Published in final edited form as:

Vet Immunol Immunopathol. 2013 September 1; 155(0): 87–97. doi:10.1016/j.vetimm.2013.06.008.

Ovine skin-recirculating $\gamma\delta$ T cells express IFN- γ and IL-17 and exit tissue independently of CCR7

Skye A. Geherin^a, Michael H. Lee^a, R. Paul Wilson, and Gudrun F. Debes

University of Pennsylvania, School of Veterinary Medicine, Department of Pathobiology, 380 South University Avenue, Philadelphia, PA 19104, USA

Abstract

$\gamma\delta$ T cells continuously survey extralymphoid tissues, providing key effector functions during infection and inflammation. Despite their importance, the function and the molecules that drive migration of skin-recirculating $\gamma\delta$ T cells are poorly described. Here we found that $\gamma\delta$ T cells traveling in the skin-draining afferent lymph of sheep are effectors that produce IFN- γ or IL-17 and express high levels of the skin- and inflammation-seeking molecule E-selectin ligand. Consistent with a role for chemokine receptor CCR7 in mediating T cell exit from extralymphoid tissues, conventional CD4 and CD8 T cells in skin-draining lymph were enriched in their expression of CCR7 compared to their skin-residing counterparts. In contrast, co-isolated $\gamma\delta$ T cells in skin or lymph lacked expression of CCR7, indicating that they use alternative receptors for egress. Skin-draining $\gamma\delta$ T cells were unresponsive to many cutaneous and inflammatory chemokines, including ligands for CCR2, CCR4, CCR5, CCR8, CCR10, and CXCR3, but showed selective chemotaxis toward the cutaneously expressed CCR6 ligand CCL20. Moreover, IL-17⁺ $\gamma\delta$ T cells were the most CCL20-responsive subset of $\gamma\delta$ T cells. The data suggest that $\gamma\delta$ T cells survey the skin and sites of inflammation and infection, entering via CCR6 and E-selectin ligand and leaving independent of the CCR7-CCL21 axis.

Keywords

$\gamma\delta$ T cells; sheep; skin; inflammation; cell migration; chemokines

1. Introduction

T cell recirculation through extralymphoid tissues is critical to immunosurveillance, host defense, and inflammation. During recirculation, memory/effector T cells enter extralymphoid organs from the blood via a multistep adhesion cascade involving adhesion molecules and chemokines (reviewed in (Ley et al., 2007; Picker and Butcher, 1992)). Memory/effector T cells subsequently exit the skin or other extralymphoid tissues by entering afferent lymph vessels and migrating into draining lymph nodes (Mackay et al.,

Corresponding author: Gudrun F. Debes, gdebes@vet.upenn.edu, Hill Pavilion Room 317, 380 South University Avenue, Philadelphia, PA 19104, Telephone: 215-573-9167, Fax: 215-746-2295.

^aThese authors contributed equally to this work

Conflict of interest statement

The authors declare that they have no conflicts of interest.

1990). After leaving lymph nodes in the efferent lymph, they return to the blood via the thoracic duct and continue surveying the body.

As a barrier organ, the skin is constantly exposed to mechanical, chemical, thermal, or vector-mediated threats and thus constantly threatened by infection following barrier breach. The skin is also a common target of inflammation during allergy and autoimmunity. In addition to conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells reside in the dermis and epidermis of mammalian skin. $\gamma\delta$ T cells are innate-like T cells with unconventional T cell receptors that are not major histocompatibility complex (MHC) restricted and are hypothesized to bind a diverse set of foreign and self antigens (reviewed in (Born et al., 2011; Morita et al., 2007)). Ruminants have exceptionally large numbers of these T cells, particularly early in life, suggesting that they rely more heavily on $\gamma\delta$ T cells for host defense relative to other mammals (Hein and Mackay, 1991). $\gamma\delta$ T cells provide a rapid, early response to viral, bacterial and fungal infection by secretion of effector cytokines such as IFN- γ , IL-4, and IL-17, and several subsets of $\gamma\delta$ T cells have been described based on their localization, function, and thymic T cell receptor (TCR) – ligand interactions (reviewed in (Hayday, 2000; Shibata, 2012)). For example, dendritic epidermal T cells (DETCs), a well-studied sessile population of mouse $\gamma\delta$ T cells, are named for their morphology and localization. Recently, it has been shown that mouse epidermal $\gamma\delta$ T cells provide IL-17 in response to *Staphylococcus aureus* infection (Cho et al., 2010). However, others found that motile dermal $\gamma\delta$ T cells, but not DETCs, rapidly express high levels of IL-17 and are therefore critical in cutaneous infection and inflammation in mice (Cai et al., 2011; Gray et al., 2011; Sumaria et al., 2011). Thus, it is important to understand how different cytokine producing $\gamma\delta$ T cells are mobilized to the skin to promote optimal host immunity or to regulate inflammation.

DETCs upregulate skin-homing receptors after TCR ligation in the thymus (Xiong et al., 2004) and akin to skin homing CD4 T cells (Mora and von Andrian, 2006; Sigmundsdottir and Butcher, 2008), require ligands for E- and P-selectin, as well as CCR4 and CCR10 for their migration into the epidermis (Jiang et al., 2010; Jin et al., 2010). While conventional $\alpha\beta$ T cells depend on CCR7 to egress from extralymphoid tissues, such as the skin, and enter afferent lymph vessels (Bromley et al., 2005; Brown et al., 2010; Debes et al., 2005), the molecules used by recirculating $\gamma\delta$ T cells to enter and exit the skin remain to be identified.

In addition to effector T cell properties, one subset of human $\gamma\delta$ T cells was proposed to function as a professional antigen-presenting cell (APC) (Brandes et al., 2005). Specifically, upon stimulation human V δ 2⁺ T cells upregulate MHC class II (MHCII) and costimulatory molecules as well as the “tissue exit receptor” CCR7 to induce primary responses in naïve $\alpha\beta$ T cells in draining lymph nodes (Brandes et al., 2005). It remains unknown if this finding also applies to $\gamma\delta$ T cells of other species.

Many studies focus on $\gamma\delta$ T cells as they reside in the tissue or in the blood; however, little is known about tissue-recirculating $\gamma\delta$ T cells. Although all jawed vertebrates have $\gamma\delta$ T cells, the particularly large number of recirculating $\gamma\delta$ T cells of ruminants, including sheep and cattle (Shekhar et al., 2012), make them an ideal model to study migratory $\gamma\delta$ T cells.

While ruminants and humans have epidermal and dermal $\gamma\delta$ T cells, both species lack prototypic DETCs and therefore rely on a mobile surveillance of the skin by $\gamma\delta$ T cells (Gorrell et al., 1995; Groh et al., 1989; Shekhar et al., 2012). In this study, we found that ovine $\gamma\delta$ T cells that have just left the inflamed or uninfamed skin and are traveling in the afferent lymph, exhibit an effector phenotype opposed to antigen-presenting capabilities. Furthermore, we show that these cells express high levels of L-selectin and E-selectin ligand, suggesting that lymph-borne $\gamma\delta$ T cells are easily deployed into skin and sites of inflammation. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells are able to exit the skin in a CCR7-independent manner. Finally, while many $\gamma\delta$ T cells migrate to the cutaneously expressed CCR6 ligand, CCL20, IL-17 producing $\gamma\delta$ T cells were highly enriched in the responsive fraction, suggesting that IL-17⁺ $\gamma\delta$ T cells utilize CCR6 to recirculate through the skin.

2. Material and Methods

2.1. Animals, lymph cannulations, and induction of skin inflammation

Mixed breed intact ewes or wethers, 5–10 mo of age, were purchased from 3/D Livestock (Woodland, CA), the University of California, Davis (Davis, CA), Animal Biotech Industries (Danboro, PA), or Pine Ridge Dorsets (East Berlin, PA). Some blood samples from 5–36 month old mixed breed sheep were kindly provided by Thomas Schaefer (Department of Clinical Studies-New Bolton Center). Pseudoafferent lymph vessels were generated by surgical removal of prefemoral (subiliac) lymph nodes and were cannulated as previously described (Brown et al., 2010; Young et al., 1997). Briefly, six to twelve weeks post-lymphectomy, pseudoafferent lymph vessels were surgically cannulated using heparin-coated catheters (Carmeda), and afferent lymph was continuously collected into sterile, heparinized (APP Pharmaceuticals) bottles. Every 1–12h, lymph collection bottles were changed, and lymphocytes stained for $\gamma\delta$ T cells. A total of 0.3–0.5 ml Complete Freund's Adjuvant (Sigma-Aldrich), emulsified 1:1 with sterile saline, was injected subcutaneously into the drainage area of the prefemoral node to induce chronic (21-d old) inflammation, as described (Brown et al., 2010). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.2. Cell isolation

Leukocytes were isolated as previously described (Geherin et al., 2012). Briefly, cells were released by grinding lymph nodes through a cell-dissociation sieve and passed through a 40- μ m cell strainer (BD Biosciences). Cells were isolated from shaved skin by mechanical disruption, followed by enzymatic digestion with Liberase TM and DNase I (Roche). Subsequently, the cell suspension was filtered through a cell-dissociation sieve or a French press coffee filter (Bodum) and washed. Blood was collected by venipuncture and mixed with heparin. Blood leukocytes were separated from red blood cells by either gradient centrifugation with Histopaque-1077 (Sigma-Aldrich) or lysis with red blood cell lysing buffer (Sigma-Aldrich). Leukocytes collected from ovine lymph were washed with RPMI (Invitrogen).

2.3. Flow cytometry

Nonspecific staining was minimized by preincubation with mouse and sheep IgG (Jackson ImmunoResearch). After blocking, the cells were labeled with biotinylated or fluorochrome-conjugated (FITC, PE, Alexa Fluor 647, allophycocyanin, PE-cyanin 7; Alexa Fluor 700, Pacific Blue, Pacific Orange) monoclonal antibodies (mAbs). The following mouse anti-sheep mAbs were used: CD4 (44.38; Serotec), CD8 (38.65; Serotec), CD45 (1.11.32; Serotec), $\gamma\delta$ TCR (86D (Mackay et al., 1989); VMRD), MHCII molecule DR (TH14B; VMRD), L-selectin (DU1-29; VMRD). Mouse anti-ovine CD11c hybridoma supernatant (17-196; (Sigmundsdottir et al., 2007) and mouse anti-ovine pan-B-cell marker (2-104; (Geherin et al., 2012)) were kindly provided by Alan Young (South Dakota State University). Mouse anti-human mAb 1G1 (BD Biosciences) was used to stain for CCR4. Some mouse mAbs were labeled prior to staining using Zenon labeling kits according to the manufacturer's instructions (Invitrogen). B7.1/B7.2 expression was detected using a human CTLA4-Ig chimeric protein (ID Labs). E-selectin ligand expression was assessed by testing the binding capacity to a recombinant mouse E-selectin human IgG chimeric protein (R&D Systems) in HBSS containing Ca^{2+} and Mg^{2+} . Specificity of the binding was shown by staining in 30–50 mM EDTA buffer, which inhibits the Ca^{2+} -dependent binding to E-selectin. CCR7 was stained using a mouse CCL19 human Fc chimeric protein (eBioscience) as described (Debes et al., 2005). Primary incubation with either E-selectin or CCL19 chimeric protein was followed by biotinylated F(ab)₂ donkey anti-human IgG (Jackson ImmunoResearch). For cell surface staining with biotinylated Abs, allophycocyanin- (BD Bioscience), PerCP-Cy5.5- (BD Biosciences), Pacific Orange- (Invitrogen), PE-cyanin 7- (eBioscience) or Alexa Fluor 405-conjugated streptavidin (Invitrogen) was used as a secondary reagent. When necessary, LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen) was used according to the manufacturer's instructions. Staining of digested skin was performed in parallel with that of digested and undigested lymph node samples to verify that antigens were not cleaved during the cell-isolation process. Intracellular staining was performed on lymphocytes stimulated with phorbol 12-myristate 13-acetate and ionomycin (Sigma-Aldrich) for 4 h prior to staining as described (Brown et al., 2010). Brefeldin A (Sigma-Aldrich) was added for the last 2 h. Surface staining for $\gamma\delta$ TCR was followed by fixation with 2% paraformaldehyde. Fixed cells were then permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with the following mouse mAbs recognizing sheep antigens (Brown et al., 2010): anti-bovine IFN- γ (CC302; Serotec), anti-bovine IL-4 (CC303; Serotec), anti-human IL-17A (eBio64DEC17; eBioscience). Samples were acquired on a BD LSRII or FACSCalibur using FACSDiva or CellQuest software (BD Biosciences), respectively, and analyzed with FlowJo software (Tree Star, Ashland, OR). Gates were set according to appropriate isotype/control staining.

2.4. Chemotaxis assay

The assay was performed and analyzed as described (Debes et al., 2005; Debes et al., 2006). Briefly, whole lymph was collected 0–12h prior to the assay and incubated in RPMI containing 0.5% bovine serum albumin (Invitrogen) for 1 h. $5\text{--}10 \times 10^5$ cells in 100 μl were added to 5- μm -pore-sized, 24-well tissue culture inserts (Corning costar). Recombinant mouse CCL2, CCL17, CCL20, CCL21, and CXCL9 and recombinant human CCL1, CCL4, CCL20, and CCL28 (all from R&D Systems) were titrated in triplicates, and cells were

allowed to migrate for 90 min. Leukocytes in the migrated and input wells were quantified using a fixed number of 15 μ m polystyrene beads as a standard (Polyscience Inc.) combined with flow cytometric analysis of $\gamma\delta$, CD4⁺ and ($\gamma\delta$ TCR⁻) CD8⁺ T cells. No difference was noted in the efficacy of mouse or human CCL20 to attract ovine lymphocytes. To assess the chemotaxis of cytokine producing cell subsets to CCL20 at its optimal concentration of 10 nM (Fig. 4), we combined the analysis of total $\gamma\delta$ T cell migration with the frequency of cytokine-producing cells in input and migrated population (Debes et al., 2006). Specifically, a fraction (**F**) of the input or migrated well was added to a fixed number of beads (**B**). The ratio of cells to beads (**c/b**) was determined for each well by flow cytometry. Absolute number of cells (**C_{sample}**) per well = **B*(c/b)/F**. The remainder of each well was stimulated and stained for T cell subsets and cytokine production as described above. To ensure sufficient cell numbers for intracellular cytokine staining, 6–12 wells were pooled for staining. The percent (**P**) of each T cell and cytokine⁺ subset was determined by flow cytometry. The migration for each subset was calculated and expressed as % of input = $100 * (C_{\text{sample}} * P_{\text{sample}}) / (C_{\text{input}} * P_{\text{input}})$.

2.5. Alignment of chemokine amino acid sequences

Amino acid sequences were downloaded from the National Center for Biotechnology Information (NCBI) website and aligned using MacVector (MacVector, Inc.). Identity and similarity were defined as identical and identical plus equivalently charged amino acids, respectively. Percent identity and similarity of sequences shown in Table 1 were based on the recombinant chemokines used in the chemotaxis assays and calculated using the MacVector pair-wise analysis application.

2.6. Statistical analysis

All statistical analyses were calculated using GraphPad Prism software. Unless otherwise indicated, all values are reported as mean $\pm$ SD, and statistical significance was determined by student's t test, paired when indicated. P values < 0.05 were considered statistically significant.

3. Results

3.1. Skin-draining ovine $\gamma\delta$ T cells are effectors rather than APCs

Previously, a subset of human $\gamma\delta$ T cells was proposed to function as an APC that upregulates MHCII and costimulatory molecules, and by inducing expression of the tissue exit receptor CCR7, migrates to draining lymph nodes via the lymphatics to prime naïve $\alpha\beta$ T cells (Brandes et al., 2005). Therefore, we addressed whether ovine $\gamma\delta$ T cells leaving extralymphoid tissues via afferent lymph display characteristics of professional APCs. By using an ovine model of afferent lymph vessel cannulation (Young et al., 1997), we were able to examine skin-draining lymph from uninflamed and chronically inflamed skin in each animal, which allowed for the analysis and comparison of $\gamma\delta$ T cells in the steady state and in the presence of chronic inflammation elicited by Complete Freund's Adjuvant (Brown et al., 2010; Chin and Hay, 1980). We stained $\gamma\delta$ T cells draining skin for molecules that indicate the ability to present antigen and provide costimulatory signals and compared expression levels to that of co-isolated dendritic cells in the draining lymph. Expression

levels of MHCII in the control and granuloma skin-draining lymph were generally low and percentages of positive $\gamma\delta$ T cells were highly variable, ranging from 1.01–16.07% and 2.12–16.97%, respectively (Fig. 1A, top). While we could detect CTLA-4 binding (indicating CD80/86 expression), the expression intensity was uniformly low (Fig. 1A, bottom). In contrast, co-isolated dendritic cells draining the skin were uniformly MHCII^{hi} and expressed high levels of CD80/CD86 (Fig. 1A).

In addition to presenting antigen, subsets of $\gamma\delta$ T cells in humans, mice, and cattle are capable of producing a wide variety of cytokines (Guzman et al., 2012; Pang et al., 2012). To determine whether skin-recirculating $\gamma\delta$ T cells were capable of producing effector cytokines, we polyclonally stimulated lymphocytes from lymph draining uninflamed and granulomatous skin with PMA and ionomycin and stained for intracellular cytokines. We detected populations of IFN- γ (0.80 – 12.85%), IL-17 (0.29 – 20.76%), and IL-4 (0.059 – 2.95%) secreting $\gamma\delta$ T cells (one example staining is shown in Fig. 1B; Fig. 1C). Although, fewer $\gamma\delta$ T cells expressed IFN- γ compared to what we previously found for ovine CD4 T cells traveling in afferent lymph (10.5 – 39%), IL-17 expression of $\gamma\delta$ T cells was similar to that of CD4 T cells (2 – 6.5%) ((Brown et al., 2010), Fig. 1B and C). Thus, skin-recirculating $\gamma\delta$ T cells appear better suited to secrete pro-inflammatory cytokines rather than perform functions analogous to professional APCs and are capable of contributing to host defense but potentially also autoimmune inflammation.

3.2. Ovine $\gamma\delta$ T cells are well equipped to recirculate through skin

To determine whether skin-draining ovine $\gamma\delta$ T cells are equipped with molecules that allow for rapid recruitment into tissues, we stained them for L-selectin (CD62L) and E-selectin ligand. While E-selectin ligands mediate migration into skin and sites of inflammation, L-selectin binds to peripheral node addressin (PNAd) and is essential to lymphocyte homing into lymph nodes via the blood. However, in innate leukocytes, such as neutrophils, L-selectin is central to migration into acutely inflamed tissues by binding to heparan sulfate presented on reactive endothelial cells (Wang et al., 2005) and other ligands (Rosen, 2004). As expected (Hein and Mackay, 1991; Mackay and Hein, 1989; Young, 1999), almost all blood- and lymph-borne $\gamma\delta$ T cells expressed high levels of L-selectin (Fig. 2A, C). Furthermore, blood and skin-draining $\gamma\delta$ T cells also expressed E-selectin ligand (Fig. 2A, B), indicating a propensity of skin draining $\gamma\delta$ T cells to efficiently return to the skin. However, significantly fewer $\gamma\delta$ T cells residing in the immunized skin expressed L-selectin when compared with $\gamma\delta$ T cells from blood ($p = 0.0002$), control lymph ($p = 0.0183$), or granuloma lymph ($p = 0.0118$) (Fig. 2A, C). As this difference in L-selectin expression was due to a physiological decrease rather than the enzymatic cell isolation (Fig. 2D), the data suggest the existence of a less mobile, potentially tissue-resident, population of $\gamma\delta$ T cells in ovine skin. Our data are in line with studies in cattle, showing that blood-borne bovine $\gamma\delta$ T cells express E- and P-selectin ligand and L-selectin (Jutila et al., 1994; Walcheck and Jutila, 1994; Walcheck et al., 1993). Thus, ruminant $\gamma\delta$ T cells in general exhibit a phenotype that suggests superior capacity to enter sites of inflammation.

3.3. $\gamma\delta$ T cells exit the skin in a CCR7-independent manner

Mouse CD4 and CD8 T cells depend on CCR7 to exit uninflamed extralymphoid tissues (Bromley et al., 2005; Debes et al., 2005), as its ligand, CCL21, is constitutively expressed by afferent lymphatics (Gunn et al., 1998; Nakano and Gunn, 2001; Saeki et al., 1999). Consistent with a role of CCR7 as a tissue exit receptor for ovine T cells, CD4 and CD8 T cells in the skin-draining afferent lymph express CCR7 (Debes et al., 2005). Additionally, CCR7 was suggested to mediate human $\gamma\delta$ T cell migration from extralymphoid tissue into afferent lymphatics (Moser and Brandes, 2006). Thus, we addressed whether CCR7 is a potential skin exit receptor for skin-recirculating $\gamma\delta$ T cells. Ovine CD4 and CD8 T cells that have just left the uninflamed or chronically inflamed skin and entered the draining lymph were enriched in their CCR7 expression compared with their skin residing counterparts (Fig. 3A and B), suggesting that CCR7 acts as a tissue exit receptor for ovine $\alpha\beta$ T cells. However, only few $\gamma\delta$ T cells expressed CCR7 in the blood, skin, or skin-draining lymph and their chemotactic responses to the CCR7 ligand, CCL21, did not exceed basal level responsiveness (Fig. 3A–C). From these data, we conclude that the majority of $\gamma\delta$ T cells must be able to egress from the skin in a CCR7-independent manner.

3.4. Lymph-borne $\gamma\delta$ T cells are unresponsive to ligands for skin-homing and inflammation-seeking chemokine receptors and selectively responsive to the CCR6 ligand CCL20

To reveal the migratory potential of skin-recirculating $\gamma\delta$ T cells, we tested their chemotactic responsiveness to ligands for chemokine receptors involved in $\alpha\beta$ T cell recruitment into skin and/or sites of inflammation (Bromley et al., 2008), CCL1, CCL2, CCL4, CCL17, CCL20, CCL28, and CXCL9, ligands for CCR8, CCR2, CCR5, CCR4, CCR6, CCR10, and CXCR3, respectively. Crossreactivity with ovine cells and bioactivity of the ligands was confirmed by assessing chemotaxis of conventional CD4 and CD8 T cells in parallel (Supplemental Figure 1A), and amino acid alignments of predicted and actual ovine chemokines showed 62–92% similarity between ovine and the used recombinant mouse or human chemokines (Table 1, Supplemental Figure 2). Ligands for chemokine receptors that target human and mouse T cells to the skin, such as for CCR4, CCR8, and CCR10 also attract ovine skin-recirculating CD4⁺ T cells (Supplemental Figure 1A, (Geherin et al., 2012)), indicating that these pathways are highly conserved between mammalian species. While CCR4 and CCR10 are also important in the migration of DETCs to mouse skin (Jiang et al., 2010; Jin et al., 2010), ovine skin-recirculating $\gamma\delta$ T cells did not respond to ligands for CCR4, CCR8, and CCR10 (Fig. 4A). Consistent with their lack of chemotaxis to the CCR4 ligand CCL17, skin-draining $\gamma\delta$ T cells did not stain for surface CCR4 using a monoclonal antibody (Supplemental Figure 1B and C). Ovine lymph-borne $\gamma\delta$ T cells also lacked responsiveness to many inflammatory chemokines, such as ligands for CCR2, CCR5, and CXCR3 (Fig. 4A), receptors that are expressed by murine dermal and human peripheral blood $\gamma\delta$ T cells (Brandes et al., 2005; Cai et al., 2011). Of note, all of the tested chemokines were able to attract ovine lymph-borne CD8 and/or CD4 T cells (Supplemental Figure 1A). However, ovine skin-recirculating $\gamma\delta$ T cells, and to a much lesser extent blood-borne $\gamma\delta$ T cells, were selectively responsive to CCL20 (Fig. 4A and B), the ligand for CCR6, which is highly expressed on mouse dermal $\gamma\delta$ T cells (Cai et al., 2011; Gray et al., 2011). Importantly, CCL20 is constitutively expressed in human cutaneous vasculature

(Charbonnier et al., 1999) and the regulation of CCR6 is critical to proper epidermal localization of DETCs (Hu and Xiong, 2013). Thus, our data suggest that skin recirculating ovine $\gamma\delta$ T cells rely on CCR6 rather than the prototypical skin homing chemokine receptors (CCR4, CCR8, and CCR10) used by $\alpha\beta$ T cells for their skin tropism.

3.5. IL-17 producing $\gamma\delta$ T cells are highly responsive to the CCR6 ligand CCL20

It is well established that ($\alpha\beta$) Th17 cells express CCR6 (McGeachy and Cua, 2008), and CCR6 was recently found on mouse and human IL-17 expressing cutaneous $\gamma\delta$ T cells (Cai et al., 2011; Gray et al., 2011; Hu and Xiong, 2013; Mabuchi et al., 2013; Sumaria et al., 2011). Having found that skin-recirculating $\gamma\delta$ T cells express IL-17 (Fig. 1B and C) and migrate in response to the CCR6 ligand CCL20 (Fig. 4A), we therefore aimed to test whether IL-17⁺ $\gamma\delta$ T cells preferentially migrate to CCL20. To address this we combined PMA and ionomycin stimulation and intracellular cytokine staining with a chemotaxis assay and found that IL-17⁺ $\gamma\delta$ T cells enriched in the CCL20-responsive fraction relative to input (Fig. 4C). In a quantitative analysis, we found that IL-17⁺ $\gamma\delta$ T cell were significantly more responsive to CCL20 than were IL-17⁻ (IFN- γ ⁺ [$p = 0.006$] or IFN- γ ⁻ [$p = 0.004$]) $\gamma\delta$ T cells (Fig. 4C and D). IL-4 producing cells were too rare to reliably analyze for their chemotaxis (Fig. 1B and C). Thus, the expression of receptors for CCL20 is conserved between IL-17 producing cutaneous $\gamma\delta$ T cells from different mammalian species, suggesting a key role for CCL20 in driving IL-17-dominated $\gamma\delta$ T cell responses in the skin.

4. Discussion and Conclusions

$\gamma\delta$ T cells are innate-like T lymphocytes that act as critical responders in infection and inflammation. Recent studies show that $\gamma\delta$ T cells are involved in the control of bacterial, viral, and fungal infections (Cho et al., 2010; Romani et al., 2008; Sumaria et al., 2011), as well as in a murine models of autoimmunity (Sutton et al., 2012), many of which affect the skin. Thus, understanding the functional capacity and trafficking patterns of skin-recirculating $\gamma\delta$ T cells can illuminate novel targets for the treatment of both infectious and autoimmune skin diseases.

Using a sheep model of lymph cannulation, we examined $\gamma\delta$ T cells in the skin-draining lymph and skin under homeostatic and chronic inflammatory conditions. As a subset of $\gamma\delta$ T cells in humans activates $\alpha\beta$ T cells (Brandes et al., 2005), we evaluated MHCII and CD80/86 expression on skin and found that compared to co-isolated and -stained dendritic cells, $\gamma\delta$ T cells in skin-draining afferent lymph expressed very low to negligible levels of MHCII and costimulatory molecules (Fig. 1A). Consistent with our data, Hopkins et al. (1993) found MHCII expression by both ovine $\alpha\beta$ and $\gamma\delta$ T cells without a significant ability to stimulate other T cells with soluble antigens (Dutia et al., 1993; Hopkins et al., 1993). Taken together, we conclude that unlike dendritic cells, ovine skin-draining $\gamma\delta$ T cells lack sufficient expression of key molecules that define professional APCs. Additional data support that $\gamma\delta$ T cells in afferent lymph of ruminants are not traveling to draining lymph nodes to activate $\alpha\beta$ T cells in the T-cell zone, because these $\gamma\delta$ T cells rapidly pass through the lymph node to re-enter the efferent lymph (Young et al., 2000) and recirculate back into skin (Mackay and Hein, 1989; Vrieling et al., 2012).

$\gamma\delta$ T cell-derived IL-17 plays a crucial role in neutrophil accumulation at the effector site during infections with pathogens such as *Escherichia coli* and *S. aureus* in mice (Cho et al., 2010; Shibata et al., 2007). Moreover, IL-17 and IFN- γ produced by mouse $\gamma\delta$ T cells are required for the subsequent CD4 T cell accumulation at sites of mycobacterial infection and infection with herpes simplex virus, respectively (Nishimura et al., 2004; Sumaria et al., 2011; Umemura et al., 2007). When evaluating the capacity of skin recirculating $\gamma\delta$ T cells to produce pro-inflammatory cytokines, we found that PMA and ionomycin stimulated $\gamma\delta$ T cells in the skin-draining lymph produced IFN- γ and IL-17 (Fig 1B–C). Consequently, the population of cytokine-positive, skin-recirculating $\gamma\delta$ T cells described here is likely important in the mobile surveillance of the skin to combat infection with pathogens that require rapid, localized responses. Additionally, cytokine production by mouse $\gamma\delta$ T cells is often transient and $\alpha\beta$ T cells that respond at later time points in the infection often produce the same cytokine signature as early $\gamma\delta$ T cell responders (Ferrick et al., 1995; Nishimura et al., 2004; Romani et al., 2008; Umemura et al., 2007). On the other hand, cytokine production by $\gamma\delta$ T cells is detrimental if not tightly regulated. For example, mice infected with *Aspergillus fumigatus* succumb to immunopathology if IL-17 production by $\gamma\delta$ T cells is dysregulated (Romani et al., 2008). Therefore, proper control of $\gamma\delta$ T cell function and localization, including the regulation of their migration, is imperative for protective immune responses against pathogens.

As not only function but also the migratory capacity determines the full effector potential of a T cell in an immune response, we also examined skin and skin-draining $\gamma\delta$ T cells for the expression of chemokine receptors involved in the exit from and entry into the skin. Interestingly, unlike co-isolated ovine CD4 T cells, $\gamma\delta$ T cells traveling in the skin draining lymph were unresponsive to ligands for CCR4, CCR8, CCR10, typical skin-attracting chemokines (Bromley et al., 2008). Our data contrast the finding that bovine skin draining $\gamma\delta$ T cells express low transcripts of CCR4 and CCR10 (Vrieling et al., 2012). As mRNA expression does not always correlate with functional chemokine receptor expression, it is possible that bovine $\gamma\delta$ T cells also lack responsiveness to CCR4 and CCR10 ligands. Alternatively but less likely, ovine T cells could employ a different set of chemokine receptors to recirculate through the skin as do their bovine counterparts. Unlike skin-draining CD4 and CD8 T cells, which rely on CCR7 to exit extralymphoid tissues (Bromley et al., 2005; Debes et al., 2005), ovine $\gamma\delta$ T cells in the skin and skin-draining lymph lacked surface expression of CCR7 and responsiveness to the CCR7 ligand CCL21 (Fig. 3). These findings are in line with a recent study, showing that bovine $\gamma\delta$ T cells in the skin-draining afferent lymph lack CCR7 (Vrieling et al., 2012) and with findings in the mouse that exclude a role for CCR7 in dermal $\gamma\delta$ T cell homeostasis (Sumaria et al., 2011). Interestingly, during the chronic phase of inflammation, mouse $\alpha\beta$ T cells can also exit the skin independently of CCR7 (Brown et al., 2010). It is possible that alternative skin exit receptors used by $\gamma\delta$ T cells are shared with other leukocytes, such as CD4 and CD8 T cells during chronic inflammatory conditions. Thus, targeting exit receptors could be a novel approach to manipulate accumulation of $\gamma\delta$ T cells, and potentially other leukocytes, thereby modulating tissue inflammation and host responses to infection. Surprisingly, ovine skin-recirculating but not blood-borne $\gamma\delta$ T cells were selectively responsive to the CCR6 ligand CCL20 (Fig. 4A–B). Not only is CCL20 upregulated in the inflamed epidermis and

other sites of inflammation, CCL20 is also constitutively expressed in human cutaneous lymphatic and blood vasculature (Charbonnier et al., 1999; Schutyser et al., 2003), and CCR6 has been implicated in human Langerhans cell precursor migration to the skin (Charbonnier et al., 1999). Furthermore, in a mouse model of psoriasis, a subset of $\gamma\delta$ T cells utilizes CCR6 to migrate into the inflamed epidermis (Mabuchi et al., 2013). Thus, a subset of $\gamma\delta$ T cells in sheep likely also uses the CCR6-CCL20 axis to enter the dermis and/or sites of inflammation, to migrate from the dermis to the inflamed epidermis, or to egress from the dermis through the afferent lymph.

Our finding that ovine skin-recirculating $\gamma\delta$ T cells with the capacity to make IL-17 upon stimulation were enriched in their CCL20 responsiveness relative to other lymph-borne $\gamma\delta$ T cell subsets ($\text{IFN}\gamma^+$ or IL-17^- , or $\text{IFN}\gamma^-$ $\gamma\delta$ T cells) is consistent with recent data in the mouse showing that dermal $\gamma\delta$ T cells are poised to make IL-17 and express CCR6 (Cai et al., 2011; Gray et al., 2011; Sumaria et al., 2011). Thus, the link of IL-17 production by cutaneous $\gamma\delta$ T cells and expression of CCR6 is evolutionarily conserved. It is tempting to speculate that ovine skin-recirculating $\gamma\delta$ T cells are homologous to the IL-17^+ dermal $\gamma\delta$ T cells in the mouse. Dermal $\gamma\delta$ T cells of mice and skin-recirculating ovine $\gamma\delta$ T cells not only share the ability to produce IL-17 and express CCR6, they also both express variant TCRs (Hein and Dudler, 1993; Hein and Dudler, 1997; Sumaria et al., 2011). Similarly, data suggest that mouse dermal $\gamma\delta$ T cell can recirculate because they also localize to the skin-draining lymph node in the subcapsular sinus (Gray et al., 2011), where afferent lymph enters the lymph node. However, the potential for murine dermal $\gamma\delta$ T cells to recirculate has yet to be assessed.

In cattle, $\gamma\delta$ T cells express the transmembrane glycoprotein WC1, the isoform of which marks distinct functional subsets of $\gamma\delta$ T cells (Rogers et al., 2005). For example, $\text{IFN}\gamma$ and IL-10 production by bovine $\gamma\delta$ T cells are associated with specific WC1 isoform usage (Hoek et al., 2009; Rogers et al., 2005). Most $\gamma\delta$ T cells in adult sheep also express this molecule (also known as T19; (Mackay et al., 1989)), and although ovine isoforms are less well-characterized (Walker et al., 1994), it would be interesting to investigate WC1 expression by IL-17^+ $\gamma\delta$ T cells.

As previously mentioned, TCR diversity differs between skin-resident DETCs and motile dermal $\gamma\delta$ T cells. DETCs have restricted TCR usage, while dermal $\gamma\delta$ T cells exhibit significantly more diverse TCRs, enabling them to respond to a greater repertoire of unique antigens than their sessile counterparts (Cho et al., 2010; Hein and Dudler, 1993; Hein and Dudler, 1997; Sumaria et al., 2011). The ability to divide the workload of continuous immunosurveillance between resident and recirculating subsets of $\gamma\delta$ T cells would be advantageous to achieving consistent and robust protection from exogenous threats. While resident $\gamma\delta$ T cells would be guaranteed to be near the site of barrier breach, cell number limitations and receptor diversity could restrict their ability to combat infection. Accordingly, reinforcing the protection provided by resident cells with a highly mobile, receptor-diverse population that can also rapidly respond would permit the robust response required for barrier surfaces, such as the skin, that are under constant microbial attack.

In conclusion, we show that migratory $\gamma\delta$ T cells capable of producing IL-17 and IFN- γ constantly provide immunosurveillance of the skin. They egress from the skin in a CCR7-independent manner, and potentially use CCR6 and E-selectin ligand for efficient cutaneous recirculation. Together, these properties make $\gamma\delta$ T cells ideal first responders to cutaneous infection, bridging the gap between innate and adaptive immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank University Laboratory Animal Resources surgical staff for technical assistance, Drs. Carty and Wiltshire for veterinary support, Jack Hay for surgical instruction, and Shruti Naik, Sarah Fintushel, and Meghan Brown for preliminary experiments. We are indebted to Alan Young for antibodies, Tom Schaer and Alexander Fairman for sheep blood samples, Carolina Lopez for access to MacVector software, and to Sunny Shin, Charles Mackay, and Kirk Jensen for critical reading of the manuscript. This work was supported by NIH grant AR056730 (to GFD) and T32AI007532 (to SAG).

List of abbreviations

DETC	dendritic epidermal T cell
MHCII	MHC class II
NCBI	National Center for Biotechnology Information

References

- Born WK, Zhang L, Nakayama M, Jin N, Chain JL, Huang Y, Aydinoglu MK, O'Brien RL. Peptide antigens for gamma/delta T cells. *Cell Mol Life Sci.* 2011; 68:2335–2343. [PubMed: 21553233]
- Brandes M, Willmann K, Moser B. Professional antigen-presentation function by human gammadelta T Cells. *Science.* 2005; 309:264–268. [PubMed: 15933162]
- Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat Immunol.* 2005; 6:895–901. [PubMed: 16116469]
- Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol.* 2008; 9:970–980. [PubMed: 18711434]
- Brown MN, Fintushel SR, Lee MH, Jennrich S, Geherin SA, Hay JB, Butcher EC, Debes GF. Chemoattractant receptors and lymphocyte egress from extralymphoid tissue: changing requirements during the course of inflammation. *J Immunol.* 2010; 185:4873–4882. [PubMed: 20833836]
- Cai Y, Shen X, Ding C, Qi C, Li K, Li X, Jala VR, Zhang HG, Wang T, Zheng J, Yan J. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity.* 2011; 35:596–610. [PubMed: 21982596]
- Charbonnier AS, Kohrgruber N, Kriehuber E, Stingl G, Rot A, Maurer D. Macrophage inflammatory protein 3alpha is involved in the constitutive trafficking of epidermal langerhans cells. *J Exp Med.* 1999; 190:1755–1768. [PubMed: 10601351]
- Chin W, Hay JB. A comparison of lymphocyte migration through intestinal lymph nodes, subcutaneous lymph nodes, and chronic inflammatory sites of sheep. *Gastroenterology.* 1980; 79:1231–1242. [PubMed: 7439631]
- Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, Magorien JE, Blauvelt A, Kolls JK, Cheung AL, Cheng G, Modlin RL, Miller LS. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest.* 2010; 120:1762–1773. [PubMed: 20364087]

- Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, Butcher EC. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat Immunol.* 2005; 6:889–894. [PubMed: 16116468]
- Debes GF, Dahl ME, Mahiny AJ, Bonhagen K, Campbell DJ, Siegmund K, Erb KJ, Lewis DB, Kamradt T, Hamann A. Chemotactic responses of IL-4-, IL-10-, and IFN-gamma-producing CD4+ T cells depend on tissue origin and microbial stimulus. *J Immunol.* 2006; 176:557–566. [PubMed: 16365450]
- Dutia BM, McConnell I, Bird K, Keating P, Hopkins J. Patterns of major histocompatibility complex class II expression on T cell subsets in different immunological compartments. 1. Expression on resting T cells. *Eur J Immunol.* 1993; 23:2882–2888. [PubMed: 8223864]
- Ferrick DA, Schrenzel MD, Mulvania T, Hsieh B, Ferlin WG, Lepper H. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature.* 1995; 373:255–257. [PubMed: 7816142]
- Geherin SA, Fintushel SR, Lee MH, Wilson RP, Patel RT, Alt C, Young AJ, Hay JB, Debes GF. The skin, a novel niche for recirculating B cells. *J Immunol.* 2012; 188:6027–6035. [PubMed: 22561151]
- Gorrell MD, Townsend WL, Ladds PW. The distribution of lymphocyte subpopulations in normal and acanthotic ovine skin. *Vet Immunol Immunopathol.* 1995; 44:151–167. [PubMed: 7747398]
- Gray EE, Suzuki K, Cyster JG. Cutting edge: Identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol.* 2011; 186:6091–6095. [PubMed: 21536803]
- Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB. Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system. *J Exp Med.* 1989; 169:1277–1294. [PubMed: 2564416]
- Gunn MD, Tangelmann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc Natl Acad Sci U S A.* 1998; 95:258–263. [PubMed: 9419363]
- Guzman E, Price S, Poulosom H, Hope J. Bovine gammadelta T cells: cells with multiple functions and important roles in immunity. *Vet Immunol Immunopathol.* 2012; 148:161–167. [PubMed: 21501878]
- Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol.* 2000; 18:975–1026. [PubMed: 10837080]
- Hein WR, Mackay CR. Prominence of gamma delta T cells in the ruminant immune system. *Immunol Today.* 1991; 12:30–34. [PubMed: 1826600]
- Hein WR, Dudler L. Divergent evolution of T cell repertoires: extensive diversity and developmentally regulated expression of the sheep gamma delta T cell receptor. *EMBO J.* 1993; 12:715–724. [PubMed: 8440261]
- Hein WR, Dudler L. TCR gamma delta+ cells are prominent in normal bovine skin and express a diverse repertoire of antigen receptors. *Immunology.* 1997; 91:58–64. [PubMed: 9203966]
- Hoek A, Rutten VP, Kool J, Arkesteijn GJ, Bouwstra RJ, Van Rhijn I, Koets AP. Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo. *Vet Res.* 2009; 40:6. [PubMed: 18928784]
- Hopkins J, McConnell I, Dalziel RG, Dutia BM. Patterns of major histocompatibility complex class II expression by T cell subsets in different immunological compartments. 2. Altered expression and cell function following activation in vivo. *Eur J Immunol.* 1993; 23:2889–2896. [PubMed: 8223865]
- Hu S, Xiong N. Programmed Downregulation of CCR6 Is Important for Establishment of Epidermal gammadelta T Cells by Regulating Their Thymic Egress and Epidermal Location. *J Immunol.* 2013; 190:3267–3275. [PubMed: 23420888]
- Jiang X, Campbell JJ, Kupper TS. Embryonic trafficking of gammadelta T cells to skin is dependent on E/P selectin ligands and CCR4. *Proc Natl Acad Sci U S A.* 2010; 107:7443–7448. [PubMed: 20368416]

- Jin Y, Xia M, Sun A, Saylor CM, Xiong N. CCR10 is important for the development of skin-specific gammadeltaT cells by regulating their migration and location. *J Immunol.* 2010; 185:5723–5731. [PubMed: 20937851]
- Jutila MA, Bargatze RF, Kurk S, Warnock RA, Ehsani N, Watson SR, Walcheck B. Cell surface P- and E-selectin support shear-dependent rolling of bovine gamma/delta T cells. *J Immunol.* 1994; 153:3917–3928. [PubMed: 7523509]
- Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol.* 2007; 7:678–689. [PubMed: 17717539]
- Mabuchi T, Singh TP, Takekoshi T, Jia GF, Wu X, Kao MC, Weiss I, Farber JM, Hwang ST. CCR6 is required for epidermal trafficking of gammadelta-T cells in an IL-23-induced model of psoriasiform dermatitis. *J Invest Dermatol.* 2013; 133:164–171. [PubMed: 22895364]
- Mackay CR, Beya MF, Matzinger P. Gamma/delta T cells express a unique surface molecule appearing late during thymic development. *Eur J Immunol.* 1989; 19:1477–1483. [PubMed: 2528463]
- Mackay CR, Hein WR. A large proportion of bovine T cells express the gamma delta T cell receptor and show a distinct tissue distribution and surface phenotype. *Int Immunol.* 1989; 1:540–545. [PubMed: 2535142]
- Mackay CR, Marston WL, Dudler L. Naive and memory T cells show distinct pathways of lymphocyte recirculation. *J Exp Med.* 1990; 171:801–817. [PubMed: 2307933]
- McGeachy MJ, Cua DJ. Th17 cell differentiation: the long and winding road. *Immunity.* 2008; 28:445–453. [PubMed: 18400187]
- Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. *Trends Immunol.* 2006; 27:235–243. [PubMed: 16580261]
- Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev.* 2007; 215:59–76. [PubMed: 17291279]
- Moser B, Brandes M. Gammadelta T cells: an alternative type of professional APC. *Trends Immunol.* 2006; 27:112–118. [PubMed: 16442347]
- Nakano H, Gunn MD. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. *J Immunol.* 2001; 166:361–369. [PubMed: 11123313]
- Nishimura H, Yajima T, Kagimoto Y, Ohata M, Watase T, Kishihara K, Goshima F, Nishiyama Y, Yoshikai Y. Intraepithelial gammadelta T cells may bridge a gap between innate immunity and acquired immunity to herpes simplex virus type 2. *J Virol.* 2004; 78:4927–4930. [PubMed: 15078977]
- Pang DJ, Neves JF, Sumaria N, Pennington DJ. Understanding the complexity of gammadelta T-cell subsets in mouse and human. *Immunology.* 2012; 136:283–290. [PubMed: 22385416]
- Pickler LJ, Butcher EC. Physiological and molecular mechanisms of lymphocyte homing. *Annu Rev Immunol.* 1992; 10:561–591. [PubMed: 1590996]
- Rogers AN, VanBuren DG, Hedblom E, Tilahun ME, Telfer JC, Baldwin CL. Function of ruminant gammadelta T cells is defined by WC1.1 or WC1.2 isoform expression. *Vet Immunol Immunopathol.* 2005; 108:211–217. [PubMed: 16144715]
- Romani L, Fallarino F, De Luca A, Montagnoli C, D'Angelo C, Zelante T, Vacca C, Bistoni F, Fioretti MC, Grohmann U, Segal BH, Puccetti P. Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature.* 2008; 451:211–215. [PubMed: 18185592]
- Rosen SD. Ligands for L-selectin: homing, inflammation, and beyond. *Annu Rev Immunol.* 2004; 22:129–156. [PubMed: 15032576]
- Saeki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol.* 1999; 162:2472–2475. [PubMed: 10072485]
- Schutysse E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* 2003; 14:409–426. [PubMed: 12948524]

- Shekhar S, Milling S, Yang X. Migration of gammadelta T cells in steady-state conditions. *Vet Immunol Immunopathol.* 2012; 147:1–5. [PubMed: 22520944]
- Shibata K, Yamada H, Hara H, Kishihara K, Yoshikai Y. Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol.* 2007; 178:4466–4472. [PubMed: 17372004]
- Shibata K. Close link between development and function of gamma-delta T cells. *Microbiol Immunol.* 2012; 56:217–227. [PubMed: 22300310]
- Sigmundsdottir H, Pan J, Debes GF, Alt C, Habtezion A, Soler D, Butcher EC. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat Immunol.* 2007; 8:285–293. [PubMed: 17259988]
- Sigmundsdottir H, Butcher EC. Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking. *Nat Immunol.* 2008; 9:981–987. [PubMed: 18711435]
- Sumaria N, Roediger B, Ng LG, Qin J, Pinto R, Cavanagh LL, Shklovskaya E, Fazekas de St Groth B, Triccas JA, Weninger W. Cutaneous immunosurveillance by self-renewing dermal gammadelta T cells. *J Exp Med.* 2011; 208:505–518. [PubMed: 21339323]
- Sutton CE, Mielke LA, Mills KH. IL-17-producing gammadelta T cells and innate lymphoid cells. *Eur J Immunol.* 2012; 42:2221–2231. [PubMed: 22949320]
- Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y, Matsuzaki G. IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. *J Immunol.* 2007; 178:3786–3796. [PubMed: 17339477]
- Vrieling M, Santema W, Van Rhijn I, Rutten V, Koets A. gammadelta T cell homing to skin and migration to skin-draining lymph nodes is CCR7 independent. *J Immunol.* 2012; 188:578–584. [PubMed: 22156593]
- Walcheck B, Watts G, Jutila MA. Bovine gamma/delta T cells bind E-selectin via a novel glycoprotein receptor: first characterization of a lymphocyte/E-selectin interaction in an animal model. *J Exp Med.* 1993; 178:853–863. [PubMed: 7688790]
- Walcheck B, Jutila MA. Bovine gamma delta T cells express high levels of functional peripheral lymph node homing receptor (L-selectin). *Int Immunol.* 1994; 6:81–91. [PubMed: 7511930]
- Walker ID, Glew MD, O'Keeffe MA, Metcalfe SA, Clevers HC, Wijngaard PL, Adams TE, Hein WR. A novel multi-gene family of sheep gamma delta T cells. *Immunology.* 1994; 83:517–523. [PubMed: 7875732]
- Wang L, Fuster M, Sriramaraio P, Esko JD. Endothelial heparan sulfate deficiency impairs L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses. *Nat Immunol.* 2005; 6:902–910. [PubMed: 16056228]
- Xiong N, Kang C, Raulet DH. Positive selection of dendritic epidermal gammadelta T cell precursors in the fetal thymus determines expression of skin-homing receptors. *Immunity.* 2004; 21:121–131. [PubMed: 15345225]
- Young, AJ.; Hein, WR.; Hay, JB. Cannulation of lymphatic vessels and its use in the study of lymphocyte traffic. In: Levkovits, I., editor. *Manual of immunological methods: the comprehensive source book of techniques.* Academic Press; San Diego: 1997. p. 2039-2059.
- Young AJ. The physiology of lymphocyte migration through the single lymph node in vivo. *Semin Immunol.* 1999; 11:73–83. [PubMed: 10329494]
- Young AJ, Marston WL, Dudley L. Subset-specific regulation of the lymphatic exit of recirculating lymphocytes in vivo. *J Immunol.* 2000; 165:3168–3174. [PubMed: 10975831]

Figure 1. Skin-draining $\gamma\delta$ T cells are effectors rather than APCs

Leukocytes from uninflamed (control) and chronically inflamed (granuloma) skin-draining lymph were analyzed. **(A)** Flow cytometric analysis of $\gamma\delta$ T cells and dendritic cells (MHCII⁺, CD11c^{hi}, pan-B⁻) for the expression of MHCII and CD80/86. One representative animal of 2 (control) or 3 (granuloma) individually analyzed animals is shown. Shaded histograms indicate isotype control staining. **(B–C)** Flow cytometric analysis of IFN- γ , IL-17, and IL-4 expression on gated PMA and ionomycin stimulated $\gamma\delta$ T cells from skin granuloma draining lymph for one representative animal. **(C)** Percentage of cytokine-positive $\gamma\delta$ T cells after PMA and ionomycin stimulation for uninflamed (control, filled symbols) and chronically inflamed (granuloma, open symbols) skin-draining lymph from all animals analyzed (N=9 sheep). Each symbol represents an individually analyzed sheep.

Figure 2. L-selectin and E-selectin ligand expression by $\gamma\delta$ T cells

$\gamma\delta$ T cells from blood, control and chronically inflamed (granuloma) skin-draining lymph, and granulomatous skin were analyzed. (A) Flow cytometric analysis of L-selectin and E-selectin ligand on gated $\gamma\delta$ T cells. One representative animal is shown. Staining control for control lymph is shown and is representative of all tissues. (B and C) Percentage of $\gamma\delta$ T cells expressing E-selectin ligand (B) or L-selectin (C) for each tissue from all animals analyzed (N=3–8). Each individually analyzed sheep is represented by a unique symbol. (D) L-selectin expression levels by $\gamma\delta$ T cells after mechanical or enzymatic (digest) isolation from lymph nodes. * $p < 0.05$.

Figure 3. $\gamma\delta$ T cells exit the skin independently of CCR7

T cell subsets from uninflamed and granulomatous skin and skin-draining lymph, as well as blood, were analyzed. (A–B) Flow cytometric analysis of gated CD4, CD8, and $\gamma\delta$ T cells for expression of CCR7. One example staining with isotype controls shown as shaded areas (A), or means of 7–10 (lymph) or 3–5 (skin) individually analyzed sheep (B) are depicted. (C) Chemotaxis of lymph-borne $\gamma\delta$, CD4 and CD8 T cells to CCL21 in an ex-vivo Transwell chemotaxis assay. Data are expressed as the percentage of cells of each T cell subset that migrated to the lower chamber, and data represent the mean $\pm$ SD of triplicate wells at each concentration. One representative animal of at least 4 animals analyzed for each condition is shown.

*** $p < 0.005$; ** $p < 0.01$.

Figure 4. Chemotactic responses of lymph- and blood-borne $\gamma\delta$ T cells

(A) Chemotaxis of lymph-borne $\gamma\delta$ T cells draining uninflamed (Control) or chronically inflamed skin (Granuloma) and (B) blood-borne $\gamma\delta$ T cells to human (h) CCL1, CCL4, CCL20 and CCL28 and mouse (m) CCL2, CCL17, CCL20, and CXCL9 was tested in an ex-vivo Transwell chemotaxis assay. One representative chemotaxis profile for 2 (CCL2 and CCL4, control lymph) or 3–6 individually analyzed sheep is shown. (C and D) Chemotaxis of $\gamma\delta$ T cells from granulomatous skin-draining lymph to 10nM CCL20 was assessed by gating on $\gamma\delta$ T cells by flow cytometry. One out of two experiments with similar results is shown. (C) IL-17 and IFN- γ expression by $\gamma\delta$ T cells of the input population and of $\gamma\delta$ T cells that migrated to CCL20 or media alone. (D) Chemotaxis quantification of $\gamma\delta$ T cell subsets (IFN- γ ⁺ and IL-17⁺ as well as IFN- γ /IL-17 double-negative (non-producers)) in response to CCL20 or media. (A, B, and D) Data are expressed as the percentage of input cells of the respective subset that migrated to the lower chamber, and represent the mean $\pm$ SD of triplicate wells at each concentration. (C and D) Cytokine production was assessed after migration by PMA and ionomycin stimulation. ** $p < 0.01$.

Table 1
Cross-species comparison of amino acid sequences between used recombinant and *Ovis aries* chemokines

Amino acid sequences for chemokines of each species (*H. sapiens*, *M. musculus* and *O. aries*) corresponding to the amino acid sequence of the used recombinant proteins were aligned and analyzed. Identity is defined as identical amino acids, and similarity includes identical as well as amino acids with similar charge. The species (*H. sapiens* or *M. musculus*) from which the recombinant protein was derived is bolded.

Chemokine fragment	Species, utilized fragment	NCBI accession number	Identity (%)	Similarity (%)	Accession number for predicted <i>Ovis aries</i> sequence
CCL1	<i>H. sapiens</i> ₂₄₋₉₆	EAW80204.1	41	62	XP_004013255.1
	<i>M. musculus</i>	AAI20807.1	33	54	
CCL2	<i>H. sapiens</i>	EAW80212.1	67	84	XP_004012520.1
	<i>M. musculus</i> ₂₄₋₉₆	AAH55070.1	50	69	
CCL4	<i>H. sapiens</i> ₂₄₋₉₂	AAI04228.1	84	92	XP_004012496.1
	<i>M. musculus</i>	AAI19258.1	82	90	
CCL17	<i>H. sapiens</i>	AAI12069.1	51	66	XP_004015612.1
	<i>M. musculus</i> ₂₄₋₉₃	EAW70878.1	47	67	
CCL20	<i>H. sapiens</i> ₂₇₋₉₆	AAH28504.1	67	84	XP_004005034.1
	<i>M. musculus</i> ₂₇₋₉₆	AAH27918.1	58	79	
CCL21	<i>H. sapiens</i>	AAH27918.1	73	86	XP_004004168.1
	<i>M. musculus</i> ₂₄₋₁₃₃	NP_035254.1	69	80	
CCL28	<i>H. sapiens</i> ₃₃₋₁₂₇	ABB13417.1	78	90	ABB13417.1*
	<i>M. musculus</i>	AAH55864.1	59	76	
CXCL9	<i>H. sapiens</i>	AAH95396.1	72	81	XP_004004168.1
	<i>M. musculus</i> ₂₂₋₁₂₆	AAH03343.1	61	73	

* actual sequence