore, monocytes were identified as a source of IFN-γ and TNF-α, i.e., molecules involved in macrophage activation. In addition, monocytes may attract NK cells and actively initiate adaptive immune reactions in *E. bovis* infected hosts as they showed enhanced gene transcription of CXCL10 after stimulation with EbAg, a chemokine that acts on NK cells (Muller et al., 2001; Lande et al., 2003) and T cells (Taub et al., 1993).

Primary bovine macrophages phagocytised sporozoites even at serum-free conditions, indicating their ability to fight against these parasitic stages in the first encounter. Sporozoite uptake occurred irrespective of the viability of the parasite, as heat-inactivated sporozoites and viable ones were both phagocytised. Thus, active invasion by the parasite cannot be excluded, but appears of minor importance. Hughes et al. (1987) reported on development of *E. bovis* macromeronts in a macrophage-like cell line. In our experiments we could not observe development of sporozoites, neither in a bovine macrophage cell line (BoMac, unpublished data) nor in primary bovine macrophages, but the cells were only cultured for up to 8 days. Owing to the fact, that parasite invasion seemed rather a rare event, we judged reactions induced by invasion as neglectable.

Macrophages reacted upon exposure to viable sporozoites by upregulation of IFN-γ and IL-12 mRNAs and, in consequence, can play an active role in activating NK cells (Subauste et al., 1992; Trinchieri and Scott, 1995; Trinchieri, 1995, 1998a,b; Biron et al., 1999) and in the transition of innate to adaptive immune reactions as these cytokines are well known to trigger Th1 associated immune responses. Th1 dominated responses have recently been reported for *E. bovis* infected calves in prepatency (Taubert et al., 2008). Similar situations are well known in other *Eimeria* infections (Rose et al., 1989, 1991b; Smith and Hayday, 2000; Shi et al., 2001) and seem to be a key feature of control (for review, see Ovington and Smith, 1992). IFN-γ, which was more strongly induced in macrophages after stimulation with EbAg, may additionally activate other macrophages, which, in turn, can generate reactive oxygen intermediates, that have been reported to kill sporozoites of *Eimeria* spp. (Hughes et al., 1989; Michalski and Prowse, 1991). In addition, the detrimental effect of IFN-γ on intracellular *Eimeria* spp. replication is well documented (Hughes et al., 1989; Kogut and Lange, 1989a,b; Rose et al., 1991a; Ovington and Smith, 1992) with NO being assumed as the effector molecule (Ovington and Smith, 1992). However, in contrast to sporozoite-triggered reactions of avian macrophages (Dalloul et al., 2007), bovine macrophages failed to upregulate gene transcripts for iNOS. Even prestimulation of macrophages with IFN-γ, as successfully used in studies on *Babesia bovis* (Stich et al., 1998), did not lead to iNOS induction by sporozoites or EbAg (unpublished data).

In general and in contrast to PMN-mediated reactions (Behrendt et al., 2008), co-culture of macrophages with sporozoites led to stronger reactions than stimulation with EbAg as measured on the transcriptional level. Besides cytokines, sporozoite encounter also induced upregulation of different chemokines, such as CXCL1, CXCL8 and CXCL10, in macrophages. In all likelihood, the attraction of both, cells of the innate immune system and T cells to the site of infection should be the consequence.

In summary, the presented data emphasize the role of macrophages and respective precursor cells in *E. bovis* induced immune reactions. Enhanced phagocytic and oxidative burst activities and increased macrophage densities in gut mucosa of *E. bovis* infected calves indicate *in vivo* relevance of these cells. *In vitro* analyses show both

antibody-dependent and -independent phagocytosis of sporozoites and point at parasite induced gene transcription of immunoregulatory molecules, that influence both the chemotaxis of cells of the innate and adaptive immune system and the development of Th1 dominated immune responses.

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7.4 "Antigen-induced cytokine production in lymphocytes of *Eimeria bovis* primary and challenge infected calves"

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Antigen-induced cytokine production in lymphocytes of *Eimeria bovis* primary and challenge infected calves

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ABSTRACT

Cellular immune responses against *Eimeria bovis* are highly specific and a key factor for the development of protection against challenge infections. In this study we investigate the cellular immune responses of *E. bovis* primary and challenge infected calves stimulated *in vitro* by *E. bovis* merozoite I-antigen. Primary infection was accompanied by an increase of IFN-γ and IL-2 gene transcription in whole blood samples, peaking during prepatency (8–12 days p.i.) and declining thereafter, whereas IL-4 gene transcription was induced predominantly in patency. IL-10 mRNA was not influenced by *E. bovis* infection. Both $CD4^+$ and $CD8^+$ T cells were identified as source of IFN-γ gene transcripts, whilst IL-2 and IL-4 gene transcription was enhanced mainly in $CD4^+$ T cells. Increased levels of IFN-γ transcripts and protein were also found in lymphocytes isolated from ileocaecal lymph node biopsy 8 days p.i., and in cell culture supernatants obtained from antigen-stimulated peripheral blood mononuclear cells (PBMC) at days 8 and 12 p.i., respectively. Challenge infections of calves influenced neither IFN-γ nor IL-2 gene transcription in peripheral blood or in lymph node-derived lymphocytes. In contrast, IL-4 gene transcription was increased in lymphocytes isolated from draining lymph nodes.

Besides antigen-specific reactions we also found an infection-triggered induction of the non-specific activation state of PBMC in the course of primary infection as measured by the intracellular IFN-γ and IL-4 content of phorbol-12-myristate-13-acetate/ionomycin-stimulated PBMC. This may represent a new mechanism of immune cells of *E. bovis*-infected calves contributing to ongoing immune reactions.

1. Introduction

Cattle coccidiosis caused by *Eimeria* spp. has a high impact on animal health and profitability of the cattle industry (Fitzgerald, 1980; Daugschies et al., 1998; Daugschies and Najdrowski, 2005). *Eimeria bovis* and *Eimeria zuernii* represent the most pathogenic species causing diarrhoea, reduced weight gains and overall poor health conditions. Clinical symptoms occur predominantly in calves as infections generally result in protective immunity against subsequent challenges with the same *Eimeria* species.

Several investigations dealing with rodent model systems suggest that responses to primary infections are predominantly controlled by $CD4^+$ T cells with Th1-associated T cell reactions being key to the control of primary infection, whilst cytotoxic $CD8^+$ T cells seem to be the major effector cell type against challenge infections (Rose et al., 1992a; Findly et al., 1993; Ovington et al., 1995; Smith and Hayday, 2000; Shi et al., 2001a). IFN-γ inhibits intracellular replication of *Eimeria* spp. *in vitro* (Rose et al., 1991a; Ovington et al., 1995; Lillehoj and Choi,

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1998; Dimier et al., 1998; Heriveau et al., 2000) and appears to play a crucial role in the abrogation of primary infections (Rose et al., 1989, 1991b; Smith and Hayday, 2000; Shi et al., 2001b).

However, data generated in rodent models may only be of limited value for the ruminant system, as most of the pathogenic *Eimeria* species in ruminants (e.g. *E. bovis*, *E. zuernii*, *E. bukuensis*, *E. arloingi*, and *E. ninakohlyakimovae*) develop differently from the rodent ones with respect to primary host cells, the formation of macromeronts and duration of replication, features, that will surely influence developing immune responses. In addition, Dalloul et al. (2007) recently showed unique cytokine-based immune responses of macrophages to different avian *Eimeria* species pointing at species-specific reactions. However, detailed analyses of cellular immune responses in lifestock experiencing clinical coccidiosis are relatively rare. Occasional reports of cellular immune responses to *E. bovis* infection in calves exist. Hughes et al. (1988, 1989b) and Hermosilla et al. (1999) showed enhanced lymphocyte proliferation in response to specific antigen, although there were conflicting data on the time course of T cell response during a primary infection. Hermosilla et al. (1999) demonstrated an increase of peripheral $CD4^+$ and $CD8^+$ T cell subpopulations during prepatency of primary infection; the $\gamma\delta$-TCR^+ T cell subset was not influenced. Increased levels of IL-2, but not of IL-4 gene transcripts were found in gut lymph nodes (Hermosilla et al., 1999) after primary infection, whilst peripheral blood mononuclear cells (PBMC) of immune animals and an antigen-dependent T cell line/clone failed to upregulate IL-2 production after stimulation with *E. bovis* oocyst antigen (Hughes et al., 1988). Nonetheless, PBMC and the T cell line/clone responded with moderate and high levels of IFN-γ, respectively. However, detailed cytokine analyses covering the course of primary or challenge of *E. bovis* infections are lacking.

This study was undertaken to characterise the cellular immune responses of *E. bovis*-infected calves on the level of IFN-γ, IL-2 and IL-4 gene transcription in peripheral blood and in intestinal lymph node cells in the course of a primary infection and after challenge infection. IFN-γ and IL-4 expression was also determined intracellularly in PBMC and IFN-γ in supernatants of antigen-stimulated PBMC. In addition, we investigated $CD4^+$ and $CD8^+$ T cells as potential sources of IFN-γ, IL-2 and IL-4 gene transcription.

2. Materials and methods

2.1. Calves

Holstein Frisian calves were purchased from a local farmer at the age of 2 weeks, treated with Baycox® (Bayer) and Halocur® (Intervet) in the second week after birth, assessed for parasitic infections, and when deemed parasite free, maintained under parasite-free conditions in autoclaved stainless steel cages (Woetho) until experimental *E. bovis* infection. They were fed with milk substitute (Hemo Mischfutterwerke) and commercial concentrates (Raiffeisen). Water and sterilized hay were given *ad libitum*.

2.2. Parasite maintenance

The *E. bovis* strain H used in the present study was maintained by passages in Holstein Frisian calves. For the production of oocysts, calves were infected at the age of 10 weeks with 5×10^4 sporulated oocysts each. Excreted oocysts were isolated from faeces beginning 19 days p.i. according to the method of Jackson (1964). Sporulation was achieved by incubation in a 2% (w/v) potassium dichromate (Sigma) solution at room temperature. Sporulated oocysts were stored in this solution at 4 °C until further use.

Sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al., 2002). For *in vitro* infections bovine umbilical vein endothelial cells (BUVEC, Taubert et al., 2006b), grown to confluence, were infected with freshly isolated sporozoites (10^6 sporozoites/75 cm^2 culture flask). Culture medium (endothelial cell growth medium, PromoCell) was changed 24 h p.i. and thereafter every second day. From day 18 p.i. onwards, *E. bovis* merozoites I were harvested from culture as previously described (Hermosilla et al., 2002).

2.3. Infection and bleeding of the animals, biopsies and necropsies

In a first series of experiments, four groups (A–D) of three animals each were used, aged 8–12 weeks. Two groups (A, B) were orally infected on day 0 with 5×10^4 sporulated oocysts. Group B was additionally challenged on day 48 with 3×10^4 sporulated oocysts together with primary infection of Group C. Group D remained uninfected. In these experiments cytokine gene transcripts were estimated in whole blood and lymph node samples. In a second and third series of experiments ($n = 3$) we analysed (i) intracellular IFN-γ and IL-4 protein expression in PBMC, (ii) gene transcripts in $CD4^+$/$CD8^+$ T lymphocytes and (iii) IFN-γ in PBMC supernatants. In these experiments, non-infected animals were not available.

Shedding of oocysts was determined from day 18 p.i. onwards by daily faecal examination (McMaster technique).

Biopsies were performed on day 8 p.i. and 8 days after challenge. Animals were sedated with xylazine (0.1 mg/kg, intramuscular, Rompun®, Bayer) and fixed in left lateral position. The incision site at the right abdominal wall (approximately one hand proximally to the tuber coxae) was infiltrated with procaine (2%, Procasel®, Selectavet). Calves were then anaesthetised with ketamine (Orsotamin®, Serumwerk Bernburg). Laparotomy was conducted according to standard surgery procedures. The *Plica ileocaecalis* of the ileum and the *Ansa spiralis coli* were advanced for withdrawal of the *Lnn. ileocacalis* and *Lnn. colici*, respectively. Lymph nodes were fixed with a clamp, removed and submitted to sterile medium (RPMI, 1% penicilline/streptomycine). Mesentery defects were closed (Serafit®, Serag-Wiessner) and draining vessels were ligated. After flushing (0.9% NaCl, 37 °C), the gut was deposed and the wound was closed according to standard surgery procedures. Calves were treated with flunixin–meglumin (1 × 2.2 mg/kg, intravenously, Finadyne®,

Essex) and procaine–penicilline G (1 ml/10 kg, subcutaneously for 5 days, Animedica) and monitored.

Calves were necropsied on day 60, i.e., 12 days after challenge infection and the lymph nodes (*Lnn. ileocaecales*, *Lnn. colici*) were excised for immediate lymphocyte isolation.

2.4. *E. bovis* merozoite I antigen (EbAg) preparation

E. bovis merozoites I collected from culture were homogenized by repeated freezing followed by sonication (20 kHz, 5 × 15 s pulses) on ice. After centrifugation (11,000 × g, 4 °C, 20 min) the supernatants were passed through 0.2 μm sterile filters (Renner). Protein concentration was determined using the Bradford method (Bradford, 1976). The antigen (EbAg) was stored at −80 °C.

2.5. Whole blood assays, isolation of bovine lymphocytes and *in vitro* stimulation with EbAg

For whole blood assays calves were bled by puncture of the jugular vein on days −1, 4, 8, 12, 19, 26, 47, 52, 56 and 60 p.i. and blood was collected in 50 ml plastic tubes (Nunc) containing 0.1 ml heparin (Sigma). For each sample 8 wells of a 24 well plate were loaded with 500 μl 1:5 diluted whole blood and 500 μl EbAg (20 μg/ml) or plain RPMI 1640 medium (Gibco BRL) for control samples. The culture plates were incubated for 48 h at 37 °C in 5% CO_2-in-air. After resuspension, cells were collected, centrifuged (10 min, 400 × g) and contaminating erythrocytes were lysed (addition of 32 ml H_2O for 40 s, thereafter supplementation of 4 ml 10× Hank' solution). After two washings (10 min, 400 × g) cell pellets were used for RNA preparation.

For PBMC isolation, 20 ml of heparinized blood were mixed with 17 ml of 0.9% NaCl and applied on the top of 12 ml Ficoll-Paque (density = 1.077 g/l, Biochrom) in 50 ml centrifugation tubes (Nunc). After centrifugation (45 min, 400 × g) the lymphocyte layer was collected and the cells were washed three times (10 min, 400 × g, 4 °C) in RPMI. Using the Trypan blue (Sigma) exclusion test, viable cells were counted in a Neubauer chamber.

For preparation of lymphocytes, lymph nodes of infected and non-infected calves were gently teased through nylon sieves (meshes of 180 μm; Reichelt Chemietechnik) floating in RPMI. After three washings (400 × g, 4 °C, 10 min), the cells were resuspended in RPMI supplemented with 1% penicillin (Sigma), 5 mM glutamine (Gibco BRL), 10% fetal calf serum (Biochrom KG) and 1.7 μl/500 ml 2-mercaptoethanol (Serva). Using the Trypan blue exclusion test, the viable cells were counted in a Neubauer chamber. Lymphocytes (2×10^6 lymphocytes/well, 24-well microtiter plates, Nunc) were stimulated with EbAg (10 μg/ml final concentration, 6 h, 37 °C, 5% CO_2-in-air). Thereafter cells were collected, pelleted (400 × g, 10 min) and subjected to RNA preparation.

2.6. Isolation and *in vitro*-stimulation of $CD4^+$ and $CD8^+$ T cells

Following PBMC isolation $CD4^+$ and $CD8^+$ T cells were isolated on days −1 and 8 p.i. by magnetic sortment using the CELLection™ Pan Mouse IgG Kit (Invitrogen) according to the manufacturer's instructions. Briefly, 4×10^7 PBMC resuspended in 4 ml PBS/EDTA/BSA (PBS supplemented with 2 mM EDTA, 0.5% BSA) were incubated with antibodies directed against bovine CD4 (clone CC30, Serotec) or bovine CD8 (clone CC63, Serotec) for 30 min on ice. Cells were washed twice (400 × g, 4 °C, 8 min) and resuspended in 4 ml PBS/EDTA/BSA. The cells were then incubated with 50 μl anti-mouse IgG conjugated to magnetic beads (4×10^8 beads/ml) for 30 min at 4 °C under constant rotation (MACSmix™ Tube Rotator, Miltenyi Biotec). Applying a magnet (MPC®-L, Invitrogen), the cells were washed twice in 4 °C PBS/EDTA/BSA, once in 37 °C RPMI/1% FCS and resuspended in 800 μl prewarmed RPMI/1% FCS. To remove the attached beads, which are conjugated to anti-mouse IgG antibodies via a DNA linker, the cell-bead-suspension was treated with DNase (4 μl, 15 min, room temperature, constant rotating; DNase was supplied with the kit) and pipetted 6–8 times up and down. Thereafter, the beads were removed using the magnet. The cells were centrifuged, resuspended in RPMI/10% FCS and counted in a Neubauer chamber. The purity of $CD4^+$ or $CD8^+$ T cell preparations was determined by flow cytometry analysis as described elsewhere (Hermosilla et al., 1999).

Simultaneously to $CD4^+$ and $CD8^+$ T cell preparation, plastic adherent cells (PAC) were isolated from the respective animals as previously described (Goddeeris et al., 1986) to serve as antigen presenting cells. In brief, 7.5×10^7 PBMC were allowed to adhere (1 h, 37 °C) to 75 cm^2 tissue plastic flasks (Greiner) previously coated with 2% sterile gelatine solution (2 h, 37 °C, thereafter dried) and autologous plasma (1 h, 37 °C, thereafter washed twice with RPMI/PS). Non-adherent PBMC were removed and PAC were washed with prewarmed RPMI/PS. PAC were detached (5–10 min in 10 mM EDTA in Mg^{2+} and Ca^{2+}-free Hank's solution, room temperature), washed (10 min, 400 × g, 4 °C) and resuspended in 4 °C RPMI/PS. The cells were kept on ice until use and counted in a Neubauer chamber.

In preliminary assays suitable ratios of $CD4^+$/$CD8^+$ T cells: PAC were determined. A ratio of 10:1 was chosen for the final experiments. $CD4^+$ and $CD8^+$ T cells (10^5 cells/well) were cultured in the presence of plastic adherent cells and stimulated with EbAg (10 μg/ml final concentration) or medium for 4 h. Thereafter, cells were collected and total RNA was isolated applying the RNeasy Micro® kit (Qiagen).

2.7. Detection of intracellular IFN-γ or IL-4 in EbAg stimulated PBMC

Detection of intracellular IFN-γ and IL-4 was done according to Moussay et al. (2006) by flow cytometry (FCM). Therefore PBMC (plated in 12-well tissue culture trays, 5×10^6 cells/well) derived from *E. bovis* experimentally infected calves on days −7, −2, 5, 8, 12 and 19 p.i. were cultured in the presence or absence of EbAg (10 μg/ml final concentration) or phorbol-12-myristate-13-acetate/ionomycin (10 ng/ml, Orpegen and 1 μg/ml, Sigma, respectively) for 6 h at 37 °C in 5% CO_2 at 95% humidity.

Brefeldin A (10 μg/ml, Sigma-Aldrich) was added to cultures 1 h after the incubation had started. Subsequently, PBMC were resuspended and washed once in PBS-EDTA and PBS (202 × g, 7 min, 20 °C). The staining procedure was performed in 96-well tissue culture trays (Nunc) with 4×10^5 cells per well. Briefly, cells were centrifuged (150 × g, 7 min, 4 °C), fixed in paraformaldehyde 1% (Merck, 10 min, in the dark on ice), washed once and permeabilized (5 min on ice, in PBS supplemented with 0.1% saponin, 0.1% sodium azide, Merck). Cytokines were detected by probing with antibodies directed against bovine IFN-γ (clone CC302) and bovine IL-4 (CC303; all antibodies purchased from Serotec, diluted in PBS supplemented with 1% bovine serum albumin, 0.1% saponin, 0.1% sodium azide; incubation: 20 min in the dark, on ice) or with isotype controls. A goat anti-mouse IgG (H + L) antibody conjugated with FITC (3.4 μg/ml, Dianova) was used as secondary antibody. Cells were analysed with a FACSCalibur™ (BD Biosciences) flow cytometer detecting the percentage of IFN-γ or IL-4-positive cells and investigating the intensity of fluorescence of the respective cells.

2.8. Detection of IFN-γ in supernatants of EbAg-stimulated PBMC

PBMC (2×10^5/well) obtained from *E. bovis* experimentally infected calves on days −3, −7, 5, 8, 12 and 19 p.i. were dispensed as triplicates into 96-well tissue culture trays and stimulated with EbAg (10 μg/ml final concentration; 37 °C in 5% in air, 48 h). Non-supplemented medium was used as negative control for each time point. The plates were centrifuged (150 × g, 7 min) after incubation, supernatants were removed and assayed using an ELISA-kit for bovine IFN-γ (bovine IFN-γ EASIA, Biosource) according to the manufacturer's instructions. Levels of IFN-γ were calculated relative to the positive control (supplied by the kit) and expressed as corrected OD: (mean antigen or RPMI stimulated wells − test negative control)/(test positive control − test negative control).

2.9. Isolation and reverse transcription of total RNA

Total RNA was isolated from whole blood samples/lymphocytes and $CD4^+/CD8^+$ T cells using the RNeasy® Mini or Micro Kit (Qiagen), respectively, according to the manufacturer's instructions. To minimize contamination with genomic DNA and to achieve reliable photometric measurements of the RNA, an on-column-DNase I treatment (Qiagen) was applied during total RNA isolation following the manufacturer's instructions. RNA was controlled for integrity by electrophoresis on a 1% agarose gel. Since on-column-DNase I treatment was not absolutely efficient, the RNA (1 μg in the case of whole blood samples and lymphocytes, the RNA equivalent of 10^5 cultured cells in the case of $CD4^+/CD8^+$ T cells) was additionally treated with 1 U RNase-free DNase I (30 min, 37 °C; Roche). DNase I was inactivated afterwards by heating (65 °C, 6 min). Total RNA probes were stored at −80 °C until use. For cDNA synthesis M-MLV-reverse-transcriptase (Gibco) was used. DNase I-treated total RNA was mixed with 5 μl 5× RT-buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μl DTT (0.1 M), 2 μl hexanucleotides (62.5 A_{260}/ml; Hoffmann La Roche), 1 μl dNTPs (10 mM, MBI Fermentas) and 1 μl M-MLV-reverse transcriptase (200 U/μl). The reaction was carried out in a final volume of 25 μl at 37 °C for 60 min. After addition of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA; 175 μl in the case of whole blood samples and lymphocytes, 25 μl in the case of $CD4^+/CD8^+$ T cells) the probe was stored at −20 °C until further use.

2.10. Real-time PCR for the relative quantification of IL-2, IFN-γ, IL-4, and IL-10 cDNAs

The sequences of primers (purchased from MWG Biotech) and probes (purchased from Eurogentec) used are depicted in Table 1. Probes were labelled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). PCR amplification was performed on an automated fluorometer (ABI PRISM™ 5700

Table 1
Sequences of primers and probes used for real-time RT-PCR.

Specificity	Primers (5′–3′) and probes (5′–6-FAM-TAMRA-3′)	Reference
IL-2 forw	GGA TTT ACA GTT GCT TTT GGA GAA A	Leutenegger et al. (2000)
IL-2 rev	GCA CTT CCT CTA GAA GTT TGA GTT CTT	
IL-2 probe	CGT GCC CAA GGT TAA CGC TAC AGA ATT GAA	
IL-4 forw	CAT GCA TGG AGC TGC CTG TA	Waldvogel et al. (2000)
IL-4 rev	AAT TCC AAC CCT GCA GAA GGT	
IL-4 probe	TGC TGC CCC AAA GAA CAC AAC TGA GAA G	
IL-10 forw	CCA AGC CTT GTC GGA AAT GA	Moussay et al. (2006)
IL-10 rev	GTT CAC GTG CTC CTT GAT GTC A	
IL-10 probe	AGC CTG TGG CAT CAC CTC TTC CAG GTA A	
IFN-γ forw	CAG CTC TGA GAA ACT GGA GGA CTT	Waldvogel et al. (2000)
IFN-γ rev	TGG CTT TGC GCT GGA TCT	
IFN-γ probe	AGC TGA TTC AAA TTC CGG TGG ATG ATC T	
GAPDH forw	GCG ATA CTC ACT CTT CTA CCT TCG A	Taubert et al. (2006a)
GAPDH rev	TCG TAC CAG GAA ATG AGC TTG AC	
GAPDH probe	CTG GCA TTG CCC TCA ACG ACC ACT T	

Sequence Detection System, Applied Biosystems) using 96-well optical plates. Each sample was analysed in duplicate. For PCR 5 μl cDNA were used in a 25 μl PCR reaction mixture containing 12.5 TaqMan® Universal Master Mix (Applied Biosystems), 300 nM of each primer and 200 nM probe. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s.

Semiquantitative analyses used the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the manufacturer of the 5700 Sequence Detector and reported as n-fold differences in comparison to one of the samples arbitrarily chosen as the calibrator after normalizing the samples referring to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The calibrator samples used in this experiment were non-infected controls with low amounts of the respective target gene transcripts. Quantification was done referring to the result of GAPDH.

2.11. Statistical analysis

Statistical analyses used the programme package BMDP for XP, Release 8.1 (Dixon, 1993). For the description of the data arithmetical means were calculated. To describe the variability of the data standard deviations were used. As some statistical distributions of the original data were skewed to the right, if necessary, arc-sine (for data concerning IFN-γ in supernatants) or logarithmic (for all other data) transformations were performed to obtain an approximately normal distribution of the values. In accordance to the design of the experiments, data were compared by two- or three-factorial analysis of variance (ANOVA) with repeated measures (BMDP2V). Differences were regarded as significant at a level of $p \leq 0.05$.

3. Results

3.1. Cytokine gene transcription in whole blood samples in the course of *E. bovis* primary and challenge infections

As signature cytokines for Th1 and Th2 responses, the transcription of the IL-2/IFN-γ and IL-10/IL-4 genes was investigated. Concerning IFN-γ, global comparison of the different culture conditions revealed significant differences ($p < 0.001$). Transcription of the IFN-γ gene was clearly enhanced in whole blood samples of infected calves between 8 and 19 d p.i. displaying a distinct maximum at 8 d p.i. (Fig. 1). Thereafter IFN-γ gene transcription continuously declined to low levels until 26 d p.i. In contrast, there was no significant enhancement of IFN-γ-specific mRNAs in challenged animals on days 8 and 12 post challenge (=56 and 60 d p.i., respectively). Analyses of the data revealed significant differences when calculating group and stimulation-dependent dynamics of time represented by the interaction ($p < 0.05$). Also, differences induced by stimulation with EbAg within the groups were significant for both infected and the non-infected animals (both $p < 0.05$). Overall, stimulation of whole blood samples of non-infected animals with EbAg induced low IFN-γ gene transcription (Fig. 1), but, nevertheless, the values were enhanced when compared with reactions in medium controls.

IL-2 gene transcription was clearly induced in whole blood samples of *E. bovis*-infected animals between days 8 and 19 p.i. and also peaking at 8 d p.i. (Fig. 1B). Thereafter transcript levels declined almost continuously to control levels until 26 d p.i. Global comparisons of the time dynamics showed significant differences ($p < 0.05$). There was a weak, but insignificant increase of IL-2 gene transcription found after challenge infection. Stimulation with EbAg led to moderately increased levels of IL-2 mRNAs in whole blood samples of non-infected control animals when compared with medium controls (Fig. 1), but these reactions varied irregularly with the time point of investigation. Overall, ANOVA revealed significant differences when calculating group and stimulation-dependent dynamics of time represented by the interaction ($p < 0.05$).

Concerning IL-4 gene transcription global comparison of the different stimulations and time dynamics revealed significant differences ($p < 0.001$ and $p < 0.05$, respectively). The levels of IL-4 transcripts increased continuously in samples of *E. bovis*-infected calves beginning with 4 d p.i. to a distinct maximum on day 19 p.i. (Fig. 1). Thereafter consistently lower, but still enhanced levels of IL-4 mRNAs (compared to the medium controls) were observed 26–56 d p.i. On day 12 post challenge (=60 d p.i.) IL-4 gene transcription seemed upregulated again reaching almost similar levels as on day 12 of the primary infection. ANOVA revealed significant differences when calculating group and stimulation-dependent dynamics of time represented by the interaction ($p < 0.05$). Stimulation of samples of non-infected control animals with EbAg led to an increase of IL-4 transcription as well (Fig. 1), but these reactions were much weaker than those of the infected calves and independent of the time point of investigation. Thus, within the groups, differences induced by stimulation with EbAg were significant only for the infected animals ($p < 0.01$).

The transcription of the IL-10 gene was not affected by *E. bovis* infections or stimulation with EbAg throughout the investigation period (data not shown).

3.2. Cytokine gene transcription in lymph node lymphocytes

Cytokine gene transcription (IFN-γ, IL-2, IL-4, IL-10) was determined in cells isolated from *Lnn. ileocaecalis* obtained from animals 8 days after primary infection and 8 and 12 days after challenge infection. Additionally, lymphocytes isolated from *Lnn. colici* removed from challenged calves 12 days after challenge infection were exposed to EbAg to determine the transcription of the IFN-γ, IL-2, IL-4 and IL-10 genes.

IFN-γ gene transcription was stimulated by EbAg in the ileocaecal lymph node cells 8 days after primary infection, but not influenced in cells from challenged animals (Fig. 2). Global comparison of the different stimulations and time dynamics revealed significant differences ($p < 0.01$ both).

IL-2 gene transcripts appeared slightly increased in cells of primary infected calves after EbAg stimulation

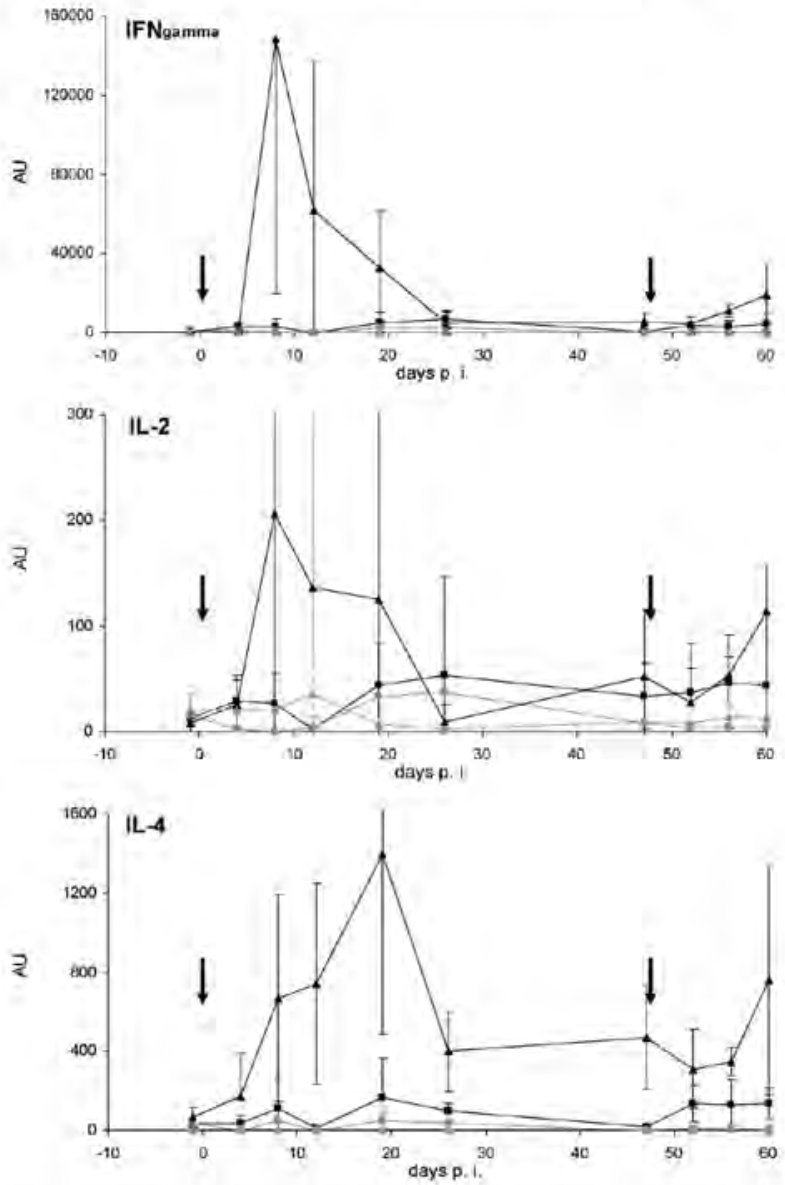

Fig. 1. IFN-γ, IL-2 and IL-4 gene transcription in whole blood samples of *Eimeria bovis* primary infected and challenged calves. Whole blood samples of primary infected (at day 0, indicated by an arrow) and challenged (at days 48 p.i., indicated by an arrow) calves (triangles) were stimulated with *E. bovis* merozoite antigen (black curves) or medium (grey curves) for 48 h. Samples of non-infected control animals (squares) were treated equally with antigen (black curves) or medium (grey curves). Total RNA was isolated on days −1, 4, 8, 12, 19, 26, 47, 52 (=4 d post challenge), 56 (=8 d post challenge) and 60 (=12 d post challenge) p.i. 1 μg total RNA was reverse transcribed into cDNA and probed with real-time RT-PCR systems for the detection of IFN-γ, IL-2 and IL-4 mRNA equivalents, respectively. Arithmetical means and standard deviations of three individuals from each group. AU, arbitrary units.

when compared with non-infected control calves, but these reactions were not significant. Early after challenge (8 days post challenge) the cells seemed almost refractory to antigen referring to IL-2 gene transcription. 12 days post challenge increased levels of transcripts were found in EbAg-stimulated lymph node cells when compared to medium controls, but this reaction did not differ from that of non-infected control animals (Fig. 2).

A different picture was obtained for the IL-4 gene transcription, which was not at all influenced 8 days after primary infection, but was increasingly enhanced 8 and 12 days after challenge (Fig. 2). Reactions induced by EbAg stimulation were significant increased in the infected group ($p < 0.05$).

IL-10 gene transcription in EbAg stimulated ileocaecal lymph node cells were neither influenced in primary nor in challenge infection (Fig. 2).

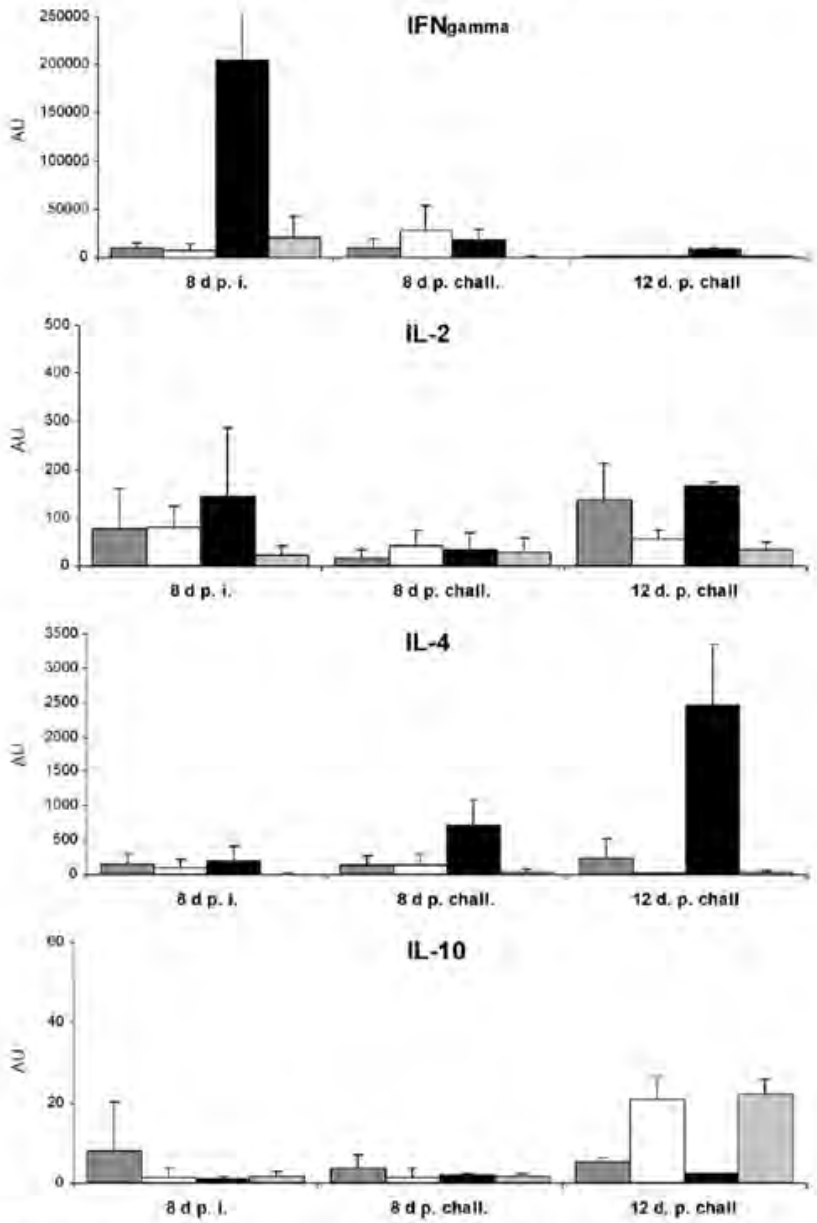

Fig. 2. IFN-γ, IL-2, IL-4 and IL-10 gene transcription in lymphocytes isolated from *Lnn. ileocaecalis* biopsies after *Eimeria bovis* primary or challenge infection. Lymphocytes isolated from the *Lnn. ileocaecalis* of *E. bovis* primary infected (8 d p.i.) and challenged (8 and 12 days post challenge) or non-infected calves were stimulated with *E. bovis* merozoite antigen (infected calves: black bars, non-infected calves: dark grey bars) or RPMI as medium control (infected calves: light grey bars, non-infected calves: white bars). Total RNA was isolated, reverse transcribed into cDNA and probed with real-time RT-PCR systems for the detection of IFN-γ, IL-2 IL-4 and IL-10 mRNA equivalents, respectively. Arithmetical means and standard deviations of three individuals from each group. AU, arbitrary units.

The data obtained from *Lnn. colici* cells for IFN-γ, IL-2, IL-4 and IL-10 gene transcripts resemble, in principle, those of the ileocaecal lymph node cells (Fig. 3), but significant differences between infected and non-infected animals were found only for IL-4 ($p < 0.001$). Concerning IL-10 gene transcription, mRNA levels seemed slightly increased in cells from challenged animals when compared with non-infected controls. However, these reactions appeared to be independent from antigenic stimulation (Fig. 3).

3.3. IFN-γ, IL-2 and IL-4 gene transcription in $CD4^+$ and $CD8^+$ T cells

To determine which T cell subtype represents a source of enhanced IL-2, IFN-γ and IL-4 gene transcripts, $CD4^+$ and

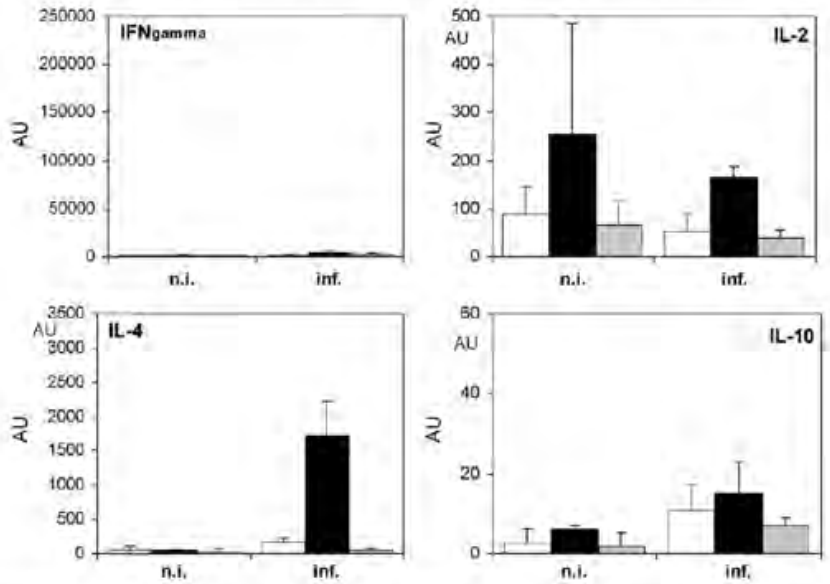

Fig. 3. IFN-γ, IL-2, IL-4 and IL-10 gene transcription in lymphocytes isolated from *Lnn. colici* 12 days after *Eimeria bovis* challenge. Lymphocytes isolated from *Lnn. colici* of non-infected control animals (n.i.) or of primary infected and challenged calves on days 12 post challenge (inf.) were either used either directly after isolation (white bars) or stimulated with *E. bovis* merozoite antigen (black bars) or plain RPMI as medium control (grey bars) for 6 h. Thereafter, total RNA was isolated, reverse transcribed into cDNA and probed with real-time RT-PCR systems for the detection of IFN-γ, IL-2, IL-4 and IL-10 mRNA equivalents, respectively. Arithmetical means and standard deviations of three individuals from each group. AU, arbitrary units.

$CD8^+$ T cells were isolated from PBMC on days −1 and 8 after primary infection and cultured in the presence of PAC with /without addition of EbAg. IFN-γ gene transcription was induced in EbAg-stimulated cells of infected animals relative to the medium controls on day 8 p.i. in both, $CD4^+$ and $CD8^+$ T cells (Fig. 4). Differences between the two cell populations were not significant. In contrast, IL-2 gene transcription was predominantly upregulated in $CD4^+$ T cells. As expected, $CD4^+$ T cells represented the sole source of IL-4 gene transcription in response to EbAg on day 8 p.i. ($p < 0.05$).

3.4. IFN-γ and IL-4 expression in PBMC of *E. bovis* primary infected calves

The proportion of PBMC of *E. bovis* primary infected calves responding upon stimulation with PMA/ionomycin by IFN-γ production was significantly enhanced during late prepatency (12 and 19 days p.i.) as reflected by the percentage of IFN-γ-positive PBMC in FCM analyses (Fig. 5A). The amount of IFN-γ/cell, as determined by the intensity of intracellular fluorescence (Fig. 5B), was increased too. In consequence, reactions induced by stimulation with PMA/ionomycin were highly significant ($p = 0.0001$). As non-infected controls, data generated on days −7 and −3 of the animals infected later on were used. Thus, the synthesis of IFN-γ is amplified in the course of infection by both the number of IFN-γ producing cells and the cytokine amount per cell. Maximum values for both data sets were found 12 days p.i., i.e., at a time when the gene transcription of IFN-γ was highly increased in whole blood samples of infected calves as well. Cells isolated on day 8 p.i. were not included in the analysis owing to technical problems. However, stimulation of PBMC with EbAg alone neither influenced the number of IFN-γ producing PBMC nor the amount of IFN-γ/cell. Addition of EbAg to PMA/ionomycin did not alter the reaction pattern when compared to non-supplemented PMA/ionomycin. This might be explained by the low numbers of antigen-specific lymphocytes which may produce sufficient IFN-γ for immunomodulatory actions *in vivo*, but might not be detected *in vitro* owing to the restricted sensitivity of FCM analyses. However, these data provide evidence that *E. bovis* primary infections influence the non-specific activation state of PBMC of infected animals by enhancing the IFN-γ production regardless of the triggering antigen.

In general, the numbers of PBMC containing IL-4 after stimulation with PMA/ionomycin were lower than IFN-γ-positive cells (Fig. 5C). Beginning at 8 days p.i. the proportion of IL-4-producing cells increased continuously, associated with an enhanced intracellular IL-4 content when compared to cells isolated on days −7 and −3 p.i., serving as non-infected controls (Fig. 5C and D). These reactions were significantly enhanced in the case of PMA/ionomycin stimulation ($p < 0.01$ for percentage IL-4-positive cells, $p = 0.0001$ for IL-4 intensity). Supplementation with EbAg or the use of EbAg alone only partially influenced IL-4 production in PBMC (supplementation: $p < 0.01$ for percentage IL-4-positive cells, $p = 0.05$ for IL-4 intensity; EbAg alone: $p < 0.001$ for percentage IL-4-positive cells, n.s. for IL-4 intensity). Thus, these data also suggest an infection-induced, but non-specific enhanced readiness for IL-4 production.

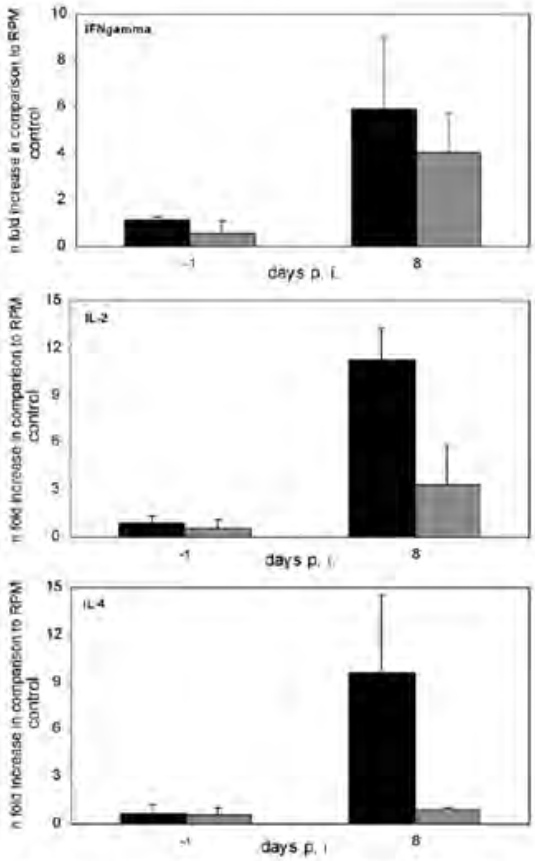

Fig. 4. IFN-γ, IL-2 and IL-4 gene transcription in CD4⁺ and CD8⁺ T cells of *Eimeria bovis* primary infected calves. CD4⁺ (black bars) and CD8⁺ (grey bars) T cells isolated by a magnetic bead technique from PBMC of *E. bovis* primary infected calves on days −1 and 8 p.i. were stimulated with *E. bovis* merozoite antigen or RPMI in the presence of autologous plastic adherent cells for 6 h. Thereafter, total RNA was isolated, reverse transcribed into cDNA and probed with real-time RT-PCR systems for the detection of IFN-γ, IL-2 or IL-4 mRNA equivalents, respectively. Arithmetical means and standard deviations of three individuals. AU, arbitrary units.

3.5. IFN-γ in supernatants of EbAg-stimulated PBMC of *E. bovis* primary infected calves

Antigen-specific IFN-γ production and secretion of PBMC isolated from *E. bovis*-infected calves is clearly enhanced in the course of *E. bovis* primary infection as detected in supernatants of EbAg-stimulated cells (Fig. 6). Global comparison of the different stimulations and the time dynamics revealed significant differences ($p < 0.01$ and $p = 0.0001$, respectively). Highest amounts of IFN-γ were found on days 8–12 p.i. (Fig. 6) confirming the IFN-γ gene transcription data. Interestingly, we also detected an enhanced, general readiness of PBMC for IFN-γ synthesis on day 8–12 p.i. as reflected by increased IFN-γ contents in supernatants of medium controls (Fig. 6).

4. Discussion

In this study we show that *E. bovis* primary infection is characterised by a sequential course of Th1- and Th2-polarized immune responses. In early prepatency Th1-associated reactions dominate as demonstrated by enhanced IL-2 and IFN-γ gene transcription. In agreement, IL-2 mRNAs had been previously found upregulated in draining lymph nodes of *E. bovis*-infected calves (Hermosilla et al., 1999), whereas Hughes et al. (1989b) had failed to detect IL-2 in sera of infected animals. The latter result may be attributed to the lower sensitivity of the method used by Hughes et al. (1989).

The data on IFN-γ match nicely in antigen-stimulated whole blood samples, lymphocytes isolated from *Lnn. ileocaecalis* by biopsies and PBMC supernatants. This overall pattern of Th1 activity is consistent with cell proliferation as observed by Hermosilla et al. (1999) and ourselves (Sühwold, unpublished data). In addition, the induction of parasite-specific IgG2 antibodies (Fiege et al., 1992), which are positively regulated by IFN-γ in the bovine system (Estes and Brown, 2002), is in agreement with our findings.

Increased levels of IFN-γ in primary infections are in concert with studies on other mammalian coccidian infections (Rose et al., 1989, 1991b; Smith and Hayday, 2000; Shi et al., 2001b) and seem to be a key feature of control (for review, see Ovington and Smith, 1992). One important effector mechanism induced by IFN-γ is considered to be the activation of macrophages, which accumulate in gut mucosa after *E. bovis* infections (Sühwold, unpublished data) and which are able to eliminate *E. bovis* sporozoites *in vitro* (Behrendt, pers. communication). Macrophages, in turn, may generate reactive oxygen intermediates, that have been reported to kill sporozoites of *Eimeria* spp. (Hughes et al., 1989a; Michalski and Prowse, 1991). In addition, the detrimental effect of IFN-γ on *Eimeria* spp. replication is well documented (Hughes et al., 1989; Kogut and Lange, 1989a,b; Rose et al., 1991a; Ovington and Smith, 1992) with intracellular NO being assumed as effector mechanism (Ovington and Smith, 1992). However, these mechanisms do not appear to be fully effective in primary infected calves, as patent infections develop.

We also found higher proportions of IFN-γ-positive cells and a higher IFN-γ content/cell in PMA/ionomycin-stimulated PBMC in early prepatency suggesting an additional infection-induced, but non-specific mechanism acting on IFN-γ expression. These reactions were also reflected by enhanced IFN-γ contents in supernatants of PBMC medium controls on days 8 and 12 p.i. However, this effect appears to be restricted on cytokine expression as non-specific proliferative activities of PBMC were not enhanced during *E. bovis* infection (Hughes et al., 1989b; Hermosilla et al., 1999). With respect to the well-known anti-replicative effect of IFN-γ on intracellular apicomplexan stages, enhanced non-specific production of this cytokine may influence ongoing *Eimeria* infections in cattle.

In this work we demonstrate that both CD4⁺ and CD8⁺ T cells react by increased antigen-specific IFN-γ gene

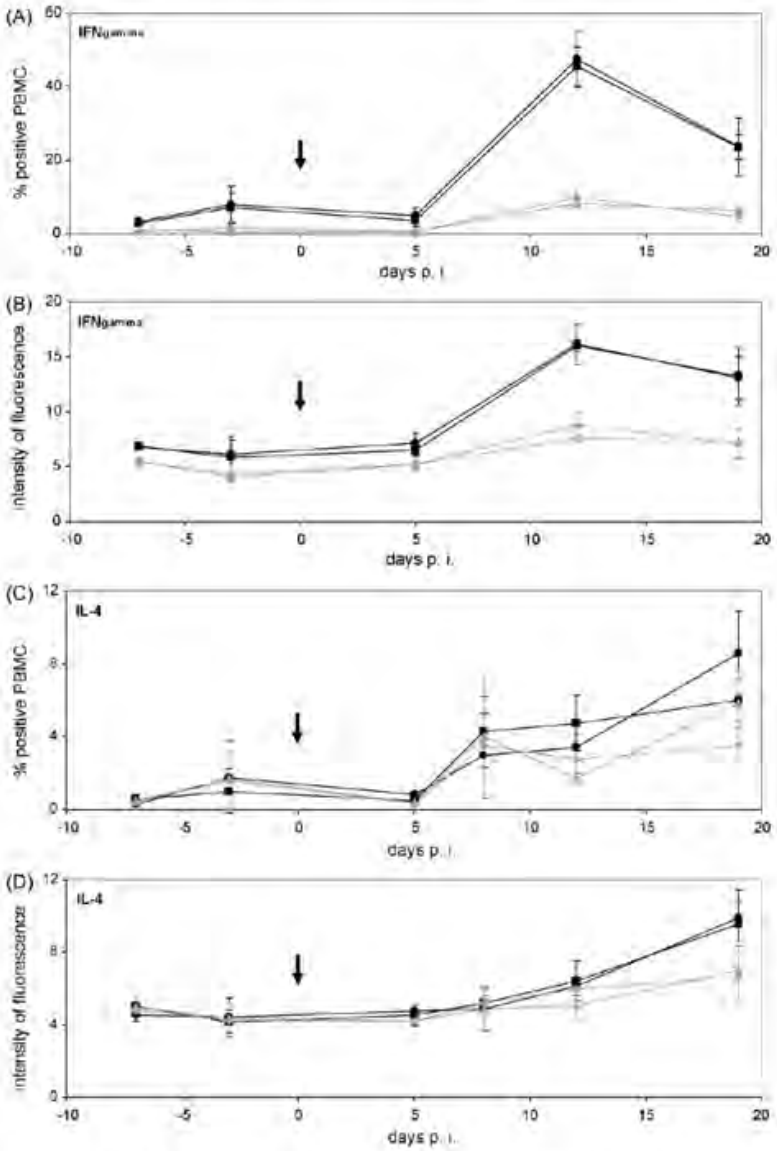

Fig. 5. Detection of intracellular IFN-γ and IL-4 in PBMC of primary *Eimeria bovis*-infected calves. PBMC were isolated from *E. bovis* primary infected calves on days −7, −2, 5, 8, 12 and 19 p.i. (day of infection is indicated by an arrow) and were stimulated with either PMA/ionomycin alone (black squares), PMA/ionomycin plus *E. bovis* merozoite I antigen (black dots), *E. bovis* merozoite I antigen alone (grey triangles) or RPMI as medium control (grey squares) for 6 h. Thereafter, PBMC were assayed by FCM analyses detecting the percentages of lymphocytes containing IFN-γ (A) or IL-4 (C) and the fluorescence intensities reflecting the cellular amount of intracellular IFN-γ (B) or IL-4 (D). Arithmetical means and standard deviations of three individuals.

transcription on day 8 p.i. Both subsets were also shown to expand in early prepatency of an *E. bovis* primary infection (Hermosilla et al., 1999). Owing to their cytotoxic capacity, $CD8^+$ T cells are often assumed to act against *Eimeria* intracellular stages, but so far, these cells appear to predominantly act in the case of challenge infection (Rose et al., 1992a; Findly et al., 1993; Ovington et al., 1995; Smith and Hayday, 2000; Shi et al., 2001a). The precise role of $CD4^+$ T cells is uncertain, nonetheless they may be important in the sense of IFN-γ producers activating macrophages, cytotoxic T cells or NK cells as effector cells. In addition, it seems probable that their protective function is expressed directly, as it has been shown for bovine $CD4^+$ T cells killing autologous *Neospora caninum*-infected cells by the perforin/granzyme pathway (Staska et al., 2003).

Upregulation of IFN-γ during primary infection appears rather transient and is limited to a few days in prepatency, coinciding with the development of early meront stages. Interestingly, this time-frame overlaps with the first-time appearance of parasite-specific antigen on the surface of

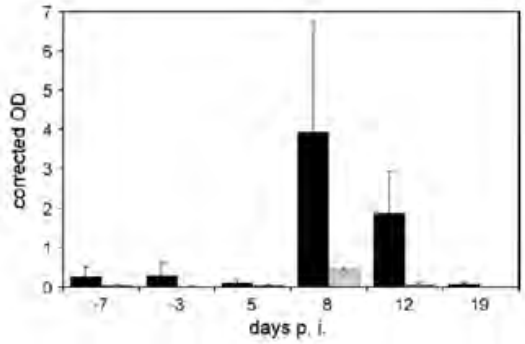

Fig. 6. PBMC-derived *in vitro* production of IFN-γ in the course of a primary *Eimeria bovis* infection. PBMC isolated from *E. bovis* primary infected calves on days −7, −3, 5, 8, 12 and 19 p.i. and stimulated with *E. bovis* merozoite antigen (black bars) or RPMI (grey bars) as medium control for 48 h. Supernatants were assayed for bovine IFN-γ using an ELISA-kit. Levels of IFN-γ were calculated relative to the positive control and expressed as corrected OD: (mean antigen or RPMI stimulated wells − test negative control)/(test positive control − test negative control). Arithmetical means and standard deviations of three individuals.

infected host cells (Badawy, personal observation). In this respect, it is noteworthy, that endothelial cells can exhibit antigen-presenting capacities (Wagner et al., 1984; Bosse et al., 1993; Knolle, 2006; Behling-Kelly and Czuprynski, 2007).

The gene transcripts of the Th2-related cytokine IL-4 were upregulated in antigen-stimulated samples in the course of primary infection and after challenge infection. Compared to Th1-associated cytokines, IL-4 induction was delayed and peaked in early patency. However, there was an overlap of increased IFN-γ/IL-2 and IL-4 gene transcripts which may be reflected in the co-induction of both IgG1 and IgG2 antibodies in *E. bovis*-infected calves (Fiege et al., 1992). We additionally observed an infection-induced, but non-specifically increased level of IL-4 expression in PBMC of infected calves, which may also contribute to the generation of parasite-specific IgG1 antibodies, as this isotype is positively regulated by IL-4 in the bovine system (Estes and Brown, 2002).

Calves experiencing challenge infection were immune and hardly shed any oocysts. However, gene transcription of Th1-related cytokines were neither upregulated in whole blood samples nor in lymphocytes obtained from lymph nodes. In agreement, Hughes et al. (1988) had also failed to detected significant levels of antigen-induced IL-2 produced by PBMC of immune animals. Overall, these reactions are consistent with PBMC proliferation displaying a lack of antigen-specific reactivity after challenge infection as well (Sühwold, unpublished data). In contrast to Th1 cytokines, mRNAs of IL-4 were consistently enhanced in lymph node-derived lymphocytes isolated from *E. bovis* reinfected calves indicating Th2-dominated responses after challenge.

Given that humoral immune responses merely reflect ongoing infections, but fail to protect against *E. bovis* (Fiege et al., 1992), it appears likely that Th1-polarized T cells act against early stages, such as sporozoites or early meronts <8 days of age, and are of major importance in parasite control after challenge infection. In concert, studies on other mammalian coccidia also suggest early stages as targets of control (Rose et al., 1992b; Shi et al., 2001a). The fact, that significantly increased levels of IFN-γ or IL-2 mRNAs were also missing in *Lnn. colici*, i.e., in lymph nodes draining the tissue of meronts II and later stages, supports the hypothesis of a prior abrogation of parasite development. This hypothesis may explain the lack of memory-associated Th1 reactions in challenge infection. In addition, we might either (i) have missed the decisive time point (earlier than 8 d post challenge.), or (ii) the critical reactions may have happened in a restricted microenvironment in gut mucosa, and in consequence were neither reflected in the periphery nor in the draining lymph nodes, or (iii) control reactions may have been mediated by an IFN-γ-independent mechanism. In agreement with the latter hypothesis, Smith and Hayday (2000) rule out IFN-γ playing a role in *E. vermiformis* challenge infections. A lack of cytokine expression after challenge infections was also described in other *Eimeria* infections (Wakelin et al., 1993; Schito et al., 1998; Smith and Hayday, 2000) and was, to some extent, attributed to a prior parasite elimination.

In conclusion, our data show distinct antigen-specific cytokine profiles in primary *E. bovis* infection reflecting both Th1- and Th2-associated reactions, with an IFN-γ-dominated prepatency and a change to IL-4-mediated immune reactions in the later phase. In contrast, challenge infection is characterised by the absence of antigen-induced, Th1-orientated cytokine mRNAs suggesting either early abrogation of parasite development or IFN-γ-independent control mechanisms. In addition we demonstrate a distinct infection-induced, but non-specific enhancement of PBMC activity as measured by IFN-γ and IL-4 production in the course of *E. bovis* primary infection. Overall, the results of this work should provide a helpful reference for the development of new strategies for the prevention and treatment of bovine coccidiosis.

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7.5 "T cell reactions of *Eimeria bovis* primary- and challenge-infected calves"

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Eigener Anteil an der Publikation

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

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ORIGINAL PAPER

T cell reactions of *Eimeria bovis* primary- and challenge-infected calves

Anke Sühwold · Carlos Hermosilla · Torsten Seeger · Horst Zahner · Anja Taubert

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Abstract *Eimeria bovis* infections commonly have clinical impact only on young animals, as homologous reinfections generally are under immunological control. So far, the nature of the immune responses delivering protection to calves has not been investigated. In this study we therefore analysed local and peripheral proliferative T cell activities of primary- and challenge-infected calves and investigated the occurrence of T cell phenotypes in the peripheral blood and in mucosal gut segments isolated either by bioptic means or by necropsies. We show that lymphocytes of *E. bovis*-infected calves exhibit effective, transient antigen-specific proliferative responses in the course of prepatency of primary infection but fail to react after homologous reinfection suggesting early abrogation of parasite development. Whilst in primary infection an expansion of peripheral $CD4^+$ T cells was observed, reinfection had no effect on the proportions of $CD4^+$, $CD8^+$ subsets or $\gamma\delta TCR^+$ T cells. In contrast, both *E. bovis* primary and challenge infections had an impact on local tissue T cell distribution. Primary infection was characterised by a $CD4^+$ T cell infiltration early in prepatency in ileum and later in colon mucosa, whereas $CD8^+$ T cells were only found accumulating in the latter gut segment. Challenge infection led to infiltration of both $CD4^+$ and $CD8^+$ T cells in small intestine and large intestine segments indicating protective functions of both cell types. In contrast, infiltration of ileum and colon mucosa with $\gamma\delta TCR^+$ T cells was restricted to primary infection.

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Introduction

Eimeria bovis infections cause important coccidian diseases of cattle severely affecting animal health and profitability of cattle industry (Daugschies et al. 1998; Daugschies and Najdrowski 2005; Fitzgerald 1980). Clinical symptoms related with bovine coccidiosis are usually restricted to primary-infected calves as they develop protective immunity against subsequent homologous infections.

In general, the termination of *Eimeria* spp. primary infections as well as the control of homologous reinfections rely on cellular adaptive immune reactions of the host (Wakelin and Rose 1990; Zahner et al. 1994). However, immunity against *Eimeria* spp. generally is species specific (Rose 1973; Rose 1987) or even strain specific (Fitz-Coy 1992; Martin et al. 1997; Norton and Hein 1976; Shirley and Bellatti 1988; Smith et al. 2002) and, in consequence, protective cross immunity is rare. So far, cellular immune responses against *Eimeria* spp. affecting cattle have scarcely been investigated. Considering the few studies available the first meront stage in *E. bovis*-infected calves—in contrast to primary *E. zuernii* infections (Stockdale 1977)—seem hardly associated with lymphocyte infiltration of the mucosa (Friend and Stockdale 1980). However, lymphocytes of *E. bovis* infected animals displayed enhanced antigen-specific proliferative activities (Hermosilla et al. 1999; Hughes et al. 1988; Hughes et al. 1989), whilst non-specific reactions

towards a mitogen seemed not influenced by infection (Hermosilla et al. 1999). Data generated in rodent models or avian eimeriosis showed gut infiltration with both αβTCR$^+$ T cells (Lillehoj 1994; Rothwell et al. 1995; Vervelde et al. 1996) and γδTCR$^+$ T cells (Findly et al. 1993; Rothwell et al. 1995). Studies with αβTCR T cell deficient animals reveal these T cells as key actors (Roberts et al. 1996; Smith and Hayday 2000b), whilst γδTCR$^+$ T cells seem of minor importance in the development of immunity (Roberts et al. 1996; Rose et al. 1996). It appears generally accepted, that primary *Eimeria* spp. infections are predominantly controlled by Th1-associated reactions of CD4$^+$ T with whilst cytotoxic CD8$^+$ T cells seem to be the major effector cell type against challenge infections (Findly et al. 1993; Ovington et al. 1995; Rose et al. 1992a; Shi et al. 2001; Smith and Hayday 2000b). However, data appear somewhat conflicting as depletion or defects of CD4$^+$ T cells did not influence primary avian *E. acervulina* or murine *E. papillata* infections (Schito et al. 1998a; Trout and Lillehoj 1996) but seemed essential for protective immunity after challenge in murine *E. vermiformis* and *E. papillata* infections (Schito et al. 1998a; Smith and Hayday 1998). Both CD4$^+$ and CD8$^+$ T cell subsets were demonstrated to expand during primary *E. bovis* infection (Hermosilla et al. 1999) and recent data showing enhanced antigen-specific IFN-γ production in prepatent *E. bovis* infections in calves suggest Th1-dominated immune responses within this period of infection (Taubert et al. 2008).

In this work, we investigate T cell-mediated immune reactions of *E. bovis* primary-infected and challenged calves with respect to antigen-specific proliferative activities and infiltration of T cell subsets in the parasite affected mucosa. We show that T cells proliferate effectively during a restricted time span during prepatency of primary infection but fail to do so after challenge suggesting early abrogation of reinfection. In accordance, we demonstrate expansion of peripheral CD4$^+$ T cells during primary infection whilst after challenge neither the proportions of CD4$^+$ or CD8$^+$ T cell subsets nor those of γδTCR$^+$ T cells were influenced. Overall, analyses of T cell infiltration into parasitized gut mucosa suggest a major involvement of CD4$^+$ T cells in the termination of primary infection and a role of both CD4$^+$ and CD8$^+$ T cells in the control of reinfections.

Materials and methods

Animals

Calves were purchased from a local farmer at the age of 2 weeks, treated with Baycox® (Bayer) and Halocur® (Intervet) in the second week after birth, tested for parasitic infections, and when deemed parasite free, maintained under parasite-free conditions in autoclaved stainless steel cages (Woetho) until experimental *E. bovis* infection. They were fed with milk substitute (Hemo Mischfutterwerke) and commercial concentrates (Raiffeisen). Water and sterilized hay were given ad libitum.

Parasite maintenance

The *E. bovis* strain H was maintained by passages in calves. For the production of oocysts, calves were infected at the age of ten weeks with 5×10^4 sporulated oocysts each. Excreted oocysts were isolated from the faeces beginning 19 days p.i. according to the method of Jackson (1964). Sporulation was achieved by incubation in a 2 % (*w*/*v*) potassium dichromate (Sigma) solution at room temperature. Sporulated oocysts were stored in this solution at 4°C until further use.

Sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al. 2002). For in vitro infections, bovine umbilical vein endothelial cells (BUVEC) isolated and grown to confluence in endothelial cell growth medium (Promocell) as described elsewhere (Taubert et al. 2006) were infected with freshly isolated *E. bovis* sporozoites (10^6 sporozoites/75 cm^2 culture flask). Culture medium was changed 24 h p. i. and thereafter every second day. From day 18 p. i. onwards, *E. bovis* merozoites I were harvested from culture as previously described (Hermosilla et al. 2002).

Infections of animals, biopsies and necropsies

Four groups (A–D) of three calves each were used, aged 8–12 weeks. Groups A and B were orally infected on day 0 with 5×10^4 sporulated oocysts and challenged on day 48 with 3×10^4 sporulated oocysts. Group C experienced only the primary infection. Group D served as non-infected control. Shedding of oocysts was determined from day 18 p.i. onwards by daily faecal examination (McMaster technique).

Biopsies were performed on days 8 and 40 after primary and 8 days after challenge infection. Animals were sedated with xylazine (0.1 mg/kg, i. m., Rompun®, Bayer) and fixed in left lateral position. The incision site at the right abdominal wall (approximately one hand proximally to the *tuber coxae*) was infiltrated with procaine (2%, Procasel®, Selectavet). Calves were then anaesthetised with ketamine (3 mg/kg, i.v., Ursotamin®, Serumwerk Bernburg). Laparotomy followed standard surgery procedures. The *Plica ileocaecalis* of the ileum and the *Ansa spiralis coli* were advanced for withdrawal of the *L. ileocaecales* and *L. colici*, respectively. Lymph nodes were fixed with a clamp, removed and submitted to sterile medium (RPMI,

1% penicilline/streptomycine) for subsequent lymphocyte isolation. Draining vessels were ligated (Serafit®, Serag-Wiessner) and mesentery defects were closed. Gut segments (ileum on days 8 after primary and challenge infection, colon on day 40 after infection) were advanced and the content in the respective area was removed by massage. The gut was then fixed with a clamp and mucosal samples were taken using a biopsy punch (ø 8 mm, Stiefel Laboratorium GmbH) and submitted to sterile medium. The mucosal defect was closed according to standard surgical methods (Serafit®, Serag-Wiessner) and checked for closeness. After flushing (0.9% NaCl, 37°C), the gut was relocated and the wound was sutured. Calves were treated with flunixin-meglumin (2.2 mg/kg, i.v., Finadyne®, Essex) and procaine-penicillin G (0.1 ml/kg, s.c. for 5 days, Animedica) and monitored.

Calves of group A and B were necropsied on days 60 and 74, respectively, i.e., 12 and 26 days after challenge infection, those of group C 26 days p.i. and group D at a corresponding age to group B. Gut mucosal tissue samples (jejunum, ileum, caecum, colon) were withdrawn for immunohistochemical analyses. Lymph nodes (*L. jejunales*, *L. ileocaecales* and *L. colici*) were excised for immediate lymphocyte isolation.

Preparation and cryoconservation of gut wall samples

Intestinal biopsies and half of the gut samples isolated at necropsies were cryopreserved. The mucosal site of the sample was applied to an equally sized cube of bovine liver, covered with a drop of OCT reagent (Tissue-Tec®, Sankura Finetec Inc.) and cooled in 2-methylbutane (15 s in liquid nitrogen). The samples were wrapped in aluminum foil and stored at -80°C until further use.

The second half of the gut samples obtained at necropsies was fixed in 4% formaldehyde (Merck) in phosphate-buffered saline for 24 h, dehydrated and embedded in paraffin according to standard procedures.

E. bovis merozoite I antigen

E. bovis merozoites I collected from culture were homogenized by repeated freezing followed by sonication (20 kHz, 5×15 s pulses) on ice. After centrifugation (11,000×g, 4°C, 20 min) the supernatants were passed through 0.2 µm sterile filters (Renner). Protein concentration was determined using the Bradford method (Bradford 1976). The *E. bovis* merozoite I antigen (EbAg) was stored at −80°C.

Isolation of peripheral blood mononuclear cells and lymphocytes

Calves were bled by puncture of the jugular vein on days 0, 4, 6, 8, 12, 15, 19, 26, 48, 49, 52, 54, 56 and 60 p.i. Blood was collected in 50 ml plastic tubes (Nunc) containing 0.1 ml heparin (Sigma). For peripheral blood mononuclear cells (PBMC) isolation, 20 ml of heparinized blood were mixed with equal parts of 0.9 % NaCl. Four ml of the mixture were applied on top of 3 ml Ficoll-Paque (density= 1.077 g/l, Biochrom) in glass tubes with an inner diameter of 12 mm. After centrifugation [45 min, 400×g, room temperature (RT)] the PBMC layer was collected and the cells were washed three times (10 min, 400×g, 4°C) in medium RPMI 1640. Viable cells (trypan blue, Sigma, exclusion test) were counted in a Neubauer chamber.

For preparation of lymph node cells, lymph nodes were cut into pieces and gently teased through sterile nylon sieves (meshes of 180 µm; Reichelt Chemietechnik) flotating in RPMI. After three washings (10 min, 400×g, 4°C), the cells were suspended in RPMI, supplemented with 1% penicillin (Sigma), 5 mM glutamine (Gibco BRL), 10% foetal calf serum (FCS, Biochrom KG) and 1.7 µl/500 ml 2-mercaptoethanol (Serva). Viable cells (trypan blue exclusion test) were counted in a Neubauer chamber.

Cells were either subsequently used in lymphocyte proliferation assays or suspended in dimethylsulphoxide (1% final concentration, Merck) in RPMI supplemented with 10% FCS (Biochrom), pre-cooled (1 h, 4°C) and cryopreserved in liquid nitrogen until required for flow cytometry analyses.

In vitro stimulation of lymphocytes with EbAg and lymphocyte proliferation assays

Freshly isolated PBMC or lymph node cells were resuspended in culture medium (CM), composed of RPMI, 2 mM L-glutamine (Sigma), 0.22% $NaCO_3$ (Merck), 1 mM 2-mercaptoethanol (Sigma), 200 UI/ml penicilline (Sigma), 50 µg/ml streptomycin (Sigma) and 10% FCS. Cells (2×10^5 lymphocytes/well, 96-well microtiter plates, Nunc) were stimulated either with EbAg (10 µg/ml, 96 h), Con A (5 µg/ml, Biochrom, 48 h) or plain RPMI. Cultures were incubated at 37°C, 5% CO_2 atmosphere and thereafter pulsed for the final 16 h with 50 µl [^{3}H] thymidine (0.5 µCi/ml, Amersham). Subsequently, cells were harvested on glass-fibre filters using a 96-well cell harvester (Skatron). After drying (60°C, 1 h), filters were saturated with scintillation fluid (Roth) and radioactivity was measured in a β-liquid scintillation counter (Tri-Carb 2700 TR, Packard Instruments).

Phenotypical characterization of T cells by fluorescence antibody cell sorting

Frozen PBMC and lymph node cells were rapidly thawn, suspended in V-shape bottomed 96-well microtiter plates

(Nunc) at a density of 1×10^5 cells/well and washed three times with RPMI (10 min, 150×g, 4°C). The cell pellet was suspended in 60 µl of monoclonal antibody solutions (IL-A 11, directed against bovine CD4; IL-A 105, directed against bovine CD8 or D86, directed against the bovine $\gamma\delta^+$TCR; all antibodies were kindly donated by C. Menge, Giessen) and incubated for 30 min on ice. Thereafter, cells were washed in 150 µl PBS and incubated in 50 µl FITC-conjugated goat anti-mouse antibodies (1:200 in PBS, 20 min on ice; Dianova) supplemented with 1 µl propidiumiodide solution (2 µg/ml), washed twice in PBS and transferred to plastic test tubes (Renner) previously filled with 300 µl PBS. Immunofluorescence staining was measured using a Coulter Epics Elite-FACS (Coulter Electronic). Tests were performed in triplicates.

Immunohistology

For the detection of $CD4^+$, $CD8^+$ and $WC1^+$ (=$\gamma\delta$TCR$^+$) T cells 4 µm cryo section were dried over night at room temperature on Superfrost plus object slides (Menzel-gläser). Samples were fixed in ice cold acetone (10 min) and dried. Endogenous peroxidase was inactivated in 0.5 % H_2O_2 (30 min, RT, Roth). After five washings in TBS (5 min), samples were probed with primary monoclonal antibodies (anti-bovine CD4, CC30; 1:5; anti-bovine CD8, CC63; 1:200; anti-bovine WC1, CC15; 1: 100; all Serotec) for 1 h (37°C, humidity chamber). After rinsing the samples thrice in PBS, they were incubated in sheep anti-mouse antibodies conjugated with peroxidase (1:50, Amersham). Following three further washings in TBS (5 min), binding was visualised by adding substrate (0.048 g DAB, Fluka, and 800 µl 3% H_2O_2 in 80 ml imidazole buffer, 3–5 min). After rinsing three times in TBS (5 min) and once in aqua dest. (5 min), the tissue probes were counterstained for 15 s in Papanicolaou solution (1:10, Merck), washed in tap water (5 min), dehydrated according to standard histological procedures and mounted in Aquatex® (Merck).

Immunostained T cells were counted in ten randomly chosen vision fields (200× magnification) placing the vision field in a way that one half comprised the tip and the other half the basis of a villus.

Statistical analysis

Statistical analyses used the programme package BMDP for XP, Release 8.1 (Dixon, 1993). For the description of the data arithmetical means were calculated. To describe the variability of the data standard deviations were used. As some statistical distributions of the original data were skewed to the right, if necessary, arc-sine or logarithmic transformation were performed to obtain an approximately normal distribution of the values. In accordance to the design of the experiments, data were compared by two or three-factorial analysis of variance with repeated measures (BMDP2V). Differences were regarded as significant at a level of $p \leq 0.05$.

Results

E. bovis challenge-infected calves are immune

All *E. bovis* primary-infected calves shed oocysts beginning on 19 days p.i. (Fig. 1). There was a rapid increase in oocyst shedding from 20 days p.i. onwards with highest amounts found 21–24 days p.i. Thereafter, oocyst shedding rapidly decreased and ceased with 29 days p.i. Primary-infected calves were immune to challenge infection and hardly shed any oocysts (difference to primary infection: $p < 0.0001$). If at all, very few oocysts were found in the faeces of challenged animals from days 20 to 24 p.i. (Fig. 1).

E. bovis primary-infected calves exhibit antigen-specific proliferative immune responses

PBMC of *E. bovis* primary-infected calves reacted to EbAg only during prepatency (Fig. 2) and exhibited a short-timed proliferative response on days 8–15 p.i. with maximum reactions on day 8 days p.i. Non-infected control animals as well as challenge calves failed to react to EbAg (difference to primary infection: $p < 0.02$; Fig. 2).

Enhanced proliferative activity of T lymphocytes in the peripheral blood corresponded to reactions found in draining lymph nodes. Thus, lymphocytes isolated from *L. ileocaecales* of primary-infected calves on day 8 p.i. showed signficantly enhanced antigen-specific T cell proliferation, but failed to do so 12 days p.i. or 8 days after challenge infection (Fig. 3). Lymphocytes isolated from *L. colici* 40 days after primary and 26 days after challenge infection also lacked antigen-specific proliferation (Fig. 3). Cells of non-infected controls never reacted to EbAg (differences between infected animals 8 days after primary infection and all other data: $p < 0.0001$ –0.001; Fig. 3).

E. bovis infection does not modulate non-specific proliferative T cell reactions

PBMC or lymph node cells responded to stimulation with the mitogen Con A with significantly enhanced proliferative activity ($p < 0.001$). Responses varied irregularly in all groups regardless of *E. bovis* infections (data not shown).

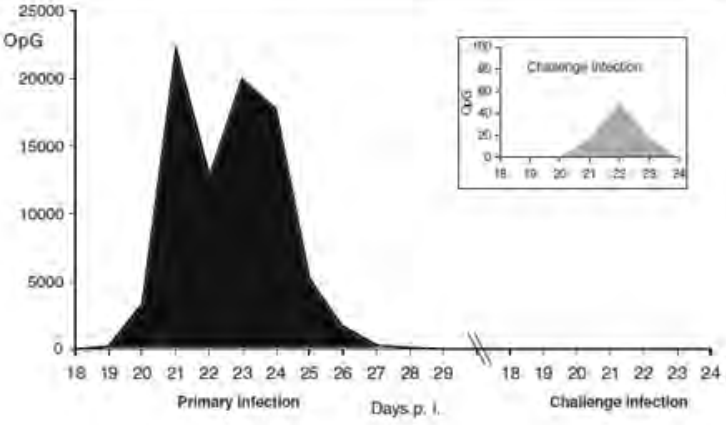

Fig. 1 Oocyst shedding of *E. bovis* primary- and challenge-infected calves. Calves ($n=3$) were infected orally (5×10^4 sporulated oocysts of *E. bovis*/animal) and challenged after 48 days (3×10^4 sporulated oocysts of *E. bovis*/animal). Oocyst counts (oocysts per gram faeces, OpG) were determined by MacMaster technique

E. bovis primary infection leads to expansion of peripheral $CD4^+$ T cells whilst challenge infection fails to influence the composition of peripheral T cell phenotypes

Phenotyping peripheral blood lymphocytes after primary infection revealed expansion of $CD4^+$ T cells beginning 15 days p. i. ($p<0.0001$), leading to a plateau-like time course from 20 days p.i. onwards (Fig. 4). Challenge infection did not influence the level of $CD4^+$ T cells and the proportions of $CD4^+$ T cells in the blood of reinfected animals remained stable ($p<0.02$, Fig. 4).

The proportions of $CD8^+$ T cells did not expand after primary or challenge infection and, however, seemed to be reduced during prepatency of the primary infection when compared to non-infected control animals ($p<0.02$, Fig. 4). Challenge infection did not influence the level of $CD8^+$ T cells (Fig. 4).

The proportions of $\gamma\delta TCR^+$ T cells, as measured by the detection of WC1 on lymphocytes, appeared decreased throughout primary and challenge infection when compared to non-infected control animals. Thus, $\gamma\delta TCR^+$ T cells declined immediately after primary infection and remained on a constantly decreased level until challenge infection (Fig. 4). After challenge, the numbers of $\gamma\delta TCR^+$ T cells increased, however, equal changes were detected in the non-infected group (Fig. 4).

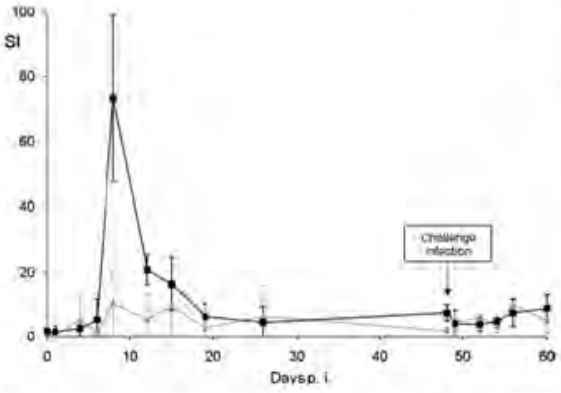

Fig. 2 Antigen-specific proliferative responses of peripheral blood mononuclear cells isolated from *E. bovis* primary- and challenge-infected calves. Peripheral blood mononuclear cells, isolated from *E. bovis*-infected calves ($n=3$, *black squares*) and non-infected control animals ($n=3$, *grey triangles*), were stimulated in vitro with *E. bovis* merozoite I-antigen (10 μg/ml, 96 h). Proliferative T cell activity was measured by ^{3}H thymidine incorporation. Arithmetical means and standard deviations. *SI* stimulation index

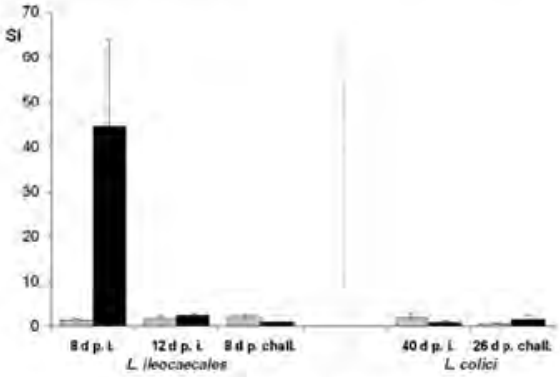

Fig. 3 Antigen-specific proliferative responses of lymphocytes isolated from *L. ileocaecales* and *L. colici* of *E. bovis* primary- and challenge-infected calves. Lymphocytes isolated from *L. ileocaecales* and *L. colici* of *E. bovis* primary- and challenge-infected calves ($n=3$, *black columns*) and non-infected control animals ($n=3$, *grey columns*), were stimulated in vitro with *E. bovis* merozoite I-antigen (10 μg/ml, 96 h). Proliferative T cell activity was measured by ^{3}H thymidine incorporation. Arithmetical means and standard deviations. *SI* = stimulation index

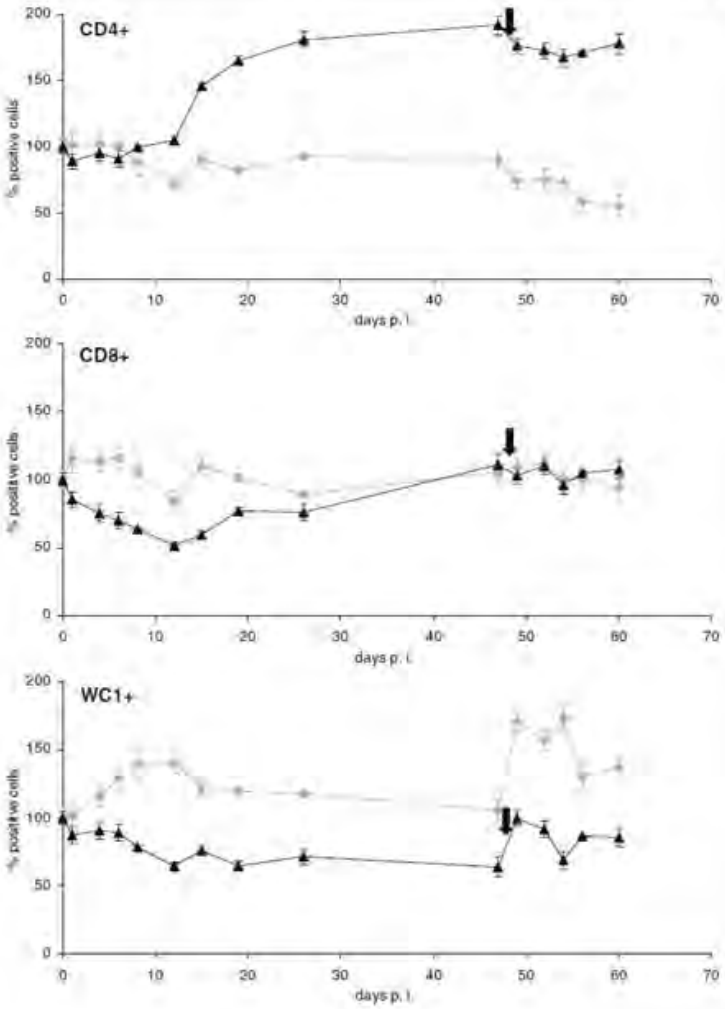

Fig. 4 T cell subpopulations in the peripheral blood of *E. bovis* primary- and challenge-infected calves. Peripheral blood mononuclear cells, isolated from *E. bovis* primary-infected and challenged calves ($n=3$, *black triangles*) and non-infected control animals ($n=3$, *grey quarderx*), were probed with antibodies directed against bovine CD4, CD8 and WC1 (=γδTCR-specific) and analysed by flow cytometry. The time point of challenge infection (48 d p. i.) is indicated by an arrow. Arithmetical means and standard deviations

E. bovis primary and challenge infections induce local cellular immune responses

Biopsies were taken to analyze local tissue responses (infiltration of $CD4^+$, $CD8^+$ and $\gamma\delta TCR^+$ T cells) to meront I formation in the ileum at 8 days after primary and challenge infection and subsequent to oocyst excretion 40 days after primary infection in the colon. The data suggest slightly increased numbers of these cell types in the ileum at 8 days after primary and, more pronounced, after challenge infection (Fig. 5) although differences generally remained below a significant level. Only the numbers of $CD4^+$ T cells in challenged animals differed significantly ($p<0.02$) from those of non-infected controls. More distinct reactions were observed 40 days after primary infection in the colon (Fig. 6). $CD4^+$ T cells ($p<0.02$) and $\gamma\delta TCR^+$ T cells ($p<0.004$) accumulated significantly when compared to non-infected controls. The numbers of $CD8^+$ T cells seemed enhanced in infected animals, too, but appeared rather variable overall.

Owing to technical reasons, analyses of tissues obtained by necropsies had to be restricted to samples isolated 26 days after primary and 12 and 26 days after challenge infection (Fig. 7). Overall, mucosal samples of challenged calves showed significant more $CD4^+$ T cells in the various gut segments ($p<0.005$–0.05) than those of primary-infected animals. There were, however, no significant differences between animals necropsied 12 and 26 days after challenge infection. Data depicted in Fig. 7 suggest a similar tendency for $CD8^+$ T cells, but significant differences between primary- and challenge-infected calves were restricted to the jejunum ($p<0.05$). $\gamma\delta TCR^+$ T cell contents

did not differ significantly between the groups and gut sections (Fig. 7).

Discussion

Infections of calves with suitable doses of *E. bovis* oocysts resulted in rapidly increasing, approximately one week lasting oocyst excretion as observed previously (Hermosilla et al. 1999). Infected animals developed effective protective immunity to homologous reinfection in accordance to Fiege et al. (1992). We furthermore show that primary infection is associated with a transient antigen-specific proliferation of PBMC during prepatency, which is in agreement with previous reports of Hermosilla et al. (1999). Transient antigen-specific proliferative activities of T cells were also reported for *E. vermiformis*-infected BALB/c mice (Wakelin et al. 1993) and *E. tenella*-infected chickens (Breed et al.

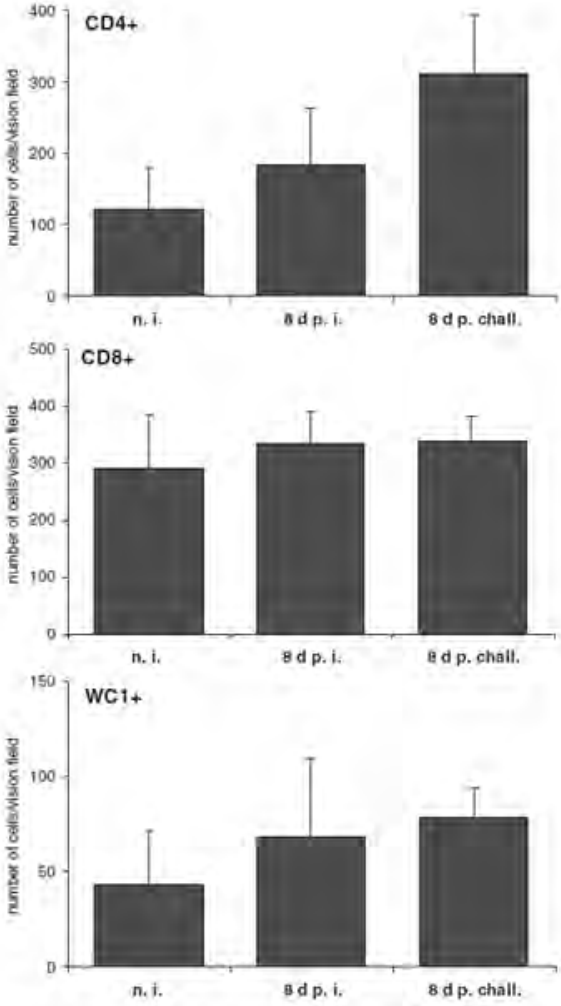

Fig. 5 T cell subtypes in the ileum mucosa of non-infected, *E. bovis* primary- and challenge-infected calves. Ileum samples of non-infected, *E. bovis* primary-infected and challenged (each group: $n=3$) calves were obtained by bioptic means at different time points p.i., fixed and subjected to immunohistological analyses using antibodies directed against bovine CD4, CD8 and WC1 (=γδ-TCR-specific). Arithmetical means and standard deviations

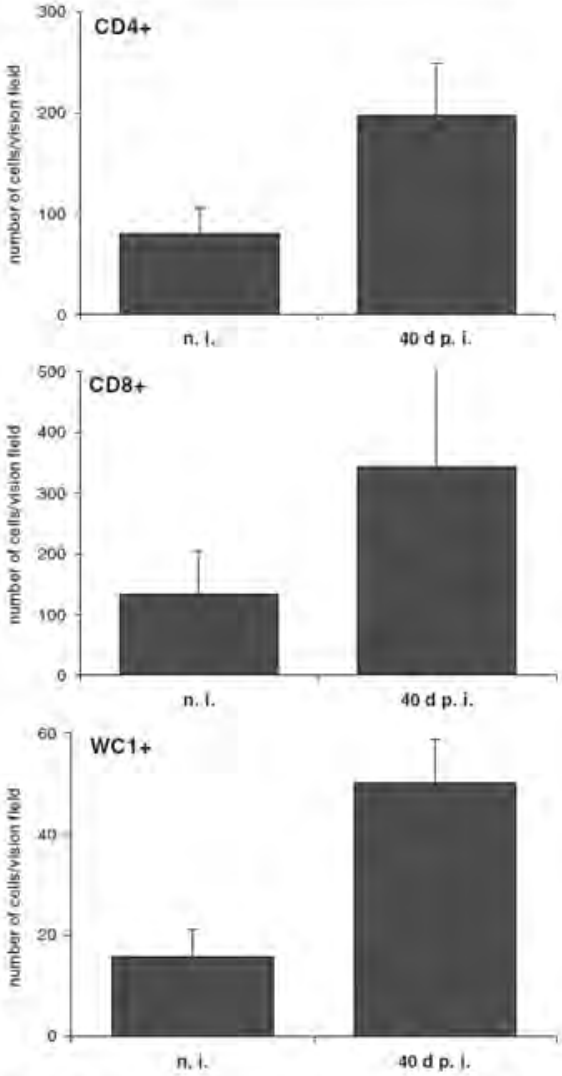

Fig. 6 T cell subtypes in biopsies of colon mucosa of *E. bovis* primary and non-infected calves. Colon samples of non-infected and *E. bovis* primary-infected (each group: $n=3$) calves were obtained by bioptic means at 40 days p.i., fixed and subjected to immunohistological analyses using antibodies directed against bovine CD4, CD8 and WC1 (=γδ-TCR-specific). Arithmetical means and standard deviations

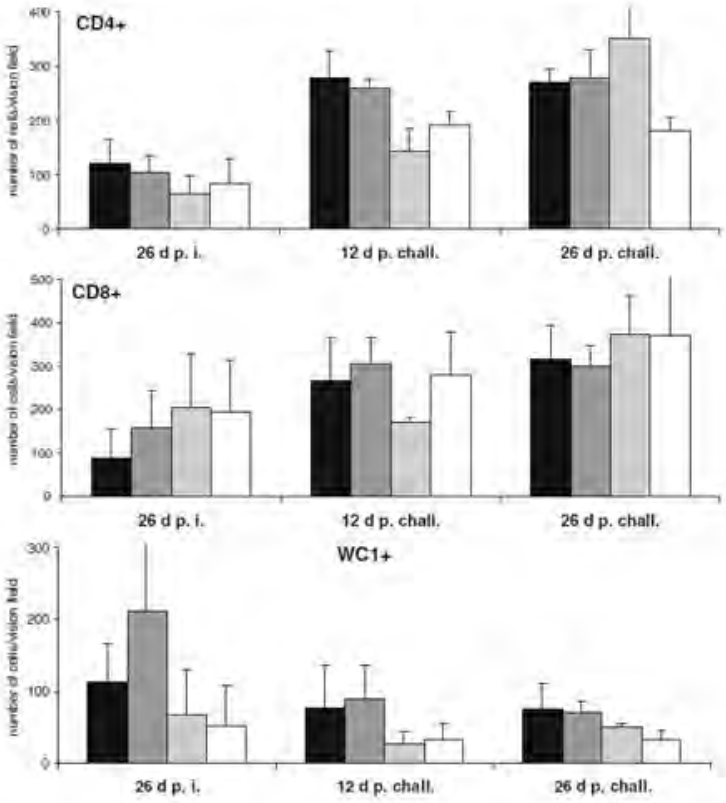

Fig. 7 T cell subtypes in intestine mucosa of *E. bovis* primary- and challenge-infected calves. Different groups ($n=3$, each) of *E. bovis* primary-infected or challenged calves were killed at different days p.i. Different gut mucosa samples (*black column*, jejunum; *dark grey column*, ileum; *bright grey column*, caecum; *white column*, colon) were fixed and subjected to immunohistological analyses using antibodies directed against bovine CD4, CD8 and WC1 (γδ-TCR-specific). Arithmetical means and standard deviations

1996) in prepatency and patency, respectively. The proliferative activity was limited to the prepatency starting after day 6 p.i. and leading to peak activity on day 8 p.i., which means that the onset of these reactions coincides with the beginning of parasite proliferation. Interestingly, this time frame also overlaps with the first-time appearance of parasite-specific antigens on the surface of infected host cells (Badawy et al. 2010). Given that the host cells of *E. bovis* during first merogony, endothelial cells, are, in principle, capable of antigen presentation (Behling-Kelly and Czuprynski 2007; Bosse et al. 1993; Knolle 2006; Wagner et al. 1984) and can activate T cells in an antigen-dependent manner (Epperson and Pober 1994; Pober and Cotran 1991; Rodig et al. 2003), these data may suggest early meront I-induced T cell reactivity.

Peripheral antigen-specific proliferative activities during prepatency coincided with respective responses of lymphocytes isolated from the draining lymph node (*L. ileocaecales*) by bioptic means and with enhanced antigen-specific IFN-γ production (Taubert et al. 2008) suggesting an overall pattern of Th1 activity in this early phase of infection.

The rapid decline of peripheral and local T cell reactivity following 8 days p.i. may form part of mechanisms allowing long term development of macromeronts in the endothelial host cell. Interestingly, in animals infected with other *Eimeria* spp. that replicate much faster and do not develop macromeronts, e. g. in *E. intestinalis*-infected rabbits, antigen-specific proliferative responses of lymphocytes indeed also coincided with the onset of meront formation, but, in contrast, were even enhanced with ongoing infection (Renaux et al. 2003).

The failure of lymphocytes to proliferate in response to antigenic stimulation after challenge infection appears surprising at a first view, although it is in concert with other studies describing an impaired antigen-specific lymphocyte proliferative activity after *E. papillata* (Schito et al. 1998b) and *E. vermiformis* (Wakelin et al. 1993) challenge infections in mice. It may be explained, however, by the fact that sporozoites or early intracellular stages represent the targets of protective immune effects in *Eimeria* spp. infections (Rose et al. 1992b; Shi et al. 2001) resulting in a lack of T cell-stimulating early meronts.

T cell subsets in primary infection were dominated by $CD4^+$ T cells which expanded beginning during late prepatency/early patency and remained on an elevated level throughout the observation period. According to these

findings and in agreement with reports in other *Eimeria* spp. infections (Rothwell et al. 1995; Shi et al. 2001; Vervelde et al. 1996), immunohistological analyses of parasitized gut mucosa revealed infiltration of $CD4^+$ T cells early after infection (8 days p. i.) in the ileum and later after infection (40 days p.i.) in the colon. The weak expansion of $CD8^+$ T cells observed in the peripheral blood of *E. bovis*-infected calves by Hermosilla et al. (1999) could not be verified in the present study. Proportions of $CD8^+$ T cells even decreased during prepatency in primary-infected calves and returned to the level of non-infected controls during patency. Accordingly, counts of $CD8^+$ T cells were not significantly elevated in the ileum mucosa at day 8 p.i. However, the late $CD8^+$ T cell infiltration of the colon mucosa after all suggests an expansion of this T cell subset.

Although challenge infection was neither associated with antigen-specific T cell proliferation nor with significant $CD4^+$ and $CD8^+$ subset expansion in peripheral blood, both subpopulations were found enhanced in the mucosa of the small and large intestine of challenge infected animals when compared with the situation after primary infection. Again, infiltration with $CD4^+$ T cells was detected earlier after reinfection on day 8 post-challenge than accumulation of the $CD8^+$ subset, which was firstly found at 12 days after challenge. Whilst $CD8^+$ T cells have often been assumed to represent the key cell type for control of challenge infections (Rose et al. 1992a; Shi et al. 2001; Trout and Lillehoj 1995; Trout and Lillehoj 1996), only some reports point at a potential role of $CD4^+$ T cells in protective immune effects against challenge infections with *Eimeria* spp. (Schito et al. 1998a; Smith and Hayday 1998). However, both subsets are known to exhibit effective cytotoxicity against intracellular apicomplexa (Denkers et al. 1993; Kasper et al. 1992; Khan and Kasper 1996; Staska et al. 2003) and may therefore both be considered as important cell types for the control of *E. bovis* challenge.

Immunhistological analyses also revealed an infiltration of $\gamma\delta TCR^+$ T cells during primary *E. bovis* infection in the ileum and colon mucosa, while they did not accumulate in the mucosal tissue after challenge infection. Gut infiltration by $\gamma\delta TCR^+$ T cells was also observed in primary avian and murine *Eimeria* infections (Findly et al. 1993; Rothwell et al. 1995). Considering that these cells contribute to 40% or more of PBMC in young calves (Wilson et al. 1996) this cell type may be of particular importance in cattle. $\gamma\delta TCR^+$ T cells have a wide range of functions, such as immunomodulation, cytokine production, cytotoxicicty and the regulation of inflammatory processes (reviewed by Pollock and Welsh 2002). Due to their general accumulation in the gut mucosa, a particular sentinel function is attributed to this cell type (De Libero 1997). However, the precise role of $\gamma\delta TCR^+$ T cells in *Eimeria* infections is still uncertain. On the one hand, Rose et al. (1996) and Roberts et al. (1996) did not find a protective role of $\gamma\delta TCR^+$ T cells in *Eimeria vermiformis* infections of mice, on the other hand Smith and Hayday (2000a) report on a higher susceptibility of *E. vermiformis*-infected $\alpha\beta TCR^-/\gamma\delta TCR^-$ mice to homologous challenge infection compared to $\alpha\beta TCR^-$ controls. Independent from this question, however, the anti-inflammatory efficacy of $\gamma\delta TCR^+$ T cells seems to play a role at least in murine coccidiosis as $\gamma\delta$TCR-deficient mice showed strongly escalated intestinal damage after primary *E. vermiformis* infection when compared with wildtype controls (Roberts et al. 1996).

In conclusion, our data show distinct antigen-specific proliferative T cell activities with exclusive reactions occurring during primary *E. bovis* infection and a failure after reinfection indicating early abrogation of parasite development after challenge. Primary infection is characterized by an early $CD4^+$ T cell infiltration into the intestine, whilst both $CD4^+$ and $CD8^+$ T cells accumulate in intestine mucosa of challenged animals. In contrast, gut infiltration with $\gamma\delta TCR^+$ T cells was restricted to primary infection. Overall, these data promote a better understanding of peripheral and local adaptive cellular immune responses of *E. bovis* infections and call for functional analyses of T cell subsets in ruminant *Eimeria* reinfections.

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7.6 "Bovine recombinant IFNγ induces endothelial cell gene transcription of immunoregulatory molecules and upregulates PMN and PBMC adhesion on bovine endothelial cells"

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- Initiative — weitestgehend eigenständig
- Projektplanung — weitestgehend eigenständig
- Durchführung der Versuche — wesentlich
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ORIGINAL ARTICLE

Bovine recombinant IFNγ induces endothelial cell gene transcription of immunoregulatory molecules and upregulates PMN and PBMC adhesion on bovine endothelial cells

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Abstract Interferon γ (IFNγ) is an important modulator of immune responses acting on multiple cell types, such as lymphocytes, macrophages or endothelial cells. We investigated the effects of recombinant bovine IFNγ on bovine umbilical vein endothelial cells (BUVEC) for the level of polymorphonuclear neutrophil cell (PMN)- and peripheral blood mononuclear cell (PBMC)-adhesion as well as the gene transcription of endothelial cell-derived adhesion molecules (E-selectin, P-selectin, VCAM-1, ICAM-1), chemokines (CXCL1, CXCL8, CXCL10, CCL2, CCL5), GM-CSF, iNOS and COX-2 in comparison to TNFα-stimulation. IFNγ strongly induced PMN and PBMC adhesion on BUVEC involving $CD4^+$, $CD8^+$ and γδ-TCR^+ ($WC1^+$) lymphocytes. Furthermore, IFNγ-stimulation led to a strong upregulation in the transcription of VCAM-1, ICAM-1, CXCL10 and CCL2 genes and to a low to moderate increase in the E- and P-selectin, CXCL1, CXCL8, CCL5, COX-2 and iNOS gene transcripts, but failed to enhance GM-CSF gene transcription. These results indicate that IFNγ can be considered an important activator of endothelial cells in the bovine system, most probably by influencing the outcome of inflammatory responses through selective upregulation of immunoregulatory molecules.

Keywords Bovine recombinant IFN-γ · Bovine endothelial cells · PMN adhesion · PBMC adhesion · Adhesion molecules · Chemokines · iNOS · COX-2 · GM-CSF

Abbreviations

BUVEC	bovine umbilical vein endothelial cell(s)
COX-2	cyclooxygenase-2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte macrophage colony-stimulating factor
ICAM-1	intercellular adhesion molecule-1

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iNOS	inducible nitric oxide synthase
IFNγ	interferon γ
NK cell	natural killer cell
PBMC	peripheral blood mononuclear cell(s)
p. stim.	post stimulation
PMN	polymorphonuclear neutrophil cell(s)
TNFα	tumour necrosis factor α
VCAM-1	vascular cellular adhesion molecule-1

Introduction

Interferon γ (IFNγ) exerts a multitude of immunoregulatory functions (for review, see Boehm et al. 1997) in the development of immune responses in all kinds of vertebrates. Being produced mainly by cells that occur in the bloodstream and in inflamed tissues, i.e., by T helper cells, cytotoxic T cells and NK cells, IFNγ influences the selective recruitment and extravasation of circulating leukocytes by modulating the expression of endothelial cell-derived chemokines and adhesion molecules, as shown in the human and other non-bovine systems. In this context, IFNγ has been demonstrated to promote the selective recruitment of lymphocytes and monocytes by inducing endothelial cell-derived CXCL10 and CCL2 (Beck et al. 1999; Raju et al. 2003), chemokines known to function as chemoattractants for these cell populations (Taub et al. 1993, 1995; Carr et al. 1994; Loetscher et al. 1994). Besides these pro-inflammatory activities, IFNγ also seems to display anti-inflammatory reactions by inhibiting the attraction of polymorphonuclear neutrophil cells (PMN) to the site of inflammation *in vivo* (Granstein et al. 1989; Johansen et al. 1996). Furthermore, in the human system IFNγ differentially upregulates endothelial cell-derived adhesion molecules, thereby enhancing the binding partners of the recruited immune cells, i.e. the integrin ligands intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1) (Doukas and Pober 1990; Thornhill et al. 1991; Melrose et al. 1998). Whereas extensive rolling of immune cells has not been reported, subsequent firm adhesion of lymphocytes (Yu et al. 1985; Thornhill et al. 1991), but not of granulocytes (Thornhill et al. 1990, 1991), seems to be a common feature on IFNγ-activated human endothelial cells. Under IFNγ control, human endothelial cells even appear to mediate a T_H1/T_H2-type selective polarization into tissue as an initiating event for cell-mediated immunity. Thus, Kawai and colleagues (1999) reported a selective diapedesis of T_H1 cells through rat endothelial cells, as promoted by IFNγ-induced CCL5 expression.

Bovine endothelial cells are widely used for *in vitro* studies on inflammatory reactions of the vascular bed, but whereas other stimulants such as TNFα were often used for activation of bovine endothelial cells (Maddox et al. 1999; Van Kampen and Mallard 2001a, b) basic work on the activating capacity of IFNγ in the bovine system covering endothelial cell-derived immune reactions is fragmentary. The present study was undertaken to obtain a rather broad insight into reactions of bovine umbilical vein endothelial cells (BUVEC) upon IFNγ stimulation. We analysed the transcription of several important chemokine (CCL2, CCL5, CXCL1, CXCL8, CXCL10) genes and genes encoding for GM-CSF (granulocyte macrophage colony stimulating factor), COX-2 (cyclooxygenase-2), iNOS (inducible nitric-oxide synthase) and adhesion molecules (E-selectin, P-selectin, ICAM-1, VCAM-1) under IFNγ control. Furthermore, we determined IFNγ-induced adhesion of immune cells (PMN and peripheral blood mononuclear cells (PBMC)) under physiological flow conditions on activated BUVEC.

Materials and methods

Isolation and maintenance of BUVEC

BUVEC were isolated from bovine umbilical cords as previously described (Taubert et al. 2006a). Cells were plated in 25 cm^2 plastic tissue culture flasks (Nunc, Wiesbaden, Germany) and incubated at 37°C under 5% CO_2. BUVEC were cultivated in endothelial cell growth medium (ECGM, supplemented with 0.1 ng/ml epidermal growth factor, 1 ng/ml basic fibroblast growth factor, 1 µg/ml hydrocortisone, 0.4% endothelial cell growth supplement/heparin and 2% foetal calf serum; all from PromoCell, Heidelberg, Germany) one day after isolation and thereafter every 2–3 days. Cells of passages 2–3 were used in the experiments. The BUVEC were characterized by typical cobblestone morphology and by incorporation of Dil-Ac-LDL (dioctadecyltetramethyl-indocarbocyanine perchlorate acetylated low-density lipoprotein) (Knook et al. 1977; Neubauer et al. 1996).

Recombinant cytokines

Recombinant bovine IFNγ was kindly provided by R. Steiger (Novartis Pharma, Basel, Switzerland). This bioactive stimulant had been produced in a baculovirus system (Gentilomi et al. 2006) and had been checked for LPS contamination by the Limulus Amebocyte Lysate test (endotoxin level=1.6 ng/µg protein). Recombinant human TNFα was purchased from Serotec, Düsseldorf, Germany (endotoxin level <1 ng/µg, PHP051A). In preliminary investigations different concentrations of IFNγ and TNFα were tested in PMN adhesion assays. From the results of these experiments, 1000 U IFNγ/ml and 10 ng TNFα/ml were applied in the present stimulation assays.

Isolation, DNase I treatment and reverse transcription of total RNA from stimulated BUVEC

Stimulated and control BUVEC (n=3) were harvested by Accutase treatment (3 ml/25 cm^2 flask, 5–15 min, 37°C; PAA Laboratories, Pasching, Austria) and two consecutive washings in M199 medium (400 g, 10 min; Gibco, Invitrogen, Karlsruhe, Germany). Stimulants were applied in a volume of 500 µl, avoiding the removal of medium and any strong movement or changing of the horizontal position of the tissue culture flasks. For each time-point of the assays, a non-stimulated control supplemented with 500 µl medium was examined in parallel.

Total RNA was isolated from cell pellets and reverse-transcribed according to Taubert et al. (2006b).

Real-time PCR for the relative quantification of adhesion molecule (E-selectin, P-selectin, ICAM-1, VCAM-1), chemokine (CXCL1, CXCL8, CXCL10, CCL2, CCL5), GM-CSF, COX-2 and iNOS gene transcription

For real-time RT-PCR assays, BUVEC were stimulated with IFNγ (1000 U/ml) or TNFα (10 ng/ml) for 0, 3, 6, 12 and 24 h. Real-time RT-PCR systems were used as published (Leutenegger et al. 2000; Taubert et al. 2006a, b) and are depicted in Table 1. Probes (purchased from Eurogentec, Cologne, Germany) were labelled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). PCR amplification was performed on an automated fluorometer (ABI PRISM 5700 Sequence Detection System, Applied Biosystems, Darmstadt, Germany) using 96-well optical

Table 1 Sequences of bovine probes and primers used for real-time RT-PCR

Target	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′ FAM–3′ TAMRA
GAPDH/1[a]	GCGATACTC ACTCTTCTA CCTTCGA	TCGTACCAGG AAATGAGCTTGAC	CTGGCATTG CCCTCAAC GACCACTT
GAPDH/2[b]	GGCGTGAAC CACGAGAA GTATAA	CCCTCCACGA TGCCAAAGT	ATACCCTCA AGATTGTCA GCAATGCCTCCT
E-selectin[a]	ACTCCCTTG GCAGTTGGACTT	AGGCGTTTCAG AAGCCAGAA	TGCTGGAGTC TCCCTTGTGA CAATACCATC
P-selectin[a]	GCCACCTAG GAACATAC GGAGTT	GATTGGACGAG GTCACCAAGA	CTGCGTTTGAC CCAAGCCCTT AAGAGAC
VCAM-1[a]	TTGGATGGTGTT TGCAGTTTCT	AGTCAGTGAAA CAGAGTCACCAATCT	AGCTTCCCAAA TCGACATATTC CCAAGTG
ICAM-1[a]	CTCTGTCCAT GGGATTCTGACA	GTTTCATGTGA CCCTGTGGTGTAG	CAGGCCTAAAT GTGGTGCTCAC TCCTTCAT
CXCL1[c]	CGCCTGTGGTCA ACGAACT	CACCTTCACGC TCTGGATGTT	CCAGTGCCTGCA GACCTTGCAGG
CXCL8[b]	CACTGTGAAAAA TTCAGAAATCA TTGTTA	CTTCACCAAAT ACCTGCACAACCTTC	AATGGAAACG AGGTCTGCTT AAACCCCAAG
CXCL10[c]	AAGTCATTCCTG CAAGTCAATCCT	TTGATGGTCT TAGATTCTG GATTCAG	CCACGTGTCG AGATTATTGC CACAATGA
CCL2[c]	CGCTCAGCCAGA TGCAATTA	GCCTCTGCATG GAGATCTTCTT	CCCAAGTCGC CTGCTGCTAT ACATTCAA
CCL5[c]	CCCTGCTGCTTTG CCTATATCT	GCACTTGCTG CTGGTGTAGAAA	CCCGCACCCA CGTCCAGGAGT
GM-CSF[c]	AATGACACAGAA GTCGTCTCTGAAA	CAGGCCGTTC TTGTACAGCTT	AACCAACGTGC CTGCAGACTCGC
COX-2[c]	GCACAAATCTGAT GTTTGCATTC	AGCTGGTCCTC GTTCAAAATCT	TTGCCCAGCA CTTCACCCA TCAATT
iNOS[c]	GGCCCAGGAAAT GTTCGAA	ACAGTGATGGC CGACCTGAT	AGACACGTGCGTT ATGCCACCAACAA

[a] According to Taubert *et al.* (2006a), the GAPDH/1 system was used for the determination of all molecules in question except for CCL5.

[b] According to Leutenegger *et al.* (2000), the GAPDH/2 system was used only for the determination of CCL5.

[c] According to Taubert *et al.* (2006b).

plates. Each sample was analysed in duplicate. For PCR 5 µl cDNA (corresponding to 25 ng total RNA) was used in a 25 µl PCR reaction mixture containing 12.5 µl TaqMan Universal Master Mix (Applied Biosystems), 300 nmol/L of each primer (purchased from MWG Biotech) and 200 nmol/L probe. Amplification conditions were the same for all targets assayed: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 45 cycles at 95°C for 15 s and at 60°C for 60 s.

In this work we performed a relative quantification of the target mRNAs by applying the comparative C_T (cycle threshold) method ($\Delta\Delta C_T$ method) according to the manufacturer's instructions and as described elsewhere (Leutenegger et al. 2000). Each sample was normalized relative to its content of GAPDH (= endogenous control) mRNA. For each test sample of TNFα- or IFNγ-stimulated BUVEC, we examined in parallel a non-stimulated control performed under identical experimental conditions. Gene transcription data were then reported as relative transcription or *n*-fold differences relative to the respective non-stimulated controls of the respective time points. The comparative C_T method is reliable only if amplification efficiencies of target and endogenous control genes are approximately equal. To determine the linear range and amplification efficiencies of the GAPDH and target-molecule TaqMan systems and to obtain standard curves, six 4-fold dilution steps were amplified in triplicate from at least two BUVEC cDNA samples containing high amounts of the target mRNAs. The differences of the slopes between standard curves obtained from GAPDH versus the respective target molecules were calculated and found to be <0.1, as demanded for reliable relative quantification assays.

Isolation of bovine PMN and PBMC

Calves (n=3) were bled by jugular venepuncture and blood was collected in 50 ml plastic tubes (Nunc) containing 0.1 ml heparin (Sigma-Aldrich, Munich, Germany) as anticoagulant. For PMN isolation, heparinized blood was centrifuged at 400 *g* for 20 min on a discontinuous Percoll gradient (Amersham, GE Healthcare, Little Chalfont, United Kingdom). PMN were washed twice with RPMI 1640 medium (Gibco) to remove Percoll, resuspended (5×10^6 cells/ml) in RPMI 1640 medium (Gibco) containing 2% FCS (Gibco) and incubated at 37°C and 5% CO_2 for at least 30 min before use.

For PBMC isolation, 20 ml of heparinized blood was mixed with 17 ml of 0.9% NaCl and applied on top of 12 ml Ficoll-Paque (density=1.077 g/L, Biochrom, Berlin, Germany) in 50 ml centrifugation tubes (Nunc). After centrifugation (45 min, 400 *g*, 4°C) the lymphocyte layer was collected using a pipette and the cells were washed three times (10 min, 400 *g*, 4°C) in RPMI 1640 medium (Gibco).

Adhesion assays under physiological flow conditions

BUVEC were stimulated with 10 ng/ml TNFα or 1000 U/ml IFNγ for 0, 3, 6, 12, 16, 24, 48 and 72 h. Adhesion assays were performed as previously described (Hermosilla et al. 2006; Taubert et al. 2006a) by perfusing 0.5 ml of a PMN (5×10^6 cells/ml) or PBMC (1×10^7 cells/ml) suspension into the system at a flow rate that resulted in a constant wall shear stress of 1.0 dyne/cm^2 (syringe pump sp 100i; World Precision Instruments, Malmö, Sweden). Interactions between BUVEC and PMN or PBMC were visualized using a phase-contrast videomicroscope (microscope DMIRB, Leica; CCD Video Color Camera, Sony) and videotaped (S-VHS; Panasonic). Quantification of PMN or PBMC adhesion was performed microscopically, determining the number of adherent cells in 5 randomly selected visual fields after 5 min of perfusion. In each adhesion assay, PMN or PBMC of three different animals were tested on two different BUVEC isolates (n=6).

Immunohistological analyses

Following PBMC adhesion assays under physiological flow conditions, cell layers with adhering PBMC were fixed for 10 min in ice-cold acetone, rinsed three times in Tris-

buffered saline (TBS) and reacted with primary antibody diluted in PBS–1% BSA (anti-bovine CD4, 1:4 (MCA 834S, Serotec); anti-bovine CD8, 1:100 (MCA 837G, Serotec); anti-bovine WC1, 1:75 (MCA 838S, Serotec)) for 1 hour at 37° C in a humidity chamber. After three washings in TBS cell layers were incubated with AP-conjugated anti-mouse (1:50, Amersham Biosciences) secondary antibody (30 min, RT, humidity chamber) and thereafter rinsed three times in TBS. Reactions were developed in 0.05% diaminobenzidine solution for 5 min and cell layers counterstained in 10% Papanicolaou solution (4 min, room temperature). After rinsing in distilled water three times for 3 min, cell layers were mounted using Aquatex (Merck Darmstadt, Germany). T-cell subpopulations adhering to stimulated BUVEC layers were quantified microscopically by determining the number of immunostained versus non-immunostained adherent PBMC in 4 randomly selected visual fields (400× magnification).

Statistical analyses

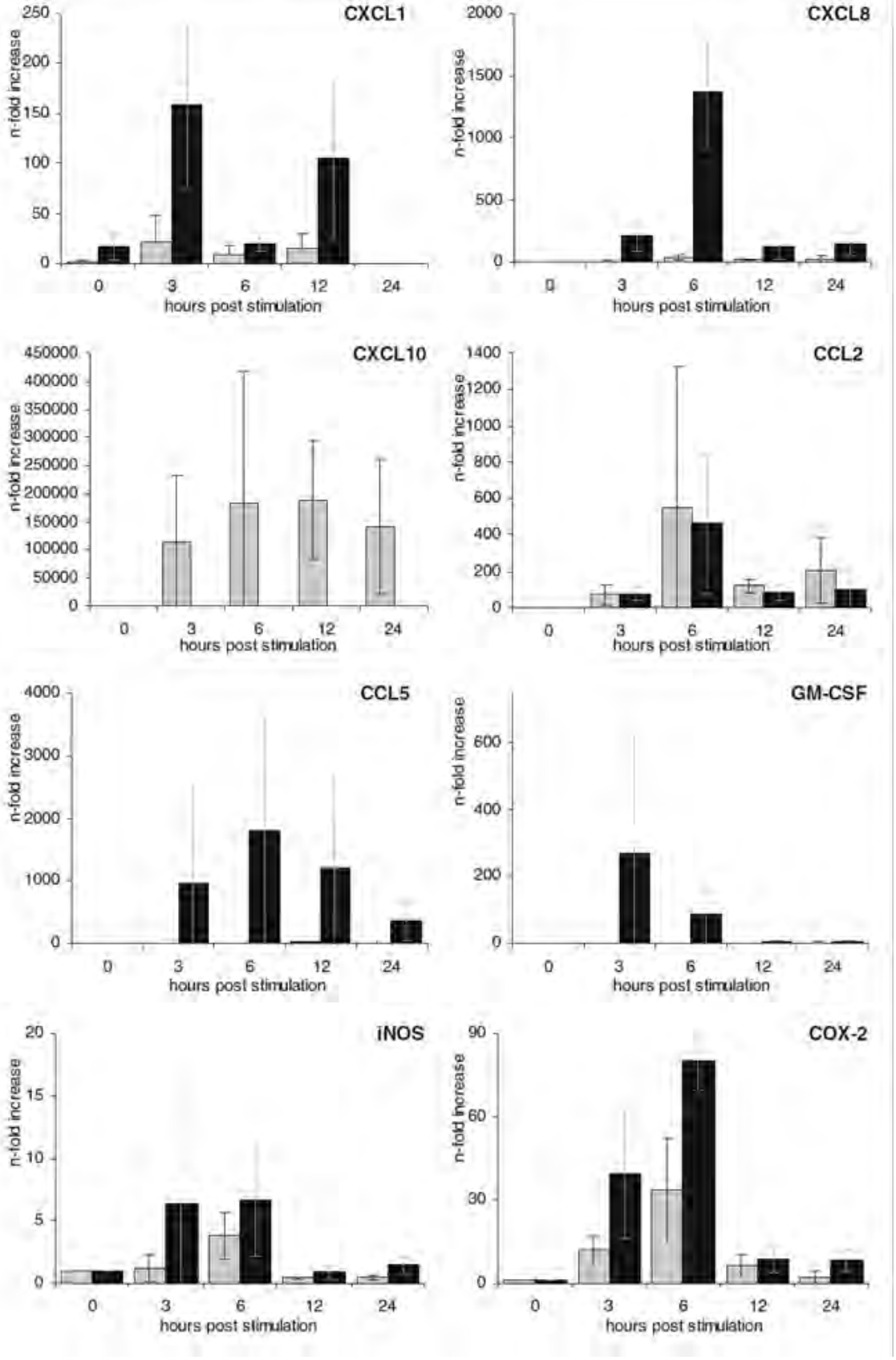
For statistical purposes data were logarithmically transformed. Analyses were performed by two-way ANOVA employing the statistical software package BMDP (Dixon 1993). Statistical differences were calculated between IFNγ and TNFα stimulation as dynamics of time; individual time-points were not analysed. Differences were considered significant at $p<0.05$.

Results

IFNγ enhances chemokine, iNOS and COX-2 gene transcription in stimulated BUVEC

IFNγ-induced activation of BUVEC was documented by clearly enhanced CXCL10, CCL2 and COX-2 gene transcription (Fig. 1). In contrast to TNFα ($p<0.001$), IFNγ efficiently stimulated CXCL10 gene transcription (Fig. 1), leading to high transcription rates from 3 h post stimulation (p. stim.) until the end of the observation period. Furthermore, IFNγ-induced CCL2 gene transcription showed comparable levels and kinetics to TNFα stimulation (Fig. 1). COX-2 gene transcription was also stimulated by IFNγ but to a lesser degree than by TNFα ($p<0.01$). Low levels of transcription were induced by IFNγ in the case of CXCL1, CXCL8, and CCL5 genes (Fig. 1), whereas TNFα promoted higher levels of gene transcription in these cases (ANOVA revealed significant differences for CXCL1 with $p<0.01$ and for CXCL8 with $p<0.001$). Stimulation with IFNγ failed to induce GM-CSF gene transcription, in contrast to TNFα ($p<0.01$) (Fig. 1). The transcription of the iNOS gene was generally affected only to a low level in BUVEC (Fig. 1) by both types of stimulation. In the case of all gene transcripts measured—except for TNFα induced CXCL-1 and GM-CSF resulting in maximum values 3 h p. stim.—maximum levels were found 6 h p. stim.; thereafter, reactions declined—except for CXCL10 with consistently high values until 24 h p. stim.—to low levels at 12–24 h p. stim.

Fig. 1 CXCL1, CXCL8, CXCL10, CCL2, CCL5, GM-CSF, iNOS and COX-2 gene transcription in BUVEC after stimulation with IFNγ or TNFα. BUVEC were stimulated with IFNγ (1000 U/ml, grey bars) or TNFα (10 ng/ml, black bars). Total RNA was isolated after 0, 3, 6, 12 and 24 h, reverse transcribed into cDNA, and probed with real-time RT-PCR systems for the detection of CXCL1, CXCL8, CXCL10, CCL2, CCL5, GM-CSF, iNOS and COX-2 mRNA equivalents. Arithmetic means of three different BUVEC isolates and standard deviations (vertical lines)


CXCL1
n-fold increase
250
200
150
100
50
0
0 3 6 12 24
hours post stimulation
CXCL8
n-fold increase
2000
1500
1000
500
0
0 3 6 12 24
hours post stimulation
CXCL10
n-fold increase
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0 3 6 12 24
hours post stimulation
CCL2
n-fold increase
1400
1200
1000
800
600
400
200
0
0 3 6 12 24
hours post stimulation
CCL5
n-fold increase
4000
3000
2000
1000
0
0 3 6 12 24
hours post stimulation
GM-CSF
n-fold increase
600
400
200
0
0 3 6 12 24
hours post stimulation
iNOS
n-fold increase
20
15
10
5
0
0 3 6 12 24
hours post stimulation
COX-2
n-fold increase
90
60
30
0
0 3 6 12 24
hours post stimulation

IFNγ upregulates adhesion molecule gene transcription in stimulated BUVEC

Stimulation with IFNγ clearly led to BUVEC activation accompanied by significantly ($p<0.05$–0.001) enhanced adhesion molecule gene transcription over time (Fig. 2). The E- and P-selectin gene transcripts were in principle induced by both stimulators ($p<0.05$) over time (Fig. 2). Stimulation with IFNγ only weakly influenced E- and P-selectin gene transcription, whereas TNFα-stimulation led to clearly enhanced levels peaking early at 3 h p. stim. followed by a decline until the end of the investigation period. In the case of VCAM-1 (Fig. 2), IFNγ-stimulation led to comparable levels and kinetics to TNFα-stimulation and consequently no significant differences were found between the stimulators. ICAM-1 gene transcription was also enhanced by both modes of stimulation.

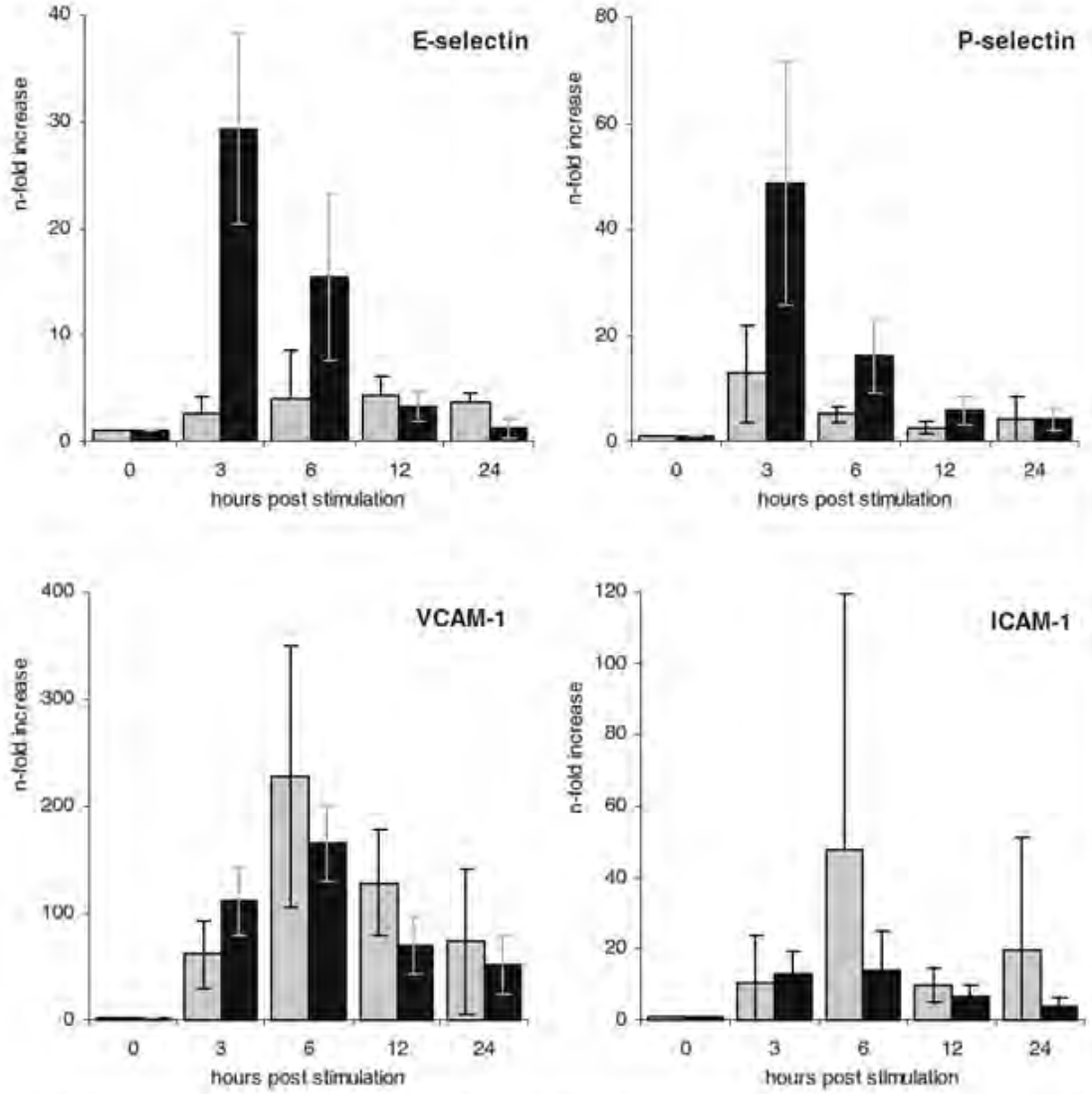


Fig. 2 E-selectin, P-selectin, VCAM-1 and ICAM-1 gene transcription in BUVEC after stimulation with IFNγ or TNFα. BUVEC were stimulated with IFNγ (1000 U/ml, grey bars) or TNFα (10 ng/ml, black bars). Total RNA was isolated after 0, 3, 6, 12 and 24 h, reverse transcribed into cDNA, and probed with real-time RT-PCR systems for the detection of E-selectin, P-selectin, VCAM-1 and ICAM-1 mRNA equivalents. Arithmetic means of three different BUVEC isolates and standard deviations (vertical lines)

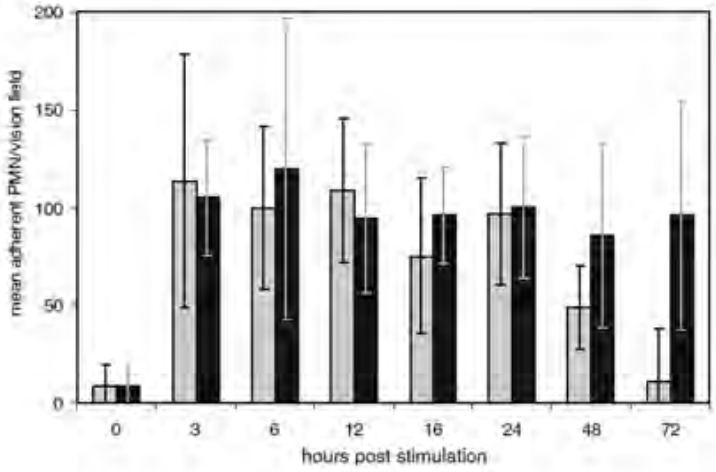


Fig. 3 PMN adhesion on BUVEC after stimulation with IFNγ or TNFα. Bovine PMN were tested for adhesion under physiological flow conditions on three different isolates of BUVEC stimulated with IFNγ (1000 U/ml, grey bars) or TNFα (10 ng/ml, black bars) for 0, 3, 6, 12, 16, 24, 48 and 72 h using the parallel-plate flow chamber. Arithmetic means of three different PMN donors tested on two different BUVEC isolates and standard deviations (vertical lines)

IFNγ induces PMN and PBMC adhesion on stimulated BUVEC

Stimulation of BUVEC with IFNγ and TNFα led to a significant upregulation of both PMN and PBMC adhesion (all $p<0.0001$) (Figs. 3 and 4) over time. PMN (Fig. 3) adhesion was immediately enhanced to high levels by 3 h p. stim., reaching a plateau phase and thereafter remaining at a high level until the end of the investigation period in the case of TNFα stimulation, while declining at 48 h p. stim. to the level of non-stimulated controls at 72 h p. stim. upon IFNγ-stimulation (IFNγ vs TNFα, $p<0.05$, measured as dynamics over time). PBMC adhesion was also induced within 3 h p. stim. by both stimulants (Fig. 4) and displayed maximum levels at 12 h p. stim. Thereafter, PBMC adhesion gradually declined upon IFNγ-stimulation in contrast to the stimulation with TNFα; however, these differences were not significant.

Involvement of T-cell subpopulations in IFNγ- and TNFα-mediated PBMC adhesion

Subtyping of PBMC adhering to stimulated BUVEC 12 h p. stim. revealed that all T-cell subsets tested adhered to BUVEC monolayers (Fig. 5b). Cells bound either as singly or as clusters (Fig. 5a). Clusters did not necessarily consist of the same T-cell subpopulation. $CD4^+$ and $CD8^+$ T cells seemed more frequently to adhere to IFNγ-stimulated BUVEC layers than to TNFα-treated ones (29.3% vs 18.1% and 41.6% vs 32.8%, respectively);

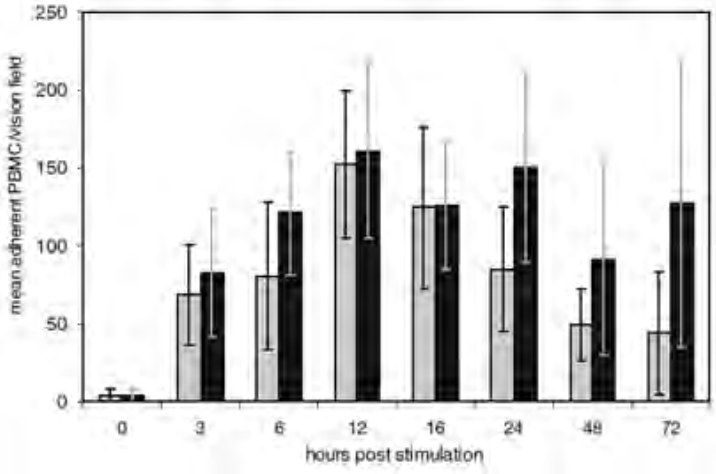
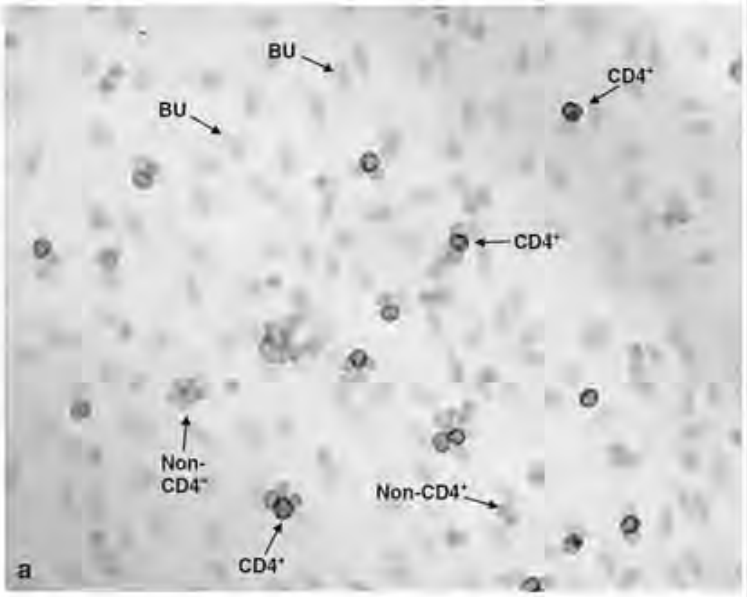


Fig. 4 PBMC adhesion on BUVEC after stimulation with IFNγ or TNFα. Bovine PBMC were tested for adhesion under physiological flow conditions on three different isolates of BUVEC stimulated with IFNγ (1000 U/ml, grey bars) or TNFα (10 ng/ml, black bars) for 0, 3, 6, 12, 16, 24, 48 and 72 h using the parallel-plate flow chamber. Arithmetic means of three different PBMC donors tested on two different BUVEC isolates and standard deviations (vertical lines)



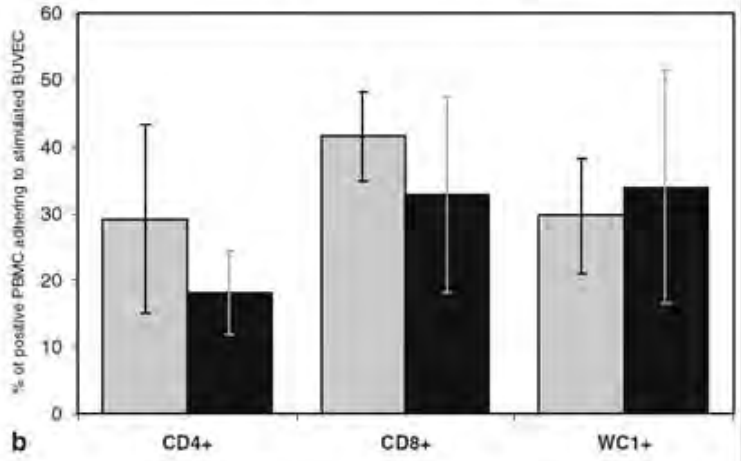


Fig. 5 Proportion of T cell subpopulations adhering to IFNγ- and TNFα-stimulated BUVEC. Bovine PBMC were tested for adhesion under physiological flow conditions to BUVEC stimulated with IFNγ (1000 U/ml; grey bars in (b)) or TNFα (10 ng/ml; black bars in (b)) for 12 h using the parallel-plate flow chamber. Thereafter, BUVEC and adherent PBMC were fixed and immunohistological assays were performed, probing with monoclonal antibodies directed against bovine CD4, CD8 and γδ-TCR (WC1). Arithmetic means of three different PBMC donors tested on two different BUVEC isolates and standard deviations (vertical lines).For illustration, $CD4^+$ T cells adhering to IFNγ-stimulated BUVEC are shown in (a). (BU=nucleus of BUVEC; $CD4^+$=$CD4^+$ T cells adhering to BUVEC; Non-$CD4^+$=PBMC adhering to BUVEC but unstained by anti-CD4 antibodies.)

however, these differences were not significant. γδ-TCR^+ T-cells adhered at comparable levels upon IFNγ- and TNFα-stimulation.

Discussion

The goal of this study was to obtain basic information on the activating capacity of recombinant bovine IFNγ on bovine endothelial cells. We therefore investigated different endothelial cell-derived activation indices in comparison to the well-known stimulator TNFα. At the level of differentially enhanced adhesion molecule, chemokine and COX-2 gene transcription, and increased PMN and PBMC adhesion we could clearly show that IFNγ is an important modulator of endothelial cell-derived immune responses in the bovine system.

We have demonstrated that IFNγ-stimulation of BUVEC led to the selective upregulation of important chemotactic mediators, such as, CXCL10 and CCL2, and should thereby be involved in immune cell recruitment to the site of inflammation in the bovine system. Besides promoting pro-inflammatory activities, IFNγ has also been demonstrated

to induce anti-inflammatory effects (Heremans et al. 1987; Granstein et al. 1989; Johansen et al. 1996), one of which is the inhibition of neutrophil recruitment. In agreement with these reports, stimulation with IFNγ, in contrast to TNFα stimulation, did not enhance GM-CSF gene transcription and, as described for human endothelial cells (Beck et al. 1999; Hillyer et al. 2003), only moderately affected mRNAs of CXCL1 and CXCL8, both mediators of PMN recruitment. In agreement with findings for human endothelial cells (Beck et al. 1999; Raju et al. 2003), IFNγ strongly induced the gene transcription of CXCL10 and CCL2, mediators that primarily attract lymphocytes or monocytes in the human system (Taub et al. 1993, 1995; Carr et al. 1994; Loetscher et al. 1994) but are inactive on neutrophils. In particular, CC chemokines interact with dendritic cells and are, therefore, as well as COX-2 which we also found upregulated after IFNγ stimulation, key molecules for the transition from innate to adaptive immune reactions (reviewed by: Zlotnik and Yoshie 2000; Locati et al. 2002). Thus, it seems clear that treatment of BUVEC with bovine IFNγ probably leads to a selective recruitment of lymphocytes and monocytes to the site of inflammation and promotes the transition from innate to adaptive immune responses.

At the level of adhesion molecule gene transcription we showed that recombinant bovine IFNγ (as described for human endothelial cells (Doukas and Pober 1990; Thornhill et al. 1991; Melrose et al. 1998)) preferentially enhances the expression of VCAM-1 and ICAM-1, i.e. those molecules that mediate the firm adhesion of immune cells to activated endothelial cells (for review see Ebnet and Vestweber 1999; Wagner and Roth 2000). In contrast, E- and P-selectin, both of which are inducible upon single cytokine treatment in the bovine system (Weller et al. 1992; Bischoff and Brasel 1995; Van Kampen and Mallard 2001a) and promote the tethering and rolling of immune cells, were affected only at a comparatively low level, which is in agreement to reports for human endothelial cells (Murakami et al. 2001; Raab et al. 2002).

IFNγ-induced PMN and PBMC adhesion was consistently observed in our experiments. Subtyping of PBMC revealed that $CD4^+$, $CD8^+$ and γδ-TCR^+ T-cells all contributed to these reactions. Thus, bovine IFNγ represents a source of endothelium-promoted, broad cellular immune reactions. All adhesion experiments were performed under conditions of flow and wall shear stress that correspond to the situation in blood capillaries and may, therefore, simulate *in vivo* conditions. Although, owing to the lack of efficient selectin genes induction, rolling of immune cells should not be promoted by IFNγ stimulation, we consistently observed this phenomenon in our experiments. This may be due to either the low but detectable induction of the E- and P-selectin gene transcription or the enhanced translocation to the cell surface of preformed P-selectin protein stored in Weibel Palade-like bodies (McEver et al. 1989). PMN and PBMC are differentially bound to activated endothelial cells; whereas PMN mainly adhere by interactions with ICAM-1, lymphocytes predominantly bind to VCAM-1 (for review see Ebnet and Vestweber 1999; Wagner and Roth 2000). As both molecules are clearly enhanced by IFNγ-stimulation, comparable kinetics of PMN and PBMC adhesion were detected. The adhesion of PBMC to BUVEC may, furthermore, have been indirectly enhanced by the IFNγ-induced production of CXCL10, a molecule reported to potentiate T-cell adhesion to endothelium (Taub et al. 1993). Whereas IFNγ-induced adhesion of PBMC has also been reported for human endothelial cells (Thornhill et al. 1991), IFNγ is additionally described as an inhibitor of recruitment and adhesion of PMN (Thornhill et al. 1991; Melrose et al. 1998) in the human system. As IFNγ-induced PMN adhesion was clearly detected in our experiments, this may represent a difference between the human and bovine systems.

In summary, our results indicate that IFNγ represents an important immunomodulator acting on bovine endothelial cells by selectively inducing adhesion molecule and chemokine gene transcription, which preferentially promotes lymphocyte recruitment.

Furthermore, this cytokine-induced PMN and PBMC adhesion points to an important role in mediating endothelial cell-induced immune cell trafficking in the bovine system.

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7.7 "*Eimeria bovis* infection enhances adhesion of peripheral blood mononuclear cells to and their transmigration through an infected bovine endothelial cell monolayer *in vitro*"

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Eigener Anteil an der Publikation

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

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ORIGINAL PAPER

Eimeria bovis infection enhances adhesion of peripheral blood mononuclear cells to and their transmigration through an infected bovine endothelial cell monolayer in vitro

Anja Taubert · Horst Zahner · Carlos Hermosilla

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Abstract The first schizogony of *Eimeria bovis* takes place in lymphatic endothelial cells of the ileum, resulting in the formation of macroschizonts within 2–3 weeks. In this study, we analyse early cellular immune responses to infected host cells on the basis of peripheral blood mononuclear cell (PBMC) adhesion on and transmigration through infected bovine umbilical vein endothelial cell (BUVEC) monolayers. Adhesion of PBMC was upregulated by an *E. bovis* infection. Most marked effects were observed 1 day p.i.; thereafter, PBMC adhesion declined reaching control levels from day 8 p.i. onward. $CD8^+$ T cells adhered more frequently to infected BUVEC (42%) than $CD4^+$ T cells (25%). About one third of attached PBMC were represented by $\gamma\delta$-TCR^+ T cells. Adhesion of T cells was not restricted to parasitised host cells, but occurred almost equally on non-infected BUVEC within the same monolayer. Furthermore, we found moderately enhanced levels of PBMC transmigration through infected BUVEC monolayers, in particular on day 2 p.i. The data presented here suggest that *E. bovis* infection of BUVEC induces endothelial cell-derived proinflammatory reactions, which appear suitable for the initiation of both adaptive and innate immune responses.

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Introduction

Eimeria bovis is an intracellular apicomplexan parasite of cattle causing clinical disease in calves and high economic losses worldwide (Fitzgerald 1980; Daugschies et al. 1998). Within its life cycle, *E. bovis* sporozoites invade endothelial cells of the central lymph capillaries of the villi of the ileum, where they develop within a period of 14–18 days to large first generation macroschizonts with up to 250 µm in size (Hammond et al. 1964). *Eimeria* infections, in general, are under immunological control and a variety of studies performed on cell-mediated adaptive immune responses emphasised the crucial role of lymphocytes in *Eimeria* infections in rodents (Rose and Hesketh 1982; Rose et al. 1988a, b, 1990, 1992a, b; Shi et al. 2001) and in *E. bovis*-infected calves (Speer et al. 1985; Hughes et al. 1988, 1989; Fiege et al. 1992; Hermosilla et al. 1999). There are, however, no data available on early interactions of lymphocytes with infected endothelial cells. These host cells represent a highly reactive cell type, and they are able to produce a broad range of adhesion molecules, cytokines and proinflammatory chemokines upon activation. Consequently, they participate in initiating leukocyte trafficking, e.g. by recruiting lymphocytes or polymorphonuclear neutrophils (PMN) to the site of inflammation and by promoting leukocyte transmigration to inflamed tissues (for reviews see Tedder et al. 1995; Ebnet and Vestweber 1999; Wagner and Roth 2000). These processes are part of the innate immune system, but via released mediators, they may also play an important role in initiating adaptive immune responses.

In some recent experiments we could demonstrate the interactions of PMN with coccidian (*E. bovis*, *Toxoplasma gondii*, *Neospora caninum*) infected bovine endothelial cells. In these studies (Hermosilla et al. 2006) we showed the dynamics of PMN adhesion to infected endothelial cell layers and of the parasite-mediated transcription of host cell genes encoding for key molecules involved in these reactions. There is, however, nothing known on the participation of lymphocytes in such early, major histocompatibility complex (MHC)-independent immune responses, although, in principle, such reaction could be assumed due to the temporary upregulation of adhesion molecules, such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), on endothelial cells by *E. bovis* or other coccidia (Hermosilla et al. 2006; Taubert et al. 2006a). Interactions of corresponding ligand molecules situated on lymphocyte surfaces, such as L-selectin or P-selectin glycoprotein ligand-1 (PSGL-1), with endothelial-derived selectins lead to tethering and rolling of lymphocytes, enabling them to survey the endothelial surface for chemotactic signals. When such signals are encountered, surface integrins of the lymphocytes, such as the $\alpha_1\beta_2$- (lymphocyte function-associated antigen [LFA-1]), $\alpha_4\beta_7$- or $\alpha_4\beta_7$-integrins (very late antigen-4 [VLA-4]), are activated and promote firm adhesion of lymphocytes (see e.g. von Andrian and Mackay 2000; Yang and Hagmann 2003) by interacting with counterpart molecules, such as VCAM-1 or ICAM-1, situated on the endothelial cell surface. This adhesive process represents one prerequisite of subsequent lymphocyte transmigration through an endothelial layer.

In fact, the process of both, adhesion and transmigration, is independent of MHC presentation but may critically be influenced by the actual status of the lymphocytes, which may vary in the course of an infection. Thus, the synthesis of PSGL-1 is dependent on the interleukin-12 (IL-12)-induced expression of fucosyltransferase VII (Maly et al. 1996). LFA-1 and VLA-4 integrins are upregulated in effector and memory cells (Butcher and Picker 1996; von Andrian and Mackay 2000), and naïve CD45RA cells display lower cell adhesion capacity than mature CD45RO memory cells (Radeke et al. 2005).

To characterise early interactions between *E. bovis*-infected endothelial cells and lymphocytes we used an in vitro system with bovine umbilical vein endothelial cells (BUVEC) as host cells. Adhesion of peripheral blood mononuclear cells (PBMC) to infected BUVEC monolayers was determined under physiological flow conditions. To consider eventual changes of the lymphocyte activity in the course of an *E. bovis* infection, PBMC were withdrawn from calves simultaneously experimentally infected with the in vitro infection of BUVEC. In addition, we subtyped adherent T cells and determined PBMC transmigration through infected endothelial cell layers.

Materials and methods

Parasites

The *E. bovis* strain H used in the present study was maintained by passages in calves for oocyst production. Sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al. 2002). Free sporozoites were collected and suspended in complete endothelial cell growth medium (ECGM, PromoCell).

Host cells

BUVEC were isolated according to Jaffe et al. (1973). Umbilical veins were separated from umbilical cords of calves born by *sectio caesarea* and kept at 4°C in 0.9% Hank's balanced salt solution (*w/v*, pH 7.4, Gibco) supplemented with 1% penicillin (*v/v*, 500 U/ml) and streptomycin (*v/v*, 500 µg/ml, Sigma) until use. Under sterile conditions, one end of an umbilical cord vein was clamped shut and collagenase type II (0.025%, *w/v*, Worthington Biochemicals) diluted in Puck's saline A solution (Gibco) was infused into the lumen. After clamping the remaining open end of the umbilical vein, it was incubated at 37°C in 5% CO_2 atmosphere for 20 min. Thereafter, the umbilical vein was gently massaged, unclamped and the content was collected in 50-ml plastic tubes (Nunc) containing 1 ml foetal calf serum (Gibco) to inactivate the collagenase. The lumen of the vein was washed two times with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco). The initial content and the washes were pooled, centrifuged (400×*g*, 10 min), resuspended in complete ECGM, plated in 25-cm^2 plastic tissue culture flasks (Nunc) and incubated at 37°C and 5% CO_2. BUVEC were fed with complete ECGM 1 day after isolation and, thereafter, every 2–3 days. They were used for infection after one to two passages in vitro.

Infection of PBMC donors and isolation of bovine PBMC

Three 8 weeks old Holstein-Frisian calves, which had been reared under parasite-free conditions, were orally infected with 5×10^4 sporulated *E. bovis* oocysts on the day of BUVEC infection (see above). They were bled by puncture of the jugular vein on days 1, 2, 4, 6, 8, 12 and 14 p.i., i.e. synchronously with the age of *E. bovis*-infected BUVEC employed in the adhesion assays (see below). Blood was collected in 50-ml plastic tubes (Nunc) containing 0.1 ml heparin (Sigma) as anticoagulant. 20 ml of heparinised blood were mixed with 17 ml of 0.9% NaCl and applied on the top of 12 ml Ficoll-Paque (density = 1.077 g/l, Biochrom) in 50-ml centrifugation tubes (Nunc). After centrifugation (45 min, 400×*g*, 4°C), the PBMC layer was

collected. The cells were pelleted (10 min, 400×*g*), and after lysing the contaminating erythrocytes (dilution of resuspended cell solution 1:10 in Aqua dest., 10 s shaking), PBMC were washed three times (10 min, 400×*g*, 4°C) in RPMI 1640 and counted in a Neubauer chamber. The viability of PBMC was estimated by the trypane blue (Sigma) exclusion test.

PBMC adhesion assays under flow conditions

Three different isolates of BUVEC were grown to confluence on Thermanox® previously coverslips (Nunc) coated with bovine fibronectin (10 ng/ml, 1 h, RT, Sigma) and cultured in 4-well plates (Nunc). Cells were infected with 2.5×10^5 freshly isolated *E. bovis* sporozoites/coverslip according to Hermosilla et al. (2006). Adhesion of PBMC, collected from the three experimentally infected calves (see above), to infected BUVEC monolayers was determined on days 1, 2, 4, 6, 8, 12 and 14 p.i. Stimulation of BUVEC with recombinant bovine interferon-γ (IFN-γ; 1,000 U/ml, 24 h, kindly donated by R. Steiger, Novartis Pharma, Basel) was used as positive control. Coverslips were placed in a parallel plate flow chamber that allowed a constant and uniform laminar flow field (Lawrence and Springer 1991). Prewarmed (37°C) RPMI 1640 medium was perfused over the BUVEC for 2 to 3 min to remove soluble factors and cell debris. Subsequently, a PBMC suspension (1×10^7 cells/ml) was perfused into the system at a flow rate that resulted in a constant wall shear stress of 1.0 dyn/cm^2 (syringe pump sp 100i; World Precision Instruments). Interactions between BUVEC and PBMC were visualised using a phase-contrast videomicroscope (microscope DMIRB, Leica; CCD Video Color Camera, Sony) and videotaped (S-VHS; Panasonic). PBMC adhesion was quantified microscopically by determining the number of adherent cells in five randomly selected vision fields after 5 min of perfusion. At each time point, PBMC of the three animals were tested on the three different BUVEC isolates employed in this study (i.e. $n=9$).

PBMC transmigration assays

BUVEC were grown to confluence on transwell cell culture inserts (6-well inserts, 3-µm pore size; Greiner) previously coated with sterile gelatine (0.1%, 30 min, RT) and infected with 4×10^4 freshly isolated *E. bovis* sporozoites/insert. On days 1, 2 and 5 p.i. PBMC (2×10^6 cells in 50 µl, added to each insert) isolated from the three experimentally infected calves (see above) on the corresponding dates p.i. were allowed to transmigrate (4 h, 37°C) through the infected BUVEC monolayers. Culture inserts were removed after incubation; transmigrated cells were collected from the bottom of the culture plates, concentrated by centrifugation (10 min, 400×*g*, resuspension in a defined volume) and counted in a Jessen chamber. Stimulation of BUVEC with human recombinant tumour necrosis factor-α (TNF-α; 10 ng/ml, 24 h; Serotec) was used as positive control. Each assay was performed in duplicates (i.e. $n=6$).

Immunohistological analyses

Subsequent to PBMC adhesion assays under physiological flow conditions performed on day 1 p.i., cell layers of the three BUVEC isolates tested with PBMC of the three experimentally infected calves were fixed in acetone (10 min, 4°C), rinsed three times in Tris-buffered saline (TBS) and reacted with primary antibodies diluted in phosphate-buffered saline/1% bovine serum albumin (anti-bovine CD4, 1:4, MCA 834S, Serotec; anti-bovine CD8, 1:100, MCA 837G, Serotec; anti-bovine WC1, 1:75, MCA 838S, Serotec) for 1 h at 37°C in a humide chamber. After three washings in TBS, cell layers were incubated in alkaline phosphatase-conjugated anti-mouse secondary antibody (30 min, RT, humide chamber) and rinsed three times in TBS. The reactions were visualised by incubation in 0.05% diaminobenzidine solution for 5 min. Cells were counterstained in 10% Papanicolaou's solution (4 min, RT). After rinsing in Aqua dest. three times for 3 min, cell layers were mounted using Aquatex® (Merck). T cell subpopulations adhering to infected BUVEC layers were quantified microscopically by determining the number of immunostained vs non-immunostained adherent PBMC in four randomly selected vision fields (×400 magnification).

To determine whether PBMC adhered merely to infected or also to non-infected BUVEC, three vision fields (×400 magnification) were digitally photographed, and T cells adhering either to infected or non-infected host cells were identified by scaling up the pictures using Adobe Photoshop (version 7.0). Single T cells or T cells forming part of cell clusters that were in contact with host cells containing one or more *E. bovis* sporozoites were considered as adhering to parasitised host cells.

All experiments included PBMC of three calves tested on three different BUVEC isolates, i.e. $n=9$.

Statistical analyses

For statistical purposes, the data of the PBMC adhesion and transmigration were logarithmically transformed. Analyses were performed by analysis of variance employing the statistical software package BMDP (Dixon 1993). In case of the PBMC adhesion and transmigration, statistical differences were calculated as dynamics in time; individual time points were not analysed. Differences were considered significant at $p<0.05$.

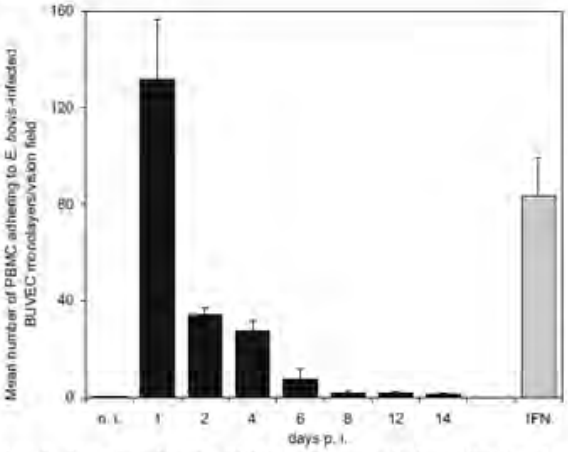

Fig. 1 Adhesion of bovine PBMC to *E. bovis*-infected BUVEC under flow conditions. BUVEC were grown to confluence and infected with *E. bovis* sporozoites (2.5×10^5/coverslip). Using a parallel plate flow chamber system, bovine PBMC isolated from simultaneously experimentally infected calves were tested for adhesion to infected BUVEC monolayers on 0, 1, 2, 4, 6, 8, 12 and 14 days p.i. Stimulation with bovine recombinant IFN-γ (1,000 U/ml, 24 h) served as positive control. Arithmetical means of flow assays probing three different BUVEC isolates with three different PBMC donors (n=9) and standard deviations (*vertical lines*)

Results

Rate of infection

BUVEC were rapidly invaded by *E. bovis* sporozoites. The majority of sporozoites was found intracellularly within 1 h p.i. In most cases, single infections of BUVEC were observed, but multiple infections of host cells were detected as well. The mean infection rates varied with 17.2±2.7%, 32±11.5% and 40.8±20.4%, depending on the batch of BUVEC used. Microscopically detectable replication of the parasites started 6 days p.i., and first mature macroschizonts of *E. bovis* were observed 14 days p.i.

E. bovis infection of BUVEC leads to increased PBMC adhesion

Infection of BUVEC with *E. bovis* sporozoites resulted in a profound, but transient cell activation as indicated by significantly (p<0.0001, determined as dynamics of time) enhanced PBMC adhesion on days 1–4 p.i. (Fig. 1) when compared to non-infected control BUVEC (= n.i.). Maximum numbers of adhering PBMC were found already on day 1 p.i. followed by a decline to almost control levels on day 8 p.i. Stimulation of non-infected BUVEC with IFN-γ, used as positive control, led to considerably enhanced PBMC adhesion, which was, however, lower than reactions to infected BUVEC on day 1 p.i.

E. bovis infection leads to enhanced transmigration of PBMC through infected BUVEC monolayers

Infection of BUVEC with *E. bovis* sporozoites resulted in enhanced transmigration of PBMC through infected monolayers (Fig. 2). Relative to non-infected controls, PBMC transmigration was increasingly enhanced on days 1 and 2 p.i. Within 5 days p.i., reactions had declined to control levels. However, these differences, determined as dynamics of time, were not significant. Stimulation of non-infected BUVEC with TNF-α, serving as positive control, led to strongly increased PBMC transmigration.

$CD4^+$, $CD8^+$ and γδ-TCR^+ T cells adhere to *E. bovis*-infected BUVEC monolayers

Subtyping of PBMC adhering to infected BUVEC on day 1 p.i. revealed that all T cell subsets tested adhered to infected BUVEC monolayers (Fig. 3). Cells bound either as single cells or as clusters. Clusters did not necessarily consist of the same subpopulation. $CD8^+$ T cells (42%) were found more frequently adhering to infected BUVEC layers than $CD4^+$ T cells (25%). Furthermore, γδ-TCR^+ T cells participated in adhesion, as 33% of the attached PBMC were positively stained with an anti-WC1 antibody. The overall differences in cell counts were not significant (p=0.089).

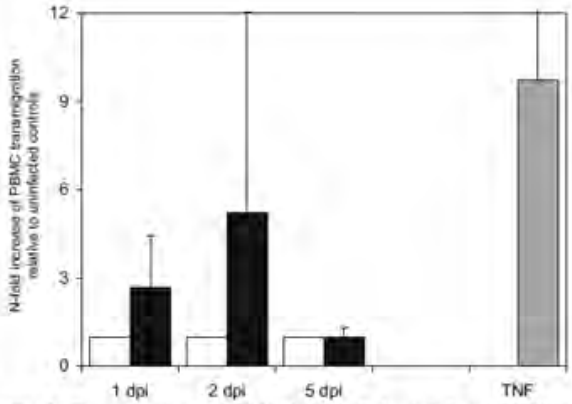

Fig. 2 Transmigration of PBMC through *E. bovis*-infected BUVEC monolayers. BUVEC were grown to confluence on transwell inserts (6-well size, 3 μm pores) and infected with 4×10^4 sporozoites of *E. bovis*. PBMC isolated from simultaneously experimentally infected calves were subjected into the transwells and allowed to transmigrate for 4 h through infected (*black bars*) and non-infected (*white bars*) BUVEC monolayers. Stimulation of BUVEC with human recombinant TNF-α (10 ng/ml, *grey bar*) was used as positive control. Arithmetical means of three different PBMC donors performed in duplicates (n=6) and standard deviations (*vertical lines*)

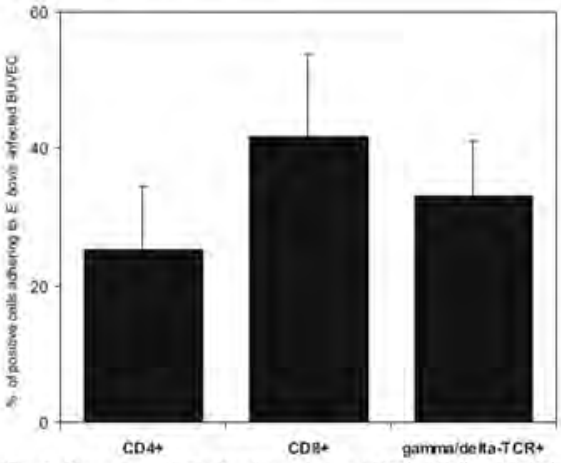

Fig. 3 Proportions of T cell subpopulations ($CD4^+$, $CD8^+$, $\gamma\delta$-TCR^+ T cells) adhering to *E. bovis*-infected BUVEC. Bovine PBMC isolated from simultaneously experimentally infected calves were tested for adhesion to *E. bovis*-infected BUVEC (2.5×10^5 sporozoites/coverslip) on day 1 p.i. under physiological flow conditions using a parallel plate flow chamber system. Coverslips with infected BUVEC monolayers and adherent PBMC were fixed and probed with monoclonal antibodies directed against CD4, CD8 and γδ-TCR (WC1) and alkaline phosphatase-conjugated secondary antibodies. Arithmetrical means of three different PBMC donors tested on three different BUVEC isolates ($n=9$) and standard deviations (*vertical lines*)

T cell subsets adhere almost equally to parasitised and non-parasitised host cells within an *E. bovis*-infected BUVEC monolayer

The infection dose used in our experiments led to an infection rate of 17.2–40.8%. To determine whether T cells adhered exclusively on infected cells, coverslips used in adhesion assays performed under flow conditions on day 1 p.i. were probed with antibodies directed against bovine CD4, CD8 and WC1. Overall, T cell adhesion was not restricted to parasitised host cells (Fig. 4a–c). We found all T cell subsets tested distributed almost equally to parasitised and non-parasitised host cells implying that non-infected host cells may have been activated by paracrine mechanisms.

Discussion

In this study we demonstrate early interactions between bovine PBMC and *E. bovis*-infected bovine endothelial cells on the level of T cell adhesion on and transmigration through host cell layers in vitro. We furthermore show that different T cell subsets are involved in these reactions.

The multistep cascade of lymphocyte extravasation represents one of the prerequisites for initiating inflammatory responses at the endothelial site and is promoted by various molecules on both, the migrating leukocyte and the activated endothelial cells (von Andrian and Mackay 2000). Different from antigen-specific lymphocyte reactions, these interactions are not MHC restricted. Corresponding studies are therefore possible using leukocytes and endothelial cells derived from allogeneic animals, as it was performed in the present experiments. As, however, the expression of receptor/ligand molecules on the PBMC surface may vary with the activation and differentiation status (von Andrian and Mackay 2000; Radeke et al. 2005), we used PBMC derived from animals which had synchronously been infected with *E. bovis* as the host cells used in our study.

PBMC adhesion to infected BUVEC monolayers was enhanced very early after infection resulting in maximum adhesion rates already on day 1 p.i. Even earlier effects cannot be excluded due to our experimental design. Thereafter, PBMC adhesion declined gradually to control levels until day 8 p.i. This early maximum reaction suggests sporozoite invasion as the major triggering event. Compared with the adhesion pattern of PMN to *E. bovis*-infected endothelial monolayers, where maximum levels were observed on day 2 p.i. (Hermosilla et al. 2006), it seems a very quick and short lasting response. It must, however, be considered that PMN or lymphocyte adhesion to endothelial cells is—at least in the human system—differentially mediated by distinct adhesion molecule usage (see Fabbri et al. 1999; Ebnet and Vestweber 1999; Wagner and Roth 2000; von Andrian and Mackay 2000).

The general upregulation of adhesion molecule gene transcription in endothelial cells by *E. bovis* and other coccidial infections was recently described by Hermosilla et al. (2006) and Taubert et al. (2006a). Strikingly, the developing meront failed to induce PBMC adhesion at all. The stimulatory activity seems to disappear with the onset of parasite replication 7–8 days p.i. (see Hermosilla et al. 1999). A likewise situation was reported in case of PMN adhesion to *E. bovis*-infected BUVEC (Hermosilla et al. 2006) and discussed by the authors as indication of an immune evasion strategy of the parasite. It could even be shown that *E. bovis* actively downregulates TNF-α-mediated PMN adhesion to infected BUVEC. Corresponding effects do not seem unlikely in the case of PBMC adhesion. In addition, the study shows that eventual infection-induced unspecific or specific activation of PBMC does not seem to influence the inefficiency of the cells to adhere to the infected endothelium with developing meronts, although they are highly reactive to specific antigens (Hermosilla et al. 1999).

By subtyping adhering PBMC, we could show that all T cell subsets tested were involved in adhesion to the infected endothelium early after infection probably triggering a complex immune response by the release of cytokines and chemokines by endothelial cells (Taubert et al. 2006b) or by

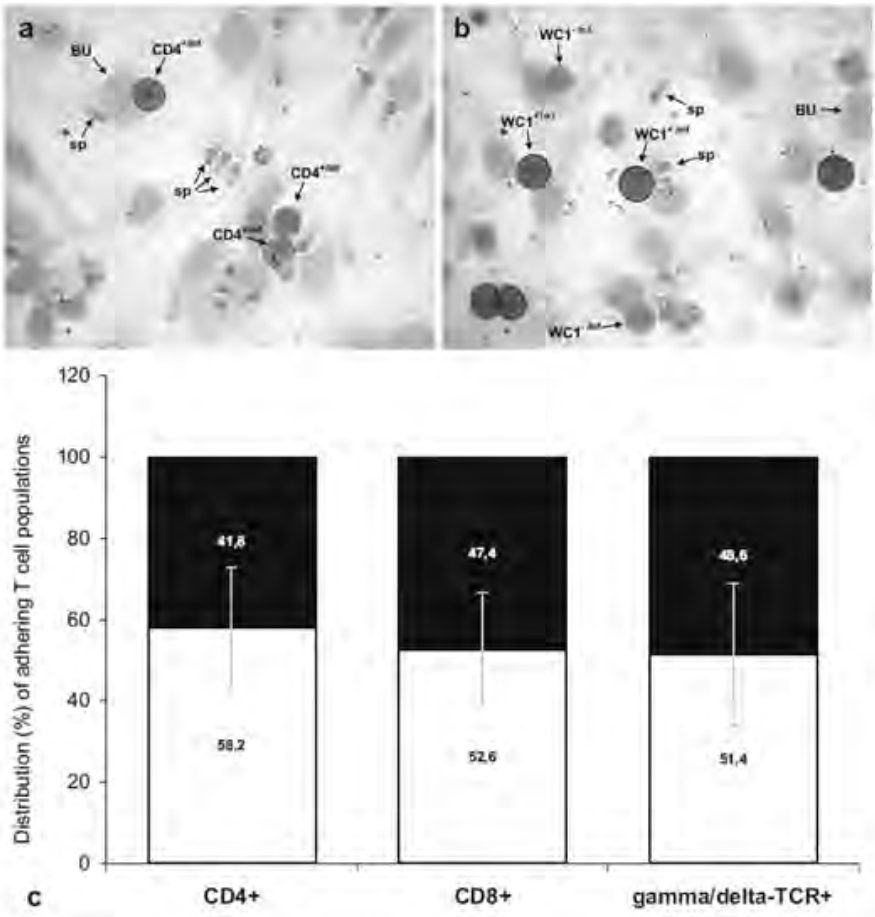

Fig. 4 $CD4^+$, $CD8^+$ and $WC1^+$ T cell subpopulations adhering to either *E. bovis*-parasitised or non-parasitised single host cells within one BUVEC monolayer. Following PBMC-adhesion assays and immunhistological tests detecting $CD4^+$ (a, c), $CD8^+$ (c) and $WC1^+$ (b, c) T cells, the proportions (%) of each subpopulation adhering either to *E. bovis*-parasitised (*black parts of the bars*) or non-parasitised (*grey parts of the bars*) single host cells (c) within one monolayer was estimated microscopically (×600 magnification). Arithmetical means of three different PBMC donors tested on three different BUVEC isolates ($n=9$) and standard deviations (*vertical lines*). For illustration, $CD4^+$ and $WC1^+$ T cells adhering to *E. bovis*-infected BUVEC are shown in (a) and (b; *BU* nucleus of BUVEC, *sp* intracellular *E. bovis*-sporozoite, $CD4^{+/inf}$ $CD4^+$ T cell adhering to an infected cell, $WC1^{+/inf}$ $WC1^+$ T cell adhering to an infected cell, $WC1^{+/n.i.}$ $WC1^+$ T cell adhering to a non-infected cell, $WC1^{-/inf}$ $WC1^-$ T cell adhering to an infected cell, $WC1^{-/n.i.}$ $WC1^-$ T cell adhering to a non-infected cell, ×1,000 magnification), respectively

PBMC themselves. There may be no extensive cytotoxic consequences mediated by adhered or transmigrated T cells for infected endothelial cells in vivo after a primary infection; however, they could play a critical role in *E. bovis* challenge infections. Studies performed so far in animals superinfected with *Eimeria* spp. show protective cytotoxic reaction predominantly affecting very early stages (see Shi et al. 2001). In this concern, it has to be considered that endothelial cell in principle, are able to display antigen-presenting function and to activate $CD4^+$ and $CD8^+$ T cells in an antigen-dependent manner (Pober and Cotran 1991; St Louis et al. 1993; Epperson and Pober 1994; Ma and Pober 1998; Rodig et al. 2003).

The study showed that adhesion of $CD4^+$, $CD8^+$ and γδ-TCR^+ T cells was not restricted to *E. bovis*-infected cells, but occurred almost equally on non-infected ones, too. Corresponding results were obtained when determining PMN adhesion to *E. bovis*, *T. gondii* and *N. caninum*-infected BUVEC (Hermosilla et al. 2006; Taubert et al. 2006a), although, in the case of *E. bovis*-infection, >90% of PMN were found attached to non-infected BUVEC. As endothelial cells generally react hypersensitive to mechanical alterations, the process of sporozoite gliding on the surface of potential host cells, which is mediated by a complex series of interactions between parasite- and host cell-derived molecules and results in the deposition of protein trails onto the underlying host cell surface (Bumstead and Tomley 2000), may have played a role in activating even non-infected endothelial cells. These effects may also have been brought about by a parasite-induced paracrine activation of non-infected bystander cells, as it has been shown for cytomegalovirus-infected human umbilical vein endothelial cells via IL-1β production (Dengler et al. 2000). In a recent work, we could demonstrate *E. bovis*-induced upregulation of the MCP-1 (endothelium-derived monocyte chemoattractant protein-1) and the RANTES (regulated upon activation, normal T cell expressed and secreted) gene transcription (Taubert et al. 2006b). Hypothetically, these chemokines may also play a role in paracrine activation processes.

In conclusion, our data suggest that *E. bovis* sporozoite infection of endothelial cells triggers the multistep machinery of lymphocyte adhesion on and transmigration through

the endothelium involving different types of T cell subpopulations. As all T cell subsets found to contribute in T cell adhesion in this study are, in principle, able to eliminate parasitised host cells by cytotoxic reactions, they may play an important role in the developing immune response, and, in consequence, the host cell itself may therefore indirectly influence the outcome of the infection.

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7.8 "Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites"

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Dynamics of transcription of immunomodulatory genes in endothelial cells infected with different coccidian parasites

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Abstract

Sporozoites of *Eimeria bovis* and tachyzoites of *Neospora caninum* and *Toxoplasma gondii* are able to invade and to replicate in endothelial cells. Here we report on responses of bovine umbilical vein endothelial cells (BUVEC) *in vitro* to these coccidial infections by determining mRNA levels of the CXC chemokines GRO-α, IL-8 and IP-10, the CC chemokines MCP-1 and RANTES and of GM-CSF, COX-2 and iNOS relative to the level of housekeeping gene (GAPDH) transcription. *T. gondii* and *N. caninum* tachyzoites caused profound transcriptional upregulation of all genes in question. In general, upregulation started 2–4 h p.i. and maximum transcript levels were observed 4 h p.i. GRO-α and IL-8 gene transcription had decreased to almost control levels by 12 h p.i.; in the case of the other chemokines enhanced transcript levels persisted longer or showed a biphasic time-course. A similar time-course to CC chemokines was observed for GM-CSF mRNA, whilst COX-2 gene transcript peaks were detected at 2–4 h p.i. and 48–72 h p.i. iNOS mRNA levels increased from 4 to 48 h p.i. In contrast, *E. bovis* sporozoites failed to induce the transcription of CXC chemokine genes and of COX-2, and only caused moderate transcription upregulation of the other genes considered. In conclusion, infections of BUVEC with these coccidian parasites result in host cell activation associated with enhanced transcription of genes encoding for proinflammatory and immunomodulatory molecules, which are important for innate immune reactions and the transition to adaptive immunity. Differences between *E. bovis* versus *T. gondii* and *N. caninum* may illustrate a particular evasion strategy of *E. bovis* sporozoites, which is related to their need to persist in the host cell for a long period of time and to the avoidance of inflammatory process-induction.

Keywords: *Eimeria bovis*; *Neospora caninum*; *Toxoplasma gondii*; Endothelial cells; Chemokines; GM-CSF; iNOS; COX-2

1. Introduction

Eimeria bovis, *Neospora caninum* and *Toxoplasma gondii* are obligate intracellular, apicomplexan parasites. They are important animal pathogens (Daugschies et al., 1998; Dubey et al., 1988, 2002), and in the case of *T. gondii* also of human importance (Tenter et al., 2000). These infections underly complex immunological regulations (Gazzinelli et al., 1993, 1994, 1996, 1998; Hermosilla et al., 1999; Rettigner et al., 2004; Innes et al., 2005; Moore et al., 2005), however, little is known on early innate immune responses to host cell invasion. Endothelial cells represent highly immunoreactive cells, which are able to synthesize a broad spectrum of immunoregulatory molecules, such as chemokines. Sporozoites of *E. bovis* and tachyzoites of *N. caninum* and *T. gondii* are all able to infect bovine endothelial cells and in these host cells development of *E. bovis* sporozoites into first schizonts lasts rather a long time whereas tachyzoites of the latter two species

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replicate rapidly. Chemokines are involved in the recruitment of immune cells by chemotaxis, in leukocyte activation as well as in the regulation of inflammatory processes by interaction with growth factors, cytokines and adhesion molecules (summarized by Locati et al., 2002). They are classified into four families according to the relative position of their cysteine residues (CXC, CC, C, CX_3C). All exert pleiotropic effects but generally CXC chemokines are predominantly attracting neutrophils and lymphocytes, whereas the CC family shows less activity on neutrophils whilst being effective on the majority of other leukocytes. The latter two families have predominantly an impact on lymphoid cells (summarized by Mantovani and Sozzani, 2000). In this concern data are exclusively available for *T. gondii* infections in several murine and human host cell types (Denney et al., 1999; Brenier-Pinchart et al., 2000; Blader et al., 2001) and, however, are varying to a certain extent, e.g., concerning the spectrum of chemokines synthesized differing results are reported even for the same host cell type such as human fibroblasts. Bovine cells have, so far, not been considered as host cells in this respect. The present study was therefore undertaken to investigate the chemokine response of bovine cells to the three above mentioned coccidian parasites. We selected bovine umbilical vein endothelial cells (BUVEC) as host cells because endothelial cells are essential for *E. bovis in vivo* and as well are suitable for the other species. In addition, they represent a highly reactive cell type. Due to the lack of specific antisera in the bovine system host cell responses were investigated at the level of gene transcription applying Realtime RT-PCR technology. In attempting to select genes from different classes of immunoregulatory molecules, we chose three chemokines of the CXC family [growth-related oncogene protein α (GRO-α), IL-8, 10 kDa interferon-inducible protein (IP-10)] and two from the CC family [monocyte chemotactic protein-1 (MCP-1), regulation upon activation normal T cell expressed and secreted (RANTES)]. In addition, we determined the transcription profiles of genes encoding for granulocyte-macrophage colony stimulating-factor (GM-CSF), cyclooxygenase 2 (COX-2) as a marker of prostaglandin production and iNOS as a marker of inducible nitric oxide synthesis.

2. Materials and methods

2.1. Parasites

The *E. bovis* strain H used in the present study was maintained by passages in Holstein Frisian calves. Sporozoites were excysted from sporulated oocysts as previously described (Hermosilla et al., 2002) and free sporozoites were collected and suspended at concentrations of 2.5×10^5/500 μl in complete endothelial cell growth medium (ECGM, PromoCell). *T. gondii* (RH strain; Sabin, 1941) and *N. caninum* tachyzoites (NC-1 strain; Dubey et al., 1988) were maintained in African green monkey kidney cells (VERO). Tachyzoites were washed off cell cultures and prepared as above.

2.2. Host cells

Bovine umbilical vein endothelial cells were isolated according to Jaffe et al. (1973). Umbilical veins were isolated from umbilical cords of calves born by *sectio caesarea* and kept at 4 °C in 0.9% HBSS-HEPES buffer (w/v, pH 7.4, Gibco) supplemented with 1% penicillin (v/v, 500 U/ml) and streptomycin (v/v, 500 μg/ml, Sigma) until use. Under sterile conditions one end of the umbilical cord veins was clamped shut and 0.025% collagenase type II (w/v, Worthington Biochemicals Corporation) in Puck's saline A solution (PSA, Gibco) was infused into the lumen. After clamping the remaining open end of the umbilical veins, they were incubated at 37 °C and 5% CO_2 atmosphere for 20 min. Thereafter umbilical veins were gently massaged, unclamped and the resulting collagenase solutions were each collected in 50 ml plastic tubes (Nunc) containing 1 ml FCS (Gibco) to inactivate collagenase. The umbilical veins lumens were washed two times with RPMI 1640 medium (Gibco). Washes were pooled, centrifuged (400 × *g*, 10 min), resuspended in complete ECGM, plated in 25 cm^2 plastic tissue culture flasks (Nunc) and incubated at 37 °C and 5% CO_2. BUVEC were fed with complete ECGM 1 day after isolation and, thereafter, every 2–3 days. They were used for infection after 1–2 passages *in vitro*.

2.3. Infection and harvesting of BUVEC

Confluent BUVEC monolayers in 25 cm^2 culture tissue flasks were infected with either 2.5×10^5 freshly isolated *E. bovis* sporozoites or *T. gondii* or *N. caninum* tachyzoites. Experiments were performed on three different BUVEC isolates. Infected BUVEC were harvested after 0.5, 1, 2, 4, 6, 24, 48 and 72 h p.i. by trypsin pretreatment (5 min, 37 °C; Sigma) followed by two washings in M199 medium (400 × *g*, 10 min; Gibco). In the case of *E. bovis* the investigation period was extended to 4, 6, 8, 10 and 14 days p.i. For each time-point non-infected BUVEC were examined in parallel as negative controls.

2.4. Isolation of total RNA and DNase I treatment

Total RNA was isolated from BUVEC pellets using the RNeasy-Kit (Qiagen) for isolation of total RNA according to the manufacturer's instructions. To minimize contamination with genomic DNA and to achieve reliable photometric measurements of the RNA, an on-column DNase I treatment (Qiagen) was applied during total RNA isolation following the manufacturer's instructions. The integrity of RNA was controlled by electrophoresis on a 1% (w/v) agarose gel. Since on-column DNase I treatment was not absolutely efficient, the extracted total RNA (1 μg) was additionally treated with RNase-free DNase I (1 U DNase I/μg RNA, 30 min, 37 °C; Hoffmann La Roche). DNase I was inactivated afterwards by heating (75 °C, 6 min). Total RNA probes were stored at −80 °C until further use.

2.5. Reverse transcription of total RNA

For cDNA synthesis M-MLV-reverse transcriptase (Gibco) was used. One μg DNase I-treated total RNA was mixed with 5 μl 5 × RT-buffer [250 mM Tris–HCl (pH 8.3, 375 mM KCl, 15 mM $MgCl_2$], 2 μl DTT (0.1 M), 2 μl hexanucleotides (62.5 A_{260}/ml; all Boehringer Mannheim), 1 μl dNTPs (10 mM, MBI Fermentas) and 1 μl M-MLV-reverse transcriptase (200 U/μl). The reaction was carried out in a final volume of 25 μl at 37 °C for 60 min. After addition of 175 μl TE buffer [10 mM Tris–HCl (pH 8), 1 mM EDTA] the sample was stored at −20 °C until further use.

2.6. Sequence analysis of a partial bovine IP-10 mRNA sequence

In order to set up an appropriate Realtime RT-PCR system for bovine IP-10, primers were selected using the ovine IP-10 (accession no. AB070717) mRNA sequence (forward primer CTGCCTTATCCTTCT-GACTCTG, reverse primer ATGCCTCTTTCTGTG-TTCGAG) and tested by PCR (amplification conditions: one cycle at 95 °C for 2 min, 30 cycles at 95 °C for 40 s, at 60 °C for 1 min and at 72 °C for 1 min, finally one cycle at 72 °C for 10 min) using cDNA from BUVEC stimulated with bovine recombinant IFN-γ (1000 U/ml; kindly donated by R. Steiger, Novartis, St. Aubin, CH) for 6 h. The resulting DNA fragment of

Table 1
Sequences of bovine probes and primers used for Realtime RT-PCR

Target	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′ FAM-3′ TAMRA	Length (nt)
GAPDH/1[a]	GCGATACTCACT CTTCTACCTTCGA	TCGTACCAGGAA ATGAGCTTGAC	CTGGCATTGCCCTCA ACGACCACTT	82
GAPDH/2[b]	GGCGTGAACCACG AGAAGTATAA	CCCTCCACGATG CCAAAGT	ATACCCTCAAGATTG TCAGCAATGCCTCCT	120
GRO-α	CGCCTGTGGTCA ACGAACT	CACCTTCACGCT CTGGATGTT	CCAGTGCCTGCAGAC CTTGCAGG	83
IL-8[c]	CACTGTGAAAAATTC AGAAATCATTGTTA	CTTCACCAAATAC CTGCACAACCTTC	AATGGAAACGAGGTC TGCTTAAACCCCAAG	113
IP-10	AAGTCATTCCTG CAAGTCAATCCT	TTGATGGTCTTAG ATTCTGGATTCAG	CCACGTGTCGAGATT ATTGCCACAATGA	103
MCP-1	CGCTCAGCCAGAT GCAATTA	GCCTCTGCATGG AGATCTTCTT	CCCAAGTCGCCTGCT GCTATACATTCAA	77
RANTES	CCCTGCTGCTTT GCCTATATCT	GCACTTGCTGCT GGTGTAGAAA	CCCGCACCCACGTCCA GGAGT	78
GM-CSF	AATGACACAGAA GTCGTCTCTGAAA	CAGGCCGTTCTT GTACAGCTT	AACCAACGTGCCTGC AGACTCGC	87
COX-2	GCACAAATCTGA TGTTTGCATTC	AGCTGGTCCTCG TTCAAAATCT	TTGCCCAGCACTTCA CCCATCAATT	80
iNOS	GGCCCAGGAAA TGTTCGAA	ACAGTGATGGCC GACCTGAT	AGACACGTGCGTTAT GCCACCAACAA	81

[a] The GAPDH/1 system was used for the determination of all molecules under question except for RANTES.
[b] According to Leutenegger et al. (2000), the GAPDH/2 system was only used for the determination of RANTES.
[c] According to Leutenegger et al. (2000).

approximately 280 bp was purified using E. Z. N. A.® Cycle pure kit (PeqLab) and sequenced.

2.7. Realtime RT-PCR for the relative quantification of chemokines (GRO-α, IL-8, IP-10, MCP-1, RANTES), GM-CSF, COX-2 and iNOS cDNA

The Realtime PCR system for IL-8 was used according to Leutenegger et al. (2000). All other primers and probes were designed using the Primer Express software (version 2.0; Applied Biosystems). As genomic DNA sequences were not available in all cases, probes were not designed as spanning the junctions of two exons. Primers (purchased from MWG Biotech) and probes (purchased from Eurogentec) are shown in Table 1. Probes were labelled at the 5′-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′-end with the quencher dye TAMRA (6-carboxytetramethyl-rhodamine). PCR amplification was performed on an automated fluorometer (ABI PRISM™ 5700 Sequence Detection System, Applied Biosystems) using 96-well optical plates. Each sample was analysed in duplicate. For PCR 5 μl cDNA (corresponding to 25 ng total RNA) were used in a 25 μl PCR reaction mixture containing 12.5 TaqMan® Universal Master Mix (Applied Biosystems), 300 nM of each primer and 200 nM probe. Amplification conditions were the same for all targets assayed: one cycle at 50 °C for 2 min, one cycle at 95 °C for 15 s and at 60 °C for 60 s.

Semiquantitative analyses used the comparative C_T method ($\Delta\Delta C_T$ method) according to the instructions of the manufacturer of the 5700 Sequence Detector and reported as *n*-fold differences in comparison to one of the samples arbitrarily chosen as the calibrator after normalizing the samples to the housekeeping gene GAPDH. The calibrator samples used in this experiment were non-infected controls with low amounts of the respective target gene transcripts. Quantification was done referring to the result of GAPDH.

To determine the linear range and amplification efficiencies of the GAPDH and chemokine cDNAs six 4-fold dilutions steps were amplified from two different cDNAs derived from TNF-α- (Serotec) or IFNγ-stimulated BUVEC in duplicate to obtain the standard curves. The differences of the slopes between standard curves obtained from GAPDH and the chemokines (which should be <0.1 for reliable quantification) were plotted against the logarithm of input total RNA and the regression line was calculated.

2.8. Statistical analysis

For statistical purposes data were logarithmically transformed. Analyses were performed by two-factorial ANOVA employing the statistical software package BMDP (Dixon, 1993). Statistical differences were calculated between all possible combinations of the three parasites as dynamics in time; individual time-points were not analysed. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of a partial bovine IP-10 mRNA sequence

The sequence of the PCR product comprised 268 bp and homology searches revealed 97% homology to *Capra hircus* IP-10 mRNA, 96% homology to *Ovis aries* IP-10 mRNA and 86% to *Homo sapiens* IP-10 mRNA. The sequence is available with the accession no. AM086628 in the EMBL Nucleotide Sequence Database.

3.2. Parasite development

BUVEC were rapidly invaded by the parasites. In the case of *T. gondii* and *N. caninum* mature replicative stages containing tachyzoites were observed 24 and 48 h p.i., respectively. From 72 h p.i. onwards most of the infected cells were found destroyed. *E. bovis* sporozoites did not develop further until 6 days p.i. Subsequently, parasite replication started and mature schizonts were found 14 days p.i. Infection rates varied and differed between the species. From the mean of infection, 25.1, 8.5 and 7.9% of BUVEC were infected with *E. bovis*, *T. gondii* and *N. caninum*, respectively.

3.3. Effects of E. bovis, T. gondii and N. caninum infections on chemokine, GM-CSF, COX-2 and iNOS gene transcription

Levels of gene transcription in *T. gondii*- and *N. caninum*-infected BUVEC were followed up to 72 h p.i., and after *E. bovis* infection, up to 14 days p.i. Since in the latter infection, however, most changes in transcript levels were limited to the first 72 h p.i., only data obtained up to this time-point were considered in the figures.

Effects on the transcription of CXC chemokine (GRO-α, IL-8, IP-10) genes varied with the parasite species but otherwise were, in principle, similar for all three genes (Fig. 1a–c). *E. bovis* hardly affected gene

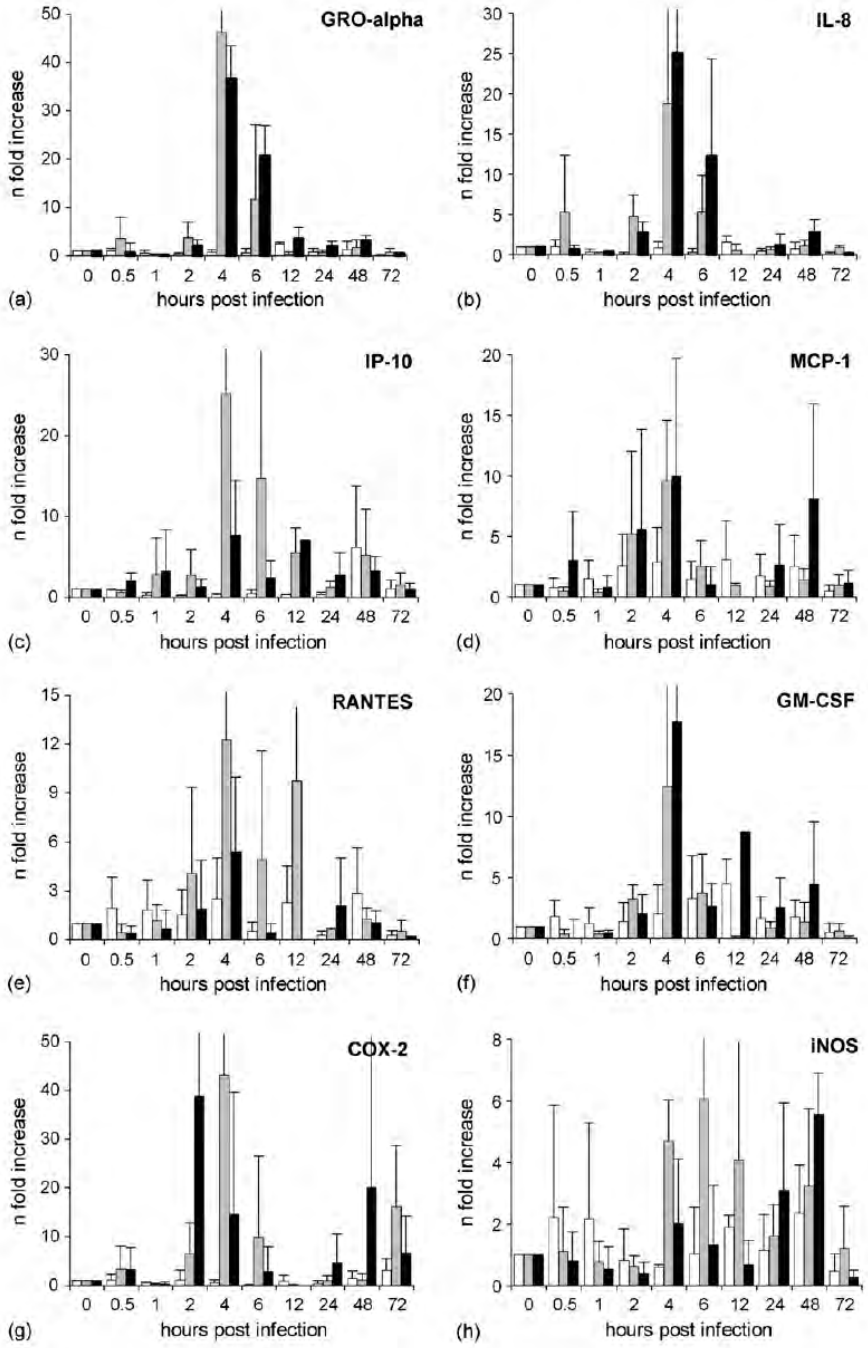

Fig. 1. Transcription of the: GRO-α (a), IL-8 (b), IP-10 (c), MCP-1 (d), RANTES (e), GM-CSF (f), COX-2 (g) and iNOS (h) genes in BUVEC during *Eimeria bovis* (white bars), *Neospora caninum* (grey bars) and *Toxoplasma gondii* (black bars) infections *in vitro*. BUVEC were grown to confluence and infected with 2.5×10^5 parasites. Non-infected controls were cultured in parallel for each time-point and results were expressed as *n*-fold increase in comparison to non-infected controls. Total RNA was isolated up to 72 h p.i. 1 μg total RNA was reversely transcribed into cDNA and probed with Realtime RT-PCR systems for the detection of the respective mRNA equivalents. Arithmetrical means of three different BUVEC isolates and standard deviations.

transcription. In contrast, *T. gondii* and *N. caninum* infections caused strong transcriptional upregulation of the three genes between 4 and 6 h p.i. [differences between time dynamics induced by *E. bovis* versus *T. gondii* and *E. bovis* versus *N. caninum* were significant ($P < 0.05$) for IL-8 and IP-10] resulting in maximum transcript levels 4 h p.i. Earlier effects 0.5–2 h p.i., if at all, were weak. Levels decreased until 6 h p.i. and in the case of GRO-α (Fig. 1a) and IL-8 (Fig. 1b) had reached control values by 12 h p.i. and expressed no further changes until the end of the observation period 72 h p.i. IP-10 (Fig. 1c) gene transcripts were found slightly increased until 48 h p.i.

In the case of the CC chemokines MCP-1 and RANTES, besides *T. gondii* and *N. caninum*, *E. bovis* also caused some upregulation of gene transcription. However, the time-course was more irregular in the latter infection (Fig. 1d–e). Compared with CXC chemokines, CC chemokine transcript levels appeared to have increased slightly earlier, i.e., already by 2 h p.i., but maximum transcript levels were as well found at 4 h p.i. Subsequently, mRNA amounts decreased but enhanced transcription levels persisted for a longer period of time than in case of GRO-α and IL-8.

Transcription of the GM-CSF gene was markedly enhanced to a distinct maximum at 4 h p.i. in *T. gondii* and *N. caninum* infections (Fig. 1f). Later on, until 48 h p.i., irregularly increased transcript levels were observed. In *E. bovis*-infected cells, GM-CSF gene transcription was moderately enhanced peaking at 12 h p.i.

Similar to CXC chemokines, *E. bovis* infection failed to enhance COX-2 gene transcription (Fig. 1g). *T. gondii* and *N. caninum* infections caused markedly increased values 2 and 4 h p.i. According to statistical analyses evaluating the time dynamics of *T. gondii*- and *N. caninum*-induced mRNA levels, COX-2 data differed significantly ($P < 0.05$). Altogether, time-courses appeared biphasic as transcript levels had dropped to almost zero by 12 and 48 h p.i. but were found enhanced again at 48 and 72 h p.i.

Transcriptions of the iNOS gene were affected by the different parasite infections, but differed with the species and were in general low (Fig. 1h). Again *E. bovis*-related effects were weaker than those induced by *T. gondii* and *N. caninum*. *E. bovis* sporozoites caused a temporary weak, but insignificant increase of transcripts at 0.5–1 h p.i. and 12–48 h p.i. In addition, we found moderately enhanced levels of iNOS gene transcripts 8–14 days after *E. bovis* infection (data not shown). The profiles of iNOS gene transcription differed in *N. caninum*- and *T. gondii*-infected BUVEC: in the case of *N. caninum*, it increased 4 h p.i. peaking at 6 h p.i. and declining thereafter to almost control levels by 72 h p.i., whereas transcriptional levels in *T. gondii*-infected cells did not peak until 48 h p.i.

4. Discussion

This study shows that *T. gondii* and *N. caninum* tachyzoites and sporozoites of *E. bovis* invade and in principle activate bovine endothelial cells as indicated by a profound upregulation of the transcription of a series of genes encoding for chemokines, GM-CSF and enzymes responsible for the synthesis of prostaglandins and NO.

Markedly enhanced transcription of the genes in question generally occurred between 2 and 6 h p.i. implying that the invasion of either *E. bovis* sporozoites, *N. caninum* or *T. gondii* tachyzoites represented the triggering event that led to BUVEC activation, as it has been described recently at the levels of adhesion molecule gene transcription in and adhesion of neutrophils to infected cells (Hermosilla et al., 2006; Taubert et al., 2006). However, it became clear that infections with different coccidian stages differentially affected endothelial cells as host cells. Thus, BUVEC generally reacted much more weakly to *E. bovis* sporozoites than to *T. gondii* and *N. caninum* tachyzoites, although, the infection rates were much lower in the latter cases. If at all, infection with *E. bovis* sporozoites induced the CC chemokine MCP-1 – a molecule that has recently been described by Rosenberg et al. (2005) to be induced in mouse dendritic cells by a 18 kDa protein conserved in *Eimeria* spp. – and GM-CSF but not the CXC chemokines. In this context, it seems interesting that only CC chemokine mRNA but not CXC chemokine mRNA could be demonstrated in jejunal and caecal extracts of *Eimeria tenella*- and *Eimeria maxima*-infected chickens prepared 7 days p.i. (Laurent et al., 2001).

The quantitative differences described here could stand for a distinct evasion strategy of *E. bovis* sporozoites, which are slowly developing stages needing 6–8 times longer than *T. gondii* and *N. caninum* tachyzoites to complete replication. This may correspond to findings in *T. gondii* infections, which showed that only the rapidly developing tachyzoites but not the slowly dividing bradyzoites upregulate MCP-1 synthesis in infected fibroblasts (Brenier-Pinchart et al., 2002). In fact, the sporozoites and tachyzoites, which we used were derived from different species and, therefore, differences should not be overestimated. However, the tachyzoites of the two different species provoked comparable reactions. In the

case of *T. gondii* this is in principle in agreement with earlier observations on chemokine gene transcription in *T. gondii*-infected HeLa cells and human fibroblasts (Denney et al., 1999; Brenier-Pinchart et al., 2000). Denney et al. (1999) reported on upregulated IL-8 mRNA synthesis in *T. gondii*-infected HeLa cells and human fibroblasts for 24 h p.i. Similar time-courses were observed by these authors concerning GRO-α whereas the transcription of the MCP-1 gene was little influenced. In contrast, Brenier-Pinchart et al. (2000, 2004) found upregulation predominantly of MCP-1 gene transcription in infected human fibroblasts, astrocytes and gliablastoma cells, which persisted at least until 24 h p.i., whereas transcripts of other chemokine genes, including RANTES, had already disappeared (Brenier-Pinchart et al., 2000). Results more similar to our data were obtained in microarray experiments by Blader et al. (2001) with infected human fibroblasts and by Knight et al. (2005) with rat retinal vascular endothelial cells. In both cases the transcription of a variety of CXC and CC chemokine genes was upregulated by 2 h p.i. and had ceased by 24 h p.i. Besides chemokines, genes encoding for GM-CSF, COX-2 and iNOS as well were found to be upregulated in BUVEC infected with *N. caninum* or *T. gondii* tachyzoites in this study, which corresponds, at least in the case of *T. gondii*, with the findings of others. In the case of GM-CSF, an overall effect of *T. gondii* on GM-CSF synthesis in host cells is known (Delemarre et al., 1995; Fischer et al., 1997; Nagineni et al., 2000) and it was also found upregulated in infected fibroblasts (Channon et al., 2002). COX-2 gene transcription was rapidly enhanced in BUVEC upon invasion by *T. gondii* and *N. caninum* tachyzoites. In the case of *T. gondii* infection this upregulation is in principle in agreement with other findings (Thardin et al., 1993; Yong et al., 1994; Channon and Kasper, 1996; Lüder et al., 1998). The gene transcription of iNOS was moderately upregulated in all infections, although, the effect was again comparatively low in the case of *E. bovis*. NO represents an important effector molecule against intracellular pathogens in phagocytes (see Ovington et al., 1995; Brunet, 2001) and may also act in endothelial cells. This, however, should not be overestimated. iNOS-deficient mice, for example, survive at least an acute *T. gondii* infection but die within 3–4 weeks p.i. (Scharton-Kersten et al., 1997).

Immunological consequences of the upregulated synthesis of inflammatory chemokines are obvious. Chemokines are essential factors in innate immune responses. CXC chemokines such as GRO-α and IL-8 act predominantly on neutrophils whereas CC chemokines such as RANTES and MCP-1 attract predominantly monocytes and T lymphocytes. CC chemokines, in particular, have an impact on dentritic cells and are, therefore, key molecules for the transition from innate to adaptive immunity (reviewed by Zlotnik and Yoshie, 2000; Locati et al., 2002). The importance of chemokines in coccidial infections *in vivo* was demonstrated by Khan et al. (2000), who showed that neutralization of IP-10 in *T. gondii*-infected mice resulted in a >1000-fold increase in parasite burden, increased host mortality and impaired antigen-specific T cell responses. Other important immunoregulatory molecules, such as prostaglandins, induce and enhance inflammatory reactions in response to infections, but also modulate immune processes and the transition from innate to adaptive responses (see Phipps et al., 1991; Zhang and Rivest, 2001). The key enzyme in prostaglandin synthesis is COX-2, which was also found to be upregulated after infection with *N. caninum* or *T. gondii* tachyzoites. As cell damage is generally associated with upregulated COX-2 expression as, e.g., shown in the brain (Nogawa et al., 1997; Li et al., 2005), a possible explanation for enhanced levels of COX-2 mRNA in infected BUVEC monolayers – besides immunomodulatory assumptions – could be increasing destruction of the cells due to the rupture of mature replicative stages within 72 h p.i.

In conclusion, infection of BUVEC with coccidian parasites resulted in host cell activation associated with enhanced transcription of genes encoding for proinflammatory and immunomodulatory molecules, which are important for innate immune reactions and the transition to adaptive immunity. Differences between host cell reactions induced by the sporozoite or tachyzoite stages – although they were not derived from the same parasite and may, therefore, not be directly comparable – may nevertheless point to a particular evasion strategy of *E. bovis* sporozoites, which is related to their need to persist in the host cell for a long period of time and to avoid inducing proinflammatory processes.

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7.9 "Microarray-based transcriptional profiling of *Eimeria bovis*-infected bovine endothelial host cells"

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Microarray-based transcriptional profiling of *Eimeria bovis*-infected bovine endothelial host cells

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Abstract – Within its life cycle *Eimeria bovis* undergoes a long lasting intracellular development into large macromeronts in endothelial cells. Since little is known about the molecular basis of *E. bovis*-triggered host cell regulation we applied a microarray-based approach to define transcript variation in bovine endothelial cells early after sporozoite invasion (4 h post inoculation (p.i.)), during trophozoite establishment (4 days p.i.), during early parasite proliferation (8 days p.i.) and towards macromeront maturation (14 days p.i.). *E. bovis* infection led to significant changes in the abundance of many host cell gene transcripts. As infection progressed, the number of regulated genes increased such that 12, 45, 175 and 1184 sequences were modulated at 4 h, 4, 8 and 14 days p.i., respectively. These genes significantly interfered with several host cell functions, networks and canonical pathways, especially those involved in cellular development, cell cycle, cell death, immune response and metabolism. The correlation between stage of infection and the number of regulated genes involved in different aspects of metabolism suggest parasite-derived exploitation of host cell nutrients. The modulation of genes involved in cell cycle arrest and host cell apoptosis corresponds to morphological in vitro findings and underline the importance of these aspects for parasite survival. Nevertheless, the increasing numbers of modulated transcripts associated with immune responses also demonstrate the defensive capacity of the endothelial host cell. Overall, this work reveals a panel of novel candidate genes involved in *E. bovis*-triggered host cell modulation, providing a valuable tool for future work on this topic.

Eimeria bovis **/ apicomplexan parasite / host cell interaction / transcriptomic / endothelial cell**

1. INTRODUCTION

Eimeriosis in cattle is an important enteric protozoan parasitosis which causes economic loss and severe clinical diseases in calves [8, 10]. As with several other pathogenic eimerian species that infect ruminants, the life cycle of *Eimeria bovis* includes the formation of macromeronts of up to 250 µm in size which develop in endothelial cells [13]. This lengthy process (14–18 days) is associated with the

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enlargement and reorganisation of the host cell (e.g. host cell cytoskeletal elements [16]). Parasite growth and proliferation within the parasitophorous vacuole (PV) requires nutrients from the host cell, as in other apicomplexa [7, 27]. Furthermore, given that endothelial cells generally represent a highly reactive cell type possessing a broad range of effector mechanisms capable of initiating pathogen elimination, *E. bovis* has to trigger a complex modulation of the host cell transcriptome and proteome to ensure its successful development. To date few details are known about the molecular mechanisms supporting long term survival of *E. bovis* or other macromeront forming *Eimeria* spp. within the host cell. Lang et al. [18] have recently shown that *E. bovis* prevents host cell apoptosis by up-regulating anti-apoptotic molecules. In avian *Eimeria* infections, modulation of epithelial host cell apoptosis was also achieved by expression of NFκB and the anti-apoptotic factor bcl-x_L [9]. Accordingly, up-regulation of NFκB was observed in sporozoite-infected, non-permissive epithelial host cells [2]. Comparative studies investigating the influence of different apicomplexa on the transcription of genes encoding for immunoregulatory molecules showed a relatively weak impact of *E. bovis* when compared to *Toxoplasma gondii* and *Neospora caninum* infections [30]. Interactions of *E. bovis*-infected endothelium with leukocytes were shown at the level of PBMC and PMN adhesion and seem to rely on infection-induced up-regulation of distinct adhesion molecules [15, 31].

Taken together, these few reports strongly suggest that *E. bovis* manipulates the host cell on a broad level involving different functional categories of host cell molecules. In order to gain a broad insight into *E. bovis*-induced host cell modulation with maximal coverage, we utilised a genome-wide approach using microarrays. We therefore analyzed *E. bovis*-induced gene regulation in in vitro infected endothelial host cells at distinct time points related to the development of the parasite, i.e., early after invasion by the sporozoite stage, at the trophozoite stage, at the beginning of parasite proliferation and when most mature meronts I were observed. Our microarray-based approach covered 23 000 known genes and uncharacterized expressed sequence tags. We show that with ongoing parasite development the number of regulated host cell gene transcripts increase and that modulated genes significantly interfere with various functions, networks and canonical pathways being mainly involved in cellular development, cell cycle, cell death, host cell immune response and metabolism of the host cell.

2. MATERIALS AND METHODS

2.1. Parasite

The *E. bovis* H strain used in the present study was maintained by passage in Holstein Friesian calves. Sporozoites were excysted from sporulated oocysts as previously described [14] and free sporozoites were collected and suspended at concentrations of 10^6/mL in complete endothelial cell growth medium (ECGM, PromoCell, Heidelberg, Germany).

2.2. Isolation, infection and harvesting of host cells

Bovine umbilical vein endothelial cells (BUVEC) used as host cells were isolated according to Taubert et al. [30]. Confluent BUVEC monolayers established in 75 cm^2 culture tissue flasks were infected with 10^6 *E. bovis* sporozoites. In order to account for individual variations and to have a rather robust setting, we worked with three different infected BUVEC isolates and respective non-infected BUVEC were analysed in parallel as negative controls. Infected BUVEC were harvested for RNA isolation 4 h, 4, 8 and 14 days post inoculation (p.i.) by direct lysis (1.2 mL lysis buffer/flask, RNeasy Mini Kit, Qiagen, Hilden, Germany).

2.3. RNA extraction

Total RNA was isolated from BUVEC using the RNeasy-Kit (Qiagen) for isolation of total RNA according to the manufacturer's instructions. To minimise contamination with genomic DNA and to achieve reliable photometric measurements of the RNA, an on-column DNase I treatment (Qiagen) was applied during total RNA isolation following the manufacturer's instructions. The integrity of RNA was controlled by electrophoresis on a 1%

(w/v) agarose gel. Since on-column DNase I treatment was not absolutely efficient, the extracted total RNA (1 µg) was additionally treated with RNase-free DNase I (0.5 µg DNase I/µg RNA, Fermentas, 15 min, room temperature). DNase I was inactivated afterwards by heating (65 °C, 10 min). Total RNA samples were then purified using the RNA cleanup protocol (RNeasy Mini Kit) and stored at −80 °C until further use.

2.4. Microarrays

BUVEC expression pattern at the respective harvest time points were assessed using Affymetrix GeneChip bovine Genome Arrays (Affymetrix, High Wycombe, UK) representing 24 016 probe sets or genes that cover 16 813 transcripts annotated to NCBI's database Entrez Gene. Preparation of antisense biotinylated RNA targets from 5 µg of total RNA was done using the GeneChip Expression 3′Amplification On-Cycle Target Labelling and Control Reagents (Affymetrix) that involve 1st and 2nd strand cDNA synthesis and simultaneous in-vitro transcription and biotin labeling. Microarray hybridisation, washing and subsequent scanning were performed using an Affymetrix hybridisation oven, fluidic station and scanner respectively. The quality of hybridisation was assessed in all samples according to the manufacturer's recommendations. Data were analysed with Affymetrix GCOS 1.1.1 software using global scaling to a target signal of 500. Data were then imported into Arrays Assist software (Stratagene, Madrid, Spain) for subsequent analysis. The data were processed with MAS5.0 to generate cell intensity files (present or absent)[1]. Quantitative expression levels of transcripts were estimated using PLIER[2] for normalisation. Paired *t*-tests were done between the samples of the two treatment groups within the respective sampling times with three biological replicates. Differences were considered significant at $p < 0.05$.

2.5. Identification of significantly over-represented host cellular functions, networks and canonical pathways

Individual, time-point-related lists of genes found to be significantly ($p \leq 0.05$) up- or down-regulated in *E. bovis*-infected BUVEC were submitted to Ingenuity Pathway Analyses (IPA; Ingenuity® Systems, Redwood City, CA, USA). Ingenuity maps gene IDs to its database and performs statistical computing to identify the most significant related functions, networks and canonical pathways over-represented in a given list when compared to the Ingenuity Pathways Knowledge Base which relies on millions of individually-modelled, peer reviewed pathway relationships. By default $p \leq 0.05$ was used in all calculations. IPA computes a score for each network according to the fit of the network to the set of genes in question. The score is derived from a *p*-value and indicates the likelihood of focussed genes in a network being found together because of random chance. Gene symbols were coloured according to the respective regulatory state (red indicating up- and green down-regulation).

3. RESULTS

3.1. *E. bovis* in vitro infection

In general, in vitro development of *E. bovis* corresponded to that described by Hermosilla et al. [14]. Infections of BUVEC with *E. bovis* sporozoites resulted in initial infection rates of 21.4 ± 5% (1 day p.i.). Infection controls conducted for up to 21 days p.i. revealed successful completion of meront I maturation and the production of merozoites I from days 17 p.i. onwards (Figs. 1E, 1F). On day 14 p.i. 7.1 ± 2.8% of BUVEC carried meronts I; intracellular sporozoites that had not undergone further development were detected in 6.9 ± 4.5% of the cells. However, some microscopic observations were noteworthy as they may directly be linked to the microarray results: immediately after invasion, intracellular sporozoites are generally situated close to the nucleus (Fig. 1A) which may indicate a direct influence on this organelle. Up to 4–7 days p.i., the nucleus of infected cells resembled that of non-infected cells showing a spotted content (Fig. 1B). From day 8 p.i. onwards, the morphology of the nucleus changed, resulting in a "fried-egg"-phenotype with increased proportions of light-coloured euchromatin and large coalescing/growing nucleoli (Figs. 1C–1F). This shape persisted until the release of merozoites I.

[1] Affimetrix GeneChip Expression Analysis. Technical Manual, 2001.

[2] Affimetrix. Technical Note. Guide to probe logarithmic intensity error (Plier) estimation, 2005.

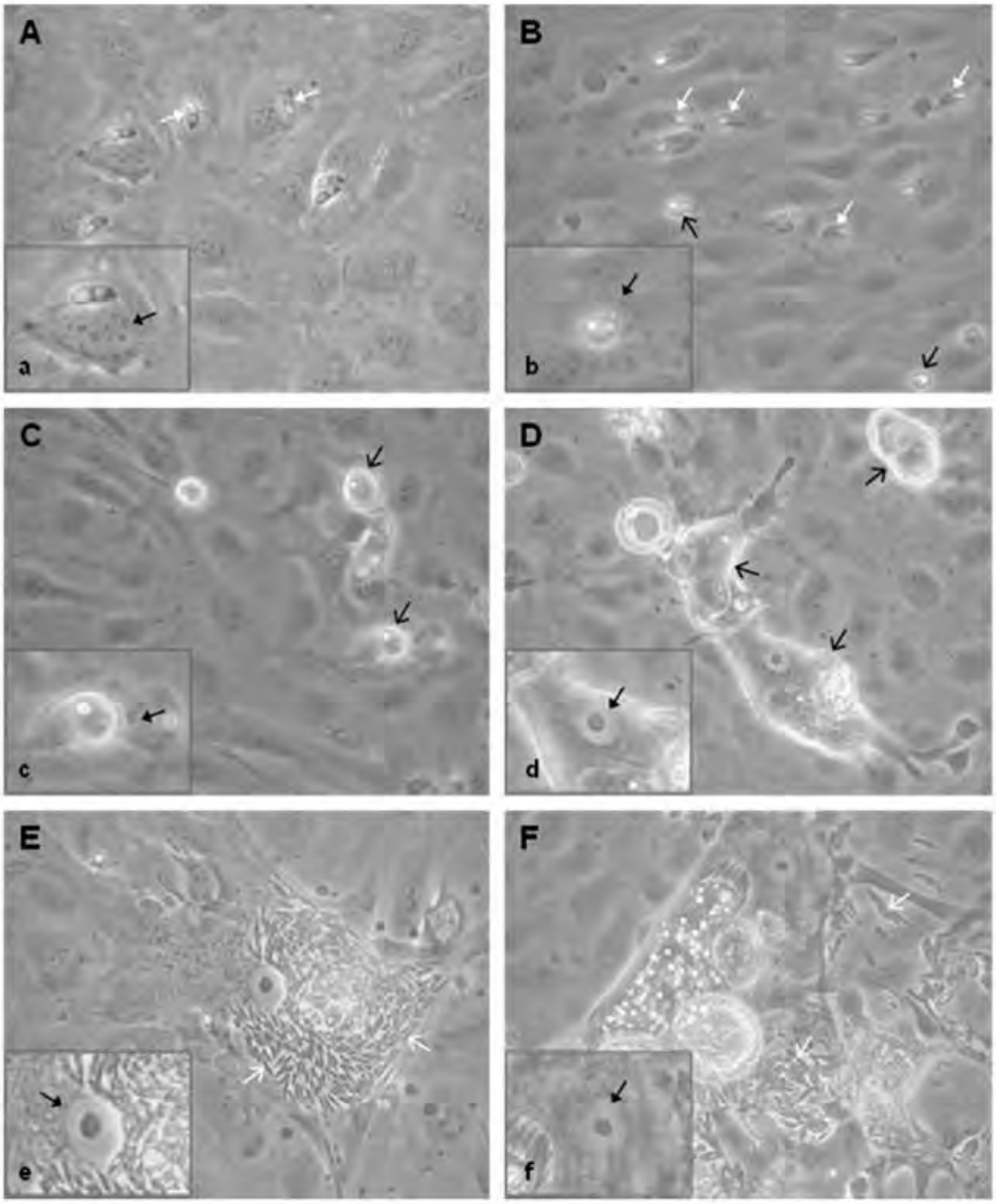

Figure 1. *E. bovis* in vitro culture used for microarray analyses. Bovine endothelial cells were infected with *E. bovis* sporozoites. Parasite development was followed microscopically, illustrated here at 4 h (A), 4 (B), 8 (C), 14 (D), 17 (E) and 20 (F) days after infection. Note the enormous enlargement of the host cells (D–F) and the change in nucleus morphology (a–f) in infected cells into a "fried egg"-type. White solid arrow = intracellular sporozoite; black opened arrow = trophozoite (4 days p.i.), immature meront I (8 and 14 days p.i.); white opened arrow = merozoite I; black solid arrow = nucleus of infected cell (all 630× magnification). (A color figure is available at www.vetres.org.)

3.2. *E. bovis*-induced overall changes in host cell gene transcription

In general, the number of regulated genes increased with ongoing development of the parasite. Microarray analyses revealed a total of 12, 45, 175 and 1184 significantly ≥ 1.5-fold parasite-modulated host cell gene transcripts at the sampled time points (selected data in Tab. I). The proportion of up- and down-regulated gene transcripts varied with time after infection. The ten most modulated gene transcripts were all up-regulated and detected at 14 days p.i.

The proportion of molecules commonly regulated in the course of infection was generally low early after infection but increased later on. FOS was the only gene transcript significantly up-regulated ≥ 1.5-fold at all four time points. Considering the last three time points, an overlapping gene subset of 17 molecules was influenced by *E. bovis* infection on days 4, 8 and 14 p.i. Comparing data generated on days 4 vs. 8 and 4 vs. 14 p.i., 7 additional molecules overlapped each. Additionally, 94 molecules overlapped when comparing regulated gene transcripts on days 8 vs. 14 p.i., with 63% of the molecules modulated at day 8 p.i. being equally influenced 6 days later. Within this set of common molecules, in 82%, the transcription was even enhanced 14 days p.i.

3.3. Functional analyses of gene sets regulated by *E. bovis*

3.3.1. Predicted functional effects

IPA revealed various host cell biofunctions to be significantly regulated by *E. bovis* infection, the majority of which dealt with general cell-related functions (Fig. 2), metabolism/biosynthesis (Fig. 3) and immune response (Fig. 4). In general, an increasing probability of involvement was associated with ongoing infection. It is noteworthy that regulation of the cell cycle was clearly enhanced in the late phase of infection on 8 and 14 days p.i. Genes associated with metabolism/biosynthesis-related functions included many involved in carbohydrate metabolism, molecular transport and small molecule biochemistry (Fig. 3). In addition, late *E. bovis* infection (14 days p.i.) significantly regulated molecules involved in lipid metabolism, nucleic acid metabolism and energy production. *E. bovis* infection also altered the host cell immune response. Whilst major reactions related to immune cell trafficking and inflammatory response were restricted to 8 days p.i., molecules involved in cell-mediated immune response and antigen presentation were significantly affected 4–14 days p.i. (Fig. 4). A more precise overview detailing the number of genes involved in host cell functions and respective *p*-values as well as identified molecules involved in different functions is given in the Supplementary Tables I and II, respectively, available on line only.

3.3.2. Predicted signaling pathways

The most significantly affected pathway was the aryl hydrocarbon receptor signalling pathway, which was significantly over-represented in *E. bovis*-infected cells at 4 h, 8 and 14 days p.i. (Tab. II). It is noteworthy that two signalling pathways referring to cell cycle regulation (G1/S checkpoint regulation, G2/M checkpoint DNA damage regulation, 8 and 14 days p.i.) and stress response (NRF2-mediated oxidative stress response, 14 days p.i.) were exclusively over-represented by regulated genes in the late phase of *E. bovis* infection.

3.3.3. Predicted gene networks

IPA constructed 1, 3, 9 and 25 interconnected gene networks that were significantly altered as a result of *E. bovis* infection 4 h, 4, 8 and 14 days after infection, respectively.

The most significant network over-represented by *E. bovis*-regulated genes at 4 days p.i. concerned cardiovascular system development/function, organ morphology and organismal functions (Supplementary Fig. 1, available online at www.vetres.org) focussing on the transcription regulators FOS and ANKRD1.

The most significant network at 8 days p.i. referred to cellular movement, immune cell trafficking and host cell inflammatory response (Fig. 5A). Out of 35 network-forming

Table I. Host cell gene transcription significantly modulated by *E. bovis* infection (selected).

Functional class	Symbol	Gene	Fold change in transcription*			
			4 h	4 d	8 d	14 d
Transcription/ Translation	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	2.45	1.69	1.97	1.82
	KLF4	Kruppel-like factor 4	–	–	2.21	2.14
	KLF5	Kruppel-like factor 5	–	–	1.51	2.60
	NR2F1	Nuclear receptor subfamily 2, gr F, member 1	–	–	−1.69	−2.55
	PRICKLE1	Prickle homolog 1	–	–	2.05	2.97
	FHL2	Four and a half LIM domains 2	–	–	1.98	3.88
	CAMTA1	Calmodulin binding transcription activator 1	–	–	–	−2.74
	CRYM	Crystallin, mu	–	–	–	4.12
	EIF4E	Eukaryotic translation initiation factor 4E	–	–	2.12	1.65
	MRPS6	Mitochondrial ribosomal protein S6	–	–	–	3.46
	PPARG	Peroxisome proliferator-activated receptor gamma	–	–	–	−2.77
Signal transduction	RND1	Rho family GTPase 1	–	–	2.28	2.34
	SOCS1	Suppressor of cytokine signaling	–	–	2.21	3.24
	DKK3	Dickkopf homolog 3	–	–	1.73	3.96
	STK38L	Serine/threonine kinase 38 like	–	–	1.69	3.05
	PPP1R1B	Protein phosphatase 1, regulatory subunit 1B	–	–	−2.14	−5.31
	PTGER4	prostaglandin E receptor 4	–	–	−1.76	−5.31
	RASD1	dexamethasone-induced ras-related protein 1	–	–	−1.61	−3.05
	FGL1	Fibrinogen-like 1	–	–	–	−4.49
	GPR116	G protein-coupled receptor 116	–	–	–	−2.65
	LGR4	Leucine-rich repeat-containing G protein-coupled receptor 4	–	–	–	−2.65
	ARHGEF11	Rho guanine nucleotide exchange factor 11	–		–	2.98
	APLP2	Amyloid beta (A4) precursor-like protein 2	–	–	–	2.60
	PTPN1	protein tyrosine phosphatase, non-receptor type 1	–	–	–	4.22
	RAB26	Ras-related oncogene protein	–	–	–	2.77
	RASGEF1B	RasGEF domain family, member 1B			–	−2.65
	RUVBL1	RuvB-like protein 1			–	2.56
	CHP	Calcineurin homologous protein	–	–	–	2.77
	SPARCL1	SPARC-like 1		–	–	−2.65
Nuclear protein	HIST2H2BE	Histone cluster 2, H2BE	–	2.13	2.13	2.82
	SNRNP25	Small nuclear ribonucleoprotein 25kDa		–	–	2.71
Metabolism						
Lipids	CYP51	Cytochrome P450, family 51	–	–	–	3.59
	CH25H	Cholesterol 25-hydroxylase			2.26	3.14
	OLR1	Oxidized low density lipoprotein receptor 1	–	–	2.14	2.72
	ELOVL5	Elongation of long chain fatty acids member 5	–	–	1.73	2.64
	ELOVL6	Elongation of long chain fatty acids member 6	–	–	–	2.85
	NAMPT	Nicotinamide phosphoribosyl-transferase	–	–	1.50	2.60
	ACOT7	Acyl-Coenzyme A thioesterase 7	–		1.57	4.46
	IDI1	Isopentenyl-diphosphate delta isomerase 1	–	–	–	7.74
	SCD	Stearoyl-Coenzyme A desaturase	–	–	–	5.26

continued on next page

Table 1. Continued.

Functional class	Symbol	Gene	Fold change in transcription*			
			4 h	4 d	8 d	14 d
	HMGCS1	Hydroxy-methylglutaryl-Coenzyme A synthase 1	–	–	–	3.51
	ACAT2	Acetyl-Coenzyme A acetyltransferase 2	–	–	–	2.99
	FASN	Fatty acid synthase	–	–	–	2.68
	FADS1	Fatty acid desaturase 1	–	–	–	3.42
	FDPS	Farnesyl diphosphate synthase	–	–	–	2.68
	FABP	Fatty acid binding protein 3	–	–	–	3.60
	PLD1	Phospholipase D1	–	–	–	−2.66
	NSDHL	NAD(P) dependent steroid dehydrogenase-like	–	–	–	2.69
	ACER3	Alkaline ceramidase 3	–	–	–	2.62
Carbohydrates	GALT	Galactose-1-phosphate uridylyltransferase	–	–	1.53	3.17
	STBD1	Starch binding domain 1	–	–	2.11	4.35
	CHPF	Chondroitin polymerizing factor	–	–	–	4.80
	SULF2	Sulfatase 2	–	–	–	3.20
	UGGT2	UDP-glucose glycoprotein glucosyltransferase 2	–	–	–	3.17
	EXT1	Exostoses (multiple) 1	–	–	–	2.51
	MTHFD1L	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	–	1.63	2.87	10.78
	MTHFD2	Methenyltetrahydrofolate cyclohydrolase	–	–	2.05	3.06
Proteins	CYP1A1	Cytochrom p450 1A1	6.35	–	–	–
	PSAT1	Phosphoserine aminotransferase 1	–	−1.52	1.93	2.96
	PSPH	Phosphoserine phosphatase	–	−1.50	1.69	3.33
	GOT1	Glutamic-oxaloacetic transaminase 1	–	–	–	3.79
	GPT2	Glutamic pyruvate transaminase 2	–	–	–	2.89
	WDYHV1	WDYHV motif containing 1	–	–	–	2.93
Nucleic acids	UPP1	Uridine phosphorylase 1	–	–	2.24	2.94
	CTPS	Cytidine 5′-triphosphate synthetase	–	–	–	3.63
	RRM2	Ribonucleotide reductase M2 polypeptide	–	–	1.83	6.70
Energy production	ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	–	–	–	3.19
	MDH1	Malate dehydrogenase 1	–	–	–	2.53
Other	KCNK1	Potassium channel, subfamily K, member 1	–	1.85	2.37	3.22
	TMEM98	Transmembrane protein 98	–	1.67	2.33	2.74
	SFXN1	Sideroflexin 1	–	–	–	3.78
Transport	SLC25A33	Solute carrier family 25, member 33	–	–	1.62	2.84
	SLC31A1	Solute carrier family 31, member 1	–	–	1.91	6.50
	SLC25A4	Solute carrier family 25, member 4	–	–	–	3.33
	SLC2A3	Solute carrier family 2, member 3	–	–	–	3.35
	TINAGL1	Tubulointerstitial nephritis antigen-like 1	–	–	–	3.51
	TFRC	Transferrin receptor	−1.54	–	–	2.51

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Table 1. Continued.

Functional class	Symbol	Gene	Fold change in transcription*			
			4 h	4 d	8 d	14 d
Cell cycle	CCND2	Cyclin D2	–	–	2.38	5.42
	CCNE1	Cyclin E1	–	–	2.05	4.76
	PMP22	Peripheral myelin protein 22			2.03	3.51
	S100A4	S100 calcium binding protein A4			2.05	2.34
	S100A2	S100 calcium binding protein A2	–	−1.53	–	2.55
	PTTG1	Pituitary tumor-transforming protein 1				2.51
Cell growth/ proliferation	TIMP2	Tissue inhibitor of metalloproteinase 2			2.31	7.96
	CYR61	Cysteine-rich, angiogenic inducer, 61	–	–	2.48	2.89
	EGR1	Early growth response 1	2.37	–	2.65	1.84
	FGF1	Fibroblast growth factor 1, acidic		2.13	2.25	2.77
	VEGFA	Vascular endothelial growth factor A			2.46	–
	VEGFC	Vascular endothelial growth factor C	–	–	2.07	22.23
	CSRP2	Cysteine and glycine-rich protein 2	–	–	1.72	11.25
	SERPINE2	Serpin peptidase Inhibitor, clade E member 2			–	2.86
	CD320	CD320 molecule	–	–	–	2.85
Cell structure	COL1A2	Collagen, type I, alpha 2	–	−2.57	–	−2.22
	FGD4	FYVE, RhoGEF and PH domain containing 4			−1.51	−3.13
	PALLD	Palladin, cytoskeletal associated protein			–	3.64
	MAP1B	Microtubule-associated protein 1B				3.23
	TUBB	Tubulin, beta			–	2.90
Cell adhesion	SELP	Selectin P	–	–	2.29	7.23
	VCAM1	Vascular cell adhesion molecule 1	–	–	2.18	–
	THBS	Thrombospondin 1	–	−1.67	2.05	1.52
	THBS2	Thrombospondin 2		−1.63	2.13	1.62
	LGALS1	Lectin, galactoside-binding, soluble, 1	–	–	1.71	11.61
	TPBG	Trophoblast glycoprotein	–	–	1.58	3.60
	CLDN16	Claudin 16			–	−2.70
	PARVB	Parvin, beta	–	–		2.71
	LAMA3	Laminin, alpha 3				−2.61
Stress response	USP2	Ubiquitin specific peptidase 2				−3.92
	SERPINH1	Serpin peptidase Inhibitor, clade H member 1	–	–	1.51	3.03
Apoptosis	DRAM	DNA-damage regulated autophagy modulator 1	–	–	−1.62	−3.20
	CYCS	Cytochrome c, somatic			–	2.90
	TIMP4	Tissue inhibitor of metalloproteinase 4	–	–	−1.65	−3.28

continued on next page

Table 1. Continued.

Functional class	Symbol	Gene	Fold change in transcription*			
			4 h	4 d	8 d	14 d
Immune response	IFI30	Interferon-gamma-inducible protein 30	–	–	1.66	24.16
	IL8	Interleukin-8	–	–	3.75	–
	CXCL5	Chemokine (C-X-C motif) ligand 5	–	–	3.63	–
	CXCL1	Chemokine (C-X-C motif) ligand 1	–	–	2.52	–
	CCL26	Chemokine (C-C motif) ligand 26	–	–	2.11	1.63
	CXCR4	Chemokine (C-X-C motif) receptor 4	–	–	–	−2.71
	SERPINE1	plasminogen activator inhibitor-1	–	−1.53	3.19	–
	PLAU	Plasminogen activator, urokinase	–	1.90	2.64	1.88
	PLAT	plasminogen activator, tissue	–	–	1.54	8.77
	C3	Complement component 3	–	–	2.54	2.60
	CFB	Complement factor B	–	–	2.78	2.50
	C1QBP	Complement component 1, q subcomponent binding protein	–	–	–	2.56
	CD200	CD200 molecule	–	–	–	6.51
	TAP1	Transporter 1, ATP-binding cassette, sub-family B	–	–	–	3.49
Unknown function	TM4SF18	Transmembrane 4 L six family member 1	–	–	–	3.28
	SYNGR2	Synaptogyrin 2	–	–	1.61	5.93
	DBNDD2	Dysbindin (dystrobrevin binding protein 1) domain containing 2	–	–	1.79	3.26
	FIBIN	Fin bud initiation factor homolog	–	–	1.61	−3.09
	GLIPR2	GLI pathogenesis-related 2	–	–	2.06	3.00
	B9D1	B9 protein domain 1	–	–	1.55	2.75
	GPM6B	Glycoprotein M6B	–	–	–	−3.17
	PALD	KIAA1274	–	–	–	−2.89
	HIGD1D	HIG hypoxia inducible domain family, member 1D	–	–	–	2.62
	SELM	Selenoprotein M	–	–	–	2.85

* Only genes with ≥ 2-fold (4 h, 4 and 8 d) and ≥ 2.5-fold (14 d) changes in at least one tested time point versus non-infected controls are considered.

molecules, 18 were modulated by *E. bovis* infection and all were up-regulated. Up-regulation especially concerned chemokines (IL-8, CXCL1, CXCL3, CXCL6) and adhesion molecules (VCAM1, ICAM1), but also focussed on the regulation of coagulation (PLAU, PLAUR, PLAT, SERPINE1).

The most significant network at 14 days p.i. concerned cellular organisation, gene expression and infection mechanisms (Fig. 5B). All network-forming genes were modulated by *E. bovis* infection resulting in a MYC-centered gene network with the majority of genes being up-regulated.

Another network significantly influenced by *E. bovis* infection referred to the regulation of cell cycle, cellular development, cellular growth and proliferation (Supplementary Fig. 2, available online at www.vetres.org). In this network a high number of transcription regulators (SOX18, KLF4, KLF5, ID3, MEF2C, PHB) were affected.

4. DISCUSSION

In this investigation we performed a transcriptional profiling of *E. bovis*-infected host

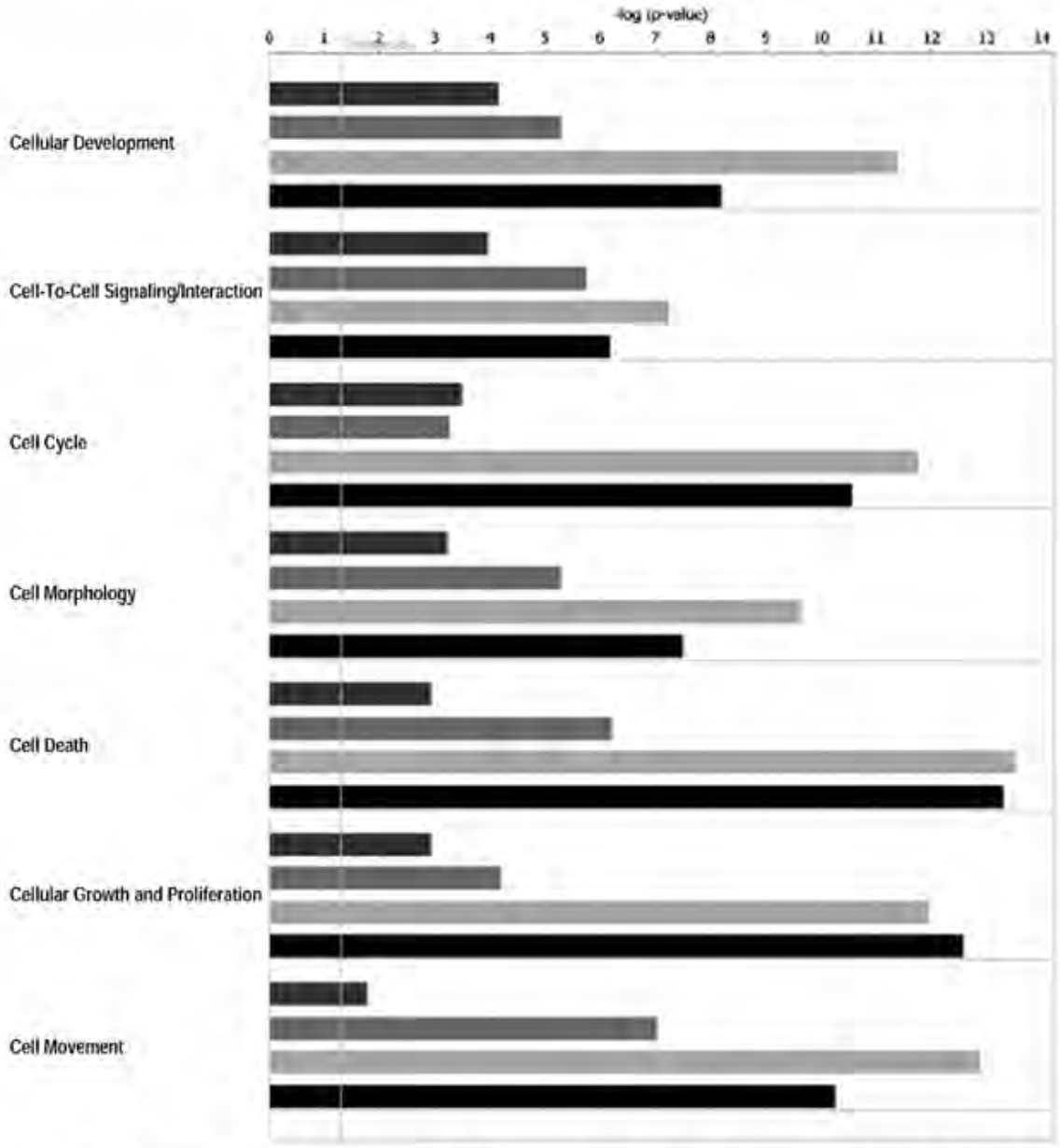


Figure 2. Biofunctions related to general cell characteristics significantly affected in endothelial host cells by *E. bovis* infection. A Fisher's exact test was used to calculate a *p*-value (bars) determining the probability that the association between the genes in the different datasets (4 h p.i. = dark blue bars, 4 days p.i. = medium blue bars, 8 days p.i. = bright blue bars, 14 days p.i. = black bars) and the respective biofunctions can be explained by random chance. The threshold (yellow line) refers to the cut off for $p \leq 0.05$. (A color figure is available at www.vetres.org.)

endothelial cells to obtain global insights into parasite-triggered host cell modulation. Considering the total number of regulated genes and the overlapping gene sets, it appears that in vitro development of meronts I can be divided into an early phase (4 h, 4 days p.i.) with poor regulatory processes on the transcriptional level and a more transcriptionally active late phase (8, 14 days p.i.) beginning with the onset of parasite proliferation.

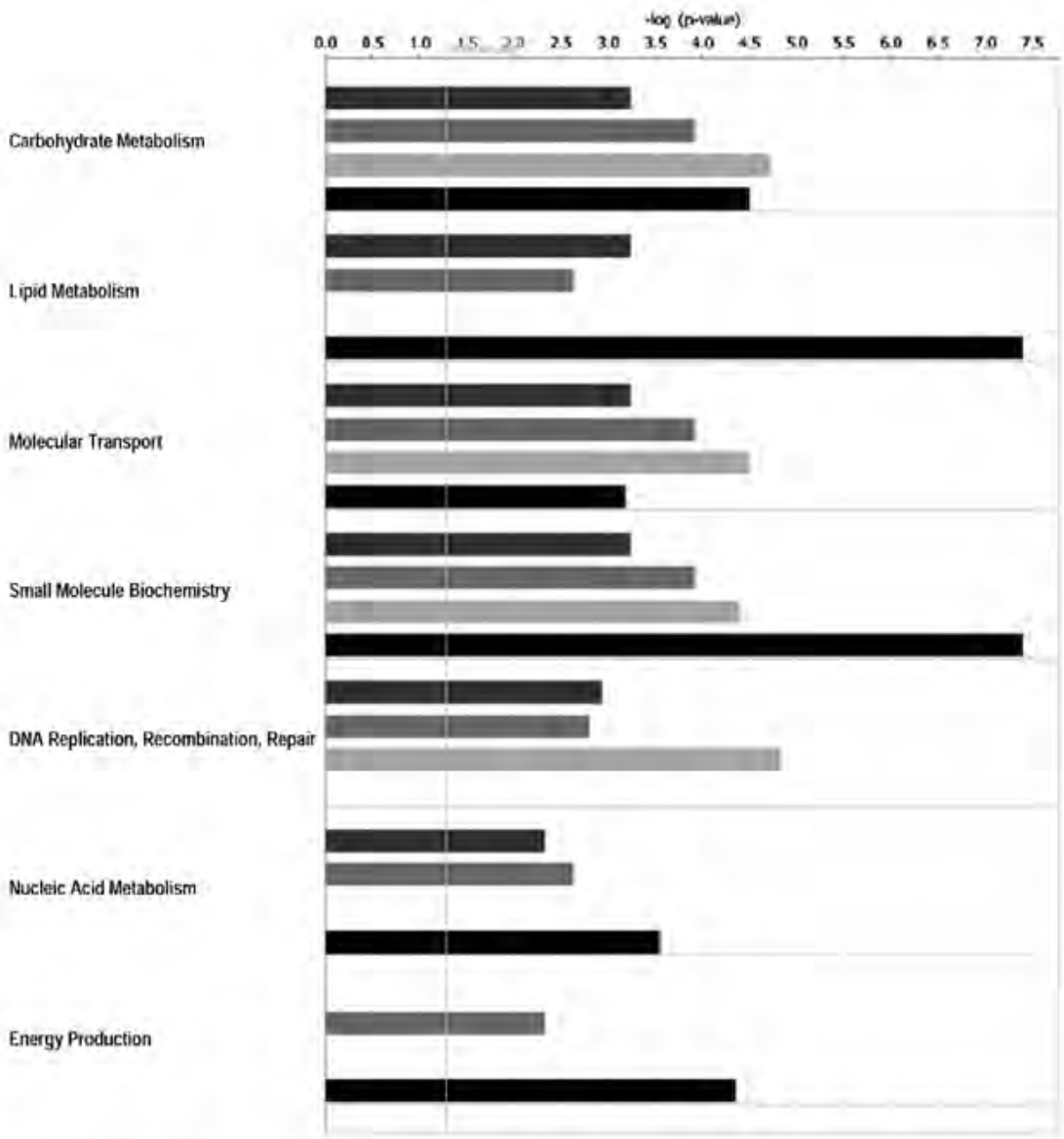


Figure 3. Metabolism-related biofunctions significantly regulated in endothelial host cells by *E. bovis* infection. A Fisher's exact test was used to calculate a *p*-value (bars) determining the probability that the association between the genes in the different datasets (4 h p.i. = dark blue bars, 4 days p.i. = medium blue bars, 8 days p.i. = bright blue bars, 14 days p.i. = black bars) and the respective biofunctions can be explained by random chance. The threshold (yellow line) refers to the cut off for $p \leq 0.05$. (A color figure is available at www.vetres.org.)

The in vitro culture used in this investigation corresponded well with the host cell type, the duration of meront I development and the resulting size of these stages to those reported in vivo [13]. The reduction of infection rates with progression of infection may result from the consistent observation that not all sporozoites having successfully invaded host cells undergo further development in vitro which agrees with ex vivo histological studies on

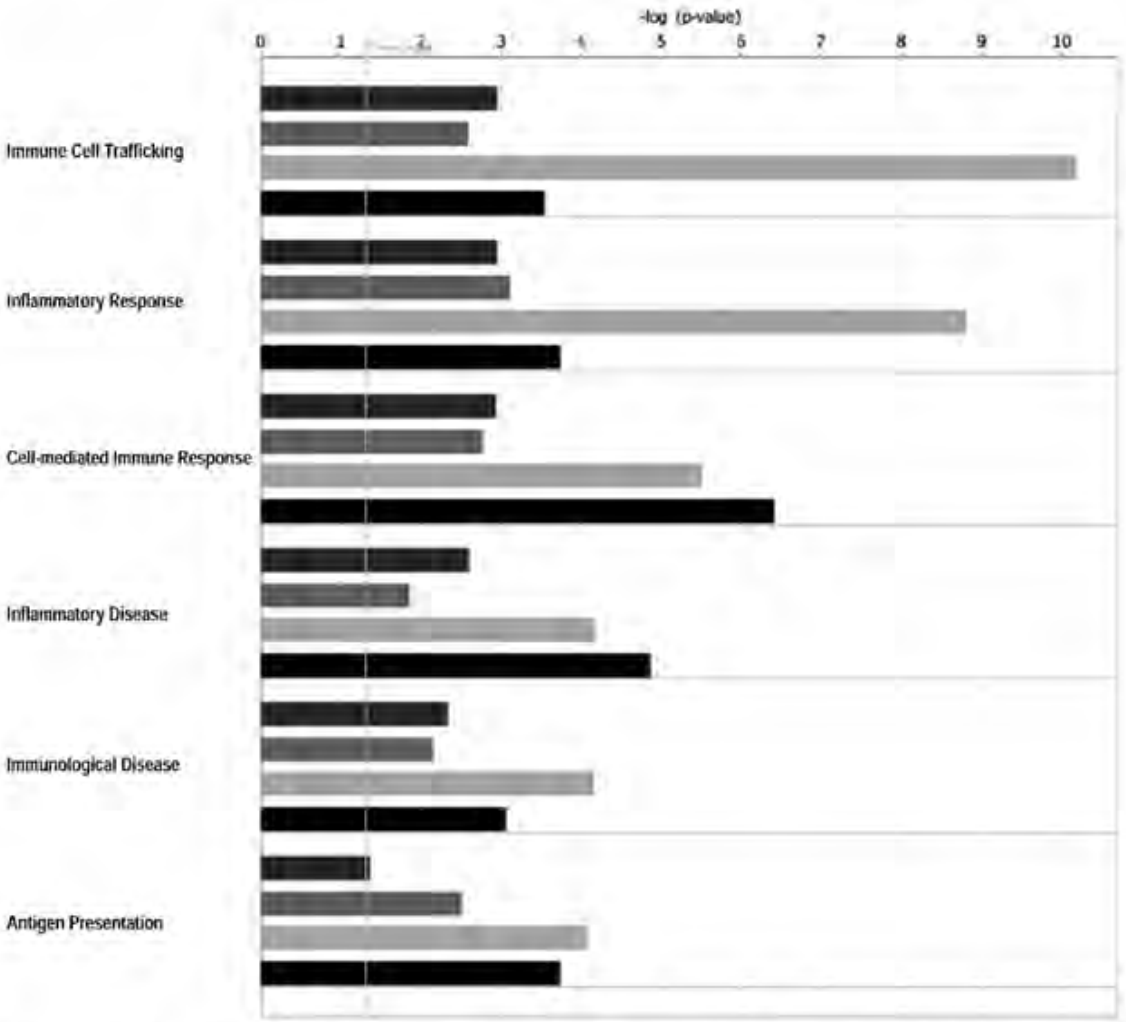


Figure 4. Biofunctions related to immune responses significantly affected in endothelial host cells by *E. bovis* infection. A Fisher's exact test was used to calculate a *p*-value (bars) determining the probability that the association between the genes in the different datasets (4 h p.i. = dark blue bars, 4 days p.i. = medium blue bars, 8 days p.i. = bright blue bars, 14 days p.i. = black bars) and the respective biofunctions can be explained by random chance. The threshold (yellow line) refers to the cut off for $p \leq 0.05$. (A color figure is available at www.vetres.org.)

E. bovis-infected ileum mucosa[3] and those reported for other *Eimeria* spp. infections [25]. Furthermore, as reported for other apicomplexan-infected host cells [29], some vital early meronts reproducibly disintegrate from the cell layer and are lost during the following cell feeding procedure. Taken together, these facts indicate that the in vitro system used in this investigation relates to the situation in vivo.

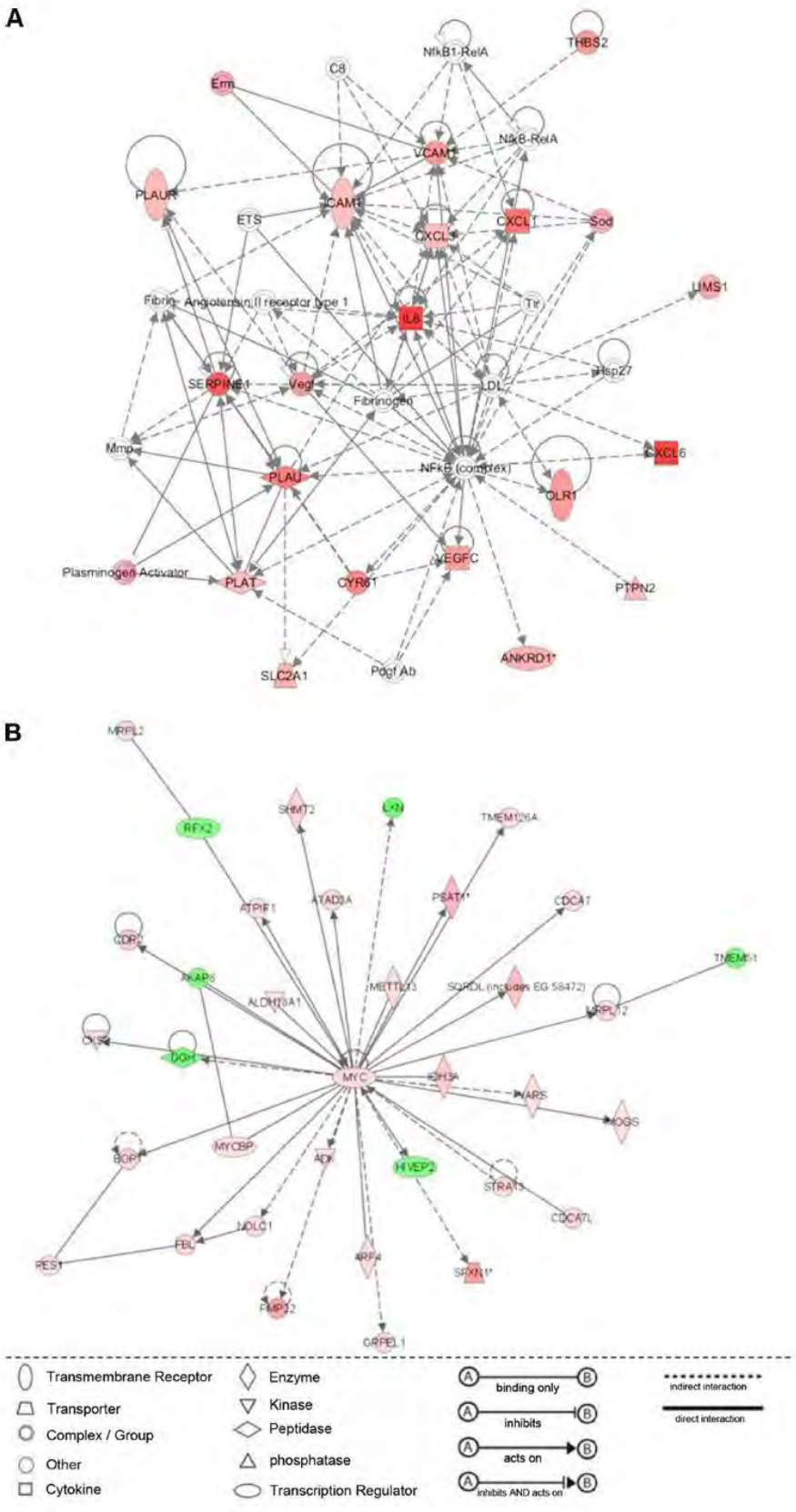
To assure that the regulation of gene transcription was parasite-induced and to exclude effects based on cell aging processes we performed non-infected control samples for every time point and every BUVEC isolate. Direct comparisons of infected BUVEC isolates with the respective non-infected cells revealed significant parasite-driven effects. Given that with progression of infection the number of regulated genes increases – although less infected cells carrying respective stages were detected – demonstrates

[3] Hermosilla C., unpublished observation.

Table II. Canonical pathways significantly over-represented by *E. bovis*-regulated genes in infected bovine endothelial host cells ($p \leq 0.05$).

Canonical Pathway[a]	p.i.	Molecules
Aryl Hydrocarbon Receptor Signaling	4 h	FOS, CYP1A1
	8 d	NR2F1, MYC, FOS, CCNE1, CCND2, TGFB2
	14 d	GSTM1, MGST1, CCNE2, GSTP1, IL6, MYC, NR2F1, FOS, CCNE1, JUN, CCND3, CCND2, SP1, CDKN1A, ALDH1A2, TGFB2, HSP90AA1, ALDH18A1, HSPB1, GSTK1
HMGB1 Signaling	4 d	FOS, SERPINE1
	8 d	IL8, FOS, VCAM1, ICAM1, SERPINE1, PLAT
	14 d	KAT2B, FOS, JUN, MAPK14, SP1, CDC42, RBBP7, MRAS, IFNGR2, PIK3R2, PLAT
HGF Signaling	14 d	MAP3K11, CDC42, PLCG1, IL6, FOS, JUN, GAB1, CDKN1A, MRAS, PRKCH, MAP3K8, PIK3R2, ELK3, MAP3K3
IGF-1 Signaling	4 d	FOS, IGFBP5, NEDD4
	14 d	FOS, YWHAG, JUN, NOV, YWHAH, MRAS, RPS6KB2, IGFBP5, PRKCH, PIK3R2, CYR61, GRB10
ERK5 Signaling	4 h	FOS
	14 d	MYC, FOS, YWHAG, YWHAH, GAB1, MRAS, RPS6KB2, MAP3K8, MEF2C, RPS6KA5, MAP3K3
NRF2-mediated Oxidative Stress Response	14 d	AKR7A2, GSTM1, GSTP1, USP14, MGST1, DNAJC3, DNAJA1, FOS, MAPK14, SOD2, JUN, SCARB1, STIP1, MRAS, UBE2K, DNAJC1, PRKCH, PIK3R2, DNAJB6, GSTK1
Protein Ubiquitination Pathway	14 d	PSMB9, UCHL3, USP14, PSMB5, PSMD7, PSMC4, USP54, THOP1, UBE2S, HSPA5, USP2, PSMB6, TAP1, PSMD8, UBE2F, UCHL1, HSPA8, USP53, PSMB2, HSP90AA1, PSMD14, USP40, UBE2E1
Acute Phase Response	8 d	SOCS1, FOS, SOD2, C1S, NOLC1, CFB, SERPINE1
Leukocyte Extravasation Signaling	8 d	TIMP4, VCAM1, ICAM1, EZR, TIMP2
	14 d	TIMP3, CDC42, CXCR4, MMP16, PLCG1, RAPGEF3, WIPF1, TIMP4, MAPK14, CDH5, CLDN1, EZR, CYBA, CLDN16, CD44, MMP11, PRKCH, PIK3R2, VCL, TIMP2
IL-8 Signaling	8 d	IL8, VCAM1, ICAM1, CCND2, CXCL1, VEGFC
Coagulation System	4 d	PLAUR, PLAU, SERPINE1
	8 d	PLAUR, PLAU, SERPINE1, PLAT
Cell Cycle: G1/S Checkpoint Regulation	8 d	MYC, CCNE1, CCND2, TGFB2
	14 d	MYC, CCNE2, CCNE1, CCND3, HDAC3, CCND2, CDKN1A, TGFB2
Cell Cycle: G2/M DNA Damage Regulation	8 d	TOP2A, CCNB2, BRCA1
	14 d	KAT2B, GADD45A, PTPMT1, CDKN1A, TOP2A, CCNB2

[a]As generated by Ingenuity Pathway Analyses program.


A
NfkB1-RelA
THBS2
C8
Erm
VCAM1
NfkB-RelA
PLAUR
CAM1
ETS
CXCL1
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Sod
UMS1
Fibrin
Angiotensin II receptor type 1
IL6
Tir
HspZ7
SERPINE1
Vegf
LDL
Fibrinogen
Mmp
CXCL6
PLAU
NFkB (complex)
OLR1
VEGFC
Plasminogen Activator
PLAT
CYR61
PTPN2
SLC2A1
Pdgf Ab
ANKRD1*
B
MRPL2
SHMT2
LYN
TMEM126A
RFX2
ATP1F1
ATAD3A
PSAT1*
CDCA7
CDR2
TMEM51
AKAP8
ALDH18A1
METTL13
SORDL (includes EG:58472)
RPL12
OXSR1
DDX1
MYC
H2AFX
WARS
HOGS
BOP1
MYCBP
ADK
HIVEP2
STRAP
CDCA7L
MDLC1
FBL
RES1
ARF4
SPATA1*
PMP22
ORPEL1
Transmembrane Receptor
Transporter
Complex / Group
Other
Cytokine
Enzyme
Kinase
Peptidase
phosphatase
Transcription Regulator
binding only
inhibits
acts on
inhibits AND acts on
indirect interaction
direct interaction

Figure 5. Molecular networks over-represented by *E. bovis*-regulated host cell molecules 8 (A) and 14 (B) days after in vitro infection. A list of significantly ($p \leq 0.05$) regulated molecules in *E. bovis*-infected endothelial host cells 4, 8 and 14 days after infection was individually subjected to Ingenuity Pathway Analyses which generated highly significant networks displaying up-regulated molecules in red and down-regulated ones in green. (A) Network on molecules involved in cellular movement, immune cell trafficking and inflammatory response (8 days p.i.). (B) Network on molecules involved in cellular assembly and organisation, gene expression and infection mechanism (14 days p.i.). (A color figure is available at www.vetres.org.)

stage-specific, parasite-triggered modulation. The fact that identical molecules regulated 8 and 14 days p.i. are mostly found to be increased in their gene transcription at the later time point strengthens enhanced parasite-driven host cell regulation towards the end of merogony I.

The observed changes in host cell nucleus morphology coincided with increasing numbers of regulated genes during the late phase of *E. bovis* infection. Enhanced transition of hetero- to euchromatin is indicative of a transcriptionally active cell and was exclusively observed from 8 days p.i. onwards. The finding that most regulated gene transcripts were detected 8 and 14 days p.i. supports this hypothesis. Among these transcripts, some key regulators of gene transcription (FOS, MYC, STAT1) and various other transcription factors (ANKRD1, ANKRD52, BHLHB2, GTF2E2, GTF2H3, EZH2, KLF4, KLF5) were up-regulated. Especially the MYC-centred gene network generated at 14 days p.i., which indicates high transcriptional activity of *E. bovis*-infected host cells.

An intriguing morphological observation was the consistent arrest of the nuclear "fried-egg"-morphology after this status had appeared. In non-infected cells this morphology indicates the interphase of a cell which changes again as soon as the cell displays mitotic activity [1]. In *E. bovis*-infected endothelial cells, the morphology remained stable irrespective of ongoing development or host cell type, an observation that has also been made for a macromeront-forming *Eimeria* species of the goat, *Eimeria ninakohlyakimovae*[4]. Overall, this phenomenon may indicate a parasite-induced arrest in cell cycle from 8 days onwards. Accordingly, the predicted influence of *E. bovis*-regulated genes on the cell cycle was of a higher significance at days 8 and 14 p.i. than at earlier time points. This was also reflected in the signalling pathways on G1/S checkpoint regulation and G2/M checkpoint DNA damage regulation which were over-represented in *E. bovis*-infected host cells. However, when considering total parasite-triggered modulation of the cell cycle regulatory machinery, both cell cycle progress and arrest seem to have been affected. Up-regulation of different cyclins (CCNB2, CCND2, CCNE1, CCNE2) and related molecules (CDK2AP1, GADD45A), as well as down-regulation of the cyclin-dependent kinase inhibitor 1C, favour cell cycle progress [22–24, 28]. In contrast, down-regulation of CCND3 and up-regulation of inhibitory molecules (CDKN1A, BRCA1) argues for a cell cycle arrest [11, 20, 24, 33, 34].

Once the parasite starts to grow and divide within the cell, it must acquire nutrients through or from the host cell. In contrast to *T. gondii*, which exhibits well-documented auxotrophies for purines [17], hypoxanthine, xanthine and other molecules [26], no data are available for the nutritional requirements of *E. bovis* or other eimerian species. Although different metabolic categories, such as carbohydrate, lipid and nucleic acid metabolism, were significantly modulated by *E. bovis* infection at almost all time points investigated, overall, most metabolism/biosynthesis-related molecules were influenced at 14 days p.i. (i.e. when parasite proliferation was fully active). Genes up-regulated in infected host cells were then involved

[4] Ruiz A., personal observation.

in pathways of carbohydrate biosynthesis and metabolism such as amino sugar, fructose/mannose, galactose, nucleotide sugar and pyrovate metabolism as well as in glycolysis/gluconeogenesis and the pentose phosphate pathway, indicating that transcriptional alteration of the basic host cell metabolism is pivotal for intracellular growth of meronts I.

T. gondii has to scavenge host cholesterol since it cannot synthesize sterols de novo via the mevalonate pathway [7, 27]. Interestingly, 16 molecules involved in cholesterol biosynthesis and metabolism were significantly up-regulated in *E. bovis*-infected host cells 14 days p.i., which may indicate comparable nutritional needs in both parasites. Most notable was the up-regulation of squalene epoxidase (SQLE), which is reported to be one of the rate-limiting enzymes in the mevalonate pathway and which was also induced in porcine and human *T. gondii*-infected host cells [6, 21].

At 14 days p.i., molecules contributing to the metabolism of malic acid (MDH1, MDH2, ME1) were also up-regulated in infected cells, indicating an increased need for energy production towards the end of merogony I.

E. bovis meront I development within the endothelial cell generally leads to enormous alteration of host cell characteristics concerning cell size and subcellular organisation. Hermosilla et al. [16] reported an increasing influence on actin- and tubulin-related reorganisation of the host cell cytoskeleton with ongoing infection. We now found enhanced gene transcription of several tubulins (TUBB, TUBB4, TUBB6) and of molecules involved in microtubule binding or cytoskeleton organisation (TPPP, DOCK7, CKAP4, DCT3) in the late phase of infection. Also vinculin and ezrin, molecules involved in actin cytoskeleton signalling, and other actin-related genes (CAPG, CNN2, TAGLN, PALLD) were up-regulated at the meront I stage and may contribute to the cytoskeleton restructuring process induced by the formation of this parasitic stage.

The vast reorganisation and enlargement of *E. bovis* infected host cells in the late phase of meront I formation causes considerable cell stress, which is reflected in up-regulation of several heat shock proteins (HSP90AA1, HSP90B1, HSP70, HSP70-3, HSP70-5, HSP27, HSPB6) and other stress-related molecules, such as SERP1 and STIP1 at day 14 p.i. The latter coordinates the functions of HSP70 and HSP90. HSP are also closely linked to host cell apoptosis, since certain members exhibit anti-apoptotic effects via both the receptor-mediated pathway and the inner, mitochondrial pathway [3, 4]. It is well known that intracellular stages of other apicomplexan parasites modulate host cell apoptosis to guarantee successful intracellular development (for review see [12]). Lang et al. [18] have recently shown that *E. bovis*-infected cells are defended from apoptosis by enhanced expression of the anti-apoptotic molecules c-FLIP and c-IAP1. The current data confirm up-regulation of c-IAP at 8 days p.i. and provide new candidates repressing apoptosis, either by their up-regulation (DDIT4, BCL2A1) or by decreased abundance (BOK). However, the modulated abundance of several other molecules rather argues for enhanced host cell apoptosis (BAG5, BAK1, BCL2L14, CYCS, COX5A, DAP3).

Previous work has shown that interactions of *E. bovis* and endothelial cells result in inflammatory processes associated with the adhesion of PMN [15] and PBMC [31] to infected cell layers and enhanced transcription of genes encoding for immune-modulatory molecules [30]. The enhanced host cell immune responses described here support these reports, most notably on day 8 p.i. when a respective gene network was generated involving various up-regulated chemokines, adhesion molecules and factors of coagulation. Interestingly, all up-regulated chemokines belonged to the CXC-family (CXCL1, CXCL3, CXCL6, CXCL8), molecules predominantly acting on PMN and/or lymphocytes [19, 35], both of which are stimulated in *E. bovis* infections [5, 32]. Up-regulation of the adhesion molecules VCAM1, ICAM1 and SELP may directly contribute to the above mentioned adhesion of leukocytes to infected cell layers [15, 31].

The precise role of molecules involved in coagulation (i.e. PLAU, PLAUR, PLAT, SERPINE1), in *E. bovis* infection is not known. Given that pro-inflammatory reactions at the endothelial site are generally accompanied by

enhanced permeability of cell layers, activation of the coagulation system may represent an additional sign of parasite-triggered endothelial cell activation. The induction of CFB, CFH, C1S and C3, molecules involved in the complement cascade, may also be seen in this sense. Overall, the inflammatory response at 8 days p.i. was also reflected in regulated canonical pathways in *E. bovis*-infected host cells. Involvement in IL-8 signalling, leukocyte extravasation signalling and in the coagulation system has already been mentioned above. Moreover, *E. bovis* infection induced HMBG1 signalling on days 4, 8 and 14 p.i., a pathway leading to the release of pro-inflammatory molecules.

In conclusion, the current investigation has identified numerous gene transcripts regulated by *E. bovis* during in vitro infection for the first time. The induction of cholesterol- and carbohydrate-related pathways shed light on *Eimeria*-associated nutritional needs for intracellular growth. Various indications from transcripts associated with host cell apoptosis, immune response and cell cycle regulation provide useful tools for more detailed, prospective studies on *Eimeria*-host cell interactions.

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7.10 "*Eimeria bovis*-induced modulation of the host cell proteome at the meront I stage"

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♦ Durchführung der Versuche	unterstützend
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Eimeria bovis-induced modulation of the host cell proteome at the meront I stage

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ABSTRACT

The proteome of *Eimeria bovis* meront I-carrying host cells was analyzed by two-dimensional gel electrophoresis (2DE) at 14 days p.i. and compared to non-infected control cells. A total of 221 protein spots were modulated in their abundance in *E. bovis*-infected host cells and were subsequently analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectometry (MALDI-TOF-MS). These analyses identified 104 proteins in total with 25 host cell proteins being up-regulated and 79 proteins being down-regulated in *E. bovis*-infected host cells. Moreover, 20 newly expressed proteins were identified exclusively in *E. bovis*-infected host cells and were most likely of parasite origin. Parasite-induced differences in protein abundance concerned distinct functional categories, with most proteins being involved in host cell metabolism, cell structure, protein fate and gene transcription. Some of the modulated molecules also indicated regulatory processes on the level of host cell stress response (HSP70, HSP90), host cell apoptosis (caspase 8) and actin elongation/depolymerization (α-actinin-1, gelsonin, tropomodulin-3, transgelin). Since merozoites I were already released shortly after cell sampling, the current data reflect the situation at the end of first merogony. This is the first proteomic approach on *E. bovis*-infected host cells that was undertaken to gain a rather broad insight into *Eimeria*-induced host cell modulation. The data processed in this investigation should provide a useful basis for more detailed analyses concerning *Eimeria*–host cell interactions.

1. Introduction

Eimeria bovis is an obligate intracellular apicomplexan parasite which represents one of the most pathogenic species in cattle coccidiosis, causing severe haemorrhagic typhlocolitis in calves and high economic losses worldwide [1]. Within the gut, freely released *E. bovis* sporozoites traverse the epithelium in order to invade endothelial cells of the central lymph capillaries of the ileal villi, where they undergo the first merogony [2]. Enclosed by a parasitophorous vacuole (PV), the sporozoites of *E. bovis* develop into ≥300 μm sized macromeronts within 14–18 days [3,4]. It appears likely that once the parasite begins growth and proliferation it must acquire nutrients from the host cell as reported for other intracellular apicomplexans [5,6]. Furthermore, the massive replication and the final enlargement of the host cell far beyond the physiological size may cause considerable stress to the host cell [7,8], and cell stress, in turn, is well known to trigger host cell defense mechanisms and apoptosis [9]. In consequence, the obligate intracellular parasite *E. bovis* must rely on several regulatory processes to actively modulate the host cell proteome to guarantee survival and first merogony development.

The general capacity of apicomplexan parasites to manipulate infected host cells for their advantage is well-documented in case of *Toxoplasma gondii*. This particular parasite acquires auxotroph nutrients from infected host cells, interacts with the host cell mitochrondria, endoplasmic reticulum and cytoskeleton [10–12] and actively interferes with the apoptotic capacity of the host cell [10,13–15].

In the case of *E. bovis* only few reports deal with its capacity for host cell modulation, although its merogony takes much longer and results in much larger intracellular meronts than *T. gondii*. Some evidence is reported on the level of distinct modulation of host cell cytoskeleton [16] and inhibition of host cell apoptosis by enhanced expression of anti-apoptotic factors [17], the latter of which is in agreement with other *Eimeria* spp. [18]. Activation of NFκB in sporozoite-infected, non-permissive epithelial host cells was shown for *E. bovis* as well [19].

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E. bovis also appears to modulate host endothelial cell-mediated immune reactions [20–23]. At least generally transcript levels of genes encoding for pro-inflammatory molecules in *E. bovis*-infected endothelial cells were significantly lower when compared with T. gondii- or N. caninum- infected cells [22].

The aim of this study was to gain a broad insight into *E. bovis*-induced host cell proteome modulation at the meront I stage. Therefore, whole cell proteomes of *E. bovis*-infected host cells and non-infected controls were analyzed by two-dimensional gel electrophoresis (2DE) followed by the identification of individual spots by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Overall, 27 host cell proteins were found up-regulated and 82 proteins down-regulated in host cells carrying mature *E. bovis* meronts I, the majority of them being involved in cell metabolism, cell structure, protein synthesis and gene transcription. In addition, we identified 20 proteins in infected cells which were most probably of parasite origin. With these data we create a basis for more detailed analyses targeting functional *E. bovis*–host cell interactions.

2. Materials and methods

2.1. Parasite

The *E. bovis* strain H used in the present study was isolated in the field in northern Germany [24] and maintained by passages in parasite-free Holstein Friesian calves. For oocysts production, calves were infected orally at the age of 10 weeks with 5×10^5 sporulated *E. bovis* oocysts. Excreted oocysts were isolated from the faeces beginning 18 days p.i. according to the method of Jackson [25]. Sporulation of oocysts was achieved by incubation in a 2% (w/v) potassium dichromate (Merck) solution at room temperature (RT). Sporulated oocysts were stored in this solution at 4 °C until further use.

Sporozoites were excysted from sporulated oocysts as previously described [26]. Free sporozoites were washed three times in phosphate buffered-solution (PBS), resuspended in complete Iscove's modified Dulbecco medium (IMDM; Gibco) and counted in a Neubauer haemocytometre as described elsewhere [27].

2.2. Host cells and parasite infection

Bovine foetal gastrointestinal cells (BFGC), an immortalized primary cell line originally isolated from *in utero* foetuses 4–6 months after conception [26], were cultured in complete IMDM medium supplemented with 500 U/ml penicillin, 50 μg/ml streptomycin (Sigma), 1% L-glutamine (w/v) and 10% heat-inactivated foetal calf serum [(v/v); FCS; Gibco]. Cells were cultured in 75 cm^2 tissue culture flasks (Nunc) and incubated (37 °C, 5% CO_2 atmosphere) until confluency. BFGC monolayers were infected with 5×10^5 freshly excysted sporozoites. Sporozoite viability was determined by trypan blue exclusion test according to Lang et al. [17]. Cell culture medium was changed 1 day after infection and thereafter every third day. One-day p.i. infection rates of the BFGC cultures were determined microscopically.

2.3. Sample preparation for the two-dimensional gel electrophoresis (2DE)

Previous transcriptome studies on *E. bovis*-infected host cells revealed most molecules being modulated at 14 days p.i. when compared to earlier time points of infection. In accordance to these results we restricted the current analysis to this time point. At 14 days p.i. whole cell preparations of *E. bovis*-infected and non-infected BFGC were subjected to 2DE. BFGC layers were washed three times with PBS. Cells were scraped off the tissue culture flasks using a sterile rubber policeman (Nunc) and resuspended in PBS. The cell suspension was pelleted (600 × g, 15 min) and washed two times with sorbitol–buffer [25 mM sorbitol, 10 mM Tris-base (v/v, pH 7); all Sigma]. Cells were lyzed in a lysis buffer [8 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, 20 mM Tris, 2% pharmalyte buffer (v/v, pH 3–10); all Sigma] and stored at −80 °C.

For 2DE 375 U of benzoase (Calbiochem) and 8 μl protease inhibitor (Sigma) for mammalian cells were added to each sample and samples were incubated (30 min, RT), sonicated twice (200 W, working time 10 s, interval time 15 s, 50 cycles, ice bath) and centrifuged (21,000 × g, 30 min, 20 °C). The supernatant was precipitated with acetone [1:7; (Merck)] and centrifuged (8510 × g, 30 min, 20 °C). The resulting pellet was washed in acetone (Sigma) and resuspended in the above-described lysis buffer. Solubilized proteins were then quantified using the Plus One™ 2D Quant Kit® technique (GE Healthcare Life Sciences) and subjected immediately to 2DE.

2.4. Iso-electro focusing (IEF) and 2DE

Proteins were solubilized by incubating the washed pellet at least 1 h at RT in iso-electro focusing rehydration buffer onto immobilized pH gradient (IPG) strips [pH 3–11 non-linear; length 11 cm; DryStrip® (GE Healthcare Life Science)]. Each strip was rehydrated overnight with 300 μg of protein sample in 220 μl lysis buffer. The IEF was carried out in a Multiphor chamber® (Amersham Biosciences) at 20 °C applying the following conditions: *Phase 1*: gradient from 0 V to 50 V for 0.25 kVh; *Phase 2*: gradient from 100 V to 3500 V for 11 kVh; *Phase 3*: 3500 V for 21 kVh. Current was limited to 0.25 mA per strip. For the second dimension the protein-loaded IPG strips were equilibrated for 15 min by rocking in DTT-equilibration buffer [10 mg/ml 1,4-dithio-DL-threitol, 20 mg/ml sodium dodecyl sulfate (SDS) in 6 M urea (all Fluka), 30% (v/v) glycerin (Sigma), 50 mM Tris–HCl (Merck), pH 8.8, 4% (w/v) SDS 0.01% bromophenol blue (Fluka)] and then washed for 15 min in jodacetamid-solution [40 mg/ml jodacetamid (Fluka), 20 mg/ml SDS in 6 M urea, 30% (v/v) glycerin, 50 mM Tris–HCl, pH 8.8, 4% (w/v) SDS 0.01% bromophenol blue (all Fluka)]. The strips were then embedded on a precast separating gel [12.5%, v/v acrylamide (Fluka)] and sealed into place with agarose-solution (Fluka). Gel electrophoresis was carried out at 25 °C applying a constant voltage of 500 V for 4.5 h in a 14.5 cm × 14.5 cm vertical gel electrophoresis chamber (Hoefer 600®; Amersham Biosciences).

The gels were then stained by colloidal Coomassie (Fluka). Thereafter, the 2DE gels were agitated for 60 min in staining solution [0.2% (w/v) Coomassie R 250, 50% (v/v) MeOH, 50% (v/v) ddH_2O] and then overnight in stripping solution [50% (v/v) MeOH, 6% (v/v) acetic acid, 44% (v/v) ddH_2O]. Analysis of 2DE gels was performed using a flatbed scanner (Powerlook 2100 XL®; Umax) with a resolution of 600 dpi. For each sample, four gels were run and analyzed in parallel.

2.5. Image analysis

Differences in protein abundance between non-infected and *E. bovis*-infected BFGC samples were detected by image analysis using the software Proteomweaver® (Version 4.0; BioRad). To prevent that proteins originating from *E. bovis*-macromeronts may falsify the total amount of host cell protein, a consistently expressed "house-keeping" protein was needed for calibration. Since recent studies showed that vimentin intermediate filaments are not altered in their abundance in *E. bovis*-infected host cells during the first merogony [16] we chose the bovine vimentin spot as internal reference to normalize the 2DE gels of infected and non-infected BFGC. Therefore five vimentin spots were identified in 2DE gels by immunoblotting (anti-vimentin, clone Vim

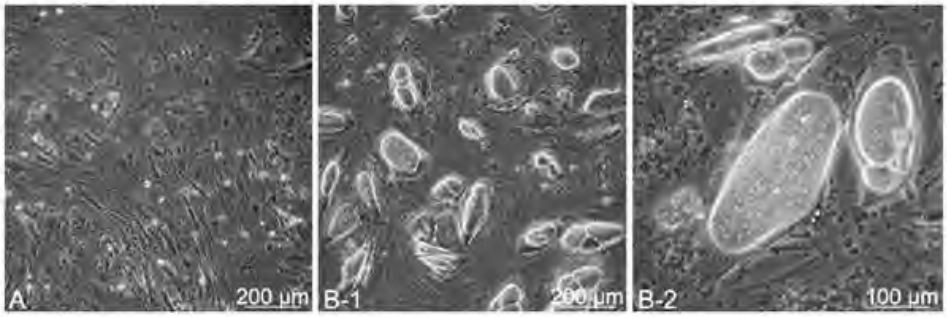

Fig. 1. *E. bovis*-infected and non-infected BFGC *in vitro*. Confluent BFGC layers were either left non-infected (A) or infected with freshly isolated *E. bovis* sporozoites (5×10^5/75 cm^2 culture flask, B1/2) and cultured for 14 days. Note huge macromeronts containing mature merozoites I in infected cell layers (B1/2).

3B4; 1:10,000, Sigma) and subsequent MALDI-TOF-MS sequence analysis. For normalization we always referred to one vimentin spot [characteristics: 60 kDa; pI: 4.8; Mowse score: 309; mass value matched: 35; seq. cov. (%): 63] consistently expressed in all probes.

The factor "infected/non-infected" was calculated from the spot average intensity of four gels and normalized to the vimentin spot average intensity of four gels by division.

The criteria for proteins being judged modulated in their abundance were the following: ≥2.0-fold up/down-regulation and each protein spot had to be detected in at least three out of four 2DE gels made from identical samples. The cut-off of >2-fold change typically applied in proteome analyses was chosen to reduce experimental variation and thereby to emphasize significant effects. Furthermore, spots required an intensity of ≥0.1 and a Student *t*-test value of ≥90% to be considered for subsequent MALDI-TOF-MS analyses.

The apparent molecular mass of the proteins was calculated using a standard 10 kDa ladder run in the gels. Additionally, the pI (iso-electro point) of the proteins was determined according to the pH value in the strips.

Indications of phosphorylation were predicted by database screening of peptide spectra of single proteins via MASCOT analysis.

2.6. In-gel digestion and peptide mass fingerprinting

Protein spots of interest were manually excised from the gels, washed in distilled water and twice in 50 mM ammonium hydrogen carbonate:acetonitrile (1:1) and acetonitrile (Fluka). Gel pieces were then incubated in 10 ng/µl trypsin solution [sequencing grade

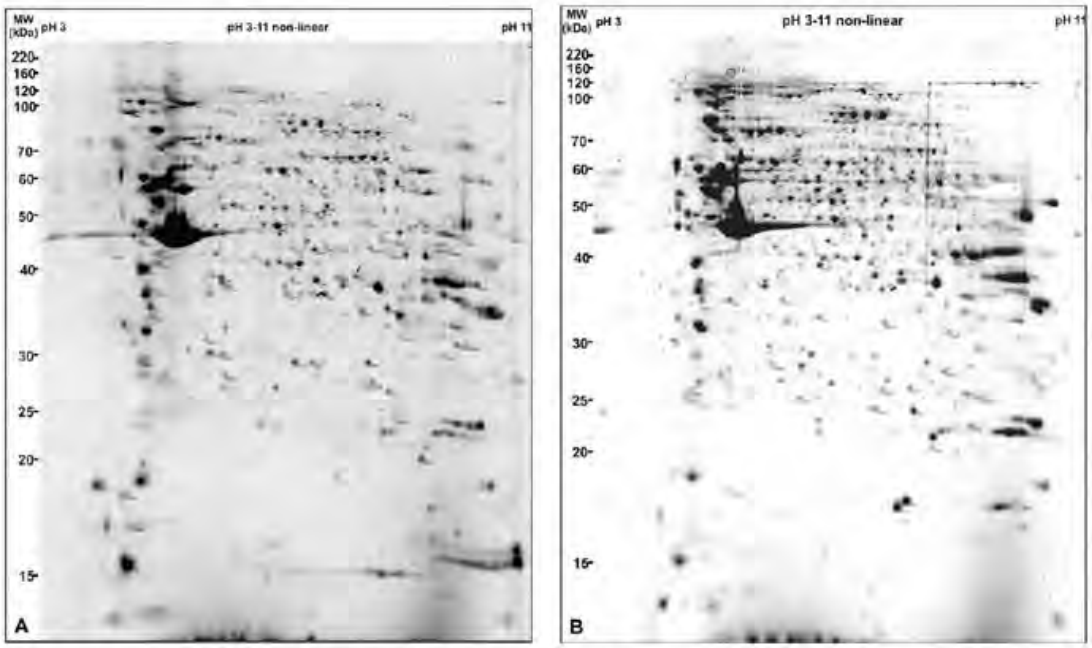

Fig. 2. Two-dimensional gel electrophoresis (2DE) of non-infected (A) and *E. bovis*-infected (B) BFGC. Confluent BFGC monolayers were infected with freshly isolated *E. bovis* sporozoites (5×10^5/75 cm^2 tissue culture flask) and sampled 14 days after infection. Non-infected BFGC were used as controls. For 2DE PAGE, 300 µg of protein were loaded per gel. After electrophoresis the gels were stained with Coomassie and scanned. Differences in protein abundance (represented as an average of the total number of gels) were detected using Proteomweaver software. Only proteins that differed by at least 2-fold in abundance when compared with non-infected controls were subjected to further analyses (peptide mass fingerprinting and MALDI-TOF-MS).

(Roche Diagnostics) in 25 mM ammonium hydrogen carbonate, 16 h, 37 °C]. Peptides were extracted with 5 µl of 1% (v/v) trifluoroacetic acid (Bruker Daltonics) containing 5 mM octylglycoside (Bruker Daltonics). Aliquots (2 µl) of the solution were applied to a thin layer of α-cyano-4-hydroxycinnamic acid on an AnchorChip® target (Bruker Daltonics). After 10 min of incubation the supernatant was removed, and the spot was washed twice with 2 µl 0.1% (v/v) trifluoroacetic acid solution (Bruker Daltonics). Mass fingerprints of tryptic digests were obtained by MALDI-TOF-MS analysis using an Ultraflex™ TOF/TOF mass spectrometer (Bruker Daltonics). Peptide mass standards (Bruker Daltonics) were used for external calibration of the mass spectra. Proteins were identified by database searching (NCBI, SWISS-PROT, TrEMBL) with peptide masses using the program 'Mascot in house®' (Version 2.1, http://www.matrixscience.com). Protein identification was completed by significance of $p < 0.05$ probability based values on Mowse Scores (≥61).

3. Results and discussion

3.1. Comparative proteome analysis by 2D-electrophoresis and MALDI-TOF-MS

The study compares the proteomes of *E. bovis*-infected and non-infected host cells 14 days after *in vitro* infection. As expected, *in vitro* culture had resulted in large macromeronts containing either almost mature or fully mature merozoites I (Fig. 1). Shortly after cell sampling, freely released merozoites I were observed in supernatants of corresponding control samples. The initial infection rate as measured on day one p.i. was 10%.

Overall, comparative 2DE of *E. bovis*-infected (Fig. 2A) and non-infected (Fig. 2B) BFGC revealed that, in total, 221 protein spots were modified in their abundance by this protozoan infection with 137 protein spots being down-regulated and 84 spots being up-regulated. Amongst the latter group 20 proteins were annotated to parasite origin (data not shown) and for 23 proteins no reference was found.

Overall, only a proportion of the modulated proteins fulfilled the criteria for subsequent MALDI-TOF-MS analyses. Consequently, a total of 104 proteins were further characterized by this method. The majority of these proteins was down-regulated ($n = 79$, Table 1), whilst only 25 proteins (Table 2) were enhanced in their abundance.

3.2. Functional distribution of differentially expressed proteins

Functional annotation analyses revealed that parasite-induced differences in host cell protein abundance concerned different functional categories. In general, it appears noteworthy to mention that grouping the different proteins in just one category may be very simplistic with respect to the multifunctional characteristics of many proteins, but had to be performed in order to achieve overview in general.

The majority of up-regulated proteins was involved in metabolism (35%), protein fate (19%) and cell structure (15%), whilst down-regulated proteins were mainly grouped into the functional categories of metabolism (20%), cell structure (23%), core proteins and transcription (17%) (see overview, Fig. 3). Moreover, some down-regulated proteins were involved in other biological functions such as signal transduction (6%), protein fate and translation (both 5%), cellular transport (4%), apoptosis (1%), immune (1%) and stress response (1%).

3.2.1. Metabolism

Host cell metabolism was by far the mostly affected functional category in *E. bovis*-infected host cells, indicating that the parasite's development is fundamentally linked to the acquisition of essential host cell molecules. It appears likely that with the onset of parasite growth and proliferation *E. bovis* needs access to host cell nutrients as described for other apicomplexan parasites [11,12,28]. Unfortunately, in contrast to *T. gondii* exhibiting well-documented auxotrophies for purines [29], hypoxanthine, xanthine and other molecules [30], no data are available for nutritional requirements of *E. bovis* or other *Eimeria* species.

Overall, modulated molecules were involved in different metabolic pathways, such as glycolysis, citric acid cycle or alcohol and lipid degradation. In total, 16 proteins related to metabolism were down-regulated and only 9 molecules were found to be up-regulated. Interestingly, the total number of modulated proteins linked to metabolism corresponded well to those reported in *T. gondii*-infected host cells [31]. In *E. bovis*-infected BFGC many down-regulated proteins play a role in glycolysis and the citric acid cycle, suggesting that this major pathway of energy generation is not or no longer exploited by the parasite. This may relate to the stage of development of the parasite at the time of sampling, 14 days p.i. At this point of time most infected cells carried already mature macromeronts filled with merozoites I, i.e. parasite replication was completed and it was therefore rather a matter of conservation of a status quo than of high energy expenditure. This is partially in accordance with proteome analyses on *T. gondii* tachyzoite-infected host cells showing that most of the host cell proteins grouped in metabolic processes were also down-regulated [31]. Admittedly, in contrast to our findings in *E. bovis*-infected cells, molecules involved in glycolysis were rather up- than down-regulated in case of *T. gondii*. It has to be considered that in the latter case analyses were performed 24 h after infection [31], i.e., during the parasite's proliferative phase – considering the much shorter merogony – and may consequently reflect a different developmental situation.

3.2.2. Cell structure

Structural molecules were the second largest group of proteins to be influenced in their abundance by *E. bovis* infection. Previous studies on *E. bovis*-triggered modulation of the host cell cytoskeleton in primary endothelial cells demonstrated that both actin filaments and microtubules were increased in their abundance and reorganized within the host cell with ongoing infection [16]. Interestingly, and in accordance with findings in *T. gondii*-infected host cells [31], several proteins known to interact with actin and involved in the elongation and depolymerization of actin filaments (alpha-actinin-1, gelsolin, actin-like protein-2, gelsolin-like capping protein, tropomodulin-3, transgelin) were also down-regulated. However, in contrast to *T. gondii*-infected host cells, myosin-related proteins (myosin-10, β-tropomyosin, myosin regulatory light chain 2) were down-regulated as well. Since tubulin-associated proteins (tubulin beta 5 and 6) were also decreased in their abundance, the modulation of these two main cytoskeleton systems suggests a general mechanism taking place at the final of merogony when macromeronts need to rupture for merozoite I release.

Lamin A/C, an intermediate filament protein of the nuclear membrane, was also down-regulated in *E. bovis*-infected BFGC. Lamin filaments represent one of the major constituents of the nuclear membrane and exhibit supportive characteristics for the nuclear double lipid layer forming a fiber network [32]. Lamins are functionally regulated by phosphorylation leading to disintegration of the nuclear membrane during mitosis [32,33]. Since several, particularly phosphorylated isoforms of lamin A/C were down-regulated in *E. bovis*-infected host cells this phenomenon could be associated with reduced host cell mitotic activity. Actually we have never observed division of *E. bovis*-infected cells *in vitro* and a similar situation seems to exist in *T. gondii*-infected cells [31]. In this context, the fact that *E. bovis* infection down-regulates the abun-

Table 1
Down-regulated host proteins in *E. bovis*-infected BFGC (14 days p.i.).

Proteins and functional categories	Accession number (SwissProt/Trembl or NCBI)	Factor inf./non-inf.	P*	Theoret. MW/pI	Seq. cov. (%)
Metabolism					
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	gi|19885261	0.452		88.4/6.42	40
Aconitate hydratase	P20004	0.342	P	86.0/7.87	48
Transketolase	Q6B855	0.438	P	68.5/7.56	36
Adenylyl cyclase-associated protein 1	Q3SYV4	0.526	P	51.6/7.16	57
Alpha-enolase	Q9XSJ4	0.377	P	47.6/6.37	53
Protein-arginine deiminase type-2	Q9Y2J8	0.537	P	76.3/5.4	23
Acyl-CoA thioesterase 9	gi|77736067	0.0		50.2/8.87	53
LOC505323 (Ornithine aminotransferase)	gi|77735431	0.449		48.4/6.1	35
Fructose-1,6-bisphosphate aldolase A	gi|119916947	0.0		52.0/9.08	44
Isocitrate dehydrogenase 3 (NAD+) alpha	gi|109939980	0.420		40.1/6.76	56
Glyceraldehyde-3-phosphate dehydrogenase	P10096	0.397		36.1/8.5	63
L-Lactate dehydrogenase A chain	P19858	0.420	P	36.9/8.12	57
L-Xylulose reductase	Q91X52	0.360		25.9/6.82	27
Phosphoglycerate mutase 1	Q3SZ62	0.466		28.9/6.67	83
Triosephosphate isomerase	Q5E956	0.271		26.9/6.45	77
Protein disulfide-isomerase A3	P38657	0.391		57.3/6.23	65
Structure proteins					
Alpha-actinin-1	Q3B7N2	0.439		103.5/5.25	63
Alpha-actinin-1	Q3B7N2	0.226	P	103.5/5.25	54
Myosin-10	Q27991	0.271		229.9/5.44	25
Actinin, alpha 4	gi|119910351	0.439		105.3/5.27	54
Gelsolin	gi|74356373	0.287	P	81/5.54	41
Moesin	Q2HJ49	0.387	P	68.1/5.9	52
Lamin A/C**	gi|77404182	0.168–0.456	±P	65.1/6.54	54–66
Lymphocyte cytosolic protein 1 (L-plastin)	gi|77736385	0.530		70.7/5.21	44
Beta-actin	P60712	0.481		42.1/5.29	32
Gelsolin-like capping protein	gi|30466254	0.272	P	39.1/6.17	58
Tropomodulin 3	gi|115496236	0.223	P	38.3/4.92	55
Beta-tropomyosin	gi|83778524	0.448		33.3/4.62	42
PDZ and LIM domain protein 1	Q5E9E1	0.239	P	36.3/6.76	44
F-actin capping protein subunit alpha-2	Q5E997	0.541		33.1/5.57	65
Transgelin	Q9TS87	0.0	P	22.6/8.87	84
Myosin regulatory light chain 2	Q5E9E2	0.0	P	19.9/4.67	80
Actin-like protein 2	P61160	0.337		45.0/6.3	42
Tubulin beta 5	gi|74204140	0.441		50.1/4.75	71
Tubulin beta 6	gi|114052148	0.441		50.3/4.76	61
Core proteins/transcription					
Matrin-3	gi|119895555	0.263		100.5/5.71	35
Heterogeneous nuclear ribonucleoprotein M	P52272	0.000		77.7/8.84	30
Heterogeneous nuclear ribonucleoprotein K	Q3T0D0	0.348		51.3/5.14	48
U4/U6 small nuclear ribonucleoprotein Prp4	Q3MHE2	0.000		59.05/7.06	41
LOC513868 (nuclear matrix protein 200)	gi|115497226	0.395	P	55.6/6.14	55
Histone-binding protein RBBP4	Q3MHL3	0.296		47.9/4.74	31
Heterogeneous nuclear ribonucleoprotein D0B isoform 6	gi|76620242	0.432		32.99/8.23	33
Heterogeneous nuclear ribonucleoprotein A/B	Q99729	0.166		36.7/9.04	34
Heterogeneous nuclear ribonucleoprotein C	gi|73977325	0.419		27.8/4.55	58
Heterogeneous nuclear ribonucleoprotein H3	gi|14141157	0.238		36.96/6.37	64
Poly(rC)-binding protein 1	Q5E9A3	0.367		37.98/6.66	74
SET (myeloid leukemia-associated)	gi|3953617	0.255	P	24.3/4.97	49
Heterogeneous nuclear ribonucleoproteins A2/B1	Q2HJ60	0.0	P	36.04/8.67	58
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	0.482		37.5/8.97	45
High mobility group protein B1	P10103	0.212		25.1/5.62	46
High mobility group protein B1	P10103	0.456	P	25.1/5.62	31
GTP-binding nuclear protein Ran	Q3T054	0.190		24.6/7.01	55
Histone H2B type 1	P62808	0.0	P	13.9/10.31	48
Nonhistone chromosomal protein HMG-14	P02316	0.0	P	10.8/9.49	46
Translation					
Elongation factor 2	Q3SYU2	0.4	P	96.3/6.41	44
Eukaryotic translation elongation factor 1 gamma	gi|95769122	0.341		50.6/6.33	68
Probable ATP-dependent RNA helicase DDX48	Q2NL22	0.318		47.1/6.3	78
40S ribosomal protein S17	P63273	0.155	P	15.6/9.85	70
Protein fate					
Heat shock protein HSP 90-alpha	Q76LV2	0.284	P	85.1/4.93	46
Heat shock protein HSP 90-beta	Q76LV1	0.0	P	83.5/4.97	52
Heat shock 70 kDa protein 5	gi|115495027	0.209	P	72.5/5.07	55
10 kDa heat shock protein	P61603	0.129		10.9/8.89	66
Signal transduction					
Integrin alpha-V	P80746	0.199	P	117.1/5.55	23
Integrin alpha-V	P80746	0.389		117.1/5.55	30
Rab GDP dissociation inhibitor alpha	P21856	0.308		51.1/5.0	44
Ras suppressor protein 1	Q5E9C0	0.0	P	31.5/8.56	52

Table 1 (*Continued*)

Proteins and functional categories	Accession number (SwissProt/Trembl or NCBI)	Factor inf./non-inf.	P*	Theoret. MW/pI	Seq. cov. (%)
14-3-3 protein theta	Q3SZI4	0.423	P	28.0/4.68	65
Annexin A2	P04272	0.407		38.9/6.92	72
Cellular transport					
Voltage-dependent anion-selective channel protein 2	P68002	0.310		32.1/7.48	72
Voltage-dependent anion-selective channel protein 3	Q9MZ13	0.383		31.1/8.95	51
Chloride intracellular channel protein 4	Q9XSA7	0.259	P	28.9/5.6	78
Apoptose					
Caspase 8	gi|114051073	0.0	P	57.4/5.51	36
Protease					
Catalase	P00432	0.0		60.1/6.78	32
26S protease regulatory subunit S10B	Q2KIW6	0.216		44.3/6.74	58
Immune response					
Annexin A1	P46193	0.416		39.2/6.37	64
Stress response					
Peroxiredoxin-4	Q9BGI2	0.383	P	30.9/6.01	57
Interaction with environment					
Annexin A5	P81287	0.324		36.1/4.86	62
Unclassified					
LOC504957 (alpha-2-macroglobulin receptor-associated protein)	gi|122692301	0.360		41.9/7.36	40
Growth hormone-like protein 1	gi|58339172	0.0	P	25.2/5.33	56
Glypican 4	gi|119919671	0.5	P	63.5/5.85	30
Protein SIX6OS1	Q9CTN5	0.409		67.3/5.45	24
Cysteine-rich protein 2	Q0VFX8	0.126	P	23.4/9.01	62

Seq. cov. (%)—sequence coverage.
* Indication of phosphorylation (Biotool).
** Several detected down-regulated protein spots.

Table 2
Up-regulated host proteins in *E. bovis*-infected BFGC (14 days p.i.).

Proteins and functional categories	Accession number (SwissProt/Trembl or NCBI)	Factor (inf./non-inf)	P*	Theoret. MW/pI	Seq. cov. (%)
Metabolism					
Glutamate dehydrogenase 1	P00366	N.A.		61.8/7.25	36
Protein disulfide-isomerase A3	P38657	4.331		57.3/6.23	31
D-3-Phosphoglycerate dehydrogenase	Q5EAD2	3.476		57.3/6.47	49
Aldehyde dehydrogenase	P20000	4.051		57.1/7.55	46
Isocitrate dehydrogenase	Q9XSG3	3.372		47.1/6.13	48
ADP/ATP translocase 1	P48962	3.610	P	33.1/9.73	35
Enoyl-CoA hydratase	Q58DM8	2.684		31.6/8.82	51
Malate dehydrogenase	Q32LG3	N.A.		36.1/8.82	67
GTP:AMP phosphotransferase	P08760	5.230	P	25.7/9.02	62
Structure proteins					
Transgelin-2	Q5E9F5	3.908		22.6/8.39	88
Transgelin-2	Q5E9F5	4.965	P	22.6/8.39	85
Desmin	O62654	7.109		53.6/5.21	64
Caldesmon 1**	gi|27806279	2.789–3.326		62.1/6.24	51
Alpha-actinin-1	Q3B7N2	2.671		103.5/5.25	41
Protein fate					
Endoplasmin (heat shock protein 90 kDa)	Q95M18	2.693		92.7/4.76	48
Heat shock cognate 71 kDa	P19120	4.010		71.4/5.49	38
Heat shock 70 kDa protein 9B	gi|77735995	N.A.	P	73.9/5.97	42
Calreticulin	P52193	2.323		48.2/4.31	26
Calumenin	Q3T0K1	2.250		37.2/4.47	49
Signal transduction					
Rab GDP dissociation inhibitor beta	P50397	2.691		50.96/5.94	50
Cellular transport					
Vacuolar ATP synthase subunit B	P31408	3.446		56.9/5.66	49
ATP synthase subunit alpha	P19483	3.317		59.8/9.21	53
Unclassified					
GRIP and coiled-coil domain-containing protein 2	Q8IWJ2	N.A.		185.5/5.08	14
Hypothetical protein Chro.70256	gi|67614925	6.090		62.6/9.17	19
Hypothetical protein XP_532413	gi|73975430	2.658		46.9/6.34	31
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	gi|73949814	N.A.		18.9/7.03	44

N.A.: a factor could not be calculated, as the respective protein spot was not detectable in non-infected samples.
* Indication of phosphorylation (Biotool).
** Several detected up-regulated protein spots.

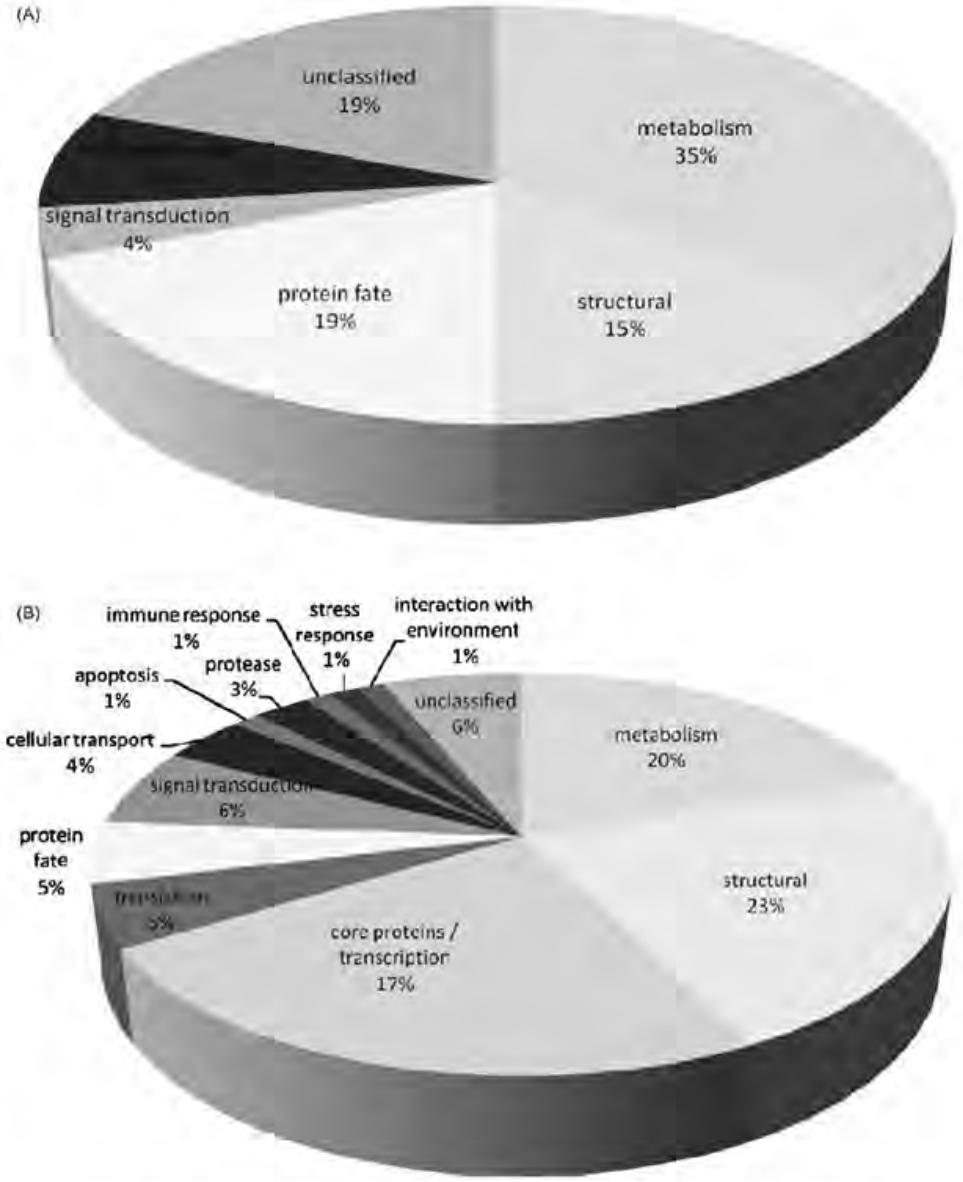

Fig. 3. Functional distribution of host cell proteins up-regulated (A) and down-regulated (B) by *E. bovis* infection (14 days p.i.). Whole cell preparations of *E. bovis*-infected BFGC were subjected to 2DE, peptide mass fingerprinting and MALDI-TOF-MS analyses and compared to non-infected controls. Significantly up-regulated (A) and down-regulated (B) host cell proteins were grouped according to annotation analyses into different functional categories and expressed relatively to the total numbers of up-regulated molecules.

dance of myosin-10, a protein known to be involved in cell division [34,35], may be of further interest.

3.2.3. Core proteins, transcription and translation factors

E. bovis infection clearly influenced the abundance of molecules involved in gene transcription and translation. Besides histone-related proteins (histone H2B type 1, histone-binding protein RBBP4) and the key molecule in nuclear ex- and import Ran [36,37], *E. bovis* also modulated several molecules of the heterogeneous nuclear ribonuclear protein (hnRNP) family, a finding that agrees with data on *T. gondii* [31]. hnRNPs play a key role in pre-mRNA processing within the nucleus [38] and in the transport of processed mRNA [39]. Overall, seven hnRNPs (hnRNP A/B, A2/B1, C, D0B isoform 6, H3, M, K; see Table 1) were decreased in their abundance in infected cells, suggesting a general role of this protein family in the regulation of *Eimeria*-induced changes in gene transcription. Moreover, molecules involved in mRNA splicing, such as the U4/U6 small ribonuclear protein Prp4 and a protein homologue to the

nuclear matrix protein 200 of *Saccharomyces cervesiae* and other mammalian species [40] and of matrin-3, which is involved in gene transcription by binding to other matrix proteins in order to form a fibrogranular nuclear network [41] suggest an impact of *E. bovis* infection on host cell gene transcription. The modified abundance of translation-related proteins (eukaryotic translation elongation factor 1 gamma, elongation factor 2, ATP-dependent RNA helicase DDX48, 40S ribosomal protein S17) in infected cells additionally indicates the parasite-triggered modulation of this cellular process.

3.2.4. Apoptosis

It is well known that intracellular stages of other apicomplexan parasites, such as *Cryptosporidium parvum* [42], *Theileria parva* [43–45], *T. gondii* [46–49,14], *Neospora caninum* [50–52] and *Eimeria* [18] modulate host cell apoptosis to guarantee successful intracellular development (for review see Ref. [53]). To date, only few details are known about the molecular mechanisms allowing for long-term survival of *E. bovis* within adequate host cells. In detailed studies Lang et al. [17] have recently shown that cells infected with viable sporozoites of *E. bovis* are protected by enhanced expression of c-FLIP and c-IAP1, anti-apoptotic molecules involved in different apoptosis signalling pathways, i. e., the inner [54–58] and the receptor-related apoptotic pathway [59–61]. Also enhanced levels of NFκB in *E. bovis*-infected cells were considered an indicator of apoptosis inhibition [18]. In addition, the current study revealed a strongly reduced abundance of caspase 8 in *E. bovis*-infected host cells, an effect which is also seen in *T. gondii*-infected cells [13]. So far, it is not known whether this is a result of *E. bovis*-triggered c-IAP1 suppression, or if caspase 8 represents an additional target of parasite-induced modulation of the Fas/Fas ligand-mediated pathway of apoptosis.

Also heat shock proteins (HSP) play a role in host cell apoptosis. Certain members of the HSP27-, HSP70- and HSP90-families exhibit anti-apoptotic effects via both the receptor-mediated pathway and the inner, mitochondrial pathway [62,63]. In the current study different HSPs belonging to the HSP70 (heat shock 70 kDa protein 5, heat shock 70 kDa protein 9B) and HSP-90 (heat shock protein 90 kDa, HSP90-alpha, HSP90-beta) families were modulated in *E. bovis*-infected host cells. This might indicate an additional mechanism used by *E. bovis* to interfere with host cell apoptosis for its benefit.

Another interesting candidate related to apoptosis – besides other functions – is the voltage-dependent anion channel protein 3 (VDAC-3) which was also found down-regulated in *E. bovis*-infected host cells. VDAC-3 forms part of a mitochondrial pore which transports adenine nucleotides through the mitochondrial membrane [64]. Especially during cell stress these pores can become permeable for cytochrome *c*, the release of which is the key factor of the mitochondrial-mediated pathway of apoptosis [65]. Further studies may show whether a direct link exists between *E. bovis*-induced lowered abundance of VDAC-3 and inhibition of host cell apoptosis.

4. Conclusion

This is the first report on the modulation of the host cell proteome by an *Eimeria* species and it should be interpreted as a first step to a better understanding of global parasite–host cell interactions. The data of this investigation reflect the situation at the very end of first merogony with down-modulatory processes being most prominent. For more detailed insights into host cell regulation induced by different developmental stages of *E. bovis* further proteomic studies comprising kinetic analyses are needed. To date, data of microarray-based kinetic studies are under evaluation and comparative analyses of data resulting from both techniques will then provide useful tools to elucidate active modulation of the host cell on the level of both the *Eimeria*-specific and, in comparison to other pathogens, the protozoan- or pathogen-specific one.

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7.11“Development of *Eimeria ninakohlyakimovae in vitro* in primary and permanent cell lines”

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Development of *Eimeria ninakohlyakimovae in vitro* in primary and permanent cell lines

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ABSTRACT

Infections with *Eimeria ninakohlyakimovae* represent important coccidian diseases of goats severely affecting animal health and profitability of goat industry. For the development of suitable vaccination strategies basic research is needed for which one important prerequisite is the establishment of *in vitro* cultures guaranteeing the availability of parasitic material. Therefore, primary cell cultures [caprine, bovine and human umbilical vein endothelial cells (CUVEC, BUVEC, HUVEC)] as well as permanent cell lines [bovine foetal gastrointestinal cells (BFGC), bovine colonic epithelial cells (BCEC), African green monkey kidney cells (VERO)] were exposed to vital sporozoites of *E. ninakohlyakimovae*. The parasites invaded all different cell types used, irrespective of their origin, but further development into macromeronts and subsequent release of viable merozoites I were restricted to ruminant cells. Mature macromeronts developed in both, eudothelial (CUVEC, BUVEC) and epithelial cells (BCEC). VERO cells were non-permissive for parasite development, nevertheless sporozoites survived for 21 days p.i. within an enlarged parasitophorous vacuole. Best *in vitro* development of *E. ninakohlyakimovae* macromeronts with respect to the production of viable merozoites I was observed in BCEC, followed by BUVEC. However, the largest macromeronts developed in CUVEC. Mature macromeronts were also detected in BFGC, but these cells were less effective concerning infection rates and productivity. The complete life-cycle of *E. ninakohlyakimovae* leading to oocyst production was not accomplished in any cell type used.

In conclusion, we established suitable *in vitro* systems for the culture of *E. ninakohlyakimovae* macromeronts, e.g., for the mass production of merozoites I, for basic studies on parasite/host endothelial cell interactions or for pharmaceutical screenings.

1. Introduction

Eimeria ninakohlyakimovae is an ubiquitously spread intracellular apicomplexan enteropathogen of goats causing severe haemorrhagic typhlocolitis with up to 30% mortality rates in goat kids (Koudela and Bokova, 1998). In some areas, more than 96% of goat kids, aged 4–10 weeks, may be affected (Ruiz et al., 2006). Clinical *E. ninakohlyakimovae* coccidiosis is influenced by different factors such as the intensification of production, immune status of animals, age, and even climate conditions (Ruiz et al., 2006). Especially in rural semi-arid areas, depending economically on goat rearing, such as the Canary Islands (Spain) or other zones in Africa or the Middle East exhibiting

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analogous climate conditions, caprine coccidiosis severely affects animal health and profitability of goat industry (Ruiz et al., 2006). For such, there is a need of basic research allowing for the development of vaccines in the future.

The endogenous development of the obligate intracellular protozoan *E. ninakohlyakimovae* comprises two merogonies, the first of which results in the formation of large macromeronts (up to 166 μm × 124 μm, Vieira et al., 1997). Macromeronts specifically develop in endothelial cells of the central lymph capillaries in the villi of the distal ileum within 10–12 days p.i. and contain >100,000 merozoites I (Vieira et al., 1997). Much smaller second meronts (17 μm × 12 μm) as well as gamonts of *E. ninakohlyakimovae* develop in epithelial cells of the crypts of the caecum and colon (Vieira et al., 1997). Occasionally, meronts and oocysts of *E. ninakohlyakimovae* have also been observed in bile duct epithelial cells of the liver and were described as a cause for hepatic coccidiosis in goats (Dai et al., 1991; Mahmoud et al., 1994).

Given that *Eimeria* spp. generally are highly host-specific and that reactions induced by different *Eimeria* spp. within the same host animal are species-specific on both the immunologic (Rose, 1987) and the host cell level (Dalloul et al., 2007), it appears essential that basic research is performed on the precise *Eimeria* species in question and the respective host cell type. However, suitable *in vitro* systems, representing one important prerequisite for parasite-specific investigations, are lacking for caprine *Eimeria* spp. whilst they have already been established for avian *Eimeria* spp. (Hofmann and Raether, 1990; Augustine, 1994; Bumstead et al., 1998; Heriveau et al., 2000), murine *Eimeria* spp. (Danforth et al., 1984) and the bovine species *Eimeria bovis* (Fayer and Hammond, 1967; Hammond et al., 1969; Speer and Hammond, 1973; Speer et al., 1985; Reduker and Speer, 1986; Speer and Whitmire, 1989; Hermosilla et al., 2002). Some of these *in vitro* systems, however, use cell types which differ from the specific host cell of sporozoites *in vivo*, and may therefore only partially mirror the unique spectrum of responses. In fact, most of the pathogenic *Eimeria* spp. in ruminants (e.g. *Eimeria bovis*, *Eimeria zuernii*, *Eimeria bakuensis*, *Eimeria arloingi*, *Eimeria christenseni*, *Eimeria ninakohlyakimovae*) differ from those infecting mice or chicken with respect to sporozoite specificity for host endothelial cells *in vivo*, macromeront formation and prolonged replication time.

The goal of this investigation was to establish an *in vitro* system for the caprine pathogen *E. ninakohlyakimovae* allowing for the production of parasite material. Therefore we tested primary caprine (CUVEC), bovine (BUVEC) and human (HUVEC) umbilical vein endothelial cells as host cells for sporozoites and compared these results with data obtained in permanent cell lines of bovine foetal gastrointestinal cells (BFGC), bovine colonic epithelial cells (BCEC) and African green monkey kidney cells (VERO) with respect to first generation macromeront formation, duration of development and the production of viable merozoites I *in vitro*. Cells of ruminant origin were identified as the most suitable cell types for studies on immunology and molecular biology of *E. ninakohlyakimovae*, whilst immortalized BCEC guaranteed the highest and easiest production of merozoites I.

2. Materials and methods

2.1. Animals

Goat kids were purchased from a local farmer at the age of 1–7 days, treated with Baycox® (Bayer) and Halocur® (Intervet), assessed for parasitic infections and, when deemed parasite free, maintained under parasite-free conditions in autoclaved stainless steel cages until experimental *E. ninakohlyakimovae* infection. Goat kids were fed with milk substitute (Bacilactol, Capisa) and commercial concentrates (Starting Concentrate, Capisa). Water and sterilized hay were given *ad libitum*.

2.2. Parasite maintenance

The *E. ninakohlyakimovae* strain GC used in the present study was initially isolated in the field from naturally infected goats in Gran Canaria Islands (Spain) and maintained by passages in goat kids for oocyst production. Briefly, excreted *E. ninakohlyakimovae* oocysts were isolated from the faeces by the use of a micromanipulator (Olympus IMT-2) and suspended in 2% potassium dichromate (w/v) according to Hermosilla et al. (2002). Sporulation was achieved by leaving this suspension at room temperature. Sporulated oocysts were stored at 4 °C until further use. For oocyst production, goat kids were orally infected at the age of 4 weeks with 2×10^5 sporulated *E. ninakohlyakimovae* oocysts.

Sporozoites were excysted from sporulated oocysts and purified by the modified method of Fayer and Hammond (1967). In brief, sporulated oocysts were suspended in sterile 0.02 M L-cysteine HCL/0.2 M $NaHCO_3$ (Merck) solution and incubated in a 100% CO_2 atmosphere at 37 °C for 20 h. Subsequently, oocysts were resuspended in Hank's balanced salt solution (HBSS; Gibco) containing 0.4% (w/v) trypsin (Sigma–Aldrich) and 8% (v/v) sterile filtered caprine bile obtained from the abattoir and incubated under microscopic control up to 4 h (37 °C, 5% CO_2 atmosphere). Excysted sporozoites were washed three times (20 min,

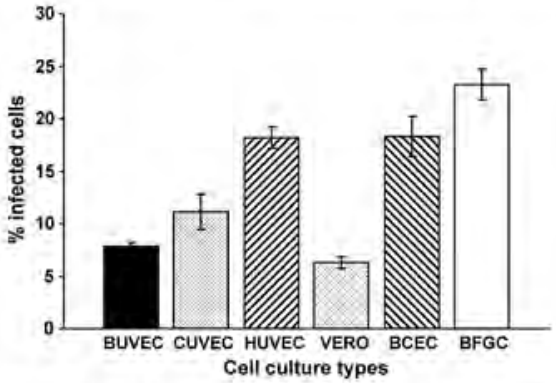
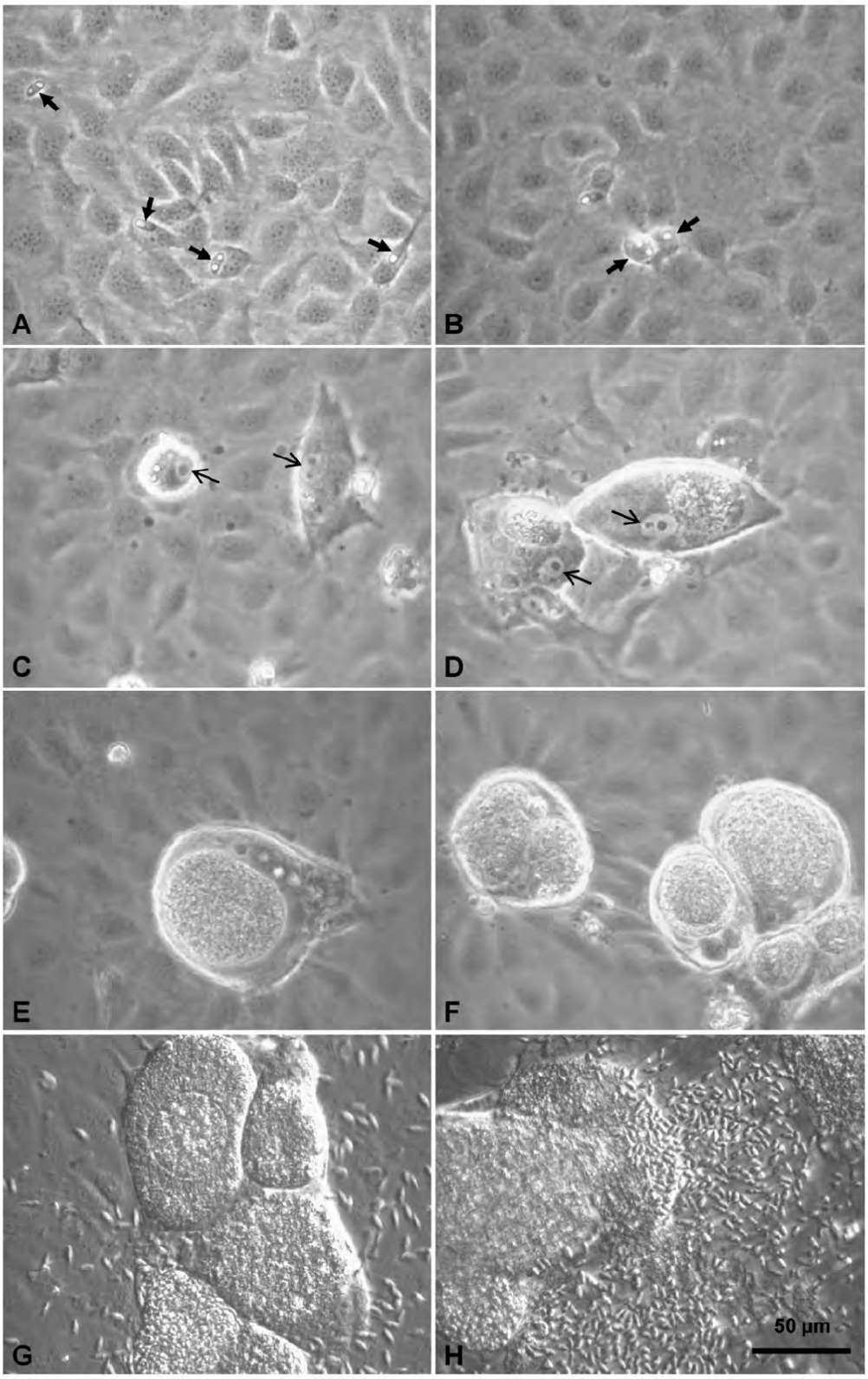


Fig. 1. Infection rates of *E. ninakohlyakimovae* sporozoite-exposed cell cultures. CUVEC, BUVEC, HUVEC, BCEC, BFGC and VERO cells (all, $n = 3$) were grown to confluence and exposed to freshly isolated *E. ninakohlyakimovae* sporozoites (3×10^5/25 cm² flask). One day after exposure the percentage of infected cells were estimated microscopically.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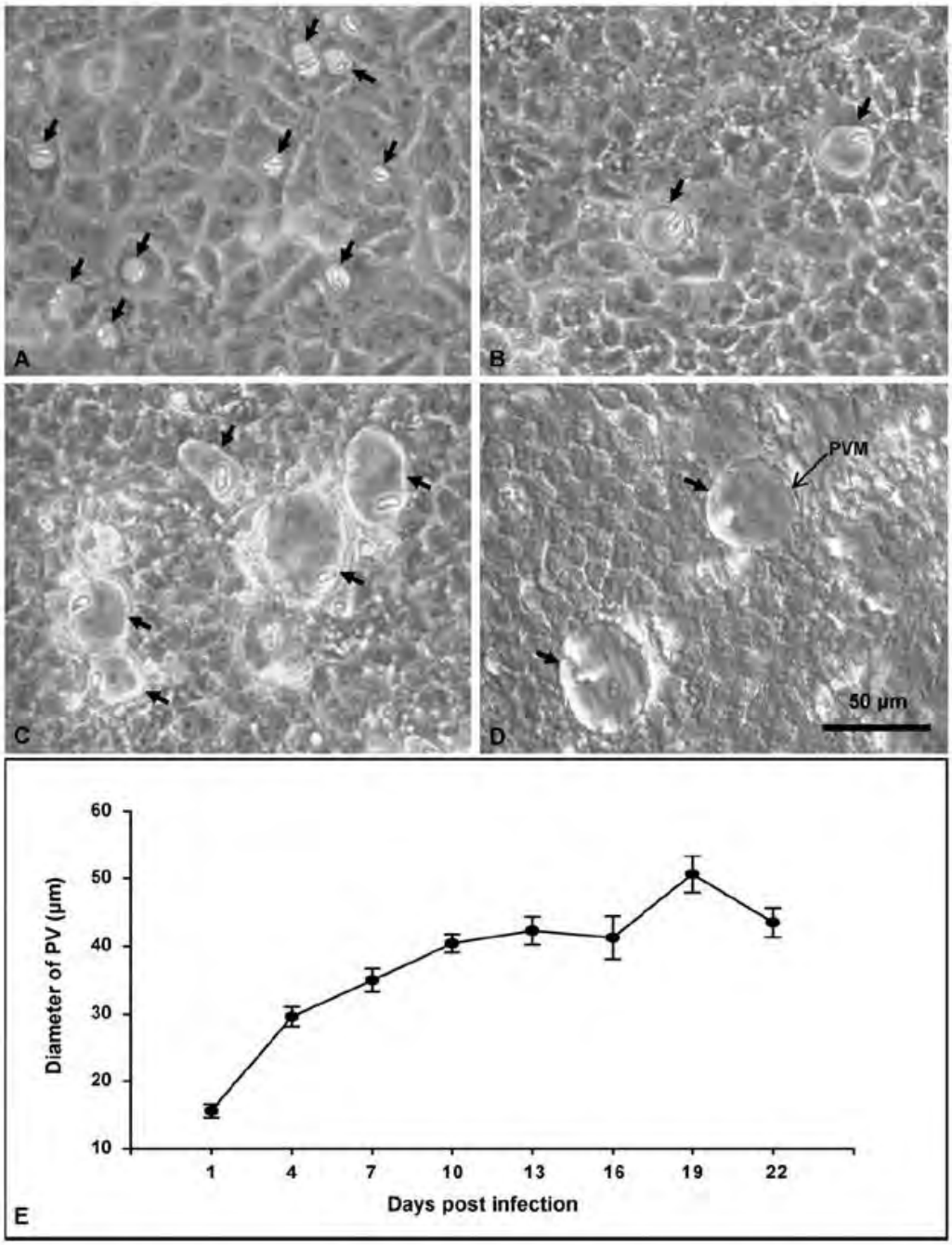

Fig. 3. Exposure of *E. ninakohlyakimovae* sporozoites to VERO cells. VERO cells were grown to confluency and exposed to freshly isolated *E. ninakohlyakimovae* sporozoites. Cell cultures were monitored daily. (A) 1 day, (B) 4 days, (C) 10 days (D) 16 days p.i., all 630× magnification, note clearly visible, enlargening parasitophorous vacuole (PV, indicated by an arrow) with parasitoporous vacuole membrane (PVM, indicated by an open arrow), (E) diameters of PV in the course of infection.

Fig. 2. Infection and development of *E. ninakohlyakimovae* in BUVEC. BUVEC were grown to confluency and infected with freshly isolated *E. ninakohlyakimovae* sporozoites. Infection and development was monitored daily for up to 22 days. (A) 1 days p.i., intracellular sporozoites are indicated by an arrow, (B) 4 days p.i., trophozoites are indicated by an arrow, (C) 7 days p.i., (D) 10 days p.i., (E) 13 days p.i., (F) 16 days p.i., (G) 19 days p.i., merozoite I release (H) 22 days p.i., merozoite I release.

1100 × g) and suspended either in tissue culture medium (1×10^5 sporozoites/ml) appropriate for the different host cell types or in tissue culture medium containing 10% dimethylsulfoxide (Merck) for cryopreservation in liquid nitrogen.

2.3. Host cells

Primary caprine (CUVEC), bovine (BUVEC) and human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins, according to the method of Jaffe et al. (1973). Umbilical cords were collected under aseptic conditions from animals born by *sectio caecaria* or from children born at the hospital (Justus Liebig University Giessen, Germany) and were kept at 4 °C in 0.9% HBSS–HEPES buffer (pH 7.4, Gibco) supplemented with 1% penicillin (500 U/ml; Sigma–Aldrich) and streptomycin (50 μg/ml; Sigma) until use. For the isolation of endothelial cells, 0.025% collagenase type II (Worthington Biochemical Corporation) suspended in PSA salt (Sigma–Aldrich) solution was infused into the lumen of the ligated umbilical vein and incubated for 20 min at 37 °C in 5% CO_2 atmosphere. After gently massaging the umbilical vein, the collagenase-cell suspension was collected and supplemented with 1 ml foetal calf serum (FCS; Gibco) in order to inactivate the collagenase. After two washings (400 × g, 10 min, 4 °C), the cells were resuspended in complete endothelial cell growth medium (ECGM; PromoCell), plated in 25 cm^2 tissue plastic culture flasks (Nunc) and kept at 37 °C in 5% CO_2 atmosphere. CUVEC, BUVEC and HUVEC were fed with complete ECGM medium (PromoCell) 1 day after isolation and, thereafter, every 2–3 days. Cells were used for infection after 1–2 passages *in vitro*. To prevent from *Mycoplasma* spp. contamination, which represents a common problem in primary caprine cell culture, CUVEC cultures were treated with Plasmocin™ (Amaxa Biosystems) from the moment of isolation onwards.

Bovine foetal gastrointestinal cells (BFGC), an immortalized primary cell line (Hermosilla et al., 2002), were grown in Iscove's modified Dulbecco culture medium (IMDM; Gibco) supplemented with 500 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS. BFGC were cultured in 25 cm^2 tissue culture flasks (Nunc) and incubated (37 °C, 5% CO_2 atmosphere) until confluency.

Bovine colonic epithelial cells (BCEC), an immortalized cell line initially isolated from bovine colonic crypts according to Föllmann et al. (2000), were kindly donated by C. Menge (Justus Liebig University Giessen). BCEC had been immortalised by lipofection with the plasmid pSVneo[3] encoding the SV40 large T antigen according to Pauly et al. (1995). BCEC were grown in IMDM medium supplemented with 500 U/ml penicillin, 50 μg/ml streptomycin and 3% FCS. Cells were cultured in 25 cm^2 tissue culture flasks and incubated until confluency.

African green monkey kidney (VERO) cells were obtained from the European Collection of Cell Cultures (ECAACC 84113001) and cultivated in RPMI 1640 culture medium (Gibco) supplemented with 2 nM L-glutamine, 500 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS until confluency.

2.4. Host cell infection and control thereof

Confluent CUVEC, BUVEC, HUVEC, BFGC, BCEC and VERO cell layers were infected with 3×10^5 freshly excysted *E. ninakohlyakimovae* sporozoites per 25 cm^2 flask. Culture medium was changed 24 h after infection and thereafter every third day. To control the long-term *E. ninakohlyakimovae*-meront I development and the release of merozoites I, infected cell cultures were analysed daily over a period of 22 days p.i. For this purpose a phase-contrast videomicroscope (DMIRB, Leica) was used.

For quantitative analyses, the total number of meront stages (immature and mature) which had developed per 25 cm^2 tissue culture flask was determined and expressed as a percentage of the numbers of sporozoites initially applied to the culture. The number of meronts was calculated over a minimum of five pictures randomly taken at 100× magnification, which represents approximately 1.5 mm^2 each. In addition, randomly selected meronts (n = 15/flask) were measured in size 10, 13, 16, 19 and 22 days p.i., using the Carl Zeiss 4.7 micro-analyzer software. Mature *E. ninakohlyakimovae* merozoites I, released into the medium were harvested at 2-day intervals as described elsewhere (Hermosilla et al., 2002) and counted in a Neubauer haemocytometer. Finally, the percentage of infected cells for each cell type was determined. For that purpose, a total of 20 pictures randomly taken at 630× magnification were carefully analyzed in order to discriminate between infected and non-infected cells.

Additionally, attempts were made to detect development of *E. ninakohlyakimovae* beyond first generation meronts. Since the sporozoite preparations were always contaminated with few unsporulated oocysts, contaminations were thoroughly quantified in the culture flasks and later taken into consideration. Furthermore, viable *E. ninakohlyakimovae* merozoites I (1×10^6/25 cm^2 tissue culture flasks) were added to CUVEC, BUVEC, BCEC and BFGC. Culture medium was changed 24 h after exposure and thereafter every third day. To control the *E. ninakohlyakimovae*-meront II and gamont development and the production of oocysts, infected cell cultures were analysed every day over a period of 1 week.

3. Results

3.1. Gliding motility of Eimeria ninakohlyakimovae sporozoites and host cell invasion

Excystation of sporozoites from sporulated oocysts was successful and resulted in viable sporozoites. After exposure to potential host cells, sporozoites showed typical movements of gliding motility on the surface of all cell types used. Sporozoites of *E. ninakohlyakimovae* invaded all cell types used, i.e., irrespective of their origin. Highest initial infection rates were observed in BFGC (23.2%), followed by BCEC (18.3%), HUVEC (18.2%), CUVEC (11.2%), BUVEC (7.9%) and VERO (6.3%) (Fig. 1). The time span from application of the sporozoites to host cell invasion varied (2–60 min) irrespective of the host cell type; most sporozoites had completed invasion after 30 min.

3.2. Early intracellular development

After invasion, intracellular sporozoites of *E. ninakohlyakimovae* were generally situated close to the nucleus (Fig. 2A). Intracellular sporozoites were relatively often observed to leave the initially invaded host cell without visibly damaging it to infect a new one within the first 48 h of infection, irrespective of the host cell type used. One day after infection sporozoites generally had changed their shape, thereby becoming shorter and slightly broader than free sporozoites. The average size of free sporozoites was 12.5 μm × 3.0 μm (n = 20), whereas intracellular sporozoites had an average size of 10.3 μm × 4.1 μm (n = 20). These morphological changes occurred irrespective of the host cell type. Multiple sporozoite infections of host cells were also observed, with some cells harbouring up to four sporozoites (data not shown).

However, not all sporozoites showed further development. In contrast, a rather high proportion of intracellular sporozoites remained as such within the host cell irrespective of the cell type used, some of them eventually egressing and re-invading other host cells later on. On days 3–4 p.i., those sporozoites that showed further development had shortened and rounded up into trophozoite stages (Fig. 2B) in all cell types used, except for VERO cells.

In VERO cells intracellular sporozoites failed to develop beyond the shortened sporozoite stage, but persisted intracellularly and survived for at least 20 days. A striking difference concerned the morphology of the parasitophorous vacuole (PV). This structure was clearly visible within two hours of infection in VERO cells and its size increased continuously for up to 19 days (Fig. 3A–D), measuring up to 50.6 ± 2.7 μm in diameter (Fig. 3E), whilst the PV remained small and often hardly detectable in all other cell types used.

3.3. Formation of macromeronts

From days 5 to 6 onwards intracellular parasite stages continuously increased in their size, developing into immature (Fig. 2C–F) and mature meronts I (Fig. 2G and H). Mature macromeronts of *E. ninakohlyakomovae* developed exclusively in cell lines of ruminant origin, i.e., in CUVEC, BUVEC, BFGC and BCEC (Supplementary Data: Fig. S1A–D). In HUVEC, occasionally small immature meronts I developed, but remained as such until the end of the observation period and never completed development into mature meronts containing merozoites I (data not shown).

The by far highest numbers of meronts I developed in BUVEC (Fig. 4) followed by BCEC. The numbers of developing meronts in BUVEC and BCEC decreased from days 13 and 16 p.i. onwards, respectively, most probably owing to the consistently observed detachment of cell containing these stages from the cell layer and the subsequent loss with the next change of medium. Generally, 5- to 15-fold more meronts I were found in BUVEC when compared to CUVEC, HUVEC or BFGC (Fig. 4). When expressed as percentage of sporozoites initially applied, a maximum of 20% of sporozoites exhibited development into macromeronts in BUVEC, whilst in CUVEC only 2% showed

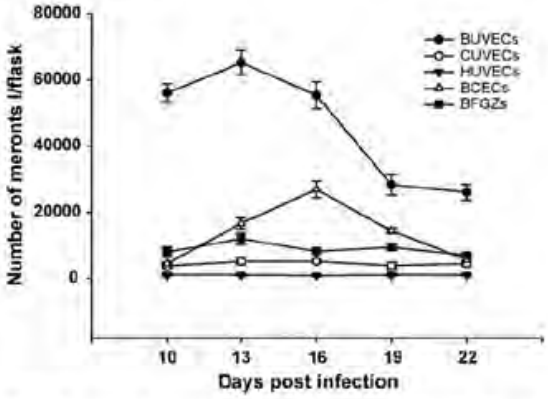

Fig. 4. Numbers of *E. ninakohlyakimovae* meronts I in different cell types. CUVEC, BUVEC, HUVEC, BCEC and BFGC (all, n = 3) were grown to confluence and exposed to freshly isolated *E. ninakohlyakimovae* sporozoites (3 × 10^5/25 cm^2 flask). The numbers of developing meronts I were estimated microscopically throughout 22 days of *in vitro* infection.

this maturation step. However, the largest macromeronts developed in CUVEC reaching 155.5 ± 10.8 μm in diameter, whilst in the other cell types meronts I were much smaller (Supplementary Data: Fig. S2). Overall, meront I maturation was not synchronous within one cell layer leading to varying meront sizes in each cell type. This observation held true for all permissive cell types used.

It is noteworthy, that macromeronts also differed in their morphology within one cell layer irrespective of the host cell type. As such, we observed macromeronts with mature merozoites I situated mainly in the periphery (Fig. 5A), clustered in rosette-like formations (Fig. 5B) or irregularly spread within the meront (Fig. 5C). In addition, meronts were either chambered (Fig. 5D) or not.

Infected host cells generally displayed a change in the morphology of the nucleus with ongoing infection. From day 7 onwards we consistently observed a hypertrophy of the nucleus (Supplementary Data: Fig. S1C) and highly decondensed chromatin structures therein (Figs. 2C–F and 4C and D). Consequently the morphology of the nucleus resembled a "fried egg" form (Supplementary Data: Fig. S1D), with mostly one but also multiple, more or less centrally situated dark dots representing the nucleolus/nucleoli and a bright, enlarged proportion of euchromatin (see Fig. 2C and D), whilst the nucleus of non-infected cells showed a rather spotted morphology (see Fig. 2A–C). These nuclear changes were consistently found in all types of host cells allowing for macromeront formation.

3.4. Release of first generation merozoites

Free first generation merozoites in cell supernatants were earliest detected in CUVEC, BUVEC and BCEC at 13 days p.i. followed by BFGC at 22 days p.i. (see Fig. 2 G and H). The number of released merozoites increased daily after their first appearance irrespective of the permissive cell type used. The total yield of merozoites I was by far the greatest in BCEC, reaching up to 40 × 10^6 merozoites I/flask

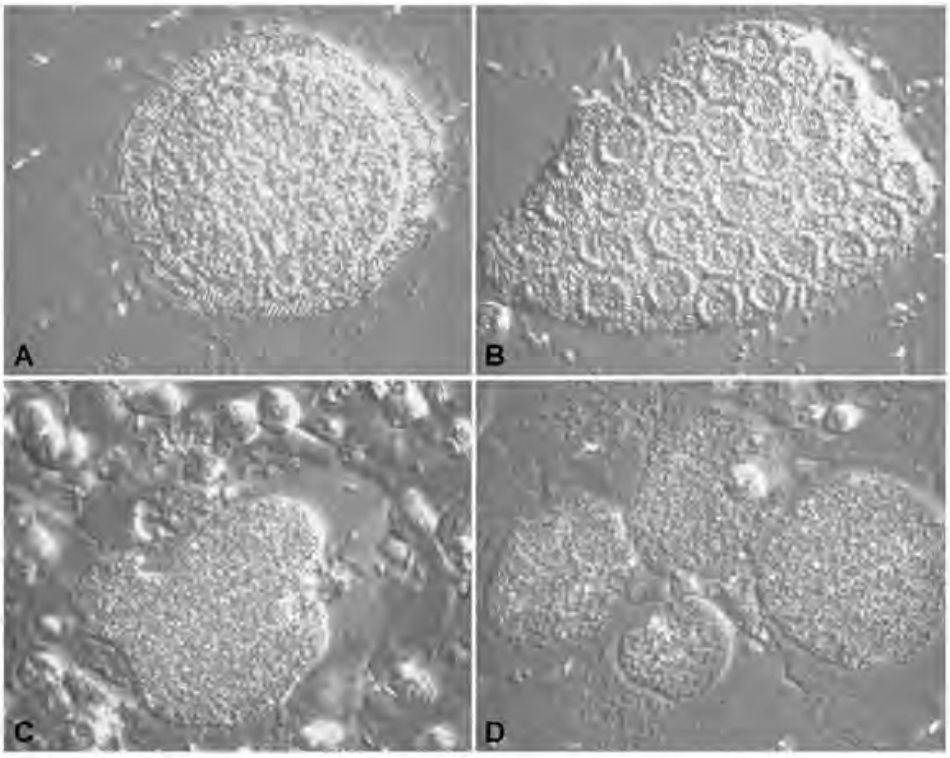

Fig. 5. Differential morphology of *E. ninakohlyakimovae* macromeronts. Host cells were grown to confluency and infected with freshly isolated *E. ninakohlyakimovae* sporozoites. Mature macromeronts showed differences in their morphology: (A) periphery-type with merozoites I assembling in a peripheral rim of the meront, (B) rosette-type with merozoites I being clustered in several rosettes within the meront, (C) non-structured-type with merozoites I diffusely spread in the meront, (D) chambered-type with clearly visible compartmentalization within the meront. (A) and (B) correspond to CUVECs and (C) and (D) to BCECs.

within 19–22 days p.i. (Fig. 6). Considerably less merozoites I were produced in all other permissive cell types used. The average number of merozoites I which developed in CUVEC and BUVEC at an infection dose of 3×10^5 sporozoites/flask during the 22-day sampling period was 1.4×10^6 and 2.9×10^6 merozoites I, respectively. In BCEC the same infection dose allowed for the production of 42.6×10^6 merozoites I whilst BFGC cultures produced only 0.14×10^6 merozoites I.

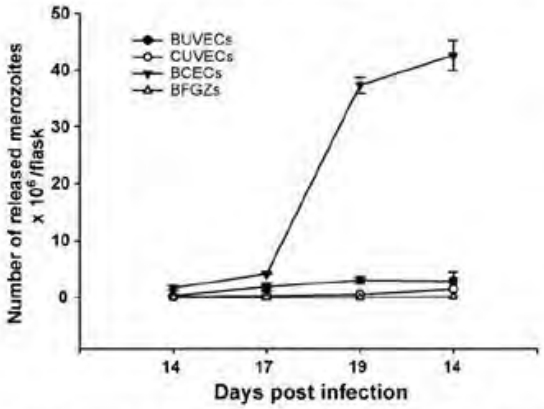

Fig. 6. Merozoite I production in different *E. ninakohlyakimovae*-infected cell cultures. CUVEC, BUVEC, BCEC and BFGC (all, $n=3$) were grown to confluence and exposed to freshly isolated *E. ninakohlyakimovae* sporozoites (3×10^5/25 cm^2 flask). The numbers of merozoites I released into the cell culture supernatant were estimated at 2-day intervals using the Neubauer haemocytometer.

3.5. Development of second generation meronts, gamonts and oocysts

Within the investigation period further endogenous development into second generation meronts, gamonts or oocysts was not observed in any of the host cell types used. In addition, infections of CUVEC, BUVEC, BCEC and BFGC cultures with viable *E. ninakohlyakimovae* merozoites I neither lead to further development nor to the completion of the life cycle of this parasite.

4. Discussion

In this investigation we successfully established *in vitro* culture systems for *E. ninakohlyakimovae* resulting the generation of merozoites I. *In vivo*, the sporozoite stage of this protozoan selectively develops in endothelial cells, mainly of the central lacteals of the ileum. Concerning the selection of suitable host cells and the invasion thereof *in vitro*, however, neither host- nor cell type-specificity was observed

since all cell types exposed to sporozoites were infected. In principle, this sporozoite behaviour was expected, as similar observations have been reported for other *Eimeria* species (Fayer and Hammond, 1967; Hermosilla et al., 2002; Behrendt et al., 2004).

Sporozoites of *E. ninakohlyakimovae* left their infected host cells and invaded a new one relatively often within the first 48 h. Considering that the ability to egress from once-infected host cells has also been reported for sporozoites of other *Eimeria* species (Danforth et al., 1992; Hermosilla et al., 2002; Behrendt et al., 2004, 2008) and that intracellular sporozoites of coccidia have frequently been found devoid of a PV in the cytoplasm shortly after invasion both *in vivo* and *in vitro* (Danforth et al., 1992; Chobotar et al., 1993), this capacity to traverse cells appears a common feature in the Eucoccidiida. Since no enhanced death of potential host cells of various cell types was observed microscopically after experimental infection, it seems that this kind of egress is not associated with severe damage to the abandoned host cell, as it was previously reported after Ca^{2+}-dependent egress of *Toxoplasma gondii* (Endo et al., 1982; Stommel et al., 1997). It rather resembles the alternative invasion process reported for *Plasmodium falciparum* (Mota et al., 2001; Mota and Rodriguez, 2001) and *E. bovis* sporozoites (Behrendt et al., 2004) which relies on invasion and egress of host cells by breaching the host cell plasma membrane followed by rapid repair. The authors speculate that this mode of migration through cells may reflect the need to cross tissue barriers to reach the final host cell. In fact, it is still unknown how sporozoites of *E. ninakohlyakimovae* cross the epithelial layer to invade the central lacteal endothelium of the ileum and this feature may explain the non-specificity of host cell invasion.

In contrast to the non-specific invasion process with respect to host cell type and origin, the development of *E. ninakohlyakimovae* macromeronts appeared rather host cell-specific as it exclusively occurred in cells of ruminant origin. Accordingly, mature meronts I were neither formed in VERO cells nor in HUVEC. However, macromeront formation was not restricted to endothelial cells but also occurred in cells of epithelial origin, i.e., in BCEC. It is noteworthy that this permanent cell line was initially isolated from bovine colonic crypt cells (Stamm et al., 2008), but, after immortalisation cells gradually seemed to change into a more macrophage-like cell type (C. Menge, personal communication). As such, the general cell transformation beyond the epithelial character may have facilitated macromeront development. As described for *E. bovis* (Hermosilla et al., 2002), *E. ninakohlyakimovae* macromeronts also developed in the permanent BFGC, which represent a mixture of cells initially isolated from bovine foetal gut tissue. Endothelium-specific staining of this cell line with DiI-Ac-LDL (Taubert, unpublished data) proved a certain content of endothelial cells, probably enabling the parasite's development.

Mature *E. ninakohlyakimovae* macromeronts displayed different morphologies even within one cell layer, irrespective of the type of permissive host cells. Besides varying sizes we also found morphological differences in the sense of the periphery-, rosette- and non-structured type of macromeronts. Identical phenomena were observed in *E. bovis in vitro* culture (personal observation) but the cellular basis of this phenomenon is not yet understood. Further studies should be done to determine whether these different shapes are artifacts of cell cultures or are also present in the host.

We consistently observed a change in the morphology of the nucleus of infected cells. Earliest at 7 days p.i., which most possibly marks the onset of proliferation, the nucleus morphology changed from a more spotted one to a phenotype we called "fried-egg"-type, i.e., the proportion of light-coloured euchromatin increased and was spread over almost the whole area of the nucleus, whilst the nucleoli coalesced and grew to form either single or multiple large nucleoli. Since the nucleolus is the site for the processing of rRNAs and assembly into ribosome subunits, the morphological changes of the nucleoli indicate cells producing large amounts of protein. In addition, the increased transition of heterochromatin to euchromatin indicates a transcriptional active host cell. Since we never observed cell division of *E. ninakohlyakimovae* infected host cells, it appears likely that these features are parasite-induced. Comparable observations are reported for host cells carrying *E. bovis* macromeronts and the transcriptional active status of those cells is furthermore underlined by the increasing numbers of modulated mRNAs as measured by microarrays (Taubert, unpublished data). In the prolonged maturation phase we also observed a hyperplasia of the nucleus which may also suit the latter hypothesis. However, this feature was only visible in a proportion of macromeront-carrying host cells and needs further investigation, especially on the level of transmission electron microscopy.

Accessibility of macromeronts of *E. ninakohlyakimovae in vitro* is of particular scientific interest for various reasons: (i) Enduring *E. ninakohlyakimovae* macromeront maturation within a highly immunoreactive host endothelial cell strongly suggests that the parasite must rely on several regulatory processes in order to guarantee this massive replication. Consequently it was shown that *E. bovis* modulates the host cell cytoskeleton (Hermosilla et al., 2008), inhibits its host cell apoptosis (Lang et al., 2009) and down-regulates pro-inflammatory reactions of host cells (Hermosilla et al., 2006). (ii) There is some evidence that first generation meront stages represent major targets for protective immune reactions in animals immune to *Eimeria* spp. (Rose et al., 1992; Shi et al., 2000, 2001; Taubert et al., 2008). (iii) Mature meront I cultures should be useful for pharmaceutical screenings and (iv) Guarantee accessibility of merozoites I, which may be needed for various applications, such as specific parasite preparations. Especially referring to the last item we established a suitable *in vitro* system for merozoite I mass production by using BCEC cultures, which, as a permanent cell line, exhibit clear advantages compared to primary cells considering the significant lower effort of work and economical aspects of this cell culture.

Overall and compared to other life stock, the goat is an often neglected animal with respect to basic and clinic research, although in certain climatic areas it represents the only reliable and economically viable source

of meat and milk. Yet upcoming foundations supporting research on caprine infection (e.g., CAPARA of the COST action FA0805) now pay tribute to the general value of this particular animal. Accordingly, the establishment of *E. ninakohlyakimovae in vitro*-cultures should deliver a valuable basic tool for future research on caprine coccidiosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.05.023.

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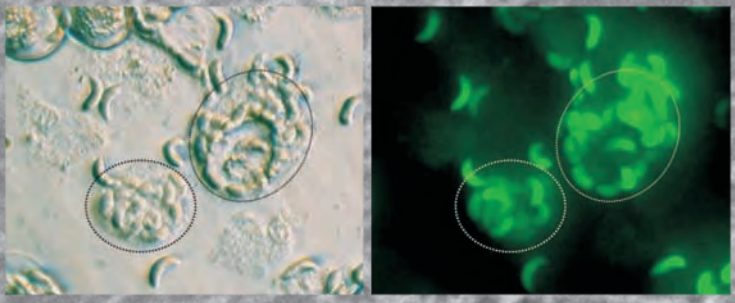

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