

Downregulation of the goat β -defensin-2 gene by IL-4 in caprine intestinal epithelial cells infected with *Eimeria* spp.

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Abstract Defensins are antimicrobial peptides that are produced by leukocytes and epithelial cells. Recent advances indicate that these peptides play an important role in innate immune responses. Nonetheless, the role of defensins in caprine eimeriosis remains unknown. Therefore, this study investigated the expression of a goat β -defensin, named GBD-2 in caprine intestinal epithelial cells (CIEC) stimulated with recombinant bovine interferon-gamma (IFN- γ) in the presence or absence of recombinant bovine interleukin-4 (IL-4) by a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. GBD-2 mRNA was clearly expressed in IFN- γ -stimulated CIEC. On the other hand, the direct addition of IL-4 showed no significant effect on GBD-2 expression in CIEC. However, when supernatants from peripheral blood mononuclear cells (PBMC) cultured with IL-4 were added to CIEC, the expression of GBD-2 decreased. To elucidate if IFN- γ functions as a signaling molecule that facilitates the generation of GBD-2 against *Eimeria* spp. in goats, anti-IL-4 was added to PBMC from *Eimeria*-infected goats and levels of IFN- γ in culture supernatants were determined by an enzyme-linked immunosorbent assay test. Results showed that IFN- γ secretion increased when anti-IL-4 was added to PBMC. It then appears safe to suggest that IL-4 may be a further factor in the pathogenesis of goat coccidiosis and its induction may be part of an evasion strategy of the parasite to avoid pro-inflammatory responses.

Introduction

Goat coccidiosis is caused by the species of the genus *Eimeria* and is one of the most common enteric diseases of goats (Agyei et al. 2004). Goat kids are very susceptible to *Eimeria* infections (Penzhorn et al. 1994). Coccidiosis in goats is an important disease caused by high mortality rates and decrease in production of milk, hair, or meat, and it is most frequently caused by *Eimeria ninakohlyakimovae*, *Eimeria arloingi*, and *Eimeria caprina* (Koudela and Boková 1998). Recently, there have been several studies regarding the importance of antimicrobial peptides and proteins as components of innate immunity against microscopic pathogens. Defensins comprise a major subclass of the family of antimicrobial peptides. Depending on their size and pairing of their cysteine residues, defensins of higher vertebrates are classified as α -, β -, and θ -defensins (Zhao et al. 1999). The α and β defensins have been thoroughly studied and it has been shown that they are expressed in the gastrointestinal tract mostly by epithelial cells (Cunliffe and Mahida 2004). All characterized β -defensins have broad-spectrum antimicrobial activity. The induction of the expression of β -defensins near sites of inflammation in vivo supports a role for β -defensins in mucosal host defense (Schonwetter et al. 1995). Homologs of bovine β -defensins have been identified in chickens (Harwig et al. 1994), mice (Huttner et al. 1997), and humans (Huttner and Bevins 1999). Zhao et al. (1999) identified two β -defensin precursors in goats, preproGBD-1, expressed mainly in the tongue and respiratory tract, and preproGBD-2, expressed throughout the intestine, where coccidian parasites are mainly located in infected goats. Luenser et al. (2005) studied the molecular evolution and variability of β -defensins of goats and sheep by sequence analysis of defensin introns, and results showed 13 discrete β -defensin coding sequences.

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It has been generally accepted that protection against protozoan parasites is frequently associated with a response of activated T-helper-1 (Th1) cells (Steinfeldt et al. 2005), which produce mainly interleukin 2 (IL-2) and interferon gamma (IFN- γ). On the other hand, Th2 cells produce other cytokines like IL-4 and IL-10, which are not associated with a protective response to challenge. Several studies have reported cytokines secreted during *Eimeria* spp. infection (Rose et al. 1989; Wakelin et al. 1993; Ovington et al. 1995; Hériveau et al. 2000; Lillehoj et al. 2004). Furthermore, it has been demonstrated that human β -defensin 2 induces or upregulates a number of cytokines involved in the adaptive immune response (Boniotto et al. 2006) and, the other way around, that cytokines are transcriptional inducers or upregulate human β -defensins (Varoga et al. 2006; Bajaj-Elliott et al. 2002). There is a paucity of information about the role and regulation of the expression of the intestinal GBD-2 gene in goat coccidiosis and, in particular, if cytokines modulate its expression. In the present study, the expression of mRNA GBD-2 was studied by a reverse transcriptase-polymerase chain reaction (RT-PCR) assay in intestinal epithelial cells infected with *Eimeria* spp. Since previous studies have demonstrated that cytokines provide a stimulus that may upregulate defensins, this study investigated the effect of exposure to IL-4 and IFN- γ on GBD-2 expression by intestinal epithelial cultured cells.

Materials and methods

Parasites

Eimeria oocysts were obtained from fecal samples collected from a naturally infected herd with a history of goat coccidiosis. Fecal samples were concentrated and examined using Sheather's sugar-flotation technique and *Eimeria* species were determined after sporulation of feces in 2.5% potassium dichromate for 2 weeks at 25°C. *Eimeria* species were identified according to the descriptions of Norton (1986), with *E. ninakohlyakimovae* and *E. caprina* being the most frequent species. Measurements were performed in a light microscope using a micrometer scale in the eyepiece. Sporozoites were excysted as previously described (Hermosilla et al. 2002), collected, and suspended at concentrations of $2.5 \times 10^5/500 \mu\text{l}$ in complete endothelial cell growth medium (BD™).

Cell culture

Caprine intestinal epithelial cells (CIEC) were isolated based on the protocol by Hashim et al. (2004). Briefly, goat intestinal tissue was removed from dairy goats immediately after slaughter. Tissue was placed in RPMI 1640 (Sigma)

supplemented with fetal bovine serum (FBS), streptomycin, penicillin, and amphotericin B (Sigma). Once in the laboratory, cells were placed in Hanks balanced salt solution (Sigma) with ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (Sigma) and incubated at 37°C for 10 min with vigorous shaking. Supernatant was removed and tissue was placed in RPMI 1640 containing collagenase (Sigma), incubated at 37°C for 15 min with vigorous shaking. Supernatant was removed; cells were centrifuged at $800 \times g$ for 5 min and resuspended in RPMI 1640 with FBS and collagenase. Repetitions of this step were performed until cell isolation was completed. Cells were placed in Costar culture plates on 13 mm plastic coverslips with Dulbecco modified Eagle medium-Ham's F-12 plus 10% (vol/vol) FBS, 8 $\mu\text{g/ml}$ insulin, 10 $\mu\text{g/ml}$ gentamycin/ml, 50 $\mu\text{g/ml}$ hydrocortisone, 100 $\mu\text{g/ml}$ streptomycin, 100 U/ml penicillin, and 2.5 $\mu\text{g/ml}$ of amphotericin B. Cells were grown for 24 to 48 h at 37°C in a 5% CO₂–95% air-humidified incubator.

Infection of caprine intestinal epithelial cells

After removing maintenance medium from the cells, 2.5×10^5 *Eimeria* sporozoites were added to them. After incubation for 3 h, infected cells were washed with phosphate-buffered saline (PBS) to remove unexcysted oocysts and toxic materials. Five hundred microliters of growth medium supplemented with 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin was added. Infected CIEC were harvested 1 and 6 h after sporozoite inoculation (ASI). Thereafter, harvesting was performed everyday for 2 weeks ASI. Uninfected CIEC were examined for each time-point as negative controls.

Ribonucleic acid preparation

Total ribonucleic acid (RNA) was extracted from cells using a total RNA isolation reagent (TRIZOL reagent; Invitrogen) according to manufacturer's protocol. The RNA preparation was dissolved in 50–100 μl RNase-free water depending on the size of pellet. Digestion of genomic DNA possibly contaminating RNA samples was performed by using DNase I (Gibco) before reverse transcription for some samples. The concentration and purity of RNA samples were spectrophotometrically determined. RNA was reverse-transcribed to cDNA using the oligo(dT) primer (Sigma-Aldrich), and reverse transcriptase products were amplified using a PCR kit (Sigma-Aldrich) and a thermal cycler (Applied Biosystems). According to Zhao et al. (1999), to detect GBD-2, the sense primer 5'-ACTCAAGGAATAATAATCA-3', corresponding to nucleotides 81 to 100, and the antisense primer 5'-CATTTTACTGGGGGCCCGTG-3', complementary to nucleotides 177 to 196 of the GBD-2 cDNA

sequence, were used. Primer for the housekeeping gene β -actin (Applied Biosystems) was 5'-CGTGGCCATC CAGGCTGTGCTGTCC-3', and antisense primer, 5'-GCGATGCCAGGGTACATGGTGGTCC-3' (Zhao et al. 1999). Changes in gene expression were reported as fold increases relative to uninfected cells. Thermal cycling conditions were a 5-min denaturation step at 96°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 3 min. PCR products were verified by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet light. Band intensities were estimated using Image J 1.37 software (National Institutes of Health, USA). All experiments were performed in triplicate.

Cytokines

Recombinant bovine interferon-gamma, recombinant bovine interleukin-4, and mouse anti-bovine interferon-gamma were from Serotec.

Cytokine stimulation

For cytokine stimulation experiments, CIEC were obtained, cultured, and infected as mentioned previously and the culture was maintained in serum-free media for 16 h before the addition of IFN- γ at 200 U/ml and stimulated for 24 h in the presence or absence of 50 ng/ml IL-4 (Nomura et al. 2003). The indirect effect of IL-4 on GBD-2 expression was determined by adding the culture supernatant from peripheral blood mononuclear cells (PBMC) in the presence or absence of IL-4 to CIEC. RNA was isolated according to the manufacturer's instructions.

Isolation of peripheral blood mononuclear cells and effect of IL-4 on GBD-2 expression

Heparinized blood (5 ml) was taken from goats isolated from the herd at birth and fed with a milk substitute. These animals tested coproscopically negative for *Eimeria* spp. Blood was diluted 1:2 in PBS and 10 ml of diluted blood was subject to density gradient centrifugation over Histo-paque-1077 (Sigma) according to the manufacturer's instructions. PBMC were collected and washed with phosphate-buffered saline. PBMC were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin (Sigma), 100 μ g/ml streptomycin (Sigma), in the presence of 10 μ g/ml brefeldin A (Sigma), 1 μ g/ml ionomycin (Sigma), and 20 ng/ml phorbol-12-myristate-13-acetate (Sigma) (Pedersen et al. 2002). PBMC were seeded at 1×10^6 cells/ml in 24-well plates (Nunclon) in the presence or absence of 50 ng/ml of IL-4 and cultured

for 24 h. The supernatant was collected after the incubation and added to cultured caprine intestinal epithelial cells.

Determination of IFN- γ levels in whole blood by an enzyme-linked immunosorbent assay

Blood was collected from *Eimeria*-infected goats and uninfected controls diagnosed by means of coproparasitological examinations. PBMC were isolated from heparinized blood and cultured for 24 h, at which time 1 μ g/ml of mouse anti-bovine IL-4 was added to the PBMC and IFN- γ levels in the culture supernatants were determined using a commercial enzyme-linked immunosorbent assay (ELISA)-based kit (Bovigam, CSL) within a single lot. Optical densities of kit standards and test samples were read at 450 nm using an ELISA plate reader (Fisher). Positive and negative controls were provided with the kit. PBMC without anti-bovine IL-4 were included in this assay as controls.

Statistical analysis

Comparison of GBD-2 mRNA expression among the uninfected, infected, and cytokine-stimulated cells was performed using the Mann–Whitney *U* test.

Results

GBD-2 mRNA expression in caprine intestinal epithelial cells

Figure 1 shows that GBD-2 mRNA expression was undetectable in non-stimulated CIEC, independent if they had been infected or not with *Eimeria* sporozoites. However, when IFN- γ was added to infected cells, GBD-2 mRNA was significantly expressed ($p < 0.05$) as from

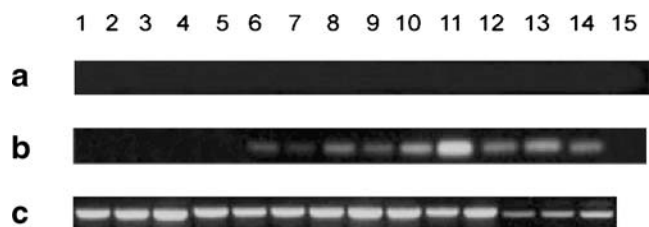


Fig. 1 The effect of *Eimeria* infection on GBD-2 expression in caprine intestinal epithelial cells was measured by a RT-PCR assay. **a** Expression of GBD-2 mRNA was not observed in IFN- γ -untreated cells (lanes 1–14, days after culture infection; lane 15, negative control). **b** RT-PCR analysis demonstrated that IFN- γ upregulated GBD-2 expression as from 6 days after sporozoite inoculation. No expression of GBD-2 mRNA was observed at early culture times (lanes 1–14, days after culture infection; lane 15, negative control). **c** A housekeeping gene, β -actin, was used as an internal standard. Lanes 1–14, days after culture infection

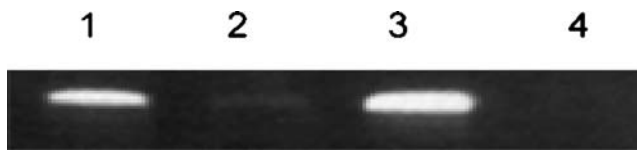


Fig. 2 Lane 1, RT-PCR analysis revealed that supernatants from PBMC in the absence of IL-4 increased the expression of GBD-2. Lane 2, when IL-4 was added to cultured cells, the expression of GBD-2 significantly decreased. Lane 3, β -actin was used as an internal standard. Lane 4, negative control

day 6 after infection. When IL-4 was directly added to CIEC, no significant increase or reduction ($p > 0.05$) of the GBD-2 mRNA expression levels was observed (data not shown). Samples of the β -actin gene were all clearly visualized. On the other hand, Fig. 2 shows that, in the absence of IL-4, supernatants from PBMC significantly increased ($p < 0.05$) the expression of GBD-2, whereas in the presence of IL-4, PBMC supernatants strongly reduced ($p < 0.05$) the expression of GBD-2.

Interleukin-4 effect on the interferon-gamma response

To investigate the possibility that the downregulatory activity of IL-4 on GBD-2 expression might be due to a decrease of cytokines produced in a Th1-type response, namely, a reduction in the secretion of IFN- γ in the presence of IL-4, anti-IL-4-treated-PBMC collected from *Eimeria*-naturally infected goats and uninfected controls were assayed for detecting IFN- γ secretion levels by an

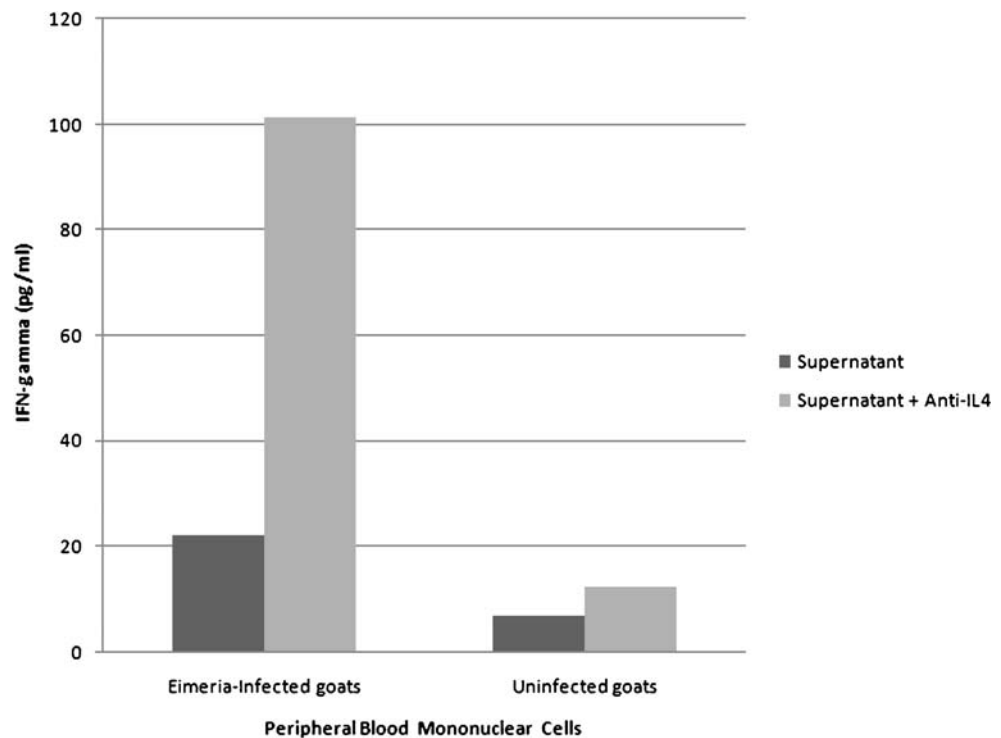
ELISA test in culture supernatants. Figure 3 illustrates that anti-IL-4-treated PBMC released significantly ($p < 0.05$) higher levels of IFN- γ to the supernatant, in contrast to untreated PBMC.

Discussion

Defensins and cathelicidins are the most dominant antimicrobial peptides that are found in neutrophils and epithelia as components of the early host defenses of mammals against infection. These peptides have potent microbicidal activity against prokaryotic and eukaryotic pathogens as well as viruses, including protozoans like *Leishmania* (Kulkarni et al. 2006), *Cryptosporidium* (Zaalouk et al. 2004), and *Giardia* (Eckmann 2003). The expression of β -defensins may be induced by infection and inflammation (Cunliffe and Mahida 2004), for example, the levels of an enteric β -defensin mRNA are elevated in calves infected with *Cryptosporidium parvum* (Tarver et al. 1998). However, to the best of our knowledge, there are no studies exploring the significance of defensins and their regulation by cytokines in the pathogenesis of goat coccidiosis. In this study, the expression of GBD-2 mRNA was assessed by infecting CIEC with *Eimeria* sporozoites. Nonetheless, this study failed to detect GBD-2 expression by RT-PCR in these cells.

In previous studies, IFN- γ , IL-1 β , and TNF- have been shown to upregulate the expression of human β -defensins (Nomura et al. 2003; O'Neil et al. 1999). Bajaj-Elliott et al.

Fig. 3 Supernatants from cultured peripheral blood mononuclear cells, infected or not with *Eimeria* spp., were harvested and analyzed for IFN- γ secretion levels by an ELISA test. IFN- γ concentrations significantly increased ($p < 0.05$) in supernatants from PBMC treated with anti-IL-4 (gray), in contrast to supernatants from PBMC without the antibody (black). Results shown represent the means of cultures and are representative of three experiments performed



(2002) reported that IFN- γ increases defensin induction in intestinal epithelium, although these referred studies were carried out focusing on the antibacterial properties of defensins. Other studies have shown that IFN- γ plays an important role in the immune response against *Eimeria* spp. despite the fact that it does not have a role in protection against a secondary infection in animal models of eimeriosis (Steinfeldt et al. 2005). However, it does inhibit *E. tenella* development in vitro (Lillehoj et al. 2004). In the present study, CIEC infected with *Eimeria* spp. were stimulated with recombinant IFN- γ , and GBD-2 expression significantly increased, thus helping confirm that IFN- γ increases defensin expression in goat intestinal epithelial cells. Nevertheless, GBD-2 was not expressed until 6 days postinfection.

This finding suggests that the expression of GBD-2 mRNA in CIEC might be related to the time it takes for the protozoan to accomplish a certain developmental stage. A recombinant IL-4 was also included in this study to assess the role played by a cytokine of the Th2-type response on the induction of antimicrobial peptides in goat coccidiosis. In this study, it was shown that the direct addition of IL-4 to CIEC failed to significantly induce or reduce GBD-2 mRNA expression levels, suggesting that IL-4 does not act directly on intestinal GBD-2 expression. However, when supernatants of PBMC cultured in the presence of IL-4 were added to CIEC, the expression of GBD-2 mRNA decreased. A stimulatory activity of IFN- γ on GBD-2 induction was observed in this study, so we envisaged that the reduction of GBD-2 expression could be due to a reduction of the IFN- γ secretion levels.

Previous studies have demonstrated that IL-4 enhances some T-cell functions but also suppresses many monocyte responses such as expression and secretion of TNF- α , PGE₂, and IL-6 (Hart et al. 1991). Moreover, the present study showed that the indirect addition of IL-4 to CIEC downregulates GBD-2 expression. Hence, to address the hypothesis that IFN- γ may function as a signaling molecule that facilitates the generation of GBD-2 and that IL-4 reduces its stimulatory activity, an antibody against IL-4 was added to PBMC isolated from normal and *Eimeria*-infected goats to determine IFN- γ concentrations by ELISA in culture supernatants. It was demonstrated that the addition of a neutralizing antibody against IL-4 increased IFN- γ secretion levels in PBMC culture supernatants.

To the best of our knowledge, this study is the first to identify that intestinal epithelial cells are able to recognize *Eimeria* spp. and trigger an inflammatory response that aims at clearing the pathogen. However, the production of IL-4 is thought to contribute in part to the pathogenesis of *Eimeria* infections in goats as it downregulates the secretion of IFN- γ and therefore the production of GBD-2. The induction of IL-4 and the inappropriate expression of

a defensin in intestinal cells appear to play major roles in the pathogenesis of goat eimeriosis; perhaps this might be part of a particular evasion strategy of *Eimeria* parasites to avoid immune or inflammatory responses and persist a longer time within the host. Future studies are suggested to focus on the interaction of several cytokines with protective mucosal factors in coccidiosis.

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