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

Anja Taubert [a,*], Carlos Hermosilla [b], Anke Sühwold [a], Horst Zahner [a]

[a] *Institute of Parasitology, Justus Liebig University Giessen, Rudolf-Buchheim-Str. 2, D-35392 Giessen, Germany*
[b] *Royal Veterinary College, Department of Pathology and Infectious Diseases, Hawkshead Lane, Hatfield AL9 7TA, UK*

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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,

* Corresponding author. Tel.: +49 641 9938475; fax: +49 641 9938469.
E-mail address: Anja.Taubert@vetmed.uni-giessen.de (A. Taubert).

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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. bakuensis*, *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 γδ-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®,