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Differential chemokine and cytokine production by neonatal bovine γδ T-cell subsets in response to viral toll-like receptor agonists and *in vivo* respiratory syncytial virus infection

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Summary

γδ T cells respond to stimulation via toll-like receptors (TLR). Bovine γδ T cells express TLR3 and TLR7, receptors that are key for the recognition of viruses such as bovine respiratory syncytial virus (BRSV); however, responses of γδ T cells to stimulation via these receptors, and their role during viral infections, remains unclear. Here, we demonstrate that neonatal bovine γδ T cells exhibit robust chemokine and cytokine production in response to the TLR3 agonist, Poly(I:C), and the TLR7 agonist, Imiquimod. Importantly, we observe a similar phenotype in γδ T-cell subsets purified from calves infected with BRSV. Bovine γδ T cells are divided into subsets based upon their expression of WC1, and the response to TLR stimulation and viral infection differs between these subsets, with WC1.1$^+$ and WC1^{neg} γδ T cells producing macrophage inflammatory protein-1α and granulocyte–macrophage colony-stimulating factor, and WC1.2$^+$ γδ T cells preferentially producing the regulatory cytokines interleukin-10 and transforming growth factor-β. We further report that the active vitamin D metabolite 1,25-dihydroxyvitamin D3 does not alter γδ T-cell responses to TLR agonists or BRSV. To our knowledge, this is the first characterization of the γδ T-cell response during *in vivo* BRSV infection and the first suggestion that WC1.1$^+$ and WC1^{neg} γδ T cells contribute to the recruitment of inflammatory populations during viral infection. Based on our results, we propose that circulating γδ T cells are poised to rapidly respond to viral infection and suggest an important role for γδ T cells in the innate immune response of the bovine neonate.

Keywords: bovine γδ T cells; respiratory syncytial virus; toll-like receptors; vitamin D.

Introduction

γδ T cells are a subset of CD3$^+$ T cells that share many described characteristics of both innate and adaptive immune cells. They respond to unprocessed or non-proteinaceous antigens independent of classical MHC I or MHC II restriction.[1] γδ T cells are found in all vertebrate species examined, including humans, where their frequencies are low, representing < 5–10% of the circulating lymphocyte population.[1] In contrast, γδ T cells are more abundant in ruminant species, where they constitute up to 70% of the circulating lymphocytes in very young animals.[2,3] This number slowly declines with age, resulting in 10–20% of circulating lymphocytes in the adult bovine. Given their abundance in peripheral blood and the ease with which they can be obtained and isolated, the bovine makes an excellent model for studying γδ T cells and understanding their role in innate and adaptive immunity.

Abbreviations: 1,25D$_3$, 1,25-dihydroxyvitamin D3; 24-OHase, 24-hydroxylase; APC, antigen-presenting cells; BRDC, bovine respiratory disease complex; BRSV, bovine respiratory syncytial virus; LN, lymph node; PBMC, peripheral blood mononuclear cells; P.I., post infection; Poly(I:C), polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; RSV, respiratory syncytial virus; TCR, T-cell receptor; TLR, Toll-like receptor; SRCR, scavenger receptor cysteine-rich superfamily; VDR, vitamin D receptor; WC1, workshop cluster 1

Despite extensive research, the function of $\gamma\delta$ T cells in immunity remains poorly understood. Activation of $\gamma\delta$ T cells via the T-cell receptor (TCR) elicits inflammatory chemokine and cytokine production including interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and granzymes.[3,4] As further evidence of their plasticity in the immune system, $\gamma\delta$ T cells can also play a regulatory role. In humans, FoxP3$^+$ $\gamma\delta$ T cells have been shown to inhibit the proliferation of autologous CD4 T cells *in vitro*.[5,6] Similarly, bovine $\gamma\delta$ T cells have been shown to produce interleukin-10 (IL-10) and inhibit autologous T-cell proliferation; however, this population does not express FoxP3.[7]

Pattern recognition receptors (PRRs) are key for innate recognition of pathogens. Human, murine and bovine $\gamma\delta$ T cells have recently been shown to express PRRs and to respond directly through PRR stimulation.[3,8] Bovine and human $\gamma\delta$ T cells both express Nod2 and respond to the bacterial Nod2 agonist muramyl dipeptide.[9] Further, $\gamma\delta$ T cells from both species also express Toll-like receptor (TLR) 2 and TLR4 and respond to the bacterial TLR agonists peptidoglycan and lipopolysaccharide.[10] Together, these results clearly demonstrate that $\gamma\delta$ T cells possess the innate ability to recognize and respond to bacterial challenge. It has recently been shown that bovine and human $\gamma\delta$ T cells also express TLR3 and TLR7,[8,10–12] both endosomal receptors that recognize double-stranded and single-stranded RNA, respectively, and are key for the innate recognition of viruses and their replication products. Despite this evidence, little is known about the responsiveness of $\gamma\delta$ T cells to TLR3 or TLR7 stimulation, or about the specific role of $\gamma\delta$ T cells following virus infection. Wesch *et al.*[12] demonstrated that human $\gamma\delta$ T-cell IFN-γ production, induced by TCR cross-linking, is enhanced by direct treatment with the TLR3 agonist Poly-inosinic:polycytidylic acid [Poly(I:C)], whereas recent evidence indicates that $\gamma\delta$ T-cell clones from adult and neonatal humans respond to TLR3 stimulation with robust IFN-γ production.[11] The capacity of bovine $\gamma\delta$ T cells to respond to TLR3 or TLR7 stimulation is currently unknown.

Bovine $\gamma\delta$ T cells are divided into subsets based upon their expression of the receptor Workshop Cluster 1 (WC1). WC1$^+$ $\gamma\delta$ T cells are the predominant subset in circulation, whereas WC1^{neg} $\gamma\delta$ T cells are most numerous in the spleen, intestinal mucosa and mesenteric lymph nodes. WC1^{neg} $\gamma\delta$ T cells are thought to play a role in immune surveillance and promoting tissue quiescence, whereas WC1$^+$ $\gamma\delta$ T cells are clearly more pro-inflammatory, producing robust amounts of IFN-γ in response to experimental challenge.[13–17] WC1$^+$ $\gamma\delta$ T cells can be further divided into multiple serologically defined populations: WC1.1$^+$, WC1.2$^+$ and WC1.3$^+$.[18–20] Recent reports have suggested that these populations likely play unique functional roles in the immune response of cattle. WC1.1$^+$ $\gamma\delta$ T cells are robust producers of IFN-γ following mitogen stimulation and experimental challenge with *Leptospira* or *Mycobacterium*.[21–23] WC1.2$^+$ $\gamma\delta$ T cells are thought to be more regulatory, producing little IFN-γ, but robust amounts of IL-10.[7] Interestingly, however, WC1.2$^+$ $\gamma\delta$ T cells have been reported to produce IFN-γ in response to challenge with the bovine rickettsial pathogen *Anaplasma marginale*,[24] suggesting that this subset probably has some functional plasticity. Although it is clear that WC1.1$^+$ and WC1.2$^+$ $\gamma\delta$ T cells are unique in their immune functions, the physiological role of each in response to experimental challenge remains poorly defined. Further, the unique response of each subset following stimulation by TLR agonists or in response to viral infection remains unknown.

Bovine respiratory disease complex (BRDC) is a disease complex afflicting young cattle resulting in significant economic loss to both beef and dairy industries each year.[25,26] Exacerbated by stress from handling, weaning and shipping, BRDC is often initiated by infection with a primary viral agent such as bovine respiratory syncytial virus (BRSV), bovine herpes virus-1 or bovine viral diarrhoea virus (BVDV).[27] These infections are thought to predispose to secondary bacterial pneumonias caused by agents such as *Mannheimia haemolytica* and *Pasteurella multocida*.[27] We are interested in understanding the immune response to BRDC, and more specifically, in understanding BRSV and its role in initiating the immune modulation that often leads to secondary bacterial pneumonias.

Infection with BRSV in calves serves as an excellent model for understanding RSV infection in children. Many features of BRSV infection in cattle parallel those observed in humans including age-dependent susceptibility, microscopic lesions and numerous changes in innate and adaptive immune responses including up-regulation of pro-inflammatory mediators such as IL-8.[28–30] In humans, RSV is a leading cause of serious acute lower respiratory tract infections in young children worldwide.[31] Few studies have examined the role of $\gamma\delta$ T cells in the immune response to RSV. In mice, depletion of $\gamma\delta$ T cells before RSV infection reduces lung inflammation and disease severity, as well as increases peak viral replication.[32] A study by Taylor *et al.*[33] suggests that depletion of WC1$^+$ $\gamma\delta$ T cells from neonatal gnotobiotic calves infected with BRSV does not affect clinical signs or viral clearance, but results in significant increases in IgM and IgA in the bronchiolar alveolar lavage. However, experimental numbers from this study were limited and control animals did not exhibit significant clinical signs of BRSV infection. Although these results suggest a role for $\gamma\delta$ T cells in altering lung pathology and viral clearance following RSV infection, the potential innate role of $\gamma\delta$ T cells in regulating early virus recognition and lymphocyte recruitment needs to be investigated.

Currently, we have limited knowledge of the response of bovine γδ T cells to stimulation through virus-sensing PRRs, and, more importantly, the response of γδ T cells to viruses themselves. Therefore, we sought to elucidate the ability of bovine neonatal γδ T cells to respond to stimulation via TLR3 or TLR7, as well as to determine their ability to respond to BRSV, a pathogen affecting neonatal calves and known to initiate BRDC. Given the potentially distinct roles of γδ T-cell subsets in cattle, we further sought to understand the unique functions of $WC1.1^+$, $WC1.2^+$ and $WC1^{neg}$ γδ T cells in the context of anti-viral immunity. Here we demonstrate that γδ T-cell subsets purified from peripheral blood of neonatal calves exhibit robust chemokine and cytokine production in response to stimulation with the TLR3 agonist Poly(I:C) and the TLR7 agonist Imiquimod. These responses differed between γδ T-cell subsets, with $WC1.1^+$ and $WC1^{neg}$ subsets producing the chemokines macrophage inflammatory protein-1α (MIP-1α; CCL3) and granulocyte–macrophage colony-stimulating factor (GM-CSF), and the $WC1.2^+$ subset preferentially producing the regulatory cytokines IL-10 and transforming growth factor-β (TGF-β). We observed a similar phenotype in γδ T-cell subsets purified from calves infected with BRSV. Interestingly, despite epidemiological evidence for the importance of vitamin D in regulating immunity to respiratory infections,[34] the active vitamin D metabolite 1,25-dihydroxyvitamin D3 ($1,25D_3$) did not affect γδ T-cell responses to viral TLR agonists or BRSV.

Together, these results describe a previously unrecognized ability of bovine γδ T cells to respond to stimulation via TLR3 or TLR7 and indicate a unique division of labour between γδ T-cell subsets for differential chemokine and cytokine production elicited by PRR stimulation. These results also provide the first detailed description of the functional capacity of γδ T cells isolated from BRSV-infected cattle and are the first to suggest that γδ T-cell subsets contribute to the recruitment of inflammatory populations during viral infection. We propose that circulating γδ T cells are poised to recognize and respond rapidly to viral infection, suggesting that γδ T cells play an important role in anti-viral immunity in the bovine neonate.

Materials and methods

Animals

A total of 24 neonatal male Holstein calves were used in two independent experiments ($n = 12$ calves/experiment). Calves arrived at the centre at 3–5 days of age and were randomly assigned to milk replacer diets with differing levels of vitamin D that resulted in two groups of calves that had normal or deficient levels of circulating 25(OH)D_3, similar to the approach previously described by Sacco *et al.*[29] No significant differences were observed in the responses of γδ T cells isolated from calves with deficient versus normal levels of 25(OH)D_3. Further, no significant differences in BRSV infection were observed in the calves with deficient versus normal levels of 25(OH)D_3. Hence, the samples described herein were obtained from calves with both low and normal levels of circulating 25(OH)D_3. Pre-infection samples were collected weekly via jugular vein starting at 2 weeks of age. Before the study calves were healthy and not vaccinated against BRSV. Calves were inoculated with BRSV at 5–6 weeks of age. Samples were collected on days 3 and 10 post-infection (p.i.) via the jugular vein. All animal procedures were conducted according to federal and institutional guidelines and were approved by the National Animal Disease Center Animal Care and Use Committee.

BRSV inoculum and aerosol challenge model

Bovine RSV strain 375 was prepared from virus stock re-isolated from the lung of an infected animal and passaged fewer than four times on bovine turbinate cells. Tissue culture infectious dose ($TCID_{50}$) was determined using standard plaque assay. Calves were inoculated via aerosol challenge with approximately 10^4 $TCID_{50}$/ml of BRSV strain 375 as previously described.[29] Briefly, calves were fitted with an aerosol mask (Trudell Medical International, London, ON, Canada) that was modified with a rubber gasket to ensure a secure fit on the muzzle, and 5 ml of virus inoculum was delivered over 10–15 min via nebulization using compressed air (1.758 kg/cm^2). The viral inoculum was determined free of contaminating BVDV by PCR as previously described.[35]

Peripheral blood collection and mononuclear cell preparation

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat fractions of peripheral blood collected from the jugular vein into 2 × acid citrate dextrose. Contaminating red blood cells were removed using hypotonic lysis. Cells were washed and resuspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 1% antibiotic–antimycotic solution, 50 mg/ml gentamicin sulphate, 1% non-essential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50 μM 2-mercaptoethanol and 10% (volume/volume) fetal bovine serum.

For FACS purification of the γδ T-cell subsets, PBMC were resuspended at 10^7 cells/ml in cRPMI and incubated for 20 min on ice with 10 μg/ml of the following primary antibodies: mouse anti-bovine γδ TCR (TCR1-N24, δ chain-specific; Clone GB21A, Isotype IgG2b), mouse anti-bovine WC1-N3 (Clone CACTB32A, Isotype IgG1) and

mouse anti-bovine WC1-N4 (Clone BAG25A, Isotype IgM) (all from VMRD, Pullman, WA). Cells were washed once and resuspended at 10^7 cells/ml in cRPMI with 5 μg/ml of the following secondary antibodies: goat anti-mouse IgG2b-Cy5, goat anti-mouse IgG1-phycoerythrin and goat anti-mouse IgM-FITC (all from Southern Biotech, Birmingham, AL). The PBMC were incubated for 20 min on ice, then washed once and resuspended at approximately 10^8 cells/ml in cRPMI for FACS sorting. $\gamma\delta$ T-cell subsets were sort-purified based on surface expression of WC1 and the $\gamma\delta$ TCR using the representative gating strategy outlined in Fig. S1 (see Supplementary material): WC1.1$^+$ $\gamma\delta$ cells (GB21A$^+$/CACTB32A^{neg}/BAG25A$^+$), WC1.2$^+$ $\gamma\delta$ cells (GB21A$^+$/CACTB32A$^+$/BAG25A^{neg}) and WC1^{neg} $\gamma\delta$ cells (GB21A$^+$/CACTB32A^{neg}/BAG25A^{neg}). Subsets were sorted to > 90% purity using a BD FACS Aria Cell Sorting System (BD Biosciences, San Jose, CA).

Monocytes were isolated by plastic adherence as previously described.[36] Briefly, PBMC were suspended in cRPMI and allowed to adhere to plastic Petri dishes for 2 hr at 37°. Non-adherent cells were removed by washing with warm cRPMI. Adherent cells were collected by washing with ice-cold PBS and gentle scraping.

In vitro $\gamma\delta$ T-cell stimulation

FACS-purified $\gamma\delta$ T-cell subsets were plated at a concentration of 2×10^6 cells/ml (100 μl/well) in sterile, round-bottom, 96-well tissue-culture-treated plates (BD Biosciences). For experiments requiring antigen-presenting cells (APC) (Figs 5–8), monocytes were plated at a ratio of 1 : 5 with purified $\gamma\delta$ T-cell subsets (4×10^4 cells/well). The TLR agonists Poly(I:C) and Imiquimod (both from Invivogen, San Diego, CA), were used at concentrations of 50 and 10 μg/ml, respectively. The $1,25D_3$ (Sigma-Aldrich, St Louis, MO) was used at a concentration of 4 ng/ml as previously described.[37] For experiments using plate-bound anti-CD3 (Fig. 3), 96-well tissue culture-treated plates were coated overnight at 4° with 10 μg/ml mouse anti-bovine CD3 (Clone MM1A, Isotype IgG1; VMRD), then washed once before use. For *in vitro* re-stimulation with BRSV (Figs 5–8), cells were incubated at a 0·1 multiplicity of infection (MOI) with BRSV Strain 375 for 90 min at 37°, washed once and resuspended in cRPMI for the remaining incubation period.

At 24 or 48 hr post-stimulation, $\gamma\delta$ T-cell subsets were pelleted by centrifugation and cell culture supernatants were collected and stored at −80°. Cell pellets were then lysed with Buffer RLT (Qiagen, Valencia, CA) containing 2-mercaptoethanol and stored at −80°. When necessary, $\gamma\delta$ T-cell subsets were removed from adherent monocytes by pipetting before centrifugation and lysis.

Real-time PCR

Total RNA was extracted using the RNeasy Mini RNA Isolation kit (Qiagen) according to manufacturer's instructions. Contaminating genomic DNA was removed using the RNase-Free DNase digestion set (Qiagen) as per manufacturer's instructions. Total eluted RNA was reverse transcribed into cDNA using Superscript III Reverse Transcriptase and Random Primers (both from Invitrogen, Life Technologies, Carlsbad, CA) following the manufacturer's instructions.

Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Forward and reverse primers used in the study are listed in Table 1.[29,37–41] Reactions were performed on a 7300 Real-Time PCR System (Applied Biosystems, Life

Table 1. Primers used for real-time PCR

Gene (alternative name)	Accession number	Strand	Sequence (5′–3′)	Reference
IL-10	NM_174088.1	Forward	TTACCTGGAGGAGGTGATG	29
		Reverse	GTTCACGTGCTCCTTGATG	
IFNγ	NM_174086.1	Forward	AGAATCTCTTTCGAGGCCGGAG	29
		Reverse	TATTGCAGGCAGGAGGACCATTAC	
MCP-1 (CCL2)	NM_174006.2	Forward	CGCCTGCTGCTATACATTCA	38
		Reverse	GCTCAAGGCTTTGGAGTTTG	
MIP-1α (CCL3)	NM_174511.2	Forward	TGGTGTCATCTTCCAGACCA	38
		Reverse	GTAGCTATGGAGGCGACAGG	
GM-CSF	NM_174027.2	Forward	AGTAATGACACAGAAGTCGTCTCTG	39
		Reverse	GCCGTTCTTGTACAGCTTCAGG	
TGF-β	NM_001166068.1	Forward	CTGAGCCAGAGGCGGACTAC	29
		Reverse	TGCCGTATTCCACCATTAGCA	
24-Ohase (CYP24A1)	NM_001191417	Forward	GAAGACTGGCAGAGGGTCAG	29,37,40,41
		Reverse	CAGCCAAGACCTCGTTGATT	
RPS9	NM_001101152.1	Forward	CGCCTCGACCAAGAGCTGAAG	29
		Reverse	CCTCCAGACCTCACGTTTGTTCC	

Technologies, Carlsbad, CA) with the amplification conditions: 2 min at 50°, 10 min at 95°, 40 cycles of 15 seconds at 95° and 1 min at 60°, and a dissociation step (15 seconds at 95°, 1 min at 60°, 15 seconds at 95°, 15 seconds at 60°). Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method[42] with RPS9 as the reference housekeeping gene.[29,37,40,41]

ELISA and multiplex cytokine immunoassay

The Bovine monocyte chemoattractant protein 1 (MCP-1; CCL2) VetSet ELISA Development kit was purchased from Kingfisher Biotech, Inc. (St Paul, MN). The Bovine MIP-1α ELISA kit and Bovine GM-CSF ELISA kit were both manufactured by TSZ ELISA (Framingham, MA) and purchased from Biotang Inc. (Waltham, MA). The human TGF-β_1 DuoSet was purchased from R&D Systems (Minneapolis, MN) and is cross-reactive with bovine TGF-β_1. All ELISA were performed using cell culture supernatants according to the manufacturer's instructions.

The ELISA for bovine IL-10 was adapted from a protocol described by Kwong *et al.*[43] Recombinant bovine IL-10 was received as a kind gift from Joanna LaBresh at Kingfisher Biotech, Inc. Briefly, 96-well microtitre plates were coated overnight with 5 μg/ml mouse anti-bovine IL-10 (Clone CC318; AbD Serotec, Raleigh, NC). Plates were washed with PBS/1% BSA/0·5% Tween-20, then blocked for 2 hr with PBS/5% BSA/0·5% Tween-20. Sample cell culture supernatants or standard serial dilutions of recombinant IL-10 were added to the wells and incubated for 1 hr at room temperature. After washing, secondary biotinylated mouse anti-bovine IL-10 (Clone CC320; AbD Serotec) was used at a concentration of 2 μg/ml. Plates were incubated for 1 hr at room temperature, then washed and incubated for 30 min with Streptavidin-HRP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Plates were developed for 10–20 min using the Pierce 1-Step Ultra TMB Substrate (ThermoScientific Pierce, Rockford, IL). The reaction was stopped with the addition of 0·2 M H_2SO_4 and plates were read at optical density at 450 nm minus the optical density at 540 nm using an automated plate reader (Flex Station 3l; Molecular Devices, Sunnyvale, CA). Concentrations of IL-10 were calculated using a standard curve.

Concentrations of IFN-γ, IL-4, IL-2, IL-6, IL-1β and TNF-α were determined using 96-well, immobilized antibody 6-plex array plates (SearchLight; Aushon Biosystems, Billerica, MA) and assays were conducted according to the manufacturer's instructions. Plates were read using a SearchLight Plate Reader (Aushon Biosystems) and the cytokine concentrations were determined from standard curves using SEARCHLIGHT ARRAY ANALYST SOFTWARE v 2.6.2.0 (Aushon Biosystems).

Statistical analysis

$\Delta\Delta C_t$ values were used in the statistical analyses of relative gene expression. $\Delta\Delta C_t$ values ± SEM were transformed ($2^{-\Delta\Delta C_t}$) and are shown as the expression relative to unstimulated control samples.[42]

Analyses to compare relative gene expression and secreted protein concentrations compared with unstimulated controls within γδ T-cell subsets were performed using a two-tailed Student's *t*-test statistic (PRISM; GraphPad, LaJolla, CA). Analyses to compare relative gene expression and secreted protein concentrations between γδ T-cell subsets were performed using one-way ANOVA (PRISM; GraphPad).

Results

Bovine γδ T cells respond to direct stimulation by the TLR3 agonist Poly(I:C) and the TLR7 agonist Imiquimod

Bovine γδ T cells express several PRRs including TLR1–10, and can respond to direct stimulation through at least some of these receptors, in particular TLR2 and TLR4.[10] It is unknown, however, if bovine γδ T cells can respond to stimulation through TLR3 or TLR7, as well as what role this response may play in immunity to viral infections. Therefore, we first sought to determine if purified bovine γδ T cells respond to direct stimulation through TLR3 and TLR7. To this end, γδ T cells were FACS sorted from the peripheral blood of uninfected neonatal Holstein calves into three groups based upon their expression of the WC1 receptor (see Supplementary material, Fig. S1): $WC1.1^+$ γδ T cells, $WC1.2^+$ γδ T cells and $WC1^{neg}$ γδ T cells. The purified subsets were then cultured for 24 hr in the presence or absence of the TLR3 agonist Poly(I:C) (Fig. 1a,c) or the TLR7 agonist Imiquimod (Fig. 1b,d). As shown in Fig. 1, $WC1.1^+$ γδ T cells responded to stimulation by Poly(I:C) with up-regulation of mRNA for both MIP-1α (Fig. 1a) and GM-CSF (Fig. 1c). A similar, although slightly reduced, response was also observed for the $WC1.1^+$ subset stimulated by the TLR7 agonist, imiquimod (Fig. 1b,d). Interestingly, the response to TLR stimulation appeared to vary between the subsets, as $WC1^{neg}$ γδ T cells up-regulated MIP-1α, but not GM-CSF expression in the presence of Poly(I:C) and Imiquimod, whereas $WC1.2^+$ γδ T cells did not alter their expression of either chemokine.

Human γδ T cells express the nuclear vitamin D receptor (VDR) and treatment with the active vitamin D metabolite $1,25D_3$ inhibits phospholigand-induced γδ proliferation and IFN-γ production.[44] Bovine $CD3^+$ T cells modulate their response in the presence of $1,25D_3$;[37] however, the effect of vitamin D on responses of individual bovine T-cell subsets, and specifically γδ

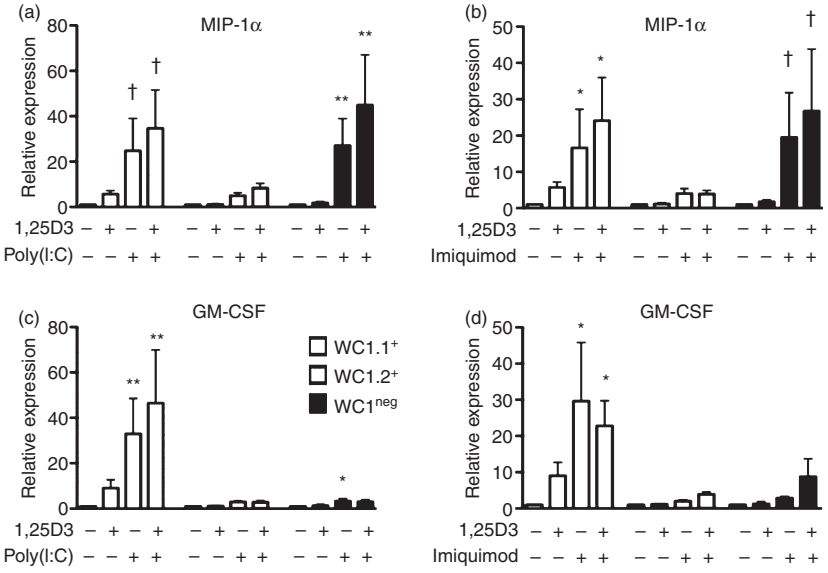

Figure 1. Bovine $\gamma\delta$ T-cell subsets produce the chemokines macrophage inflammatory protein 1α (MIP-1α) and granulocyte–macrophage colony-stimulating factor (GM-CSF) in direct response to the viral Toll-like receptor (TLR) agonists Poly(I:C) and Imiquimod. WC1.1$^+$ $\gamma\delta$ T cells (white bars), WC1.2$^+$ $\gamma\delta$ T cells (light grey bars) and WC1^{neg} $\gamma\delta$ T cells (dark grey bars) were FACS purified from the peripheral blood of healthy neonatal calves as described in Materials and methods. Purified $\gamma\delta$ T-cell subsets were cultured for 24 hr ± the active vitamin D metabolite 1,25-dihydroxyvitamin D3 ($1,25D_3$) and the viral TLR agonists Poly(I:C) (a and c) or Imiquimod (b and d). Cells were then analysed by real-time PCR for MIP-1α (a and b) and GM-CSF (c and d) mRNA expression. Results were normalized to the housekeeping gene RPS-9, and then expressed relative to unstimulated control cultures. Data are pooled from two independent experiments and represent means ± SEM ($n = 4$ to $n = 8$ animals/group). †$P < 0{\cdot}1$, *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with unstimulated control cultures.

T cells, is less clear. We therefore cultured the WC1.1$^+$ $\gamma\delta$ T cells, WC1.2$^+$ $\gamma\delta$ T cells and WC1^{neg} $\gamma\delta$ T cells in the presence or absence of $1,25D_3$ during TLR3 and TLR7 stimulation and evaluated changes in responses as measured by real-time PCR for MIP-1α (Fig. 1a,b) and GM-CSF (Fig. 1c,d) mRNA expression. We did not observe any significant changes in MIP-1α or GM-CSF expression by any of the purified $\gamma\delta$ T-cell subsets in the presence of $1,25D_3$ alone or when coupled with TLR stimulation (Fig. 1).

The lack of MIP-1α and GM-CSF expression by the WC1.2$^+$ $\gamma\delta$ subset following TLR3 or TLR7 stimulation was unexpected. While little work has been performed to elucidate functional differences between $\gamma\delta$ T-cell subsets, the WC1.2$^+$ subset has been shown to produce IL-10 in response to mitogen stimulation and suppress CD4 T-cell proliferation *in vitro*.[7] Therefore, we next sought to determine if purified WC1.2$^+$ $\gamma\delta$ T cells instead produce the regulatory cytokines IL-10 and TGF-β in response to TLR3 and TLR7 stimulation. As shown in Fig. 2, both Poly(I:C) and Imiquimod induced robust regulatory cytokine expression by purified WC1.2$^+$ $\gamma\delta$ T cells (Fig. 2a–d), supporting the hypothesis that this subset has a regulatory role in the bovine immune response. We observed little IL-10 or TGF-β expression by WC1.1$^+$ or WC1^{neg} subsets following Poly(I:C) or Imiquimod treatment (Fig. 2).

Vitamin D promotes the development and enhanced function of FoxP3$^+$ T regulatory cells in mice and humans.[45] Given their potential role as a regulatory cell in the cattle, we sought to determine if $1,25D_3$ treatment had a similar positive effect on IL-10 and TGF-β production by the WC1.2$^+$ $\gamma\delta$ subset. As seen in Fig. 2 $1,25D_3$ treatment neither enhanced nor suppressed the regulatory function of the WC1.2$^+$ $\gamma\delta$ subset as measured by IL-10 or TGF-β mRNA expression. Further, $1,25D_3$ treatment had no effect on TLR3- or TLR7-induced regulatory cytokine production by WC1.1$^+$ or WC1^{neg} T-cell subsets (Fig. 2).

TCR stimulation selectively enhances TLR-agonist-induced cytokine production, but not chemokine production by $\gamma\delta$ T-cell subsets

Bovine, as well as human and murine $\gamma\delta$ T cells respond to direct TLR stimulation by up-regulation of activation markers and increased cytokine expression (Figs 1 and 2).[3,8,12] However, previous studies have suggested that this cytokine expression is enhanced when in the presence of specific antigen or TCR stimulation.[9–12] Therefore, we next sought to determine if the response of bovine $\gamma\delta$ T-cell subsets to the TLR3 and TLR7 agonists Poly(I:C) and Imiquimod was similarly enhanced by stimulation with plate-bound αCD3. Peripheral blood $\gamma\delta$ T-cell

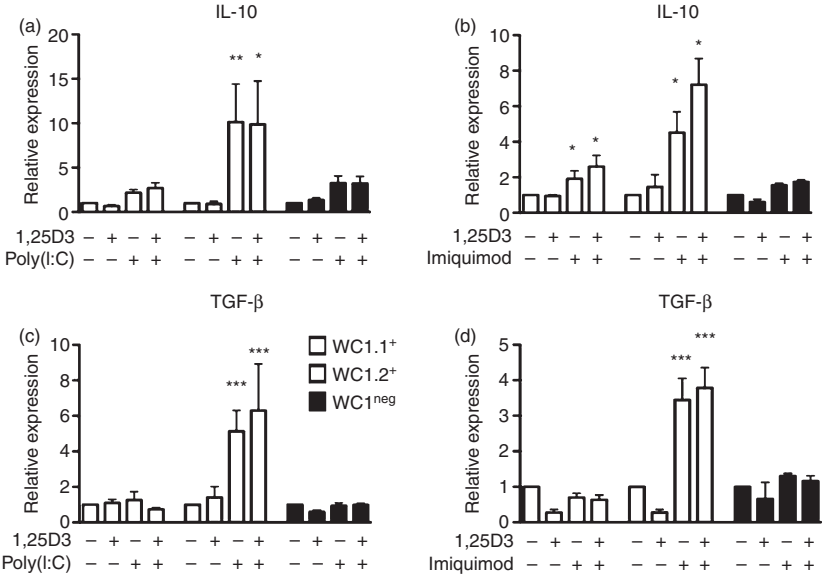

Figure 2. Bovine WC1.2$^+$ γδ T cells produce the regulatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in direct response to the viral Toll-like receptor (TLR) agonists Poly(I:C) and Imiquimod. WC1.1$^+$ γδ T cells (white bars), WC1.2$^+$ γδ T cells (light grey bars) and WC1^{neg} γδ T cells (dark grey bars) were FACS purified from the peripheral blood of neonatal calves as in Fig. 1. Purified γδ T-cell subsets were cultured for 24 hr ± 1,25D$_3$ and Poly(I:C) (a and c) or Imiquimod (b and d). Cells were then analysed by real-time PCR for IL-10 (a and b) and TGF-β (c and d) mRNA expression. Results were normalized to the housekeeping gene RPS-9 and expressed relative to unstimulated control cultures. Data are pooled from two experiments and represent means ± SEM ($n = 4$ to $n = 8$ animals/group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ compared with unstimulated control cultures.

subsets were purified and cultured with Poly(I:C) or Imiquimod and plate-bound anti-bovine CD3 + 1,25D$_3$. After 24 hr, cells were harvested and analysed by real-time PCR for mRNA expression of MIP-1α (Fig. 3a), GM-CSF (Fig. 3b), IL-10 (Fig. 3c) and IFN-γ (Fig. 3d). Surprisingly, TCR stimulation did not alter or enhance the TLR agonist-induced expression of MIP-1α or GM-CSF by purified WC1.1$^+$ (white bars), WC1.2$^+$ (light grey bars) or WC1^{neg} (dark grey bars) γδ T cells (Fig. 3a,b). Shown in Fig. 3(a) is MIP-1α expression in response to Poly(I:C) treatment and in Fig. 3(b) is GM-CSF expression in response to Imiquimod treatment; responses to both TLR agonists were similar.

In contrast to γδ T-cell MIP-1α and GM-CSF production (Fig. 3a,b), we observed a significant effect of plate-bound anti-CD3 on IL-10 and IFN-γ expression by all three subsets of γδ T cells (Fig. 3c,d). WC1.1$^+$ γδ T cells (white bars) produced very little IL-10 in response to TLR stimulation alone; however, they significantly up-regulated IL-10 expression in the presence of TCR stimulation. The TCR stimulation also enhanced IL-10 expression by the WC1.2$^+$ regulatory subset and to a lesser extent expression by the WC1^{neg} subset (dark grey bars) (Fig. 3c). Stimulation with TLR alone did not induce significant IFN-γ production by any of the purified subsets; however, TCR cross-linking increased expression by all three groups (Fig. 3d). Figure 3(c,d) shows the response to Poly(I:C) stimulation. Similar trends were observed in response to Imiquimod (data not shown). As in Figs 1 and 2, no significant changes were noted in γδ T-cell chemokine or cytokine production with the addition of 1,25D$_3$ (Fig. 3).

Bovine γδ T cells up-regulate the vitamin D catabolic enzyme 24-hydroxylase in the presence of 1,25D$_3$

The direct effects of vitamin D treatment on bovine T cells, and specifically, the γδ T-cell compartment, remain unclear. Given the lack of detectable response to 1,25D$_3$ treatment by our purified bovine γδ T-cell cultures (Figs 1–3), we wanted to verify that the 1,25D$_3$ treatment was effective and confirm that γδ T cells can respond directly to 1,25D$_3$ treatment. We therefore chose to analyse expression of the vitamin D catabolic enzyme 24-hydroxylase (24-OHase), a molecule that is highly induced in the presence of 1,25D$_3$.[37] γδ T-cell subsets were again purified as in Figs 1–3, and stimulated for 24 hr with TLR agonists ± 1,25D$_3$. Poly(I:C) (Fig. 4a) and Imiquimod (Fig. 4b) treatments alone induced a measurable, but not significant, increase in 24-OHase expression by the three purified γδ subsets. However, the addition of 1,25D$_3$ in the presence of Poly(I:C) stimulation resulted in a significant up-regulation of 24-OHase expression by WC1.1$^+$, WC1.2$^+$ and WC1^{neg} γδ T cells (Fig. 4a). The addition of 1,25D$_3$ in the presence of Imiquimod stimulation resulted in a similar trend (Fig. 4b).

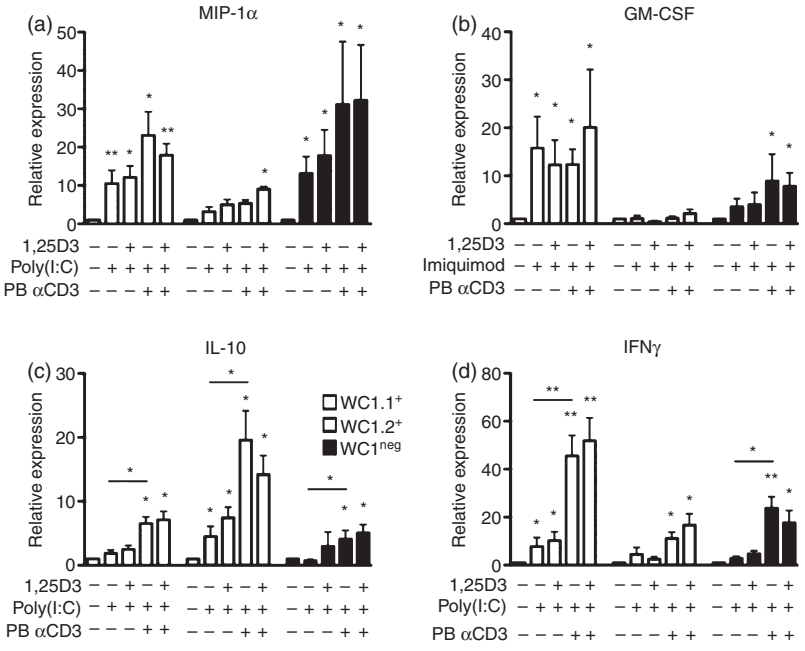

Figure 3. T-cell receptor (TCR) stimulus in the presence of the Toll-like receptor (TLR) agonists Poly(I:C) or Imiquimod results in selective enhancement of cytokine, but not chemokine, production by bovine $\gamma\delta$ T-cell subsets *in vitro*. WC1.1$^+$ $\gamma\delta$ T cells (white bars), WC1.2$^+$ $\gamma\delta$ T cells (light grey bars) and WC1^{neg} $\gamma\delta$ T cells (dark grey bars) were FACS purified as in Fig. 1. Purified $\gamma\delta$ T-cell subsets were then cultured for 24 hr ± 10 μg/ml plate-bound mouse anti-bovine CD3 ± 1,25D$_3$ and Poly(I:C) (a and c) or Imiquimod (b and d). Cells were then harvested and analysed by real-time-PCR for macrophage inflammatory protein-1α (MIP-1α) (a), granulocyte–macrophage colony-stimulating factor (GM-CSF) (b), interleukin-10 (IL-10) (c) and interferon-γ (IFN-γ) (d) mRNA expression. Results were normalized to the housekeeping gene RPS-9, and then expressed relative to unstimulated control cultures. Data are representative of two to four independent experiments and represent means ± SEM (n = 4 to n = 8 animals/group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with unstimulated control cultures unless otherwise indicated.

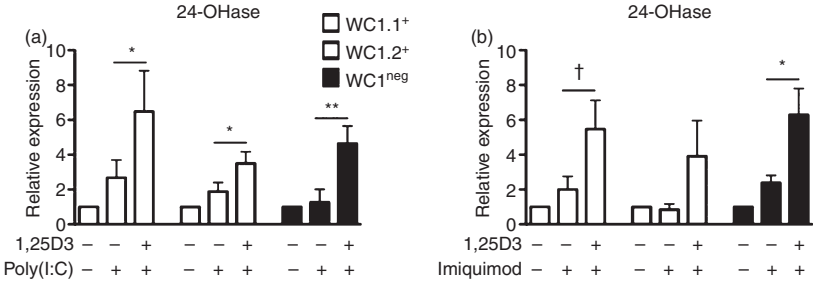

Figure 4. Effects of 1,25D$_3$ on mRNA expression in purified $\gamma\delta$ T-cell cultures. WC1.1$^+$ $\gamma\delta$ T cells (white bars), WC1.2$^+$ $\gamma\delta$ T cells (light grey bars) and WC1^{neg} $\gamma\delta$ T cells (dark grey bars) were FACS purified as in Fig. 1. Purified $\gamma\delta$ T-cell subsets were then cultured for 24 hr ± 1,25D$_3$ and Poly(I:C) (a) or Imiquimod (b). Cells were then harvested and analysed by real-time PCR for 24-OHase mRNA expression. Results were normalized to the housekeeping gene RPS-9, and then expressed relative to unstimulated control cultures. Data are representative of two independent experiments and represent means ± SEM (n = 4–6 animals/group). †$P < 0{\cdot}1$, *$P < 0{\cdot}05$, **$P < 0{\cdot}001$.

These results suggest that while bovine $\gamma\delta$ T cells are capable of responding to 1,25D$_3$ treatment, it does not significantly alter their responses to viral TLR stimulation.

Neonatal $\gamma\delta$ T cells produce chemokines early in the response to BRSV

Much research has been conducted to investigate the role of $\gamma\delta$ T cells in immunity to bacterial and parasitic infections in the bovine system;[3,23,46–48] however, less is known about the role of $\gamma\delta$ T cells in the immune response to viral infections. Given our results demonstrating robust bovine $\gamma\delta$ T-cell responses to viral TLR agonists Poly(I:C) and Imiquimod, we decided to investigate the role of these cell subsets in the immune response to a viral infection. Because $\gamma\delta$ T cells may play a role in first-line host defence, i.e. the innate recognition of viruses, before the development of adaptive immunity, we

examined the anti-viral response of γδ T cells from uninfected calves. To this end, we sort-purified γδ T-cell subsets from the peripheral blood of uninfected calves as described in Figs 1–4, and stimulated them *in vitro* for 48 hr with a 0·1 MOI of BRSV-375. The specific antigens and antigen-presenting molecules that are necessary for γδ T-cell activation remain unclear; however, γδ T cells appear to require the presence of APC during stimulation.[4] Therefore, in parallel, we also co-cultured purified WC1 γδ T-cell subsets in the presence of autologous monocytes ± 0·1 MOI BRSV-375. The γδ T cells were then harvested and analysed by real-time PCR for mRNA expression of the chemokines MCP-1 (CCL2) (Fig. 5a) and MIP-1α (Fig. 5b) and the regulatory cytokines IL-10 (Fig. 5c) and TGF-β (Fig. 5d). *In vitro* BRSV stimulation induced increased expression of both MCP-1 and MIP-1α by WC1.1^{+} and WC1^{neg} γδ T-cell subsets (Fig. 5a,b), but not increased expression of TGF-β (Fig. 5d), similar to the effects observed with Poly(I:C) or Imiquimod stimulation. Also, like the results observed using the TLR agonists, BRSV stimulation induced increased expression of TGF-β by the more regulatory WC1.2^{+} γδ subset (Fig. 5d). The addition of APC to the cultures had no significant effect on the expression of MIP-1α, MCP-1 or TGF-β by any of the subsets. Interestingly, although there was a trend, *in vitro* stimulation with BRSV, in the presence or absence of APC, did not induce a significant change in expression of GM-CSF (data not shown) or IL-10 (Fig. 5c) by any of the subsets (data not shown). Levels of IFN-γ expression remained undetectable in all three subsets following BRSV stimulation (data not shown). These results demonstrate that, upon initial encounter with BRSV, γδ T cells produce some inflammatory chemokines, but are not induced to express the more adaptive cytokines IFN-γ or IL-10.

Given the evidence that bovine γδ T cells are known to respond following *in vivo* experimental challenge to viral pathogens such as BVDV, bovine leukaemia virus and foot-and-mouth-disease virus,[49–52] we next wanted to examine the capacity of bovine γδ T cells isolated from BRSV-infected neonatal calves to respond to viral re-stimulation *in vitro*. Healthy Holstein calves, 5–6 weeks old, were infected with BRSV strain 375 via aerosol inoculation as described in the Materials and methods. On day 3 p.i., γδ T-cell subsets were purified from the peripheral blood based upon their expression of the WC1 receptor and cultured for 48 hr ± monocytes and ± 0·1 MOI BRSV-375 as indicated. γδ T cells were then harvested and analysed by real-time PCR for mRNA expression of the chemokines MCP-1 (Fig. 6a), MIP-1α (Fig. 6c) and

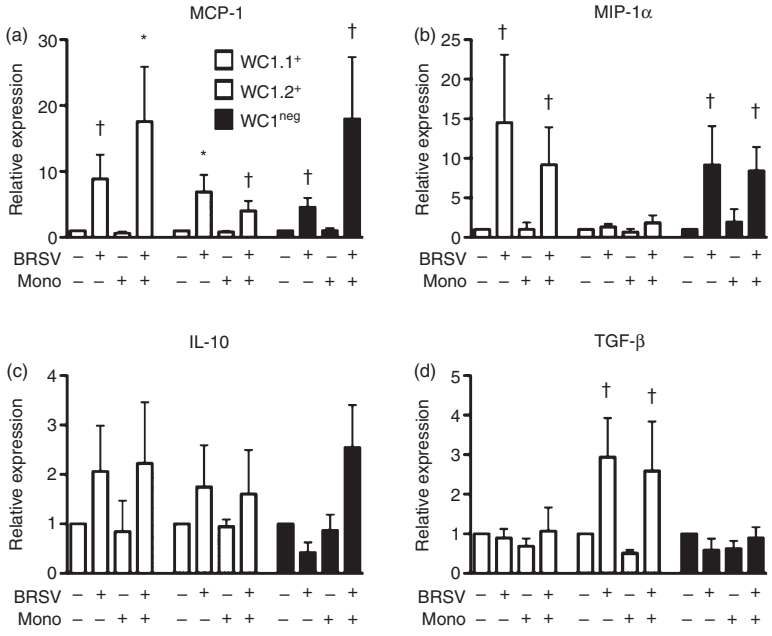

Figure 5. γδ T cells from healthy calves produce macrophage inflammatory protein 1 (MIP-1α) and monocyte chemoattractant protein 1 (MCP-1) in response to *in vitro* stimulation with bovine respiratory syncytial virus (BRSV). Peripheral blood was collected from uninfected neonatal calves and WC1.1^{+} γδ T cells (white bars), WC1.2^{+} γδ T cells (light grey bars) and WC1^{neg} γδ T cells (dark grey bars) were FACS purified as in Fig. 1. Peripheral blood monocytes were enriched by adherence as described in Materials and methods. γδ T-cell subsets were then cultured *in vitro* in the presence or absence of monocytes for 48 hr ± stimulation with BRSV. Cells were collected and analysed for mRNA expression by real-time PCR (left panels) for the chemokines MCP-1 (a), MIP-1α (b) and transforming growth factor-β (TGF-β) (c). For real-time PCR analysis, results were normalized to the housekeeping gene RPS-9, and expressed relative to unstimulated control cultures. Data are from one independent experiment and represent means ± SEM (n = 5 animals/group). †$P < 0·1$, *$P < 0·05$ compared with unstimulated control cultures.

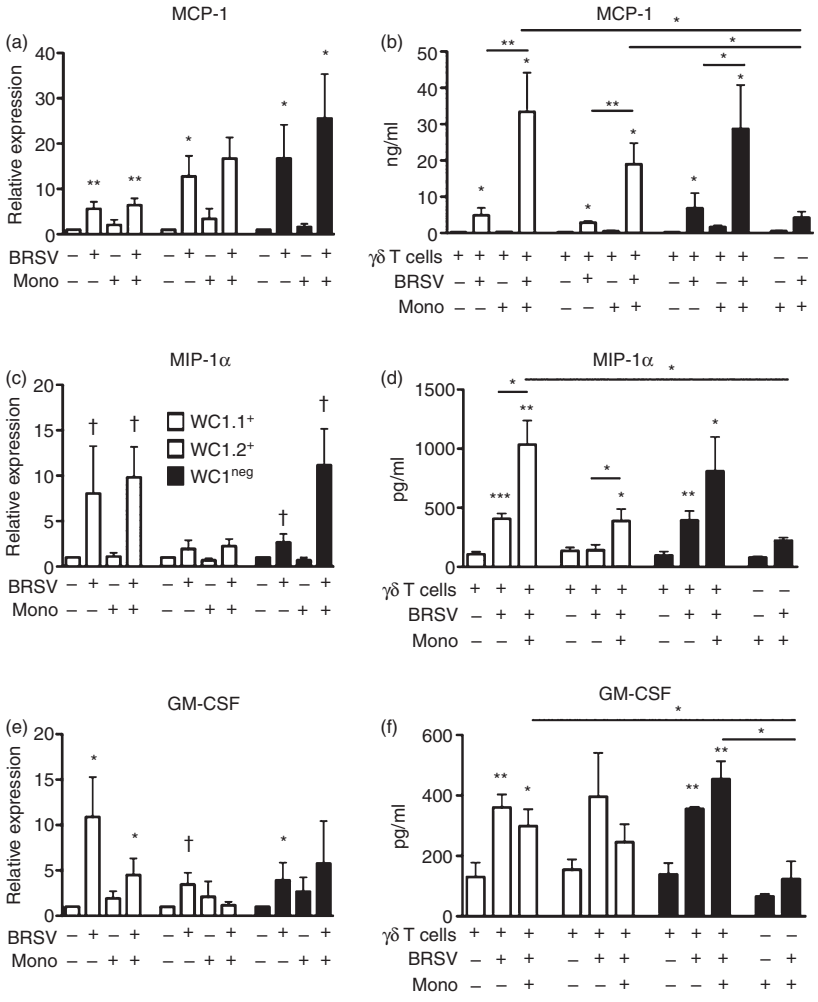

Figure 6. Bovine $\gamma\delta$ T-cell subsets produce chemokine mRNA and protein early in response to bovine respiratory syncytial virus (BRSV) infection. Neonatal calves were challenged by aerosol inoculation with BRSV strain 375 as described in Materials and methods. On day 3 post-infection (p.i.), peripheral blood was collected and WC1.1$^+$ $\gamma\delta$ T cells (white bars), WC1.2$^+$ $\gamma\delta$ T cells (light grey bars) and WC1^{neg} $\gamma\delta$ T cells (dark grey bars) were FACS purified as in Fig. 1. Peripheral blood monocytes were enriched by adherence as described in Materials and methods. $\gamma\delta$ T-cell subsets were then cultured *in vitro* in the presence or absence of monocytes for 48 hr ± stimulation with BRSV. Cells and supernatants were collected and analysed for mRNA expression by real-time PCR (left panels) and protein secretion by ELISA (right panels) for the chemokines monocyte chemoattractant protein 1 (MCP-1) (a and b), macrophage inflammatory protein 1α (MIP-1α) (c and d), and granulocyte–macrophage colony-stimulating factor (GM-CSF) (e and f). For real-time PCR analysis, results were normalized to the housekeeping gene RPS-9, and expressed relative to unstimulated control cultures. Data are pooled from two independent experiments and represent means ± SEM ($n = 7$ animals/group). $^{\dagger}P < 0{\cdot}1$, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with unstimulated control cultures unless otherwise indicated.

GM-CSF (Fig. 6e). Supernatants were also collected from these cultures and analysed by ELISA for secretion of MCP-1 (Fig. 6b), MIP-1α (Fig. 6d) and GM-CSF (Fig. 6f). As seen in Figs 6(a,b), *in vitro* re-stimulation by BRSV induced up-regulation of MCP-1 mRNA expression (Fig. 6a) and protein secretion (Fig. 6b) by all three WC1 $\gamma\delta$ T-cell subsets. We observed the most pronounced increase in mRNA expression in the WC1^{neg} subset (dark grey bars); however, this difference was not associated with increased protein secretion, as we observed similar levels of MCP-1 in the culture supernatants from all three WC1 subsets (Fig. 6b). Notably, the production of MCP-1 by each $\gamma\delta$ T-cell subset on day 3 p.i. did not differ significantly from that observed before infection (Fig. 5). This result supports the hypothesis that chemokine production is primarily an innate function for $\gamma\delta$ T cells, perhaps mediated through PRRs, as opposed to a BRSV-specific response. For the WC1.1$^+$ (white bars) and WC1.2$^+$ (light grey bars) $\gamma\delta$ subsets, addition of autologous APC to the purified $\gamma\delta$ cultures did result in a significant increase in MCP-1 protein concentration in the supernatants over either $\gamma\delta$ T cells cultured alone or monocytes cultured alone (black bars) (Fig. 6b); however, this effect was not synergistic. Addition of APC did not translate to a significant increase in MCP-1 mRNA expression by the $\gamma\delta$ T-cell subsets. Together, the results from Figs 5 and 6 suggest that, at least for the innate immune function of chemokine production, bovine WC1.1$^+$, WC1.2$^+$ and WC1^{neg} $\gamma\delta$ T cells from BRSV-infected calves are capable of recognizing and responding to BRSV in the absence of APC.

These findings were further supported when analysing $\gamma\delta$ T-cell MIP-1α and GM-CSF mRNA expression (Fig. 6c,e) and protein secretion (Fig. 6d,f). Chemokine mRNA expression and protein secretion was significantly induced in WC1.1$^+$ $\gamma\delta$ T cells (white bars) following BRSV re-stimulation (Fig. 6c,e), but there was no additional increase in mRNA expression in the presence of APC, nor did we observe a synergistic increase in protein secretion in these cultures (Fig. 6d,f). WC1^{neg} $\gamma\delta$ T cells (dark grey bars) also responded to BRSV with MIP-1α and GM-CSF mRNA and protein expression, but the response was not enhanced by the addition of APC. In agreement with our results from Figs 1 and 3, WC1.2$^+$ $\gamma\delta$

T cells (light grey bars) do not produce an appreciable amount of MIP-1α or GM-CSF in response to BRSV.

Neonatal γδ T-cell subsets are unique in their production of adaptive cytokines early in the response to BRSV infection

γδ T cells play a role in bridging the innate and adaptive arms of the immune response through their ability to produce chemokines such as MCP-1 and MIP-1α, as well as their ability to produce adaptive cytokines such as IL-10, TNF-α and IFN-γ. [4] To clarify the role of γδ T cells in the setting of neonatal BRSV infection, we next examined the ability of purified γδ T-cell subsets to produce the adaptive cytokines IL-10 and IFN-γ in response to BRSV re-stimulation *in vitro*. WC1.1$^+$, WC1.2$^+$ and WC1^{neg} γδ T-cell subsets were sort purified from the blood of day 3 BRSV-infected calves and cultured ± autologous monocytes with 0·1 MOI BRSV-375 as in Fig. 6. After 48 hr in culture, γδ T cells were collected and analysed by real-time PCR for mRNA expression of IL-10 (Fig. 7a) and IFN-γ (Fig. 7b) and the culture supernatants were analysed by ELISA or Multiplex Cytokine ImmunoAssay for protein secretion of IL-10 (Fig. 7b) and IFN-γ (Fig. 7d), respectively. In agreement with our results following TLR3 or TLR7 stimulation (Figs 1 and 2) WC1.2$^+$ γδ T cells express and secrete robust levels of IL-10 mRNA (Fig. 7a) and protein (Fig. 7b) in response to *in vitro* BRSV re-stimulation. Interestingly, this production was not significantly altered or enhanced by the addition of autologous monocytes to the cultures, suggesting that WC1.2$^+$ γδ T cells may not require interactions with professional APC to recognize and respond to BRSV *in vitro*. In contrast to the WC1.2$^+$ subset, the WC1.1$^+$ (white bars) and WC1^{neg} (dark grey bars) γδ T-cell subsets produced only negligible amounts of IL-10 mRNA or protein (Fig. 7a,b).

WC1.1$^+$ γδ T cells are robust producers of IFN-γ and are thought to be the primary responding γδ T-cell subset in a number of disease settings in the bovine.[21–23,48] In support of previous findings, we observed IFN-γ mRNA (Fig. 7c) and protein (Fig. 7d) production by the WC1.1$^+$ γδ T-cell subset (white bars) in response to BRSV re-stimulation. Interestingly, while the WC1.1$^+$ subset appeared capable of recognizing BRSV in culture in the absence of autologous APC, as measured by an approximately fourfold increase in IFN-γ mRNA expression (Fig. 7c), addition of APC significantly enhanced both IFN-γ mRNA and protein (Fig. 7c,d, respectively). These results suggest that while chemokine secretion may be a γδ T-cell function that occurs independent of antigen presentation, IFN-γ production requires additional signals

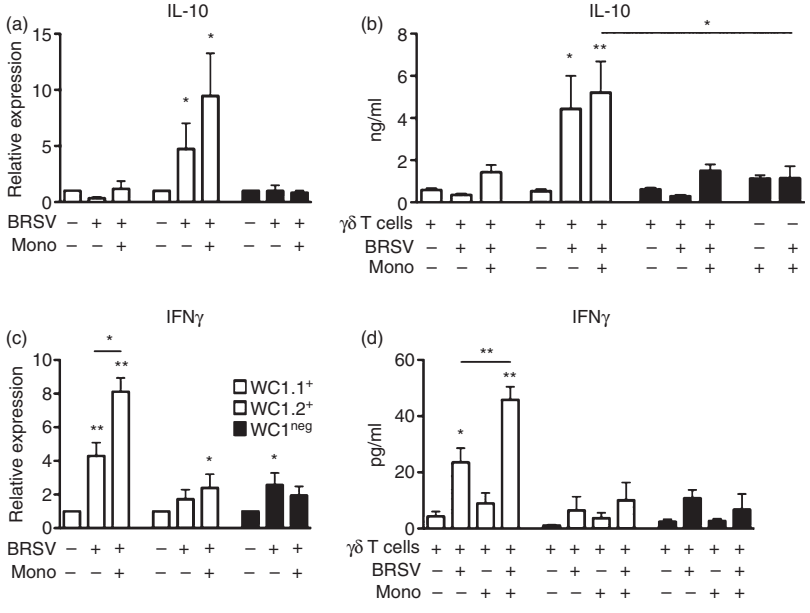

Figure 7. Bovine γδ T-cell subsets respond to early bovine respiratory syncytial virus (BRSV) infection. Neonatal calves were challenged by aerosol inoculation with BRSV strain 375 as in Fig. 5. On day 3 post-infection (p.i.), peripheral blood was collected and γδ T-cell subsets and peripheral blood monocytes were prepared as in Fig. 5. γδ T-cell subsets were then cultured *in vitro* ± monocytes for 48 hr with BRSV as indicated. Cells and supernatants were then collected and analysed for mRNA expression by real-time PCR (left panels) and protein secretion by ELISA or Multiplex Cytokine Array (right panels) for the cytokines interleukin-10 (IL-10) (a and b) and interferon-γ (IFN-γ) (c and d). For real-time PCR analysis, results were normalized to the housekeeping gene RPS-9, and expressed relative to unstimulated control cultures. Data are pooled from two experiments and represent means ± SEM ($n = 7$ animals/group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, compared with unstimulated control cultures unless otherwise indicated.

as provided by professional APC. Neither WC1.2$^+$ nor WC1^{neg} $\gamma\delta$ T cells produced significant IFN-γ in response to BRSV re-stimulation (Fig. 7c,d). In addition to IFN-γ, we also examined $\gamma\delta$ T-cell production of IL-2, IL-4, IL-6, IL-1β and TNF-α following BRSV re-stimulation by Multiplex Cytokine ImmunoAssay; however, we did not observe any significant changes in these cytokines under these culture conditions. Together, these results suggest that bovine $\gamma\delta$ T cells have different requirements for stimulation, depending upon the functional read-out, and further support the hypothesis that there are key differences between the WC1.1$^+$, WC1.2$^+$ and WC1^{neg} $\gamma\delta$ T cell subsets and their role in the bovine during respiratory viral infection.

Bovine $\gamma\delta$ T-cell subsets respond to BRSV at late time-points after infection

Given the important role of $\gamma\delta$ T cells in bridging the innate and adaptive immune responses, we hypothesized that the functional responsiveness of bovine $\gamma\delta$ T cells may change during the course of infection. Therefore, we chose to examine responses of $\gamma\delta$ T cells that were purified from the blood of BRSV-infected calves on day 10 p.i., a time when adaptive immune responses are thought to be peaking and replicating virus has been eliminated.[53,54] Bovine WC1.1$^+$, WC1.2$^+$ and WC1^{neg} $\gamma\delta$ T cells were purified from the peripheral blood of day 10 BRSV-infected neonatal calves and re-stimulated *in vitro* with a 0·1 MOI of BRSV ± autologous monocytes. After 48 hr, $\gamma\delta$ T cells were collected and analysed by real-time PCR for mRNA expression of MCP-1 (Fig. 8a), GM-CSF (Fig. 8b), MIP-1α (Fig. 8c), IFN-γ (Fig. 8d), IL-10 (Fig. 8e) and TGF-β (Fig. 8f); culture supernatants were collected and analysed by ELISA for protein secretion of MCP-1 (Fig. 9a), IL-10 (Fig. 9b) and TGF-β (Fig. 9c). As seen in Fig. 8(a–c), bovine $\gamma\delta$ T-cell subsets produced MCP-1, GM-CSF and MIP-1α in response to BRSV re-stimulation even at late time-points after infection. Interestingly, the overall magnitude of the MCP-1 response appeared to be much greater than that observed at day 3 p.i. (Fig. 6a), although the profile of the subsets seems to have changed over time, as WC1.1$^+$ and WC1^{neg} subsets were the predominant producers at day 10 p.i. (Fig. 8a), whereas all three subsets were relatively equal in their expression in response to BRSV re-stimulation at day 3

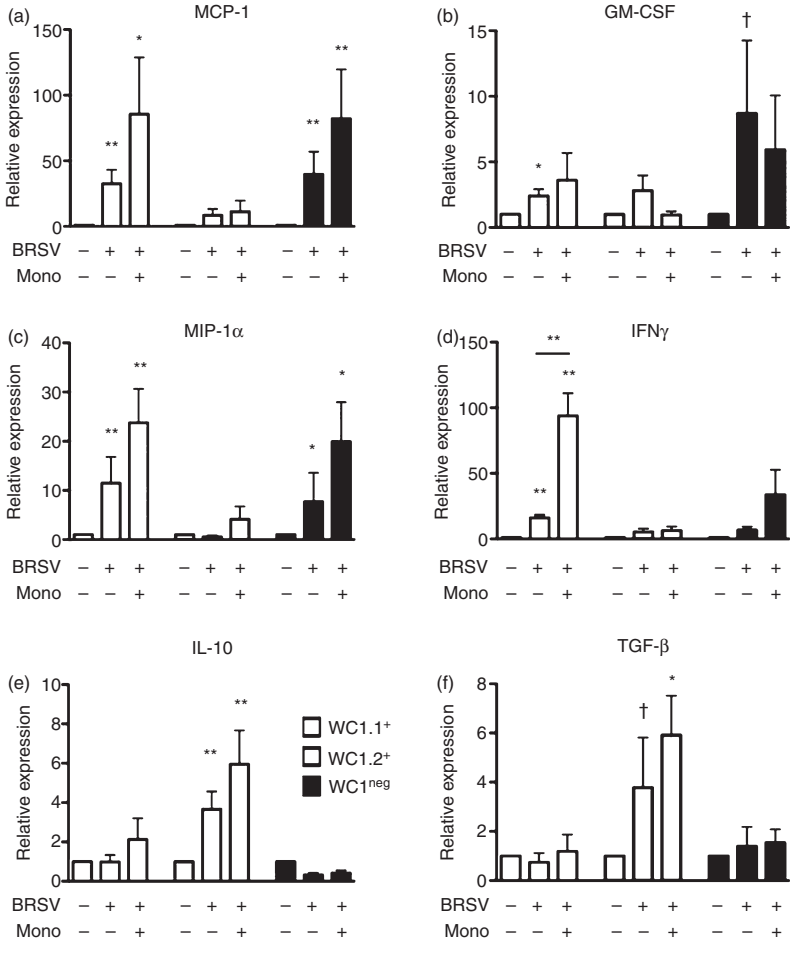

Figure 8. Bovine $\gamma\delta$ T-cell subsets respond to late bovine respiratory syncytial virus (BRSV) infection. Neonatal calves were challenged by aerosol inoculation with BRSV strain 375 as in Fig. 5. On day 10 post-infection (p.i.), peripheral blood was collected and WC1.1$^+$ $\gamma\delta$ T cells (white bars), WC1.2$^+$ $\gamma\delta$ T cells (light grey bars) and WC1^{neg} $\gamma\delta$ T cells (dark grey bars) were FACS purified as in Fig. 1. Peripheral blood monocytes were enriched by adherence. $\gamma\delta$ T-cell subsets were then cultured *in vitro* ± monocytes for 48 hr with BRSV as indicated. Cells were then harvested and analysed by real-time PCR for mRNA expression of monocyte chemoattractant protein 1 (MCP-1) (a), granulocyte–macrophage colony-stimulating factor (GM-CSF) (b), macrophage inflammatory protein 1α (MIP-1α) (c), interferon-γ (IFN-γ) (d), interleukin-10 (IL-10) (e) and transforming growth factor-β (TGF-β) (f). Results were normalized to the housekeeping gene RPS-9, and expressed relative to unstimulated control cultures. Data are pooled from two experiments and represent means ± SEM (n = 8 animals/group). $^{\dagger}P < 0{\cdot}1$, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with unstimulated control cultures unless otherwise indicated.

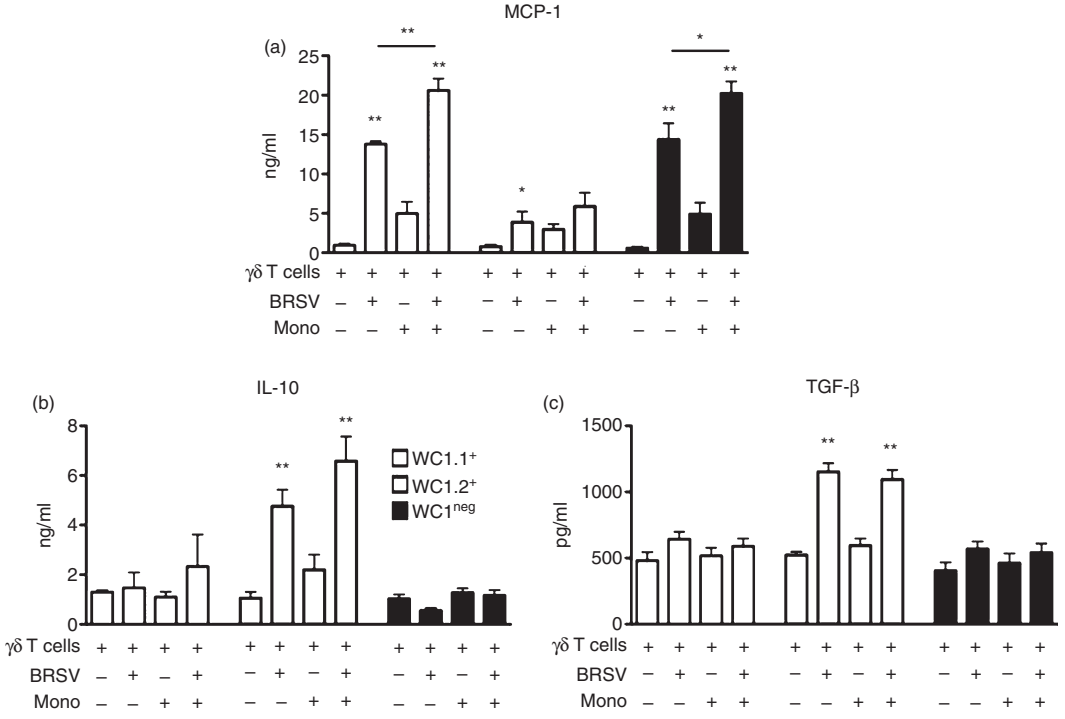

Figure 9. Bovine γδ T-cell subsets secrete cytokines in response to stimulation by bovine respiratory syncytial virus (BRSV) late following infection. Neonatal calves were challenged by aerosol inoculation with BRSV strain 375 as in Fig. 5. On day 10 post-infection (p.i.), peripheral blood was collected and WC1.1$^+$ γδ T cells (white bars), WC1.2$^+$ γδ T cells (light grey bars) and WC1^{neg} γδ T cells (dark grey bars) were FACS purified as in Fig. 1. Peripheral blood monocytes were enriched by adherence. γδ T-cell subsets were then cultured *in vitro* ± monocytes for 48 hr with BRSV as indicated. Supernatants were then harvested and analysed by ELISA for monocyte chemoattractant protein 1 (MCP-1) (a), interleukin-10 (IL-10) (b) and transforming growth factor-β (TGF-β) (c). Data are pooled from two independent experiments and represent means ± SEM (n = 8 animals/group). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with unstimulated control cultures unless otherwise indicated.

p.i. (Fig. 6a). These results were further confirmed by analysis of secreted MCP-1 protein in the culture supernatants, with both the WC1.1$^+$ (white bars) and WC1^{neg} (dark grey bars) subsets producing significantly more MCP-1 than the WC1.2$^+$ subset (light grey bars) (Fig. 9a). Similar changes were not observed in γδ subset chemokine production profiles when examining mRNA expression for GM-CSF (Fig. 8b) or MIP-1α (Fig. 8c) in response to BRSV re-stimulation on day 3 versus day 10 p.i..

We next chose to examine γδ T-cell production of the cytokines IFN-γ, IL-10 and TGF-β at a late time-point after infection. As on day 3, the WC1.2$^+$ γδ subset responds to BRSV re-stimulation with significant IL-10 (Fig. 8e) and TGF-β (Fig. 8f) expression on day 10 p.i. This expression is reflected in the culture supernatants (Fig. 9b,c). Similar to the results we observed on day 3 p.i., WC1.1$^+$ γδ were the primary IFN-γ-producing subset following BRSV re-stimulation (Fig. 9d), and this response was significantly increased in the presence of autologous APC. Interestingly, the magnitude of the response was significantly greater than that observed at day 3 p.i., with an approximately 100-fold increase in IFN-γ expression at day 10 p.i. (Fig. 8d) compared with an eight-fold increase in expression at day 3 p.i. (Fig. 7c). These results suggest enhanced sensitivity to BRSV or perhaps an increased number of circulating WC1.1$^+$ cells capable of responding to BRSV.

Discussion

We report here that neonatal bovine γδ T cells directly respond to stimulation with the TLR3 agonist, Poly(I:C) and the TLR7 agonist, Imiquimod. Although this ability occurs in human γδ T cells,[11,12] to our knowledge, this is the first description of bovine γδ T cells responding directly through these virus-sensing TLRs. In addition, we demonstrate that γδ T cells isolated from infected calves respond directly to BRSV re-stimulation. Interestingly, the response to both TLR stimulation and virus appeared to differ between γδ T-cell subsets, as we observed robust chemokine and inflammatory cytokine production by WC1.1$^+$ γδ T cells, but regulatory cytokine production by WC1.2$^+$ γδ T cells. Although previously shown in a normal animal, this is the first report to our knowledge of WC1.2$^+$ γδ T cells playing a regulatory role in the context of a viral infection. We go on to further demonstrate that while γδ T cells appeared capable of responding to the

active vitamin D metabolite $1,25D_3$, it did not alter their response to either TLR agonists or BRSV. Our results are a key first step in understanding the role of bovine $\gamma\delta$ T cells in antiviral immunity, and, more generally, in elucidating the unique functional roles played by various $\gamma\delta$ T-cell subsets in the neonatal calf.

BRSV infection in cattle shares many features with RSV infection in humans. Like children, calves infected with BRSV exhibit fever, anorexia, nasal discharge and cough.[28,30] Histopathologically, comparable microscopic lesions are observed in the lungs of both species, including bronchiolitis with interstitial pneumonia, bronchiolar neutrophilic infiltrates and extensive epithelial cell necrosis.[28–30] Calves and humans also up-regulate several pro-inflammatory cytokines in response to infection including IFN-γ, TNF-α, IL-6, IL-12p40 and IL-8.[28–30] Further, cattle have large numbers of circulating $\gamma\delta$ T cells: an average of 20% of circulating lymphocytes in adult animals, compared with only 5% in adult humans.[1–3] Hence, we believe that cattle are an ideal model for understanding the immune response to RSV infection, and in particular, the role of $\gamma\delta$ T cells in that response.

$\gamma\delta$ T cells have been clearly demonstrated to respond to an array of infectious bacterial and protozoan organisms (reviewed in refs 3,55,56). However, the role of $\gamma\delta$ T cells in immunity to viral infections is less well studied. In mice, $\gamma\delta$ T cells play a role in controlling vaccinia virus replication early after infection[57] and in protecting the host during herpes simplex virus type 1 infection.[58] In humans, $\gamma\delta$ T cells accumulate to high frequencies in the blood of patients infected with Epstein–Barr virus and HIV-1, and respond *in vitro* to cells infected with HIV, Epstein–Barr virus and human herpes simplex virus type 1 (reviewed in ref. 59). The evidence for $\gamma\delta$ T-cell responses in bovids during viral infections remains sparse. An increase in the number of circulating $\gamma\delta$ T cells has been reported in cattle infected with bovine leukaemia virus, bovine herpes-1 virus and BVDV.[49–51] A recent paper by Toka *et al.*[52] reported that numbers of circulating $\gamma\delta$ T cells expand significantly in calves infected with foot-and-mouth-disease virus, and $\gamma\delta$ cells from infected animals demonstrate increased inflammatory cytokine production and an increased ability to kill non-specific target cells.[52] Taylor *et al.*[33] reported that depletion of $WC1^+$ $\gamma\delta$ T cells from neonatal gnotobiotic calves infected with BRSV had little effect on clinical signs, pulmonary lesions or viral clearance after 10 days of infection, but resulted in a significant increase in IgM and IgA in the BAL. In mice, depletion of $\gamma\delta$ T cells before RSV infection resulted in reduced lung inflammation and disease severity, but also an increase in peak viral replication.[32] Our results clearly demonstrate that bovine $\gamma\delta$ T cells can respond to BRSV *in vitro* with robust chemokine and cytokine production, suggesting that the same recognition probably occurs *in vivo*. Future studies should be aimed at more clearly elucidating the function of $\gamma\delta$ T cells during BRSV infection including their innate role in regulating early virus recognition and lymphocyte recruitment, as well as understanding the part they may play after BRSV infection during the secondary bacterial infections associated with BRDC.

The experiments described in the present study focus on the response of $\gamma\delta$ T-cell subsets found in the peripheral blood following BRSV infection; however, it is interesting to speculate on the potential role of $\gamma\delta$ T cells in the tissue following BRSV infection. Currently, little is known about the chemokines produced by the inflamed lung during either BRSV infection in calves or RSV infection in children, or what role they play in shaping the ensuing immune response.[28] However, the balance between Th1 and Th2 type cytokine responses are particularly important in the context of RSV infection, and it is known that the early chemokine response has a significant effect on the resulting inflammatory milieu. Bovine $\gamma\delta$ T cells are known to home to sites of inflammation.[60,61] and have been described as accumulating in draining lymph nodes and lungs following both RSV infection[32,53,54] and other respiratory challenges.[48,62–64] A detailed analysis of these tissue-recruited subsets and their functional phenotype is presently lacking; however, $\gamma\delta$ T cells have significant potential for shaping the immune response both in the lymph nodes and at the site of infection. Important future studies will be aimed at examining the tissues of the respiratory tract following BRSV infection with the goal of identifying and characterizing chemokine expression therein, as well as describing the populations of $\gamma\delta$ T cells recruited to these sites of infection and elucidating their role in dictating anti-viral immune responses in the neonatal calf.

A growing body of literature has suggested a previously unrecognized role for vitamin D in regulating the immune system.[65] Epidemiological evidence has suggested a correlation between low circulating levels of vitamin D and susceptibility to respiratory infections, particularly in young children.[34] Treatment of cultured respiratory epithelial cells with $1,25D_3$ inhibits RSV-induced pro-inflammatory cytokine expression.[66] Interestingly, viral replication was not enhanced in these cells, suggesting that vitamin D may help to limit immunopathology without dampening the effective antiviral immune response.[66] Despite convincing *in vitro* evidence for vitamin D in promoting immunity to respiratory infections, the *in vivo* evidence remains conflicting.[67,68] In a study from our laboratory, we observed no correlation between clinical disease severity and circulating levels of vitamin D in a neonatal calf model of BRSV infection.[29] In fact, pro-inflammatory cytokine expression was unchanged, and in some cases, even increased in the lungs of calves with high circulating levels of $25(OH)D_3$.[29] We demonstrated in the present study that $\gamma\delta$ T cells are able to respond to

$1,25D_3$ treatment, as measured by their up-regulation of the vitamin D catabolic enzyme 24-OHase (Fig. 4), but it has little effect on their chemokine or cytokine responses to TLR agonists or virus. The unresponsiveness of γδ T cells to vitamin D treatment *in vitro*, at least as measured by the cytokines examined here, is surprising. Several groups have demonstrated that *in vitro* $1,25D_3$ treatment suppresses T-cell proliferation and pro-inflammatory cytokine production by mix-cultured T lymphocytes (reviewed in ref. 69), although these studies have primarily focused on CD4 or CD8 T cells, rather than γδ T cells. In our studies, $1,25D_3$ treatment had no effect on γδ T-cell expression of IFN-γ or the chemokines MCP-1, MIP-1α or GM-CSF. Literature also suggests that vitamin D may promote the development and enhanced function of $FoxP3^+$ T regulatory cells in mice and humans.[45] In our hands, responses of $WC1.2^+$ γδ T cells to TLR agonist or virus stimulation were not affected by vitamin D. The reason for this lack of responsiveness is unknown. Vitamin D may be modulating the expression of certain molecules that we did not examine. It is also possible that vitamin D has a bystander effect on γδ T-cell cytokine production, as opposed to a direct effect – a result that might be explained by our use of purified γδ T cells as opposed to analysing the cells in a mixed lymphocyte culture. Finally, the concentration of vitamin D, and in particular, the active metabolite $1,25D_3$, is likely to have a significant effect on the experimental outcome. Here, we used 4 ng/ml $1,25D_3$, which is approximately 10^{-8} M, and two to three orders of magnitude higher than that observed in normal serum.[37] However, this concentration is more physiologically relevant than other reports, which have commonly used concentrations as high as 10^{-7} M;[66,70,71] so the reported range of $1,25D_3$ concentrations varies dramatically and is a likely contributor to the variable results.

The requirements for γδ T-cell antigen recognition remain poorly defined. In general, γδ T cells respond to non-peptide phosphorylated molecules via a mechanism that is TCR dependent.[1,3,55] In cattle, antibody blockade of the δ chain of the TCR abrogates responses to *Babesia bovis*,[72] *Theileria parva*,[46] and *Anaplasma marginale*.[24] It is clear that γδ T-cell responses are not MHC I or II restricted; however, the majority of systems described so far require the presence of a professional APC,[1,3,55,73] and so probably some form of antigen-presenting molecule. The recent descriptions of γδ T cells responding directly via PRRs,[8–10,12] including TLRs, have added an additional layer of complexity. Freshly isolated bovine γδ T cells respond directly to lipopolysaccharide and peptidoglycan, as well as the NOD2 agonist muramyl dipeptide in the absence of APC, additional antigen or TCR signals.[9,10] Interestingly, this response, which the authors have termed 'antigen-independent priming', appears distinct from that of the response triggered by antigen presented in the context of an APC. γδ T cells stimulated with lipopolysaccharide or peptidoglycan alone exhibit minor increases in gene transcription for IFN-γ and TNF-α,[10] but the most notable changes were an increase in the production of several chemokines including IL-8, MIP-1α, MIP-1β and GM-CSF.[3,9,13] The authors propose that antigen-independent priming enables more rapid and efficient responses to secondary signals such as an encounter with the appropriate antigen in the context of an APC.[3,9] These results are in direct agreement with those presented herein, whereby we observed significant MIP-1α, GM-CSF and MCP-1 production by purified γδ T-cell subsets following stimulation with the viral TLR mimics Poly(I:C) or Imiquimod, suggesting that this form of priming is not unique to stimulation from bacterial products. In our model, we observed only minor IFN-γ up-regulation following TLR stimulation alone, while addition of a TCR signal significantly enhanced its expression (Fig. 3).

Bovine γδ T cells express the co-receptor Workshop Cluster 1 (WC1), a member of the scavenger receptor cysteine-rich (SRCR) family of proteins.[74–77] $WC1^+$ γδ T cells are negative for CD2, CD4 and CD8, whereas the majority of $WC1^{neg}$ γδ T cells express CD2 and CD8.[74–77] There are 13 WC1 genes and differential expression of these gene products can be used to divide $WC1^+$ γδ T cells into multiple serologically defined subpopulations: $WC1.1^+$, $WC1.2^+$ and $WC1.3^+$.[18,19]

Until recently, it was unknown if differential expression of the WC1 gene could be ascribed to distinct immune functions; however, several studies now suggest that the role of these subsets may be unique. Known to produce robust amounts of IFN-γ in response to mitogen stimulation,[21,22] it is now clear that $WC1.1^+$ cells are pro-inflammatory in infectious disease settings including experimental challenge with *Leptospira*[21,23] and *Mycobacterium*.[48] In contrast, $WC1,2^+$ cells undergo extensive proliferation, but produce little IFN-γ in response to mitogen stimulation.[21,22] Further, the $WC1.2^+$ subset does not respond to *Leptospira*,[21,22] but proliferates and produces IFN-γ in response to the bovine rickettsial pathogen *Anaplasma marginale*.[24] A recent report by Hoek *et al.*[7] suggests a regulatory role for the $WC1.2^+$ subset, demonstrating that cells purified directly *ex vivo* exhibit robust IL-10 production and suppress CD4 T-cell proliferation, even in the absence of additional stimuli. It currently remains unclear if this regulatory state is maintained in the face of antigenic stimulation or experimental challenge. The $WC1^{neg}$ subset, while an appreciable population in peripheral blood, is more numerous in tissues such as the spleen, mesenteric lymph nodes and intestinal mucosa.[20,60,74] Studies examining $WC1^{neg}$ responses to antigenic stimulation or infection are lacking; however, reports that have analysed $WC1^{neg}$ gene expression in a normal animal suggest an immune

sentinel role, with expression of genes promoting tissue quiescence and apoptosis.[13–15]

To our knowledge, the current study is one of the first to provide a detailed description of the unique functional differences between WC1.1$^+$, WC1.2$^+$ and WC1^{neg} $\gamma\delta$ T-cell subsets following virus infection. Thought to act as a co-stimulatory molecule, similar to CD4 or CD8 on $\alpha\beta$ T cells,[20,78,79] the function of WC1 on $\gamma\delta$ T cells has remained enigmatic until recently. A series of elegant studies by the Baldwin and Telfer laboratories have begun to elucidate the role of WC1 as a molecule that can participate directly in bovine $\gamma\delta$ T-cell antigen recognition and/or activation. Rogers *et al.* reported that bovine $\gamma\delta$ T-cell responsiveness to the spirochaete *Leptospira* was confined to a subpopulation of WC1.1$^+$ $\gamma\delta$ T cells.[21,22] Using RNA interference, Wang *et al.*[23] went on to demonstrate that experimental down-regulation of three of the WC1 genes associated with expression of WC1.1 significantly inhibits *Leptospira*-specific $\gamma\delta$ T-cell proliferation and IFN-γ production. Given that bovine $\gamma\delta$ T cells can only express a restricted set of available TCR genes, the authors suggest that the WC1 co-receptor acts as an additional regulatory element to the $\gamma\delta$ immune response lending specificity or dictating subset reactivity.[23] Our results are in agreement with these and other studies suggesting that differential WC1 expression can be correlated with differences in $\gamma\delta$ responsiveness,[7,21–24,48] but are also unique in that this responsiveness has not been previously shown with respect to viral infection or to TLR stimulation. Although we observed a correlation with WC1.1 expression and increased chemokine production, it is unclear if or how the WC1 molecule may be regulating this response. Additional research is necessary to understand how priming through TLR receptors, i.e. TLR3 and TLR7 in the case of our studies, is integrated with signals through the TCR and through the co-receptor WC1 to dictate $\gamma\delta$ T-cell responsiveness to both TLR agonists and more specific antigens such as those to BRSV or *Leptospira*.

In conclusion, we demonstrated for the first time that neonatal bovine $\gamma\delta$ T cells respond directly to stimulation through TLR3 and TLR7, as well as to direct stimulation with the bovine viral pathogen, BRSV. This response varied significantly between WC1 expressing $\gamma\delta$ T-cell subsets, with WC1.1$^+$ and WC1^{neg} populations producing pro-inflammatory chemokines, and the WC1.2$^+$ population producing regulatory IL-10 and TGF-β. We are just beginning to elucidate the unique functional roles played by bovine $\gamma\delta$ T-cell subsets, as well as the mechanisms they use to integrate signals through various receptor–ligand interactions including TLRs, WC1 and the $\gamma\delta$ TCR. Future studies should be aimed at a deeper understanding of the ways in which $\gamma\delta$ T cells recognize and respond to immune challenge, with the goal of developing more effective vaccinations and anti-viral therapies.

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Author contributions

The experiments were conceived and designed by JLM, BJN, JDL, TAR and RES and performed by JLM, BJN and RES. Data were analysed by JLM and RES and JLM, BJN, JDL, TAR and RES wrote the paper.

Disclosures

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Gating strategy for sorting $\gamma\delta$ T-cell subsets.