Two distinct populations of Bovine IL-17+ T-cells can be induced and WC1+IL-17+γδ T-cells are effective killers of protozoan parasites


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IL-17 has emerged as a key player in the immune system, exhibiting roles in protection from infectious diseases and promoting inflammation in autoimmunity. Initially thought to be CD4 T-cell-derived, the sources of IL-17 are now known to be varied and belong to both the innate and adaptive arms of the immune system. Mechanisms for inducing IL-17 production in lymphoid cells are thought to rely on appropriate antigenic stimulation in the context of TGF-β, IL-6 and/or IL-1β. Using culture protocols adapted from human studies, we have effectively induced both bovine CD4+ and WC1+γδ T-cells to produce IL-17 termed Th17 and γδ17 cells, respectively. The negative regulatory effect of IFN-γ on mouse and human IL-17 production can be extended to the bovine model, as addition of IFN-γ decreases IL-17 production in both cell types. Furthermore we show that infection with the protozoan Neospora caninum will induce fibroblasts to secrete pro-IL-17 factors thereby inducing a γδ17 phenotype that preferentially kills infected target cells. Our study identifies two T-cell sources of IL-17, and is the first to demonstrate a protective effect of IL-17 T-cells in ruminants. Our findings offer further opportunities for future adjuvants or vaccines which could benefit from inducing these responses.

IL-17, a major pro-inflammatory cytokine, has been shown to have several cellular sources indicating a multitude of roles with the immune system, including causing both pathology and providing protection1. Th17 cells, a key producer of IL-17, have been tightly linked to the outcome of multiple parasite infections, including the protozoan Toxoplasma gondii where IL-17 has been shown to dominate central nervous system (CNS) pathology during chronic infection2. Moreover Th17 cells are known to be negatively regulated by IFN-γ and not to produce IL-17 and IFN-γ simultaneously. Aside from CD4 T-cells, γδ T-cells have been described as a major source of IL-17 including during infection with Plasmodium berghei3 and Leishmania major4. Neospora caninum, a closely related intracellular protozoan, causes abortion in cattle worldwide and economic losses due to depressed production yields5. Timing of infection dictates the pathological outcome; de novo infection of pregnant animals in the 1st trimester leads to reabsorbed foetuses, during the 2nd trimester abortion can occur and in the 3rd trimester unborn calves can be congenitally infected leading to vertical transmission of the disease5. Studies have implicated IFN-γ in pathology but others report conflicting results suggesting both a protective, preventing abortion6, and a pathological, causing abortion, role7,8. This leads us to hypothesize that a source(s) of IL-17 may have an important role in protection against foetal death and host tissue damage either in isolation or combination with other cytokines.

A recent study has implicated a “cytokine storm” effect within placental tissues around the time of abortion9. Furthermore, we have recently shown that parasite limiting macrophages provoke IL-17 producing CD4 T-cells, with a Th17 phenotype equivalent to that seen in murine and human studies10. This is especially pertinent given the opposing effects that IL-17 and IFN-γ can have on each other. Given this, our hypothesis of IL-17 in protection against N. caninum and the abundance of γδ T-cells in young cattle in comparison to other mammals11, it was timely to investigate the ability of specific T-cell subsets to produce IL-17 and their functional relevance to protect against N. caninum infection.
Results

CD4 T-cells produce IL-17 under TCR and cytokine stimulation.

To test the concept that the cytokines, IL-6 and TGFβ1, can condition naïve bovine CD4⁺CD62L⁺ T-cells to differentiate into de novo IL-17 producing Th17 cells, naïve cells were stimulated in the presence of cytokines and TCR ligation by anti-CD3 for 72 hrs. The range of cytokine concentrations initially tested were IL-6 5 ng/ml–50 ng/ml and TGF-β1 2 ng/ml–16 ng/ml. Supernatants were tested for IL-17 production, which was found to correlate with increasing concentrations of IL-6 but not TGF-β1 (data not shown). The optimal concentration for maximal IL-17 production was 40 ng/ml of IL-6 and 2 ng/ml of TGF-β1 and, in line with previous findings, no IFN-γ could be detected in these cultures (Figure 1a). The addition of recombinant IFN-γ resulted in decreased IL-17 production (Figure 1b). Furthermore, these cells demonstrated elevated levels of CCR6 and IL-23R transcripts, consistent with the Th17 phenotype (Figure 1c).

γδ T-cells respond to cytokine stimuli but not TLR2 stimuli with IL-17 induction. γδ T-cells have been shown in both humans and mice to express IL-17 under various conditions. Using the above optimised IL-6/TGF-β1 concentrations above, cells were cultured both with and without anti-CD3 (Figure 1d). The results demonstrate that even in the absence of TCR ligation, cytokine stimulation is sufficient to induce IL-17⁺ WC1⁺γδ T-cells which we term γδ17 cells. Similar to our findings with CD4 T-cells, high levels of IL-17 production were consistent with little to no IFN-γ demonstrating cellular polarisation (data not shown). Likewise when IFN-γ was added to IL-6/TGF-β1 stimulated γδ T-cell cultures IL-17 production was found to be down-regulated (Figure 1e). Furthermore, γδ17 cells showed no increase in expression of IL-23R or CCR6 transcripts when tested (Figure 1f).

Murine γδ T-cells have been known to induce IL-17 under TLR2 stimulation. To test this in our system cells were cultured with Pam3CSK4, PGN or heat-killed Staphylococcus aureus; no IL-17 was induced under these conditions (data not shown).

Fibroblasts infected with Neospora caninum are susceptible to killing by γδ17 T-cells. N. caninum transitions from rapidly dividing tachyzoites to slow growing bradyzoites, which preferentially form in muscle and CNS tissue, under pressure from host immunity. Should N. caninum recrudesce it is likely they will begin to multiply within tissue fibroblasts. Investigations into the cytokine response of naïve fibroblasts to infection with N. caninum found that, after 72 hrs, there was a striking and significant increase in production of IL-6, IL-1β but not TGF-β1 (Figure 2a–c). We found no evidence for secretion of IL-10, IL-4 or IFN-γ (data not shown). This demonstrates differential production of cytokines capable of driving IL-17 responses. To further test this, we added the supernatants derived from N. caninum infected fibroblasts to...
naive γδ T-cells and tested the resulting supernatants for IL-17 and IFN-γ. Our results demonstrate that N. caninum conditioned fibroblast supernatants are capable of driving γδ T-cells with little to no IFN-γ production (Figure 2d).

To determine if γδ T-cells are capable of killing infected host fibroblasts, fibroblasts were grown on coverslips, infected and incubated with naive γδ cells or γδ17 cells for 24 hrs before microscopic examination (Figure 3a–d). The total number of fibroblasts was not significantly different between cultures incubated with unconditioned γδ and conditioned γδ17 cells (Figure 3a). When determining the percentage of infected cells per high powered field a significant decrease in infected fibroblasts cultured with γδ17 cells was observed (Figure 3b). Furthermore, examination of the number of parasites per cell revealed significant decreases between infected fibroblasts cultured with γδ and those cultured with γδ17 cells (Figure 3c). When co-cultures were conducted using transwell inserts, no significant differences in either percentage of infected cells (Figure 3d) or parasites/cell (Figure 3e) were found.

**Discussion**

Herein we show that two separate populations of bovine T-cells are capable of inducing IL-17 expression under appropriate cytokine stimulation. The CD4 population retains features of human and mouse Th17 cells with high levels of CCR6 and IL-23R expression. However, the bovine γδ17 cells produced are suggestive of a more transient, yet polarized, phenotype with no expression of CCR6 or IL-23R. We have also shown that these cells can be stimulated by supernatants collected from N. caninum-infected fibroblasts. Furthermore, the resulting γδ17 cells show strong anti-parasite effects in a co-culture system that is dependent on cell-cell contact. One striking difference between our Th17 and γδ17 cells is in the expression of CCR6 and IL-23R. The lack of IL-23R on γδ17 cells might indicate that these cells are not responsive to IL-23 and so do not undergo a stabilizing process similar to that reported in Th17 cells. The absence of CCR6 in our γδ17 may be explained by the differential use of homing chemokines dependent on the site of inflammation. Geherin et al. demonstrated that skin homing ovine γδ T-cells expressing IL-17 use CCR6 but not their blood counterparts. This is relevant given that the γδ17 cells in our studies were generated from blood. Alternatively, this difference may be a reflection of the activation processes in both cell types. γδ17 cells were generated in the absence of TCR stimulation while Th17 cells were TCR cross-linked. This may also be reflective of their function in vivo during infection. As Th17 cells will most likely be activated within a lymph node they will need to migrate to the site of inflammation whereas γδ17 cells may be already present at the site of inflammation, thus can be activated solely by cytokines without requiring guidance to the site of inflammation. Indeed there is an abundance of γδ T-cells in bovine gut tissues during the early life period.
The role of Th17 cells during N. caninum infection could possibly be related to the generation of protective antibody or a higher level function such as orchestration of neutrophil/effector cell influx. During the course of infection with the closely related *T. gondii*, mice that display heightened Th17 responses show aggravated pathology following oral infection and during CNS infection/reactivation. However, the impact of parasite strain and route of infection must be fully assessed as IL-17R-/- mice show increased mortality rates due to reduced PMN recruitment. Within *N. caninum* infection biology there already exists a contradictory role for IFN-\(\gamma\) where its function, or the outcome of its activity, would appear to be determined by timing of infection and thus timing of IFN-\(\gamma\) production. Our results suggest that IFN-\(\gamma\) can negatively regulate IL-17 production in two different T-cell types and thus may contribute to the suppression of protective or pathological responses. Certainly the γ17 cells produced here would appear to be capable of mediating cell contact dependent immunity. Given the need for cell contact it would appear that γ17 cells trigger cell autonomous killing of *N. caninum*, in an IFN-\(\gamma\)-independent mechanism. There is evidence to suggest that CD40-CD154 (CD40L) signaling can trigger autophagy in the absence of IFN-\(\gamma\). CD40 is expressed on fibroblasts and it is known that ConA stimulation causes the up-regulation of CD154 on bovine γ17 T-cells; however it remains to be determined if CD154 is expressed on bovine γ17 cells.

Our findings raise the possibility that invoking IL-17 production either short or long term might provide protection against *N. cani-
num infection, with bovine Th17 cells acting in the context of vaccine presentation. NK cells may act as a more short term or innate response to initial infection.

**Methods**

**Parasite culture.** *N. caninum* parasites (NC-Liv), a gift from Professor Diana Williams University of Liverpool, were maintained in host VERO cells and harvested as previously described.10

**T-cell separation and culture.** Healthy bovine donor tissues were assayed throughout and obtained in line with UK Home Office guidelines. CD4(-)CD26(-) cells were isolated in two steps as per Flynn & Marshall.9 Briefly, anti-CD4 (AbD Serotec clone CC30, mouse IgG1) was used at a dilution of 1/50 to label 10⁷/ml PBMC for 20 minutes. Cells were then labelled with secondary Miltenyi magnetic beads coated with anti-mouse IgG1 as per manufacturer’s instructions. The CD4(-) fraction was then isolated using a positive selection protocol using an AutoMacs separator and collected into 1%BSA-PBS. Cells, 10⁷/ml, were subsequently labelled with anti-CD62L (AbD Serotec clone CC8 mouse IgG1) at 1/30 for 20 minutes and purified using the above secondary method. γδ T-cells were isolated in a single step using anti-WC1 (AbD Serotec Clone CC15), using the same indirect labelling protocol as above, anti-WC1 antibody was used at a concentration of 1/50 to label 10⁷ cells/ml. Cells were cultured in complete media [RPMI 1640 with 10% heat-inactivated foetal calf serum, 200 U/ml penicillin, 200 µg/ml streptomycin and 1% non-essential amino acids] with cytokines as described. Where γδCD3 stimulation is indicated plate bound stimulation was used, tissue culture plates were coated with antibody (VMRD – Clone MM1A) in sterile D-PBS (Sigma-Aldrich) overnight at 37°C at a concentration of 1 µg/ml. To induce IL-17 production, IL-6 and TGF-β1 were added to T-cell cultures at the indicated concentrations in the presence or absence of plate bound αγδCD3. To polarize T-cells towards IL-17 secretion cells were cultured at a density of 2.5 × 10⁴/ml in 24 well plates for 72 hrs, unless otherwise stated. Anti-γδ T-cells were stimulated with 100 ng/ml IL-23 and 100 ng/ml IL-1α in the presence or absence of plate-bound anti-Th17 mAb, or with the Th17 cytokine cocktail. Cells were harvested at varying time points and assayed for IL-17 production by anti-IL-17A and IL-17C mAb.

**Fibroblast culture, infection and killing assays.** Fibroblasts were isolated from livers of autologous blood donors according to Dobbs et al.,9 with minor modifications. To determine cytokine production following infection, fibroblasts [10⁶/ml were grown in 48 well tissue culture plates and infected with freshly isolated parasites at a multiplicity of infection (MOI) of 1. Four hours after addition of parasites, the medium was changed to remove extracellular parasites from the culture and cells were incubated for a further 72 hrs before collection of supernatant. To test the killing ability of γδ T-cells cultured to become IL-17 secreting (γδ17 cells), fibroblasts were grown on coverslips and infected as above. 4 hrs following infection γδ cells were added to fibroblasts at a ratio of 1:1. Cultures were incubated for 24 hrs before supernatants were removed, coverslips were rinsed in D-PBS, fixed in methanol and stained with Giemsa. Coverslips were examined under a Nikon E800 microscope to determine the number of fibroblasts per high powered field of view (HPF), number of infected fibroblasts per HPF and number of parasites per infected cell. In some experiments transwell inserts, pore size 0.4 µm (Corning), were used to separate γδ cells from fibroblasts.

**ELISAs & Recombinant cytokines.** Recombinant proteins used were as follows, bovine IL-6, bovine IFN-γ (Kingfisher Biotech) and human TGF-β1 (Peprotech). Recombinant proteins were re-suspended in complete media, see above, prior to use. ELISAs were used to quantify IL-17 (Kingfisher Biotech), TGF-β1 (Promega), IL-6, IFN-γ, and IL-1β (Thermo-Scientific).

**Real-Time PCR.** mRNA was isolated by Phenol-Chloroform extraction and reverse transcription performed with GoScript Reverse Transcription System (Promega). Samples were analysed, in quadruplicate, using an ABI 7900HT Real-time PCR system for levels of IL-23r (assay ID B03817922_m1) and CCR6 (assay ID B04317064_s1) using Taqman assays (Applied Biosystems). Results are expressed as expression levels, calculated using the ΔCt method, relative to g3pdh (assay ID B03210913_g1).

**Statistical Analysis.** Data was entered into GraphPad Prism software for statistical analysis using appropriate tests (see figure legends); P-value of <0.05 was significant.