Veterinary Immunology and Immunopathology 144 (2011) 423–429

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Parasite limiting macrophages promote IL-17 secretion in naive bovine CD4$^+$ T-cells during *Neospora caninum* infection

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ARTICLE INFO

Article history:
Received 23 July 2011
Received in revised form 7 September 2011
Accepted 24 September 2011

Keywords:
Neospora caninum
IL-17
IFN-γ
Macrophage
Bovine
T-cell

ABSTRACT

Neospora caninum infects bovine hosts giving rise to pro-inflammatory immune responses that can result in foetal death or spontaneous abortion, this appears to be mediated by the actions of IFN-γ on cell activation and migration/trafficking. Yet successful vaccination or natural immunity is also strongly correlated with IFN-γ production. We utilised *in vitro* infection of bovine macrophages to prime naive T-cell responses. Naive T-cells in contact with infected macrophages produce both IFN-γ and IL-17 in a pattern that is dependent on whether the priming macrophage was protected or non-protected. Our results may explain the apparent dual role of IFN-γ during infection if a second major pro-inflammatory cytokine, IL-17, is produced simultaneously.

1. Introduction

Tachyzoites of the obligate intracellular protozoan *Neospora caninum* can cause accidental infection of bovine hosts resulting in further tachyzoite replication via either vertical or horizontal transmission. A pregnant heifer that becomes infected for the first time, or undergoes parasite recrudescence, during pregnancy will infect her unborn calf, and may give birth to a still born calf or abort the pregnancy (Williams et al., 2009). The timing during pregnancy when these events take place determines the outcomes. Experimental bovine infection produces a proto-typical type-1 (Th1) immune response that is characterised by IFN-γ production and CD4$^+$ parasite specific T-cells. Examination of the maternal tissues during pregnancy reveals that infection provokes a strong inflammatory response as demonstrated by high levels of interleukin (IL)-2, TNF-α, IL-18 and IL-12p40. Interestingly levels of IL-4 and IL-10 were also raised but not TGF-β1. This is highly suggestive of the presence of regulatory and type-2 (Th2) cytokine-secreting cells (Rosbottom et al., 2011). The production of IFN-γ during infection has been noted both at the local maternal and foetal tissue level but also in the periphery. Proliferation of peripheral CD4$^+$ cells and production of IFN-γ in response to a variety of antigens has been documented (Marks et al., 1998) (Williams et al., 2000). Profound increases in IFN-γ have also been noted in the maternal tissues during pregnancy both at the mRNA and protein levels (Rosbottom et al., 2011). The role IFN-γ plays during infection appears to be a complex one. A previous study has indicated it may be a potential mechanism of protection against abortion (Lopez-Gatius et al., 2007). In animals subjected to experimental live immunisation and subsequently challenged – protection in terms of foetal deaths was fully achieved, and it is of note that these animals also had strong antigen-specific IFN-γ responses in comparison with controls (Williams et al., 2007). Conversely there are a number of studies which suggest that IFN-γ may also mediate the pathological consequences of *N. caninum* infection. The upregulation of IFN-γ early in gestation was found to correlate with foetal death in two separate studies (Andrianarivo et al., 2000; Rosbottom et al., 2008). This conflicting role for

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doi:10.1016/j.vetimm.2011.09.008

IFN-γ may arise from a number of factors including timing of infection and foetal immunocompetence. A second factor that could affect the overall actions of IFN-γ may be the production of (an) other pro-inflammatory mediator(s). Multiple cytokines working synergistically may result in the pathology associated with infection, also the kinetics of cytokine production may change according to parasitemia and gestational period. Recent advances have led to the characterisation of a new distinct population of T-helper cells termed Th17 cells, distinguished by the production of the cytokines IL-17A, IL-17F, IL-22, and IL-23. Furthermore, they have a distinct pattern of chemokine and cytokine receptors on their surface, including the IL-23R and CCR6, and their activities have been ascribed roles in a number of infectious and non-infectious settings including arthritis and *Bordetella pertussis* infection (Weaver et al., 2007). Various members of the IL-17 family have been found in domestic animal species including IL-25 in sheep (Gossner et al., 2011), IL-17A in the horse (Tompkins et al., 2010) and IL-17 in chickens (Crhanova et al., 2011). Additionally the Th17 cells stabilising cytokine IL-23 has been also found in horses (Tompkins et al., 2010). Evidence from the closely related *Toxoplasma gondii* suggests that interplay of Th1 and Th17 type responses can give rise to the overall pathology seen during infection, during oral infection a strong Th1 response can develop leading to severe gut pathology (Oldenhove et al., 2009), while neurological inflammation caused by chronic infection was mediated by IL-17 (Stumhofer et al., 2006). This is further complicated by the fact that the IL-17 and IFN-γ promoting cytokines IL-23 (IL-12p40 and IL-23p19) and IL-12 (IL-12p40 and IL-12p35), respectively, share the cytokine subunit, IL-12p40. Infection of mice lacking either IL-12p35 or IL-23p19 showed that while both contributed to resistance against infection, only IL-12 (IL-12p35) could mediate long lasting protective immunity (Lieberman et al., 2004). It is this dichotomy of cytokine production that we have examined during *N. caninum* infection. Using a model of *in vitro* infection of primary bovine macrophages and these cells were used to stimulate cytokine production in autologous naive T-cells. Using LPS or soluble *N. caninum* antigen (SNcAg) to prime cells, whereby macrophages could restrain parasite replication, we found an explanation for the apparent dual role of IFN-γ in that IL-17 might also be induced in a system or host where restraint of parasite growth was mediated by macrophages.

2. Materials and methods

2.1. Cell isolation, parasite culture and in vitro infection

Isolation of primary bovine cells was conducted using blood samples obtained from healthy donor animals; PBMCs were purified over Ficoll–Histopaque (Sigma–Aldrich). $CD14^+$ cells, monocytes, were isolated by use of Miltenyi human CD14 microbeads, for this process direct labelling of cells with magnetic beads coated with anti-human CD14 was carried out as per manufacturer's instructions and has previously been shown to effectively isolate bovine monocytes (Crhanova et al., 2011). In direct labelling was used to purify $CD4^+$ T-cells, briefly cells were incubated with a bovine specific anti-CD4 antibody (Serotec; Clone CC30, mouse IgG1) for 20 min at 4 °C in PBS–1%BSA, cells were then washed and labelled with secondary Miltenyi magnetic beads coated with anti-mouse IgG as per manufacturer's instructions. Labelled cells were isolated using the positive selection program of an AutoMACS separator (Miltenyi Biotec) and collected in tubes containing PBS–1%BSA. A second purification step involving labelling cells with anti-CD62L (Clone CC32), coupled with anti-mouse IgG microbeads, selected naive T-cells only. Purity of $CD14^+$ cell fractions were assessed on the basis of monocyte morphology following cytospinning and Giemsa staining. $CD4^+$ T-cells were checked for purity by immunocytochemistry on cytospins using unconjugated mouse anti-bovine CD4 (Serotec; Clone CC8) detection was performed using goat anti-mouse IgG:HRP. Average purity was 98% and 99% for monocytes and $CD4^+$ T-cells, respectively.

Monocytes and $CD4^+$ T-cells were cultured in complete RPMI 1640 with 200 U/ml penicillin, 200 μg/ml streptomycin, 10% heat-inactivated foetal calf serum and 1% non-essential amino acids (all Sigma–Aldrich). Monocytes were cultured in 6-well plates for 7 days to generate monocyte-derived macrophages (MΦ), with media changes every two days. For priming of MΦ, cells were stimulated with 100 ng/ml LPS (Sigma–Aldrich) or 20 μg/ml SNcAg, prepared as described elsewhere (Zintl et al., 2006), for 24 h. To inhibit NF-κB activity in MΦ the specific inhibitor Parthenolide, 10 μM (Sigma–Aldrich) was added to cells for 30 min prior to priming.

N. caninum tachyzoites (Liverpool Strain), originally provided by Prof. Diana Williams (University of Liverpool), were maintained by infection of confluent monolayers of VERO cells as described elsewhere (Zintl et al., 2006). MΦ, following treatment as described above, were then infected with *N. caninum* at a MOI of 1. Cultures were assessed 24 h later either for parasite number/replication or supernatants were collected for cytokine measurement. For MΦ and CD4 T-cell co-culture treated MΦ were collected and mixed with CD4 T-cells at a ratio of 1:4 and incubated for a further 72 h before collection of supernatants. $CD4^+$ T-cells were added to flat-bottomed plates with or without anti-CD3 at a concentration of 2×10^5/well. Cells were allowed to settle for 1 h prior to addition of MΦ, 50,000 cells/well. IL-6 was neutralised by use of a polyclonal serum raised against IL-6 (Serotec; AHP424), sera was added at two junctures. Anti-IL-6 was added following priming of MΦ immediately prior to infection or following the mixture of MΦ with $CD4^+$ T-cells. A naive polyclonal rabbit serum was used as a negative control. All cultures were at a final volume of 250 μl/well.

2.2. Cytokine measurement, inhibition and antibody stimulation

Bovine cytokines were measured in cell free supernatants using commercially available ELISAs for IL-1β (Endogen), IL-6 (Endogen), TGF-β1 (Promega), IL-17A (Kingfisher Biotec), and IFN-γ (MAB Tech). Inhibition of IL-6 was achieved by use of a polyclonal anti-IL-6 antibody (Serotec AHP424) at a dilution of 1/250. Platebound