RECIRCULATION OF INNATE LYMPHOCYTE SUBSETS IN THE SKIN

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ABSTRACT

RECIRCULATION OF INNATE LYMPHOCYTE SUBSETS IN THE SKIN

Skye A. Geherin

Gudrun Debes

The trafficking of innate-like lymphocytes, such as γδ T cells and B-1 B cells, has garnered comparatively little attention from the immunological community relative to conventional T and B cells. However, recent studies have shown that innate-like cell subsets are critical for immune regulation and host defense. In this study, we use a classic ovine lymph cannulation model to describe the phenotype and function of γδ T cells migrating through the skin. We find that γδ T cells traveling in the skin-draining afferent lymph are IFN-γ- and/or IL-17-producing effector cells that express high levels of the skin- and inflammation-seeking molecule E-selectin ligand. Notably, they also lack expression of CCR7, indicating that they use alternative receptors for egress. Next, we analyze B cell subset composition, repertoire, and trafficking in the skin of sheep in the lymphatic cannulation model. We find a heterogeneous population of B cells in the skin and skin-draining lymph increases in inflammation that contains a subset of B-1-like B cells coexpressing IgM and CD11b. Furthermore, we show that skin accumulation of B cells and antibody-secreting cells during inflammation increases local antibody titers, which may augment host defense and autoimmunity. We then extend our findings of cutaneous B-1 B cells to the mouse, analyzing both uninflamed skin and skin with chronic inflammation from complete Freund's adjuvant, well as human tissue. We find that B-1 B cells, unlike conventional follicular B-2 B cells, efficiently enter into the inflamed skin and differentially express the trafficking molecule α4β1 integrin (VLA-4),

which facilitates their entry. Furthermore, innate B cells are a contributing source of cutaneous IL-10 in both IL-10-reporter mice and normal human skin.

These findings, initiated in the sheep model then followed up and supported by experiments in mice and human tissues, demonstrate the evolutionary similarity between mammalian species. They also validate the utilization of multiple models to allow for experimental setups not possible in all species. More importantly, the further characterization of γδ T cells and the new description of skin B and B-1 cells uncovers additional targets for regulating the cellular composition of a cutaneous immune response. In summary, the data support a model in which innate-like lymphocytes are poised to migrate into barrier sites, including the skin, where they rapidly provide requisite effector functions, such as cytokine and/or antibody production, and fulfill an emerging role in skin immunity.

PUBLICATIONS AND CONTRIBUTIONS

Part of this work was done in collaboration with Michael H. Lee. The intracellular cytokine staining (Chapter 2, Figure 1B-C) and intracellular cytokine staining in conjunction with chemotaxis assays (Chapter 2, Figure 4C-D) were done by Michael H. Lee. Chemotaxis assays, without subsequent intracellular cytokine staining (Chapter 2 Figure 3C and 4A) were done together.

The radioactive homing experiments (Chapter 4, Figure 2B) were done in together with Uta Lauer in the laboratory of Alf Hamann at the German Rheumatism Research Center, Berlin Germany.

All other experiments in Chapters 2 and 4 and all of the experiments in Chapter 3 were performed by Skye Geherin in the laboratory of Gudrun Debes.

Chapter 2 is published in the Veterinary Journal of Immunology and Immunopathology (1). It is reproduced here with their permission and can be found at http://www.sciencedirect.com/science/article/pii/S0165242713001815 .
Chapter 3 is published in the Journal of Immunology (2). It is reproduced here with their permission and can be found at http://www.jimmunol.org/content/188/12/6027.long .

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Chapter 1: Introduction

Lymphocyte Recirculation and Trafficking

Lymphocyte recirculation is critical for immunosurveillance, host-defense and site-specific immunity. Although it was historically observed that lymphocytes can be found in the lymphatics (reviewed in (3)), the source of their name, it was not until the 1950s and 60s that the importance of recirculation was experimentally determined. Gowans, using a model of rat thoracic lymph cannulation, elegantly demonstrated that if cells were collected from the lymph, there was an eventual decline in total numbers of cells in the thoracic duct lymph, and this could be rescued by returning the collected cells to the animals IV (4). It was furthermore shown that lymphocytes truly recirculate throughout the body by intravenously transferring radiolabelled cells and finding them in the thoracic lymph (5, 6). Later studies by Bede Morris and colleagues utilized an ovine cannulation model that allowed for cannulation of site-specific lymphatics over a greater length of time (7-9). Organ-specific lymphocyte recirculation was first described in this model ((10); reviewed in (11)), setting the stage for later murine studies examining lymphocyte subsets and the molecular mechanisms of recirculation.

Two general pathways of recirculation allow lymphocytes to efficiently migrate throughout the body: lymphocytes may arrive at peripheral lymph nodes either by migration from the blood circulation or from tissues via the efferent lymphatics. Primarily, blood-borne lymphocytes enter lymph nodes through high endothelial venules (HEVs) (6). Alternatively, lymphocytes recirculate through extralymphoid tissues, such as skin or gut, and exit these tissues by migrating into the afferent lymph to enter the draining lymph node. Lymphocytes in lymph nodes then return to the blood stream in the efferent

lymph via the thoracic duct (reviewed in (11)).

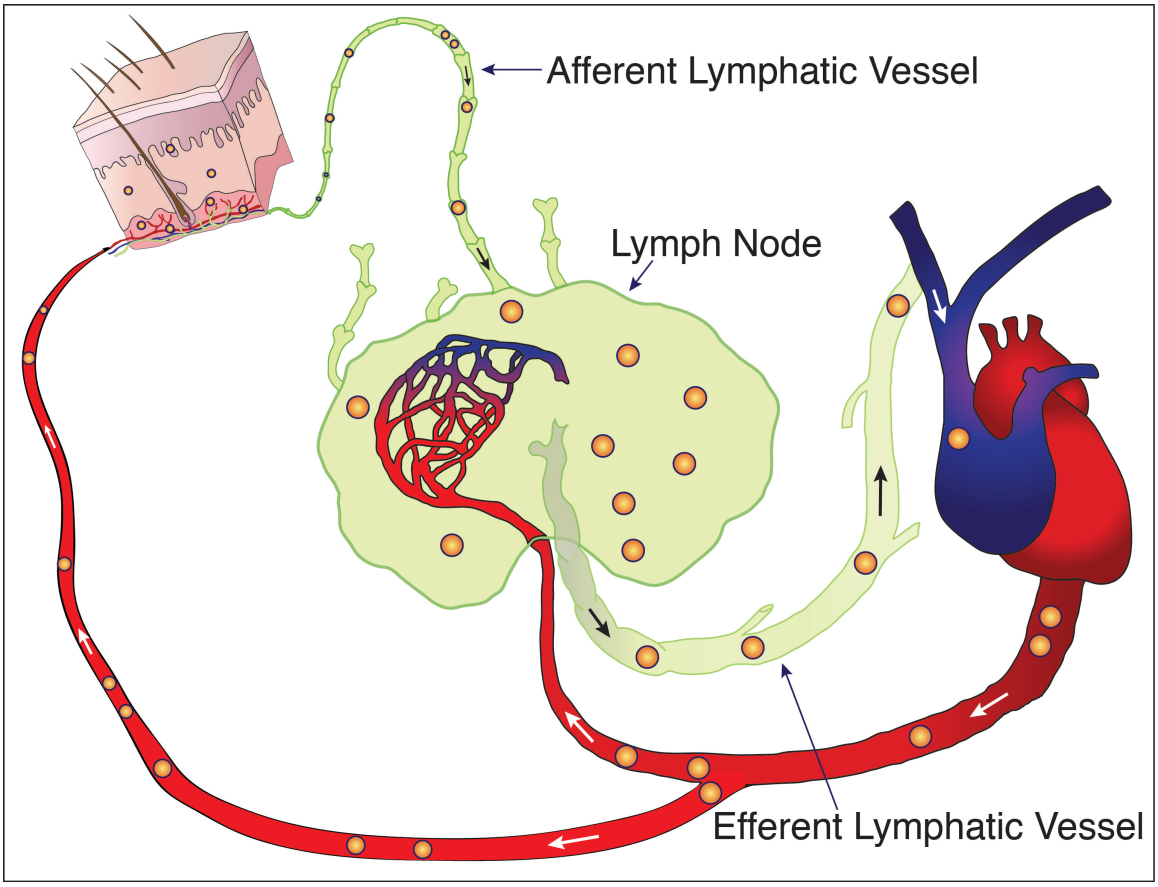


Figure 1. Recirculation pathways of lymphocytes from blood. Arrows indicate the direction of flow.

Entry into either lymph nodes or extralymphoid tissues is mediated by a multistep adhesion cascade characterized by four steps: rolling via by selectins and/or integrins; activation of integrins via chemokine receptor signaling; activated integrins mediate firm adhesion/arrest, and transmigration into the tissue. (Figure 2, (12-14)). Selectins are type 1 transmembrane glycoproteins. Three selectins have been described (E-, P-, and L-selectin), and all bind sialylated carbohydrates, require Ca^{+2} for binding, and have very similar structures. Selectin specificity is conferred by slight differences in the N-terminal

lectin domain of the protein, although there is overlap in both function and ligand (15, 16). Integrins are also type 1 transmembrane glycoproteins. These proteins form heterodimers consisting of alpha and beta subunits. Unlike the relatively few unique selectins, the integrin family is extensive with 18 alpha subunits and 8 beta subunits that can form 24 different heterodimers (17). Integrins can bind a variety of extracellular matrix proteins, such collagen and fibronectin, as well specific cellular receptors. Although integrins are widely utilized throughout the body and in development, the most important integrins in the immune system are the integrin pairs $\alpha_M\beta_2$ (Mac-1), $\alpha_L\beta_2$ (LFA-1), $\alpha_4\beta_1$ (VLA-4), $\alpha_4\beta_7$ (16, 17). The third receptor component of the multistep adhesion cascade is the chemokine receptor. Chemokine receptors are 7 transmembrane G protein-coupled receptors that can be divided into four classes based on the cysteine residue structure of a conserved region of the chemokine ligand: C, CC, CXC, and CX_3C (18). When a chemokine receptor of a cell rolling along blood vascular endothelium binds its chemokine ligand, inside-out signaling induces a conformational change in an integrin dimer from a low-affinity state to a high-affinity state. It is this activated integrin that can then firmly adhere to its ligand, also expressed on the blood vascular endothelium, and through outside-in signaling, assist in transmigration into tissues (16, 19). Combinations of selectins, integrins, and chemokine receptors together form a type of "zip code" in the body that dictates a cell's homing capacity, or the ability to specifically migrate to and enter a tissue. For example, T cells with L-selectin, $\alpha_L\beta_2$, and CCR7 are capable of entering lymph nodes, whereas B and T cells expressing $\alpha_4\beta_7$ and CCR9 may migrate to the small intestines (14, 20). The presence or absence of trafficking molecules allows for exquisite regulation of leukocyte homing and also presents pharmacological targets in the treatment of tissue specific diseases.

The zip code requirements for lymphocyte entry into the skin are best characterized for CD4 T cells. Extensive studies have revealed that CD4 T cells can utilize E-selectin ligand/CLA and $\alpha 4\beta 1$ integrin for rolling, the chemokines receptors CCR4, CCR8, and CCR10 for G protein coupled signaling leading to firm adhesion by $\alpha 4\beta 1$ or $\alpha L\beta 2$ (Figure 2, (21-28) reviewed in (20, 29, 30)).

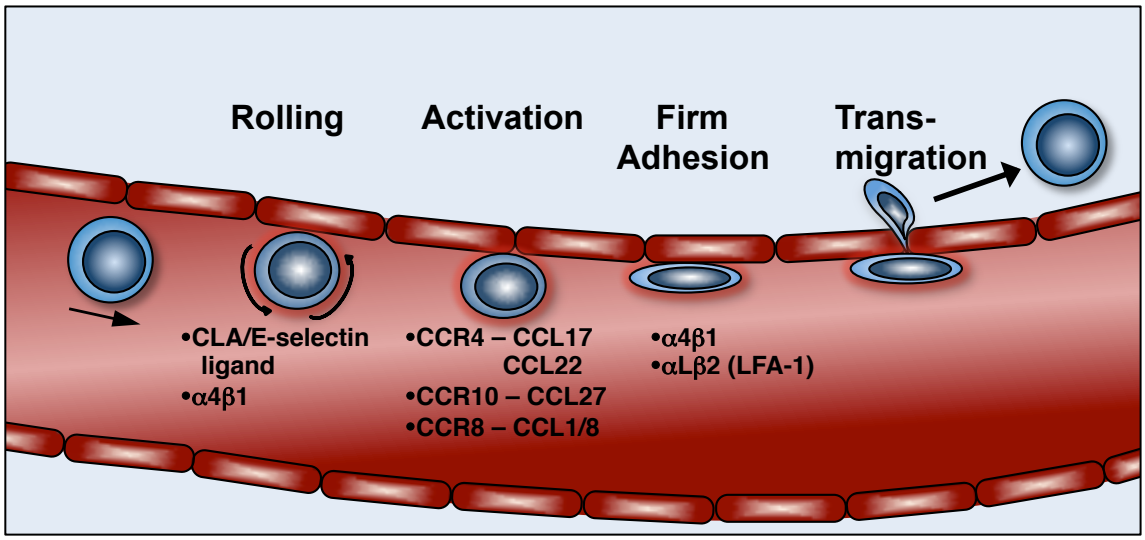


Figure 2. The multistep adhesion cascade highlighting the important trafficking receptors utilized by CD4 T cells to enter the skin.

In addition to discovering what molecules mediate T cell migration into the skin, a source of T cell programming has been identified. In human skin, previtamin D3 is produced after exposure to sunlight in the deep epidermis, which then undergoes a slow spontaneous isomerization to vitamin D (31). Although traditionally understood that vitamin D was metabolized in the liver, in recent years, keratinocytes (32), macrophages (33), and DCs (34) have been shown to break down vitamin D into it's active metabolite, $1,25(OH)_2D3$, and that both forms are capable of inducing skin-homing receptors, such as CCR10, on CD4 T cells (34). This utilization of a site-specific metabolite to program

trafficking patterns in the skin is mirrored in the gut where retinoic acid, which is acquired thorough the diet in the form of vitamin A, imprints both T (35) and B cells (36) with gut-homing programing. However, unlike vitamin A in the gut, the role of vitamin D on B cell trafficking is less fully understood. In vitro culture of human peripheral blood B cells with $1,25(OH)_2D3$ caused a downregulation of ongoing proliferation and an inhibition of plasma cell generation (37), but how this may affect the potential of B cells to migrate to the skin remains unknown as B cells are not currently considered a constituent of the cutaneous immune system (29, 38, 39).

γδ T cells

Development of γδ T cell subsets

γδ T cells are innate-like T cells with unconventional (not αβ) T cell receptors that constitute a smaller proportion of T cells than their αβ counterparts. While γδ T cells are primarily located within the barrier surfaces of the body, such as the intraepithelium of skin and intestine, in mice (40, 41) and ruminants (42, 43), γδ T cells are more homogenously distributed throughout the human immune system (44, 45). Despite differences in anatomical localization, γδ T cells across species can be divided into functional groups based upon their development and Vδ,Vγ usage, which also often distinguishes γδ T cells from different tissues (reviewed in (46)). This is in stark contrast to αβ T cells that are generally subsetted by their cytokine production profiles after antigen encounter in the periphery (47).

Murine γδ T cells develop in the thymus in functional waves with the earliest only active during fetal development. The first fetal emigrants express Vγ5. These cells

migrate to the epidermis and are termed dendritic epidermal T cells (DETCs) (40). The second wave of γδ T cells are IL-17-producing γδ T cells that migrate to the skin (48-51) and peritoneal cavity (52), and can be found in secondary lymphoid organs (51, 53). A third wave of emigrants includes γδ NKT cells, which are abundant in the spleen and liver (54, 55). The fourth and fifth waves, naive and γδ intestinal intraepithelial lymphocytes (iIELs), also develop at this time, but the production of naïve and iIEL γδ T cells continues postnatally (56, 57). Although the developmental program of human γδ T cells is less clear, first γδ T cell subset to emerge from the thymus populates intestinal epithelial tissues (58) and cytokine-producing γδ T cells can be found in umbilical cord blood (59, 60).

γδ T cells in the skin

Much of the work regarding γδ T cells in the skin has focused on the function and trafficking of murine DETCs. DETCs upregulate skin-homing receptors after TCR ligation in the thymus (61) and, akin to skin homing CD4 T cells (20, 30), require ligands for E- and P-selectin, as well as CCR4 and CCR10 for their migration into the epidermis (62, 63). Once in the epidermis, DETCs are positioned as key regulators of cutaneous responses. The DETC TCR is not MHC-restricted and is capable of responding to antigens from stressed or damaged keratinocytes (64), an interaction that appears critical for keratinocyte maintenance (65). Upon activation, DETCs are capable of producing a variety of effector molecules including keratinocyte growth factors (KGF), insulin-like growth factor-1, IL-2, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor-α, lymphotaxin, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES (66). Following injury by full skin thickness

punch, macrophage recruitment is impaired in mice lacking DETCs (67). Furthermore, wound closure is also delayed, but addition of either activated DETCs or KGF-1 restores wound healing (68). Although classical DETCs have only been observed in mice (44, 69, 70), γδ T cells in human skin are also have restricted usage Vδ usage and recently have been shown to produce IGF-1 in newly wounded skin (71), suggesting there are similarities in epidermal T cells across species.

γδ T cells in the skin also participate in cutaneous immune responses, providing a rapid, early response to viral, bacterial, and fungal infection. For example, DETCs provide IL-17 in response to *Staphylococcus aureus* infection (72). Cyster and colleagues recently observed a more abundant population of γδ T cells in skin-draining lymph nodes compared to mucosal draining lymph nodes (48). They and others found that a population of motile γδ T cells also resides in the dermis, and that these γδ T cells are unique from DETCs (48, 73, 74). These γδ T cells express a different Vδ TCR, are mostly round without dendrites, and produce large quantities of IL-17 when exposed to IL-23 or when the skin was infected with *Mycobacterium bovis* (48, 73, 74). Many studies describing DETC function employed TCRδ-deficient mice and drew conclusions on the role of DETCs assuming that there were not appreciable numbers of γδ T cells in the dermis. Furthermore, although the dermis and epidermis were often mechanically separated in the isolation of epidermal γδ T cells, the possibility of dermal contamination cannot be fully ruled out. Since we now know there is a dermal population, further studies are necessary to delineate the functions of these two populations, especially in inflammatory settings where dermal γδ T cells may have greater access than epidermis-restricted DETCs

As this newly observed dermal T cell subset is capable of modulating immune responses through IL-17 production and possibly other mechanisms, it is important to understand how these cytokine-producing γδ T cells are mobilized to the skin. While conventional αβ T cells depend on CCR7 to egress from extralymphoid tissues, such as the skin, and enter afferent lymph vessels (75-77), dermal γδ T cells do not express CCR7 (73, 78). Weninger and colleagues have demonstrated that dermal γδ T cell numbers are normal in CCR7-knockout mice (73). A similar finding by Koets and coworkers revealed that bovine γδ T cells exit the skin and enter in the skin-draining lymph without expression of CCR7 (78). Bovine skin-draining γδ T cells expressed mRNA transcripts for CCR4 and CCR10, which as previously mentioned, can mediate CD4 T cell entry into the skin; however, analysis of protein expression or function is lacking. There is currently one report in a mouse model of IL-23-induced psoriasis of an IL-17-producing γδ T cell population utilizing CCR6 to migrate from the epidermis to the dermis (79), but blocking the ligand (CCL20) only inhibited dermis to epidermis trafficking and did not affect accumulation in the dermis. As such, the molecules used by recirculating γδ T cells to enter and exit the skin remain to be identified. Chapter 1 will investigate the recirculation and functional capacity of migratory γδ T cells in the skin and skin-draining lymph of sheep.

γδ T cells as antigen-presenting cells

In addition to effector T cell properties, one subset of human γδ T cells was proposed to function as a professional antigen-presenting cell (APC) (80). Specifically, upon stimulation human $V\delta2^+$ T cells upregulated MHC class II (MHCII) and costimulatory molecules, as well as the "tissue exit receptor" CCR7, to induce primary

responses in naïve αβ T cells in draining lymph nodes (80). It remains unknown if this finding also applies to γδ T cells of other species.

B-1 B cells

Development of B-1 B cells

From extensive studies in mice, B cells can be broadly divided into two lineages: innate-like B-1 B cells and conventional, follicular B-2 B cells.[1] B-1 B cells, like their name implies, arise first in fetal development in several waves: the first wave from precursors in the para-aortic splanchnopleura and yolk sac (82, 83), followed by development from the fetal liver (84), and lastly from the bone marrow ((85), reviewed in (86)). B-1 B cells are produced more abundantly in fetal and early life, and as output of B-1 B cells decreases, the output of B-2 B cells increases. Whether this is due to the presence unique precursors at different developmental checkpoints or is regulated by environmental/developmental cues is not fully understood (87, 88). B-1 B cells are $CD19^{+}$, $CD1d^{mid}$, $CD23^{-}$, $CD43^{+}$, IgD^{low}, IgM^{hi} and can be divided into at least two phenotypic subsets: B-1a and B-1b B cells, with the former also expressing CD5. Both subsets are found primarily in the peritoneal and pleural cavities, to a smaller extent in the spleen, as well as in significantly lower numbers in the circulation and peripheral lymph nodes (81, 89). Although some differences in the developmental requirements (90) and BCR specificity for B-1a and B-1b B cells (91, 92) have been described, these subsets are phenotypically similar and self-renewing (93).

[1] B-2 B cells also include an innate-like subset called marginal zone (MZ) B cells; however, the B-2 designation is often restricted to just follicular B-2 B cells 81.
Baumgarth, N. 2011. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* 11: 34-46..

Functions of B-1 B cells

A hallmark function of B-1 B cells is the ability to secrete natural antibody (94). Natural antibody tends to bind both pathogen structures as well as endogenous antigens (95). There is some dispute as to whether peritoneal B-1 B cells are capable of secreting antibody in the peritoneal cavity without stimulation (96, 97) or whether an activation signal is required for their exit from the peritoneal cavity and subsequent antibody production in the spleen or bone marrow (98, 99). Despite debate on the cues and localization required for B-1 antibody secretion, it is widely accepted that this subset is ultimately responsible for most circulating IgM (100) as well as intestinal IgA (101, 102). B-1 B cell derived natural antibody, along with recently reported B-1-derived antigen-specific antibody (103), is critical for effective immune responses against pathogens and in inflammation. For example, B-1 derived IgM improved survival in influenza infection, even in the presence of B-2-derived IgM (104), and in *Borrellia hermsii* infection, B-1 IgM was sufficient to clear the bacteria (91). B-1 IgM is also critical in the development of contact hypersensitivity, although a more recent study questions if this IgM is perhaps produced by a unique subset of B-1 B cells (105, 106). However, the contribution of B-1 derived antibody in many infectious and/or inflammatory settings remains unclear.

B-1 derived IgM is also critical for tissue homeostasis by binding antigens expressed on apoptotic cells (107, 108) and facilitating uptake by phagocytic cells (109). Mice that are deficient in secreted IgM develop normally (110), but in a transgenic mouse model of lupus, disease is accelerated in mice also lacking circulating IgM (111), illustrating an anti-inflammatory role of secreted IgM in tissue maintenance.

B-1 B cells can exhibit effector functions beyond antibody production, and several phenotypically similar, functionally distinct populations of B-1 B cells have been characterized in recent years. Although some labs have proposed that these functions warrant the creation of additional innate B cell subsets, whether these functional groups share developmental precursors or just surface markers with B-1 B cells is unclear. Suyner and colleagues demonstrated that peritoneal B-1 cells are capable of phagocytosing bacteria and presenting it to CD4 T cells (112). Whether B-1 B cells in other anatomical locations are also phagocytic antigen-presenting cells has yet to be experimentally determined. It has been known for over two decades that B-1a B cells (then identified by expression of Ly-1, aka CD5) can produce IL-10 (113, 114). Recent studies have further characterized these cells and show that B cell-derived IL-10 can dampen T cell responses in a model of contact hypersensitivity (115) and infection with cytomegalovirus (116). Furthermore, a subset of B-1a cells is capable of producing GM-CSF, and B-cell derived GM-CSF was required for control of sepsis (117). Collectively, these studies broaden our understanding of the innate potential of B-1 B cells; however, if these cells carry out their functions beyond the peritoneum/spleen where they've thus far described requires further investigation.

Migration of B-1 B cells

As previously mentioned, B-1 B cells can exit the peritoneal cavity, migrating to the spleen or bone marrow to secrete antibody (98, 99). Furthermore, when peritoneal B-1 B cells receive LPS or a TLR4 ligand, they rapidly exit the peritoneal cavity and migrate to the spleen and small intestine (118-120). Furthermore, in the aforementioned studies on the role of B-1 B cells in influenza infection and contact hypersensitivity, peritoneal B-1s could be found in the mediastinal (92) and skin-draining lymph nodes

(105), respectively. As B-1 B cells are capable of migration into the intestine and lung, both barrier organs, it raises the question if B-1 cells (and B cells, in general) are capable of migrating into another major barrier organ, the skin.

Several groups have initiated studies on the requirements of B-1 B cell recirculation into and out of the peritoneal cavity. Cyster and coworkers conclusively demonstrated that peritoneal B cells recirculate between the peritoneal cavity and blood under homeostatic conditions in parabiotic mice (121). This exit is dependent on CXCR5-CXCL13, as mice deficient in the receptor have significantly fewer B-1s in the peritoneum (122), and B-1s in mice lacking the ligand cannot home into the peritoneal cavity (121). Unlike peritoneal B2 or CD4 T cells, B-1 B cells do not rely on CCR7 to exit the peritoneum (123), but instead are more dependent on S1P (124). Although chemokine requirements for peritoneal B-1 cell entry and exit have been fairly well characterized, the integrin requirements for B-1 cells are less clearly understood. It has been proposed that α4 integrins could play a role in peritoneal B-1 cell homing as it is used by B-2 B cells to enter the peritoneum (125). Furthermore, MADCAM$^{-/-}$/VCAM$^{-/-}$ double knockout mice exhibit significantly fewer B-1 B cells in the peritoneal cavity compared to MADCAM$^{-/-}$ single knock-outs (125). However, whether this is because of entry versus exit requirements or due to survival has not been investigated. Conversely, alpha-4 integrin has been shown to play a role in peritoneal exit. After LPS stimulation, alpha-4 and beta-1 integrins are downregulated on B-1 B cells, and this downregulation allows for their release from the peritonium (118). Chapters 2 and 3 will investigate the possibility of B-1 cell migration through the skin, identify a requirement for α4 integrin in mediating entry into the skin, and propose a resolution for the dual functionality of alpha-4 integrin in tissue exit and entry.

B-1(like) cells in humans and sheep

Although B-1 B cells are best characterized in mice, innate B cells have been described in other species, including rabbits (126), sheep (127), cattle (128), and humans (129). In sheep, B-1-like cells are prominently found in the blood as opposed to the peritoneal cavity, but they share many other characteristics of murine B-1s (127). In contrast, whether the population identified as human B-1 B cells are B-1 cells at all is hotly debated. Phenotypic overlap with circulating marginal zone B cells (130, 131) and pre-plasmablasts (132) have lead researchers to question the ontology of the proposed human B-1 B cells. Despite the subset controversy, B cells in the blood functionally similar to murine B-1 B cells, such as dominant responses to B-1 antigens (133, 134), have been described and suggest circulating innate-like B cells exist in humans. Chapters 2 and 3 will investigate B cells in the skin and skin-draining afferent lymph or just skin of sheep and humans, respectively, to determine if B cells and/or B-1-like cells are present in these tissues.

Chapter 2: Ovine skin-recirculating γδ T cells express IFN-γ and IL-17 and exit tissue independently of CCR7

Abstract

γδ 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 γδ T cells are poorly described. Here we found that γδ 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 γδ T cells in skin or lymph lacked expression of CCR7, indicating that they use alternative receptors for egress. Skin-draining γδ 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$^+$ γδ T cells were the most CCL20-responsive subset of γδ T cells. The data suggest that γδ 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.

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 (135, 136)). Memory/effector T cells subsequently exit the skin or other extralymphoid tissues by entering afferent lymph vessels and migrating into draining lymph nodes (137). 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 (138, 139)). 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 (43). $\gamma\delta$ 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 (140, 141)). 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 γδ T cells provide IL-17 in response to *Staphylococcus aureus* infection (72). However, others found that motile dermal γδ T cells, but not DETCs, rapidly express high levels of IL-17 and are therefore critical in cutaneous infection and inflammation in mice (48, 73, 74). Thus, it is important to understand how different cytokine producing γδ 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 (61) and akin to skin homing CD4 T cells (20, 30), require ligands for E- and P-selectin, as well as CCR4 and CCR10 for their migration into the epidermis (62, 63). While conventional αβ T cells depend on CCR7 to egress from extralymphoid tissues, such as the skin, and enter afferent lymph vessels (75-77), the molecules used by recirculating γδ T cells to enter and exit the skin remain to be identified.

In addition to effector T cell properties, one subset of human γδ T cells was proposed to function as a professional antigen-presenting cell (APC) (80). Specifically, upon stimulation human $V\delta2^+$ T cells upregulate MHC class II (MHCII) and costimulatory molecules as well as the "tissue exit receptor" CCR7 to induce primary responses in naïve αβ T cells in draining lymph nodes (80). It remains unknown if this finding also applies to γδ T cells of other species.

Many studies focus on γδ T cells as they reside in the tissue or in the blood; however, little is known about tissue-recirculating γδ T cells. Although all jawed vertebrates have γδ T cells, the particularly large number of recirculating γδ T cells of ruminants, including sheep and cattle (70), make them an ideal model to study migratory γδ T cells. While ruminants and humans have epidermal and dermal γδ T cells, both

species lack prototypic DETCs and therefore rely on a mobile surveillance of the skin by γδ T cells (44, 69, 70). In this study, we found that ovine γδ T cells that have just left the inflamed or uninflamed 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 γδ T cells are easily deployed into skin and sites of inflammation. In contrast to αβ T cells, γδ T cells are able to exit the skin in a CCR7-independent manner. Finally, while many γδ T cells migrate to the cutaneously expressed CCR6 ligand, CCL20, IL-17 producing γδ T cells were highly enriched in the responsive fraction, suggesting that IL-17^{+} γδ T cells utilize CCR6 to recirculate through the skin.

Material and Methods

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 Schaer (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 (77, 142). 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 γδ 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 (77). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Cell isolation

Leukocytes were isolated as previously described (2). Briefly, cells were released by grinding lymph nodes through a cell-dissociation sieve and passed through a 40-mm 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).

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), γδ TCR (86D (143); VMRD), MHCII molecule DR (TH14B; VMRD), L-selectin (DU1-29; VMRD). Mouse anti-ovine CD11c hybridoma

supernatant (17-196; (34) and mouse anti-ovine pan-B-cell marker (2-104; (2)) 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 Ca2+ and Mg2+. Specificity of the binding was shown by staining in 30-50 mM EDTA buffer, which inhibits the Ca2+-dependent binding to E-selectin. CCR7 was stained using a mouse CCL19 human Fc chimeric protein (eBioscience) as described (76). 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 (77). 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 (77):

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.

Chemotaxis assay

The assay was performed and analyzed as described (76, 144). 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. 5-10 x 105 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 μm polystyrene beads as a standard (Polyscience Inc.) combined with flow cytometric analysis of γδ, CD4+ and (γδ 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 γδ T cell migration with the frequency of cytokine-producing cells in input and migrated population (144). 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 (Csample) 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*(Csample * Psample)/(Cinput * Pinput).

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.

Statistical analysis

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

Results

Skin-draining ovine γδ T cells are effectors rather than APCs.

Previously, a subset of human γδ 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 αβ T cells (80). Therefore, we addressed whether ovine γδ T cells leaving extralymphoid tissues via afferent lymph display characteristics of professional APCs. By using an ovine model of afferent lymph vessel cannulation (142), 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 γδ T cells in the steady state and in the presence of chronic inflammation elicited by Complete Freund's Adjuvant (77, 145). We stained γδ 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 γδ 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 γδ T cells in humans, mice, and cattle are capable of producing a wide variety of cytokines (146, 147). To determine whether skin-recirculating γδ 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 γδ T cells (one example staining is shown in Fig. 1B; Fig. 1C). Although, fewer γδ 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 γδ T cells was similar to that of CD4 T cells (2 – 6.5%) ((77), Fig. 1B and C). Thus, skin-recirculating γδ 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.

Ovine γδ T cells are well equipped to recirculate through skin.

To determine whether skin-draining ovine γδ 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 (148) and other ligands (149). As expected (11, 42, 43), almost all blood- and lymph-borne γδ T cells expressed high levels of L-selectin (Fig. 2A, C). Furthermore, blood and skin-draining γδ T cells also expressed E-selectin ligand (Fig. 2A, B), indicating a propensity of skin draining γδ T cells to efficiently return to the skin. However, significantly fewer γδ T cells residing in the immunized skin expressed L-selectin when compared with γδ 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 γδ T cells in ovine skin. Our data are in line with studies in cattle, showing that blood-borne bovine γδ T cells express E- and P-selectin

ligand and L-selectin (150-152). Thus, ruminant γδ T cells in general exhibit a phenotype that suggests superior capacity to enter sites of inflammation.

γδ T cells exit the skin in a CCR7-independent manner.

Mouse CD4 and CD8 T cells depend on CCR7 to exit uninflamed extralymphoid tissues (75, 76), as its ligand, CCL21, is constitutively expressed by afferent lymphatics (153-155). 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 (76). Additionally, CCR7 was suggested to mediate human γδ T cell migration from extralymphoid tissue into afferent lymphatics (156). Thus, we addressed whether CCR7 is a potential skin exit receptor for skin-recirculating γδ 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 αβ T cells. However, only few γδ 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 γδ T cells must be able to egress from the skin in a CCR7-independent manner.

Lymph-borne γδ 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 γδ T cells, we tested their chemotactic responsiveness to ligands for chemokine receptors involved in αβ T cell recruitment into skin and/or sites of inflammation (157), 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, (2)), 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 (62, 63), ovine skin-recirculating γδ 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 γδ T cells did not stain for surface CCR4 using a monoclonal antibody (Supplemental Figure 1B and C). Ovine lymph-borne γδ 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 γδ T cells (74, 80). 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 γδ T cells, and to a much lesser extent blood-borne γδ T cells, were selectively responsive to CCL20 (Fig. 4A and B), the ligand for CCR6, which is highly expressed on mouse dermal γδ T cells (48, 74). Importantly, CCL20 is constitutively expressed in human cutaneous vasculature (158) and the regulation of CCR6 is critical to proper epidermal localization of DETCs (159). Thus, our data suggest that skin recirculating ovine γδ T cells rely on CCR6 rather than the prototypical skin

homing chemokine receptors (CCR4, CCR8, and CCR10) used by αβ T cells for their skin tropism.

IL-17 producing γδ T cells are highly responsive to the CCR6 ligand CCL20

It is well established that (αβ) Th17 cells express CCR6 (160), and CCR6 was recently found on mouse and human IL-17 expressing cutaneous γδ T cells (48, 73, 74, 79, 159). Having found that skin-recirculating γδ 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$^+$ γδ 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$^+$ γδ T cells enriched in the CCL20-responsive fraction relative to input (Fig. 4C). In a quantitative analysis, we found that IL-17$^+$ γδ T cell were significantly more responsive to CCL20 than were IL-17$^-$ (IFN-γ$^+$ [p = 0.006] or IFN-γ$^-$ [p = 0.004]) γδ 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 γδ T cells from different mammalian species, suggesting a key role for CCL20 in driving IL-17-dominated γδ T cell responses in the skin.

Discussion

γδ T cells are innate-like T lymphocytes that act as critical responders in infection and inflammation. Recent studies show that γδ T cells are involved in the control of bacterial, viral, and fungal infections (72, 73, 161), as well as in a murine models of autoimmunity (162), many of which affect the skin. Thus, understanding the functional capacity and

trafficking patterns of skin-recirculating γδ 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 γδ T cells in the skin-draining lymph and skin under homeostatic and chronic inflammatory conditions. As a subset of γδ T cells in humans activates αβ T cells (80), we evaluated MHCII and CD80/86 expression on skin and found that compared to co-isolated and -stained dendritic cells, γδ 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 αβ and γδ T cells without a significant ability to stimulate other T cells with soluble antigens (163, 164). Taken together, we conclude that unlike dendritic cells, ovine skin-draining γδ T cells lack sufficient expression of key molecules that define professional APCs. Additional data support that γδ T cells in afferent lymph of ruminants are not traveling to draining lymph nodes to activate αβ T cells in the T-cell zone, because these γδ T cells rapidly pass through the lymph node to re-enter the efferent lymph (165) and recirculate back into skin (42, 78).

γδ 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 (52, 72). Moreover, IL-17 and IFN-γ produced by mouse γδ T cells are required for the subsequent CD4 T cell accumulation at sites of mycobacterial infection and infection with herpes simplex virus, respectively (73, 166, 167). When evaluating the capacity of skin recirculating γδ T cells to produce pro-inflammatory cytokines, we found that PMA and ionomycin stimulated γδ T cells in the skin-draining lymph produced IFN-γ and IL-17

(Fig 1B-C). Consequently, the population of cytokine-positive, skin-recirculating γδ 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 γδ T cells is often transient and αβ T cells that respond at later time points in the infection often produce the same cytokine signature as early γδ T cell responders (161, 166-168). On the other hand, cytokine production by γδ T cells is detrimental if not tightly regulated. For example, mice infected with A*spergillus fumigatus* succumb to immunopathology if IL-17 production by γδ T cells is dysregulated (161). Therefore, proper control of γδ 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 γδ 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, γδ T cells traveling in the skin draining lymph were unresponsive to ligands for CCR4, CCR8, CCR10, typical skin-attracting chemokines (157). Our data contrast the finding that bovine skin draining γδ T cells express low transcripts of CCR4 and CCR10 (78). As mRNA expression does not always correlate with functional chemokine receptor expression, it is possible that bovine γδ 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 (75, 76), ovine γδ 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 γδ T cells in the skin-draining afferent lymph lack CCR7 (78) and with findings in the mouse that exclude a role for CCR7 in dermal γδ T cell homeostasis (73). Interestingly, during the chronic phase of inflammation, mouse αβ T cells can also exit the skin independently of CCR7 (77). It is possible that alternative skin exit receptors used by γδ 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 γδ T cells, and potentially other leukocytes, thereby modulating tissue inflammation and host responses to infection. Surprisingly, ovine skin-recirculating but not blood-borne γδ 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 (158, 169), and CCR6 has been implicated in human Langerhans cell precursor migration to the skin (158). Furthermore, in a mouse model of psoriasis, a subset of γδ T cells utilizes CCR6 to migrate into the inflamed epidermis (79). Thus, a subset of γδ 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 γδ T cells with the capacity to make IL-17 upon stimulation were enriched in their CCL20 responsiveness relative to other lymph-borne γδ T cell subsets (IFNγ$^+$ or IL-17$^-$, or IFNγ$^-$ γδ T cells) is consistent with recent data in the mouse showing that dermal γδ T cells are poised to make IL-17 and express CCR6 (48, 73, 74). Thus, the link of IL-17 production by cutaneous γδ T cells and

expression of CCR6 is evolutionarily conserved. It is tempting to speculate that ovine skin-recirculating γδ T cells are homologous to the IL-17$^+$ dermal γδ T cells in the mouse. Dermal γδ T cells of mice and skin-recirculating ovine γδ T cells not only share the ability to produce IL-17 and express CCR6, they also both express variant TCRs (73, 170, 171). Similarly, data suggest that mouse dermal γδ T cell can recirculate because they also localize to the skin-draining lymph node in the subcapsular sinus (48), where afferent lymph enters the lymph node. However, the potential for murine dermal γδ T cells to recirculate has yet to be assessed.

In cattle, γδ T cells express the transmembrane glycoprotein WC1, the isoform of which marks distinct functional subsets of γδ T cells (172). For example, IFNγ and IL-10 production by bovine γδ T cells are associated with specific WC1 isoform usage (172, 173). Most γδ T cells in adult sheep also express this molecule (also known as T19; (143)), and although ovine isoforms are less well-characterized (174), it would be interesting to investigate WC1 expression by IL17$^+$ γδ T cells.

As previously mentioned, TCR diversity differs between skin-resident DETCs and motile dermal γδ T cells. DETCs have restricted TCR usage, while dermal γδ T cells exhibit significantly more diverse TCRs, enabling them to respond to a greater repertoire of unique antigens than their sessile counterparts (72, 73, 170, 171). The ability to divide the workload of continuous immunosurveillance between resident and recirculating subsets of γδ T cells would be advantageous to achieving consistent and robust protection from exogenous threats. While resident γδ 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 γδ 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 γδ T cells ideal first responders to cutaneous infection, bridging the gap between innate and adaptive immunity.

Chemokine Fragment	Species$_{\text{utilized fragment}}$	NCBI Accession Number	Identity (%)	Similarity (%)	Predicted *Ovis aries* Accession Number
CCL1	***H. sapiens*$_{24-96}$**	EAW80204.1	**41**	**62**	XP_004013255.1
	M. musculus	AAI20807.1	33	54	
CCL2	*H. sapiens*	EAW80212.1	67	84	XP_004012520.1
	***M. musculus*$_{24-96}$**	AAH55070.1	**50**	**69**	
CCL4	***H. sapiens*$_{24-92}$**	AAI04228.1	**84**	**92**	XP_004012496.1
	M. musculus	AAI19258.1	82	90	
CCL17	*H. sapiens*	AAI12069.1	51	66	XP_004015612.1
	***M. musculus*$_{24-93}$**	EAW70878.1	**47**	**67**	
CCL20	***H. sapiens*$_{27-96}$**	AAH28504.1	67	84	XP_004005034.1
	***M. musculus*$_{27-96}$**	AAH27918.1	**58**	**79**	
CCL21	*H. sapiens*	AAH27918.1	73	86	XP_004004168.1
	***M. musculus*$_{24-133}$**	NP_035254.1	**69**	**80**	
CCL28	***H. sapiens*$_{23-127}$**	ABB13417.1	**78**	**90**	ABB13417.1*
	M. musculus	AAH55864.1	59	76	
CXCL9	*H. sapiens*	AAH95396.1	72	81	XP_004004168.1
	***M. musculus*$_{22-126}$**	AAH03343.1	**61**	**73**	

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. *actual sequence

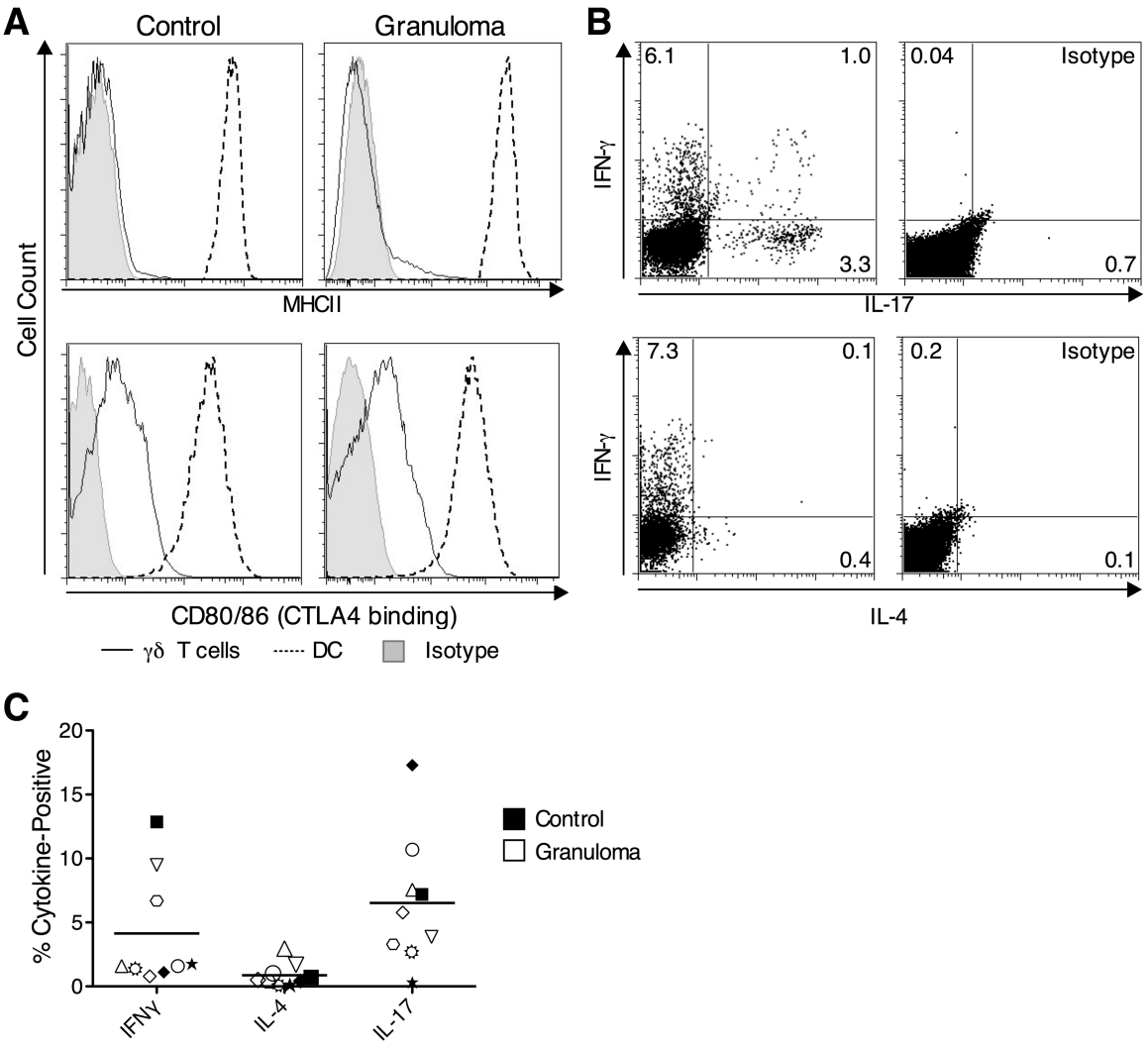


Figure 1. Skin-draining γδ T cells are effectors rather than APCs. Leukocytes from uninflamed (control) and chronically inflamed (granuloma) skin-draining lymph were analyzed. (**A**) Flow cytometric analysis of γδ T cells and dendritic cells (MHCII^{+}, CDIIc^{hi}, pan-B^{-}) for the expression of MHCII 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 γδ T cells from skin granuloma draining lymph for one representative animal. (**C**) Percentage of cytokine-positive γδ 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.

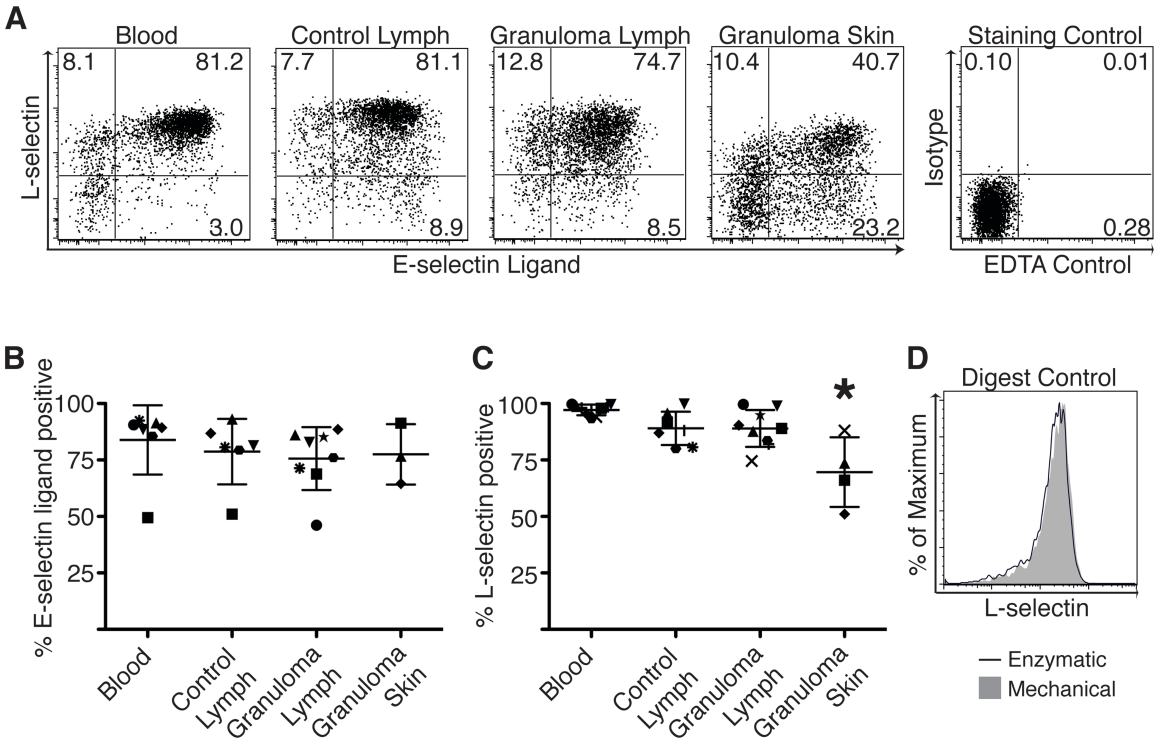


Figure 2. L-selectin and E-selectin ligand expression by γδ T cells. γδ T cells from blood, control and chronically inflamed (granuloma) skin-draining lymph, and granulomatous skin were analyzed. (**A**) Flow cytometric analysis of L-selectin and E-selectin ligand on gated γδ T cells. One representative animal is shown. Staining control for control lymph is shown and is representative of all tissues. (**B** and **C**) Percentage of γδ 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 γδ T cells after mechanical or enzymatic (digest) isolation from lymph nodes.*$p < 0.05$.

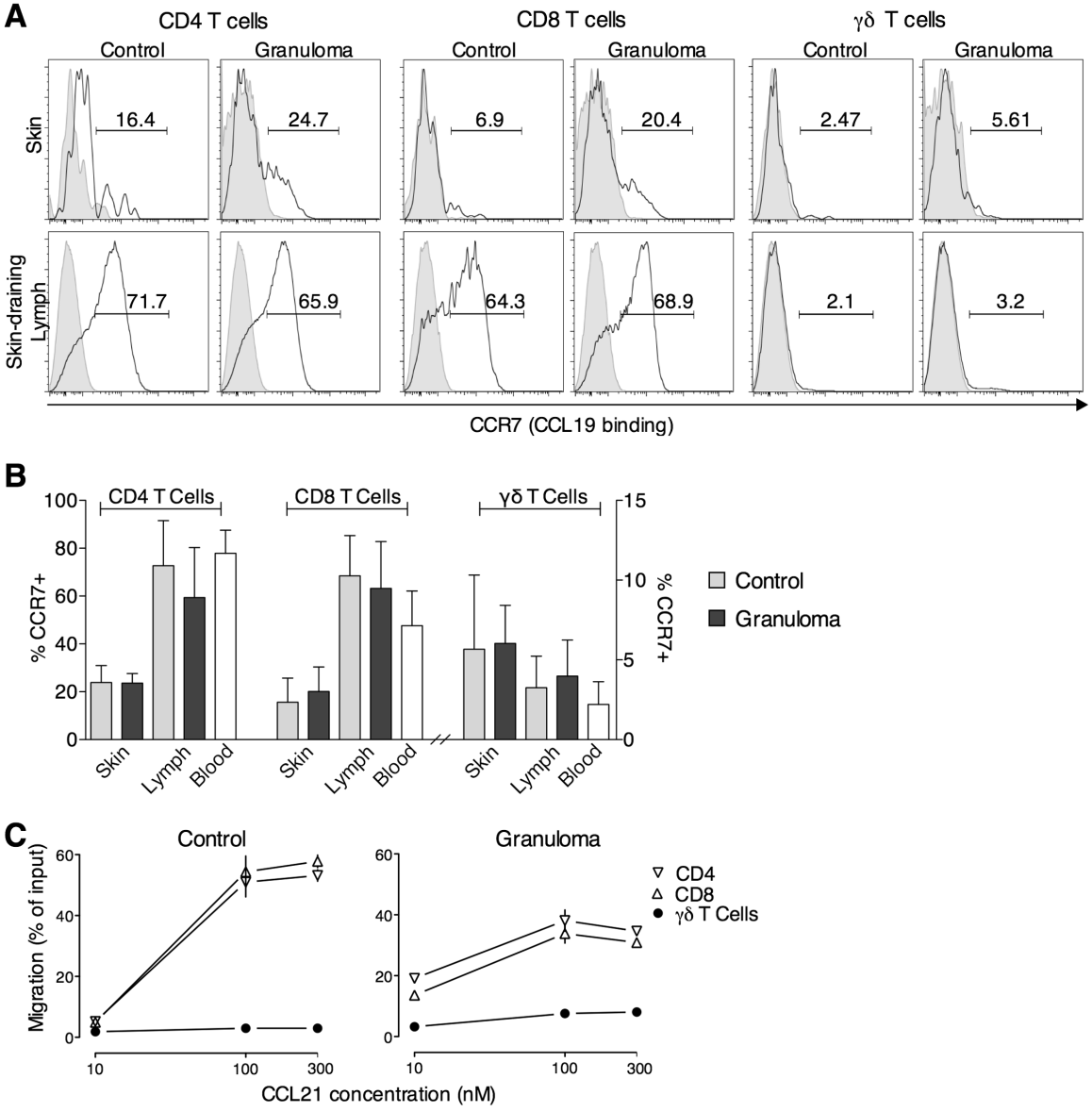


Figure 3. γδ T cells exit the skin independently of CCR7. T cell subsets from uninflamed and granulomatous skin and skin-draining lymph, as well as blood, were analyzed. (**A-B**) Flow cytometric analysis of gated CD4, CD8, and γδ T cells for expression of CCR7. One example staining with isotype controls 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 γδ, 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 ±

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.

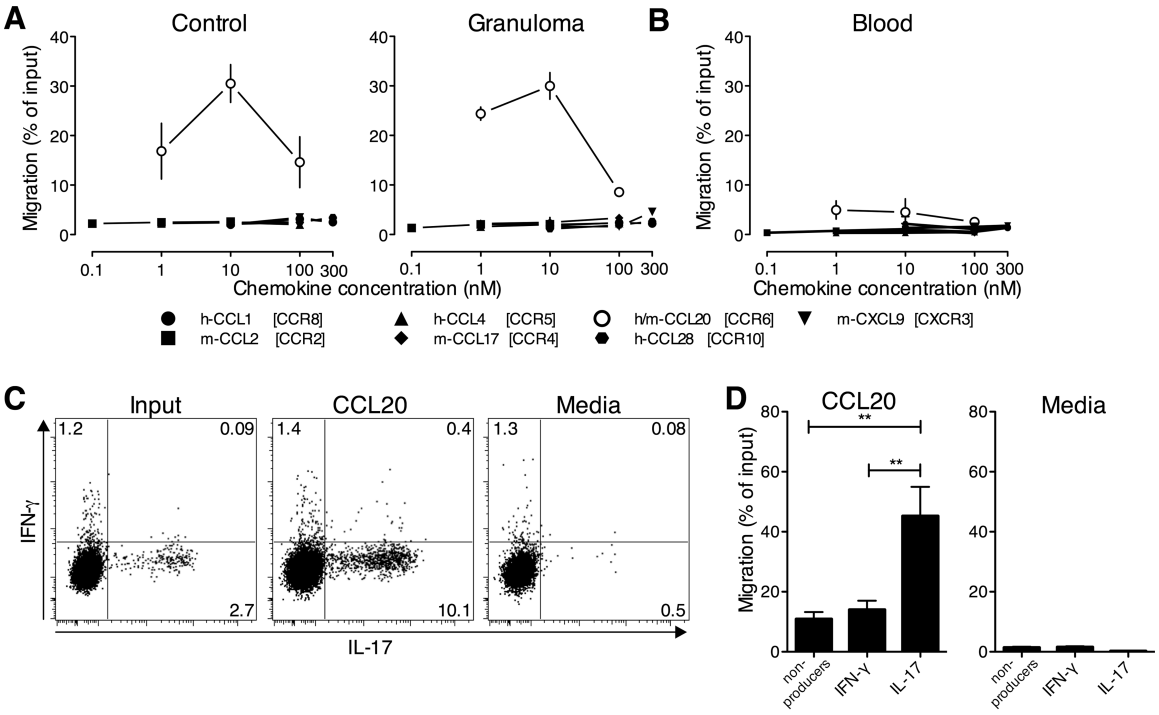
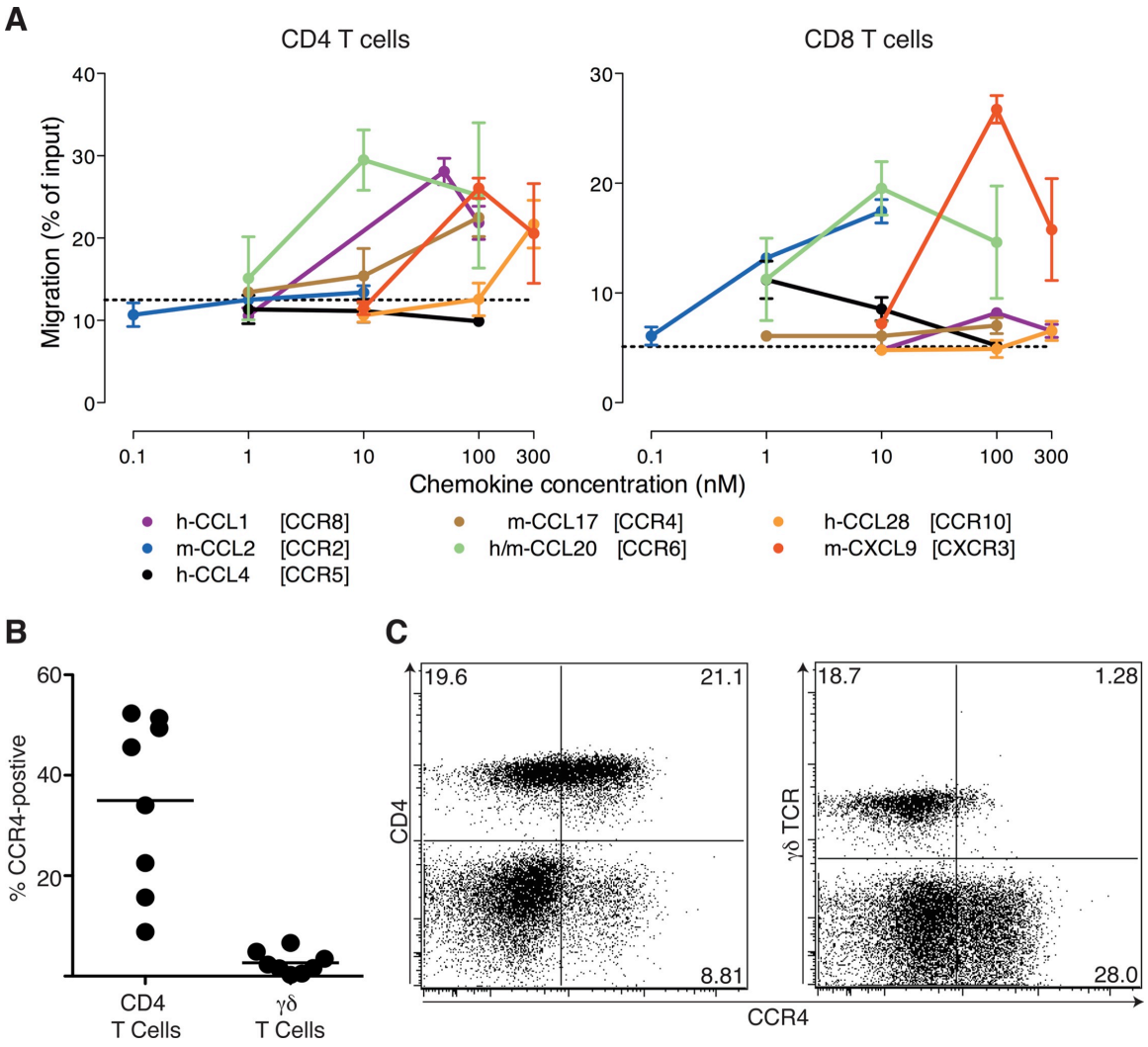
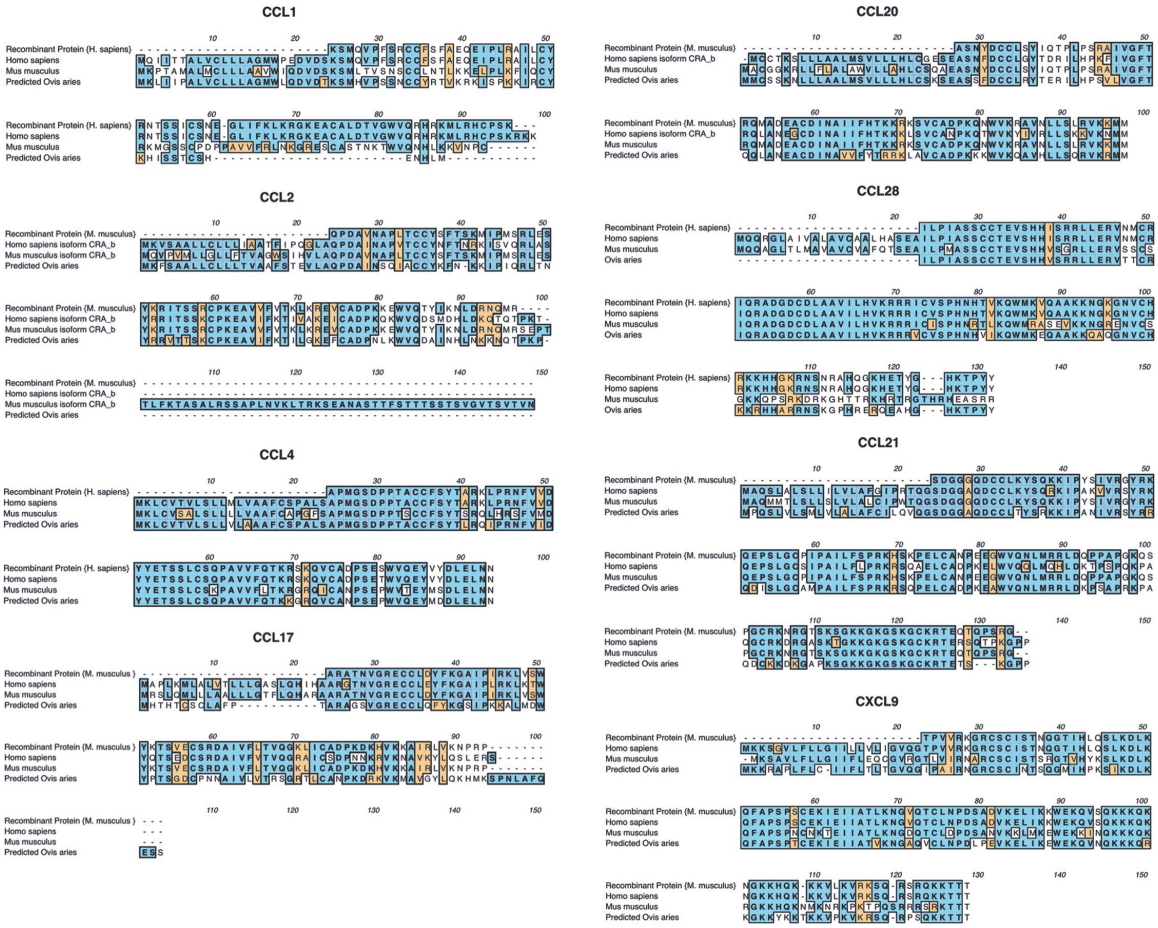


Figure 4. **Chemotactic responses of lymph- and blood-borne γδ T cells.** (**A**) Chemotaxis of lymph-borne γδ T cells draining uninflamed (Control) or chronically inflamed skin (Granuloma) and (**B**) blood-borne γδ T cells to human (h) CCL1, CCL4, CCL20 and CCL28 and mouse (m) CCL2, CCL17, CCL20, and 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 γδ T cells from granulomatous skin-draining lymph to 10nM CCL20 was assessed by gating on γδ T cells by flow cytometry. One out of two experiments with similar results is shown. (**C**) IL-17 and IFN-γ expression by γδ T cells of the input population and of γδ T cells that migrated to CCL20 or media alone. (**D**) Chemotaxis quantification of γδ 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 ± SD of triplicate wells at each concentration. (**C** and **D**) Cytokine production was assessed after migration by PMA and ionomycin stimulation.**p<0.01.



Supplemental Figure 1. Chemotactic responses of lymph-borne CD4 and CD8 T cells. (**A**) Chemotaxis of uninflamed skin draining CD4 and CD8 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. The response profile for CD4 and CD8 T cells was assessed in parallel to that of γδ T cells of the same animal shown in Figure 4A (left), and is representative of 3-6 individually analyzed sheep for each chemokine and cell subset. Data are expressed as the percentage of cells of the respective subset that migrated to the lower chamber, and represent the mean ± SD of triplicate wells at each concentration. Horizontal lines indicate migration to media alone. Similar CD4 T cell responses to CCL1, CCL17, CCL20, and CCL28 were previously

published in (2). (**B**) Surface expression of CCR4 on gated CD4 and γδ T cells from control skin-draining lymph of all animals analyzed (N=8) is shown. (**C**) One example staining of CD4 and γδ T cells from (**B**). Gates represent percent positive.



Supplemental Figure 2. Chemokine amino acid sequences are similar between mammalian species. Amino acid sequences for recombinant chemokines used in this study and the full length sequences for sheep (*Ovis aries*), mouse (*Mus musculus*), and human (*Homo sapiens*), obtained from NCBI, were aligned. Identical and similar amino acids are marked in blue and yellow, respectively.

CHAPTER 3: The skin, a novel niche for recirculating B cells

Abstract

B cells infiltrate the skin in many chronic inflammatory diseases caused by autoimmunity or infection. Despite potential contribution to disease, skin-associated B cells remain poorly characterized. Using an ovine model of granulomatous skin inflammation, we demonstrate that B cells increase in the skin and skin-draining afferent lymph during inflammation. Surprisingly, skin B cells are a heterogeneous population that is distinct from lymph node B cells, with more large lymphocytes as well as B-1-like B cells that co-express high levels IgM and CD11b. Skin B cells have increased MHCII, CD1, and CD80/86 expression compared with lymph node B cells, suggesting that they are well-suited for T cell activation at the site of inflammation. Furthermore, we show that skin accumulation of B cells and antibody-secreting cells during inflammation increases local antibody titers, which could augment host defense and autoimmunity. While skin B cells express typical skin homing receptors such as E-selectin ligand and alpha-4 and beta-1 integrins, they are unresponsive to ligands for chemokine receptors associated with T cell homing into skin. Instead, skin B cells migrate toward the cutaneously expressed CCR6 ligand CCL20. Our data support a model in which B cells use CCR6-CCL20 to recirculate through the skin, fulfilling a novel role in skin immunity and inflammation.

Introduction

The skin is a barrier organ that protects the body from external threats and thus harbors many resident leukocytes, including macrophages, dendritic cells, and T cells. During inflammation, these and additional leukocyte subsets are recruited into the skin (175). Although B cells are found in the afferent lymph draining uninflamed skin of both sheep and humans (176, 177), the widely accepted view is that B cells do not enter the skin during homeostasis (178). In contrast, B cells accumulate in the dermis during infection and autoimmunity (179-181), and B cell malignancies can manifest as cutaneous lymphomas. However, despite their association with a wide array of skin pathologies, the phenotypic and functional attributes of skin B cells remain unknown.

B cells can be divided into two lineages, B-1 and B-2 B cells. B-2 B cells include the conventional mature B cell subsets, marginal zone and follicular B cells. B-1 B cells, on the other hand, are an innate-like subset that resides in the peritoneal and pleural cavities and responds to T-independent antigens, bridging innate and adaptive immune responses (81, 182). Although their primary residence is within the coelomic cavities, B-1 B cells are capable of exiting the body cavities in response to infection (92, 183); however, they have not been described to enter the skin.

Lymphocyte recirculation is required for immunosurveillance, host defense and site-specific immunity. There are two general pathways of lymphocyte recirculation: lymphocytes may arrive at lymph nodes from either blood or extralymphoid tissues (reviewed in (11)). Primarily, blood-borne lymphocytes enter lymph nodes through high endothelial venules. Alternatively, lymphocytes recirculate through extralymphoid tissues, such as skin, and exit these tissues by migrating into the afferent lymph to enter the draining lymph node, and then return to the blood stream in the efferent lymph via

the thoracic duct. While two distinct blood-borne B cell subsets differentially recirculate through lymph node or spleen (184) and IgA^+ B cells preferentially recirculate through mucosal sites (185), little is known about B cell recirculation through non-mucosal extralymphoid tissues. To home to the skin, CD4 T cells rely on the coordinated expression of E-selectin and alpha-4 beta-1 integrin and utilize the chemokine receptors CCR4, CCR8, and/or CCR10 (reviewed in (20, 29, 30)). In contrast, the molecules involved in B cell migration to the skin remain uncharacterized.

In order to investigate B cells in the skin, we have employed a model of lymph cannulation (9) and show that B cells not only traffic through, but are also present in both uninflamed and chronically inflamed skin. We demonstrate that skin B cells are a heterogeneous population consisting of small and large lymphocytes with a subset exhibiting a B-1-like phenotype. In addition, skin B cells are well equipped for antigen presentation to T cells in situ and antibody-secreting cells, the effector stage of B cells, accumulate in the chronically inflamed skin leading to increased local antibody titers. While skin B cells express alpha-4 and beta-1 integrins and E-selectin ligands, unlike skin T cells, they do not respond to ligands for chemokine receptors associated with T cell homing into skin. Instead, skin B cells are responsive to the cutaneously expressed CCR6 ligand CCL20. These data suggest that skin B cells are key to cutaneous immunity and inflammation and that they utilize CCR6 - CCL20 to home to the skin.

Materials and Methods

Animals, lymph cannulation, and induction of skin inflammation

Intact female or wethers of mixed breed sheep, 5-10 months 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). Prefemoral (subiliac) lymph nodes were surgically removed to generate pseudoafferent lymph vessels as previously described (142). Briefly, following lymphectomy, the afferent and efferent lymph vessels anastomose, forming pseudoafferent vessels that carry afferent (prenodal) lymph (142). 6 –12 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. The cannulated lymphatics drained the skin and muscles of the rear flank (186). Every 1–12 hours, lymph collection bottles were changed, and the composition and numbers of lymph-borne leukocytes was determined by flow cytometry to calculate the hourly output of different lymphocyte subsets (B cells, CD4, CD8, and $\gamma\delta$ T cells). A total of 0.3 – 0.5 ml Complete Freund's Adjuvant (CFA) emulsified 1:1 with sterile saline was injected subcutaneously into the drainage area of the prefemoral node to induce acute (less than 24h) or chronic (greater than 21 days) inflammation as described (77). Mesenteric efferent lymph vessels were cannulated as described (145) either in parallel to cutaneous afferent lymph vessel cannulation or as an independent non-survival surgery. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Cell isolation

Cells were released by grinding lymph nodes through a cell dissociation sieve with size 40-60 mesh (Sigma Aldrich) followed by passage through a 40 μm cell strainer (BD Biosciences). Cells were isolated from shaved skin by mechanical disruption followed by three 20-min enzymatic digestion steps using 0.1 mg/ml DNase I (Roche Diagnostics) and 0.13 U/ml Liberase TM (Roche Diagnostics) in HBSS at 37°C. Between digestion steps, released cells were collected and washed with assay media [RPMI 1640 medium (Invitrogen) with 5% newborn calf serum (Hyclone Laboratories)]. Subsequently, the cell suspension was filtered through a cell dissociation sieve with size 40-60 mesh (Sigma Aldrich) or 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 lysis buffer (Sigma Aldrich). Lymphocytes collected from ovine lymph were washed with assay media.

Flow cytometry

To reduce nonspecific staining, cells were preincubated with mouse and sheep IgG (Jackson ImmunoResearch). After blocking, the cells were labeled with biotinylated or fluorochrome-conjugated (fluorescein isothiocyanate, phycoerythrin, Alexa Fluor 647, allophycocyanin, phycoerythrin-cyanine 7; Alexa Fluor 700, Pacific Blue) monoclonal antibodies. The following mouse anti-sheep antibodies were used: CD1 (20.27; Serotec), CD4 (44.38; Serotec), CD8 (38.65; Serotec), CD45 (1.11.32, Serotec,), γδ TCR (86D; VMRD), MHCII (TH14B; VMRD), L-selectin (DU1-29; VMRD), IgM (25.69; Serotec). The following mouse anti-human monoclonal antibodies that also recognize sheep integrins (22, 187) were used: alpha-4 integrin (HP2/1, Serotec), beta-1 integrin (TS2/16; Ebioscience), and beta-7 integrin (fib27; Ebioscience). Supernatants for the following

ovine antigens were produced from hybridomas: pan-B cell marker (2-104) (184, 188), CD21 (2-87-6) (165, 189), CD11b (12-5-4) (190), and CD11c (17-196) (20). Some mouse monoclonal antibodies were directly labeled prior to staining using Zenon labeling kits according to the manufacturer's instructions (Invitrogen). B7.1/B7.2 expression was detected by CTLA4-Ig binding (human chimeric protein; ID Labs). E-selectin ligand expression was tested by assessing binding capacity to a recombinant mouse E-selectin human IgG chimeric protein (R&D Systems) in HBSS containing Ca^{2+} and Mg^{2+}, followed by biotinylated $F(ab')_2$ donkey anti-human IgG (Jackson ImmunoResearch). Specificity of the binding was shown by staining in 30 mM EDTA buffer, which inhibits the Ca^{2+}-dependent binding. For cell surface staining with biotinylated antibodies, allophycocyanin- (BD Bioscience), Peridinin Chlorophyll Protein-Cy5.5 (BD Biosciences), Pacific Orange (Invitrogen) or Alexa Fluor 405-conjugated streptavidin (Invitrogen) were used as secondary reagents. When necessary, LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen) was used according to manufacturer's instructions. Staining of digested skin was performed in parallel with that of digested and undigested lymph node to verify that antigens were not cleaved during the cell isolation process. 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.

Histology and cytology

Concurrently, skin samples were frozen in OCT (Sakura Finetek), and 6-µm sections were fixed in acetone (Fisher Scientific). For immunofluorescence, sections were rehydrated for 5-10 min at room temperature with 100mM Tris-HCL (TEKnova) and blocked with immunofluorescence buffer: 10% rat serum (Equitech-Bio) in 20 mM Tris

with 0.9% NaCl and 0.05% Tween20 (Sigma Aldrich). Sections were incubated with anti-sheep IgM (25.69; Serotec), washed in IHC buffer, subsequently stained with anti-mouse IgG1 FITC (RMG1-1, Biolegend), and embedded with Prolong Gold anti-fade (Invitrogen). For cytologic analysis, lymph-borne lymphocytes were subjected to cytospins using a Shandon Cytospin 3 and subsequent Pappenheim stain (May-Grünwald and Giemsa (Sigma Aldrich) as per manufacturer instructions). For FACS-sorting of lymph-borne cells prior to cytospin, a FACSVantage Diva, Aria SORP Green, or Aria SORP was used. Fluorescence images were acquired on a Nikon Eclipse E600 microscope using a Photometrics Coolsnap EZ camera and NIS-Elements BR 3.0 software. Brightfield images were aquired using oil immersion on an Olympus BX40F4 microscope with a CC12 camera and NETCAM MicroSuite software (Olympus, Center Valley, PA.). Cell diameters were measured using NIS-Elements BR 3.0 software, and a minimum of 70 cells per population and animal were analyzed to determine the average population diameter. Contrast adjustments, applied to the whole image, were performed as needed using Adobe Photoshop.

Chemotaxis assay

The assay was performed and analyzed as described (76, 144). Recombinant mouse CCL17 and recombinant human CCL1, CCL20, and CCL28 (R&D Systems) were titrated in triplicates between 1 and 100 nM or 10 and 300 nM, and cells were allowed to migrate for 90 min.

ELISA and ELISPOT assay

For ELISAs, Immulon 4 HBX 96-well plates (Nunc) were coated overnight at 4°C with 1 µg/ml rabbit anti-ovine IgG (H+L) (Invitrogen), blocked with heat-inactivated rabbit serum

(Gibco), and washed with PBS-Tween (PBS containing 0.05% Tween-20). Sterile-filtered blood and lymph plasma samples were diluted with PBS, added to the plate, and incubated for 2 h at 37°C. Plates were washed with PBS-Tween and incubated for 1 h at 37°C with 0.2 µg/ml rabbit anti-ovine IgG (H+L) HRP conjugate (Invitrogen) diluted in PBS containing 0.1% heat-inactivate rabbit serum and 0.05% Tween-20. Plates were washed with PBS-Tween, developed for 5 min using TMB Single Solution (Invitrogen), quenched with 1 M HCl, and read immediately at OD490 using a E Max Endpoint ELISA microplate reader (Molecular Devices). A standard curve derived from whole ovine IgG (Jackson Immunoresearch) on the same plate was used to quantify antibody titers.

For ELISPOT assays, MultiScreen HTS 96-well filter plates (Millipore) were treated with 35% ethanol for 1 min and washed with PBS. To detect total Ig secreting cells, plates were coated with 5 µg/ml rabbit anti-ovine IgG (H+L, Invitrogen) overnight at 4°C, washed with PBS, and blocked with heat-inactivate rabbit serum (Gibco). Cells were plated in RPMI-1640 containing 10% heat-inactivated rabbit serum (Invitrogen) and incubated for 12 to 14 h at 37°C and 5% CO_2. Plates were then washed with PBS-Tween and incubated for 1 h at 37°C with 1 µg/ml rabbit anti-ovine IgG (H+L) HRP conjugate (Invitrogen). Next, the plates were washed with PBS-Tween followed by PBS. The plates were developed using an AEC Peroxidase Substrate Kit (Vector Laboratories) as per manufacturer's instructions, allowed to dry overnight, and the spots were enumerated using an Olympus SZ51 dissecting microscope (Olympus). The limit of detection was 5 ASC per 10^6 cells. Values below this threshold are displayed as 0. ELISPOT wells were photographed using an ImmunoSpot Reader (Cellular Technology Ltd.) and Image Acquisition software (Cellular Technology Ltd.).

Statistical Analysis

All statistical analyses were calculated using Graphpad Prism software. Unless otherwise indicated, all values are reported as mean ± SEM, and statistical significance was determined by the nonparametric Mann Whitney U test. For paired analysis, if indicated, the Wilcoxon test was used. $P<0.05$ was considered statistically significant.

Results

B cells reside in and recirculate through uninflamed skin

B cells contribute to many skin diseases, but their role in cutaneous immunity is not well characterized. In contrast to the notion that B cells are generally absent from the uninflamed dermis (177, 178), we consistently detected a population of B cells in the skin of sheep (5.9 ± 1.4% of skin lymphocytes; staining of one representative animal shown in Fig. 1A). To determine whether B cells not only reside in but also recirculate through the skin, we employed a lymph cannulation model established by Morris and colleagues (9). By cannulating the skin draining afferent lymphatics, the model allows for the analysis of lymphocytes during their physiological recirculation through uninflamed and inflamed skin (137). Due to a number of limitations, the comprehensive analysis of lymphocyte in the skin draining afferent lymph is currently not possible in rodents or humans. Consistent with Mackay *et al.* (176), we found a population of B cells in the skin-draining lymph (9.6 ± 2.5% of lymphocytes; N=9; one representative staining shown in Fig. 1A). When analyzing the cells traveling in afferent lymph, we found, as expected, mainly lymphocytes, few macrophages/dendritic cells and the occasional neutrophil (Fig. 1B). Interestingly, B cells sorted from the afferent lymph were comprised of both small and slightly larger lymphocytes (Fig. 1C). The small B cells averaged less than 10 μm in diameter with a scant rim of basophilic cytoplasm, while larger B cells greater than 11 μm contained slightly more basophilic cytoplasm, which is cytologically suggestive of an

activated phenotype. The results indicate that a heterogeneous population of B cells passes through the skin and enters lymph during its physiological recirculation through the body.

B cell traffic through the affected site increases in chronic skin inflammation

Many infectious and autoimmune diseases cause chronic skin inflammation that is characterized by granuloma formation (191). We employed an established model of granulomatous skin inflammation, in which inflammation is induced by subcutaneous injection of Complete Freund's Adjuvant (CFA) (77, 145). Draining lymph vessels were surgically cannulated 3-4 weeks later when the typical skin granulomas had formed at the injection sites. As previously shown for total lymphocytes and T cells (77), chronic inflammation also boosted the absolute numbers of B cells exiting the skin (Fig. 2A). Importantly, B cells were the only lymphocyte subsets that showed a consistent relative increase in lymph draining chronically inflamed skin relative to uninflamed (control) skin in all animals (P=0.0078; Fig. 2B; one example staining shown in Fig. 2C). The percentage of all other lymph-borne lymphocyte subsets (CD4, CD8, and $\gamma\delta$ T cells) was not consistently elevated, unchanged, or decreased (Fig. 2B). The data demonstrate that chronic inflammation particularly enhances B cell traffic through the skin.

B cells accumulate in chronically inflamed skin

Having found an increase in lymph-borne B cells draining chronically inflamed skin, we isolated lymphocytes from 3-week old skin granulomas. We observed that the percentage of B cells was significantly higher in the chronically inflamed skin compared with uninflamed skin (38.3 ± 2.2% vs. 5.6 ± 1.1%; P=0.0079) (Fig. 3A). We also detected B cells by immunofluorescent staining of frozen skin section in both uninflamed and granulomatous skin (Fig. 3B – F). While only very few IgM^+ B cells were visible in the

deep dermis of the uninflamed skin (Fig. 3B and C), the dermal and subcutaneous granuloma harbored numerous IgM^+ B cells that were dispersed throughout the tissue (Fig. 3D and E) or formed clusters in some areas of the granuloma (Fig. 3F). Thus, B cells are a major constituent of the lymphocytic infiltrate of chronically inflamed skin, suggesting a role for B cells in contributing to the inflammatory process.

Skin B cells express high levels of costimulatory molecules and MHCII

B cell traffic through and residence in uninflamed and inflamed skin raised the possibility that skin B cells may participate in local immune responses by interacting with co-localizing skin T cells. To explore whether skin B cells are equipped for efficient T cell activation, we examined antigen-presenting and costimulatory molecule expression on skin B cells. CD1 is a key antigen-presenting and regulatory molecule related to MHCI that presents lipid molecules to CD1-restricted T cells, such as NKT cells and some $\gamma\delta$ T cells, both of which are known to play important roles in the immunosurveillance and immunoregulation of the skin (192, 193). Skin B cells from granuloma-draining lymph (32 ± 6.7%), skin granuloma (42.8 ± 9.5%), and blood (59.7 ± 7.4%) were enriched in the expression of CD1 compared to B cells from control (uninflamed) skin lymph nodes (Fig. 4A). CD1 expression on skin and skin-draining B cells implies that they may interact with skin NKT or $\gamma\delta$ T cells, generating an effective cutaneous immune response.

Compared to B cells from a control (uninflamed) lymph node, B cells from uninflamed skin displayed modestly higher expression of MHCII (Fig. 4B). A more striking difference was observed for B7.1/B7.2 expression (determined by CTLA4-Ig binding). B cells from uninflamed and granulomatous skin, and even more so from skin draining lymph, expressed higher levels of B7.1/B7.2 than did lymph node B cells (Fig.

4B). Taken together, these data suggest that skin B cells are well equipped for antigen presentation to a variety of classical and non-classical T cells.

"Innate-like" B cells reside in and recirculate through uninflamed and inflamed skin.

The phenotype of skin-associated B cells could give insight to the capacity to modulate cutaneous immune responses. IgM^{hi} expression marks more innate-like B cells such as Marginal zone (MZ) and B1 cells capable of mounting efficient T-independent immune responses. Interestingly, in both the uninflamed (9.3 ± 1.5%; $P<0.0001$) and granuloma (7.9 ± 1.9%; $P=0.0006$) skin-draining lymph, we detected a significantly higher proportion of IgM^{hi} B cells compared with skin lymph node B cells, which contained only a negligible population of IgM^{hi} B cells (0.91 ± 0.21%) (Fig. 5A-B) consistent with the fact that lymph node B cells are largely (IgM^{lo}) follicular B cells. Furthermore, we found significantly more IgM^{hi} B cells in both uninflamed ($P=0.002$) and granuloma-draining ($P=0.0295$) afferent lymph than in efferent lymph (1.46 ± 0.41%; Fig. 5A). Sheep blood contains high numbers of IgM^{hi} B-1-like cells (127), and as expected, we detected a large population of B cells in the blood expressing high levels of IgM (25.33 ± 4.0%, Fig. 5A-B). Data suggests that MZ B cells recirculate in humans (131) and MZ B cells are characterized by high expression of CD1 (81). Based on the lower expression of CD1 and that of other MZ B cell markers such as CD21 and CD9 on skin B cells relative to splenic MZ B cells, we concluded that IgM^{hi} and/or $CD1^{+}$ B cells in the granuloma and granuloma lymph are not MZ B cells (data not shown). As B-1 cells express CD1 (194), we wondered if skin-associated B cells belong to this subset. In sheep, CD11b is a marker of B-1-like cells (127), and as such, we compared CD11b expression on the skin B cell populations. IgM^{hi} expressing B cells in uninflamed and granulomatous skin and skin draining lymph,

as well as blood, expressed high levels of CD11b relative to CD11b expression levels of total lymph node (follicular) B cells (Fig. 5C). Thus, skin-associated IgM^{hi} B cells are of B-1-like phenotype, constitutively traffic through skin and leave via the afferent lymph. Because ovine B-1-like cells often express CD11c (127), we determined CD11c expression by skin B cells and found that CD11c was expressed at higher frequencies than CD11b in B cells traveling in skin draining lymph (Fig. 5D). The microscopic analysis of FACS-sorted and Pappenheim-stained B cells from skin-draining lymph revealed that the double-negative cells were comprised of mature lymphocyte of approximately 10-μm diameter with a condensed chromatin and scant basophilic cytoplasm (Fig. 5E). In contrast, the slightly larger (~12 μm diameter) $CD11b^{-}/CD11c^{+}$ or $CD11b^{+}/CD11c^{+}$ lymphoid cells exhibited a more open chromatin and deeply basophilic cytoplasm that occasionally contains a fine paranuclear Golgi clearing (Fig. 5E). Systematic analysis of cell diameters confirmed that $CD11b^{-}/CD11c^{+}$ (12.08 ± 0.21 μm) and $CD11b^{+}/CD11c^{+}$ (11.92 ± 0.48 μm) B cells were significantly larger than were $CD11b^{-}/CD11c^{-}$ B cells (10.06 ± 0.40 μm; $P<0.0001$) (Fig. 5F). The cytomorphology suggests that CD11b or CD11c positive cells are more activated lymphoid cells in contrast to the more quiescent morphology of the dual negative cells.

Antibody titers and ASCs increase locally during chronic inflammation.

Antibodies secreted by effector stage B cells, antibody-secreting cells (ASC), are critical to host defense but also to autoimmunity and allergy. Having found that B cells accumulate in the chronically inflamed skin, we wondered whether an increase in localized antibody titers was a functional consequence. To address this, we compared total Ig levels in the plasma of blood and lymph and found that while blood titers were always highest, antibody titers in the granuloma draining lymph were significantly higher

than those of the uninflamed skin draining lymph (Fig. 6A, *p=0.016*). To confirm that the increased titers correlated with antibody production in the tissue, we enumerated ASCs by ELISPOT assays and found that ASCs also accumulated (~400-fold on average) in the chronically inflamed skin relative to control skin of the same animals (Fig. 6B-C). Antibody titers in the draining lymph did not increase to the same extent as the ASCs in the tissue. This is not unexpected as the cannulated lymphatics drain a much larger site than just the granuloma region, causing the antibody titers to be a diluted average of the entire drainage site.

Even though chronic inflammation is generally not associated with increased vascular leakage, we excluded that the increased antibody titers were not simply a result of increased blood vascular permeability. We analyzed Ig levels in skin-draining lymph plasma during acute CFA-induced inflammation (<24h), which is characterized by drastic blood vascular leakage and edema, and found that antibody titers did not increase (data not shown). This verifies that poor vessel integrity was not responsible for the increased Ig titers in the granuloma lymph. Based on these data we conclude that B cell accumulation in the chronically inflamed skin leads to increased localized antibody production and titers that are potentially important in the defense against skin pathogens but that could also be harmful during autoimmunity.

CD21 and L-selectin expression on skin-associated B cells

Expression of CD21 and L-selectin marks a pool of B cells that preferentially recirculates between blood and lymph nodes, leading to their enrichment in efferent lymph (184). In contrast, the lack of CD21 and L-selectin is associated with preferential migration to the spleen, a low ability to leave the blood, and an ensuing enrichment in the blood (184). We compared B cells in the afferent lymph draining the skin to these B cell pools and

found that the majority of B cells in afferent lymph were positive for CD21 (55.72 ± 6.94%) and L-selectin (70.64 ± 6.67%), as was a smaller population B cells in the blood (CD21, 37.54 ± 5.31; L-selectin, 54.38 ± 8.92) (Fig. 7). In contrast, only a small population of B cells isolated from the skin expressed either CD21 (16.5 ± 3.17%) or L-selectin (13.35 ± 3.34%) (Fig. 7). Thus CD21 and L-selectin expression does not clearly delineate skin B cells into known recirculating and non-recirculating B cell pools.

Skin B cells utilize a unique repertoire of trafficking receptors

We next examined adhesion molecule expression that denotes skin homing versus gut homing lymphocytes. As previously shown and specific to skin-tropic T cells (22), CD4 T cells in skin-draining lymph expressed high levels of both alpha-4 and beta-1 integrins as well as low levels of beta-7 integrin (Fig. 8A, top row). As expected (22), CD4 T cells in the blood contained a population of alpha-4 and beta-7 high expressing cells, consistent with the presence of gut homing T cells (Fig. 8A). Unexpectedly, lymph-borne B cells showed equal or lower expression of alpha-4 and beta-1 but higher levels of beta-7 integrin relative to co-isolated skin-draining CD4 T cells (Fig. 8A). CD4 T cells from the blood and skin lymph had distinct integrin expression patterns, which are consistent with known phenotypes of skin ($\alpha_4\beta_1^{hi}$, $\alpha_4\beta_7^{lo}$) versus gut ($\alpha_4\beta_1^{lo}$, $\alpha_4\beta_7^{hi}$) homing. However, B cells from blood and lymph had near identical expression patterns of the integrins examined (Fig. 8A). To address whether the integrin expression pattern for B cells is homogenous throughout the body, we examined B cells traveling in the mesenteric efferent lymph (Fig. 8B). We found that mesenteric lymph B cells, but not blood or skin lymph B cells, were uniformly high in the expression of beta-7 integrin (Fig. 8A-B), which, when paired with alpha-4, is required for gut homing. Thus, B cells at different anatomic sites exhibit distinct integrin expression patterns such as known gut versus skin homing

phenotypes. Furthermore, whereas approximately half (57.1 ± 9.5) of the skin-draining CD4 T cells expressed E-selectin ligand, B cell expression of this skin homing molecule was enriched compared with blood B cells but significantly lower relative to skin T cells (14.8 ± 4.9, *P*=0.02, paired t test, Fig. 8C).

The chemokine receptor requirements for entry into the skin have been well studied for some leukocyte subsets. For T cells CCR4, CCR8, and CCR10 are important in mediating entry into skin in mice and humans (20, 30). Data implies that CCR6 mediates the migration of Langerhans cell precursors into skin (158, 195). We therefore tested if skin-associated B cells migrate in response to ligands for these skin-associated chemokine receptors in an in vitro chemotaxis assay. In contrast to co-isolated CD4 T cells, B cells traveling in skin draining lymph were not responsive to CCL17, CCL1, and CCL28, ligands for CCR4, CCR8, and CCR10, respectively (Fig. 8D). Surprisingly, skin B cells migrated well in response to the CCR6 ligand CCL20. Taken together, the data suggest that B cells use CCR6 or alternative chemoattractant receptors for their recirculation through skin.

Discussion

B cells are capable of many effector functions beyond antibody production; however, their role in most extralymphoid tissues has yet to be defined. Recent studies show that B cells accumulate in the inflamed skin in a variety of diseases, including cancer, autoimmunity and infection (180, 196, 197). To shed light on the role of skin-associated B cells, we examined the migration and phenotype of cutaneous B cells.

It is often assumed that B cells do not reside in or recirculate through the skin in the absence of inflammation (178). In contrast to that assumption, we found that B cells

are present in both uninflamed skin-draining afferent lymph and the uninflamed flank skin itself (Fig 1A); thus B cells continuously traffic through the skin and are a steady-state population of this organ. Studies by others showed that B cells travel in the afferent lymph draining from uninflamed human skin (177), suggesting that B cells are characteristic of mammalian skin.

Our study further revealed that, during chronic inflammation, skin-draining B cells exhibit the most consistent and greatest relative increase of all lymphocyte subsets studied (CD4, CD8, and γδ T cells, and B cells) (Fig. 2). Importantly, B cells dramatically accumulated in the inflamed skin to constitute nearly half of all lymphocytes in the granuloma (Fig. 3). Large numbers of B cells are often found in *M. tuberculosis* granulomas of the lung, in which they play a protective role by aiding in the recruitment of other immune cells (198). B cells may fulfill a similar role in chronic skin inflammation. We found increased expression of antigen-presenting and costimulatory molecules on skin-associated B cells relative to lymph node B cells (Fig. 4). Consequently, B cells in afferent lymph may migrate into lymph nodes to activate naïve T cells, whereas B cells in the granuloma may be capable of stimulating effector/memory T cells at the site of inflammation, thereby boosting the inflammatory response. In that regard B cell antigen presentation could be of critical importance because B cells are able to present their cognate antigen efficiently at very low concentrations relative to other APCs (199).

B cells are key players in many autoimmune diseases, even in those that were primarily considered T cell-mediated, such as multiple sclerosis (180, 200). Furthermore, B cell depletion in humans has revealed a role for B cells in cutaneous disorders, including bullous skin diseases (180) and atopic eczema, a condition not typically associated with autoantibody (201). Moreover, in some systemic autoimmune disorders

with cutaneous manifestations, such as systemic lupus erythematosus, the clinical improvement following B cell depletion does not always correlate with a decline in autoantibody titers, suggesting that B cell functions other than antibody production, i.e. antigen presentation and cytokine production, are critical in cutaneous immune responses (180).

B-1 cells are typically located within the peritoneal and pleural cavities, sites of constant microbial exposure. By producing natural antibody and rapidly mounting T-independent immune responses that result in the production of broadly specific, cross-reactive IgM, B-1 cells are important early in the immune response against pathogens (81). Our analysis revealed that a subset of skin and skin-draining lymph B cells is IgM^{hi} $CD11b^{hi}$ (Fig. 5), and thus resembles B-1-like cells that were previously described in sheep blood (127). 90% of efferent lymph lymphocytes are blood-derived and only 10% originate from the afferent lymph (8, 11). Therefore, the appearance of IgM^{hi} $CD11b^{+}$ B-1-like B cells in skin, skin draining lymph and blood in combination with their relative absence in lymph nodes and efferent lymph (Fig. 5) suggests that a population of B-1-like B cells migrates between blood and skin and egresses via the afferent lymph and that only few B-1-like B cells recirculate between blood and lymph nodes.

We propose that B-1 like cells recirculate through skin as a first line of defense against pathogens that invade via the epidermis. In line with the notion of a skin surveying B-1 like B cell, B-1 cells were shown to recirculate between the body cavities and blood (121) and to migrate into the lung and draining lymph nodes during pulmonary infection (92, 202). Additionally, B-1 cells migrate from the peritoneal cavity to the skin-draining lymph nodes during the course of cutaneous contact hypersensitivity (105). Our study extends these findings by showing that B-1-like cells recirculate through the skin

itself, thus, are well positioned to participate in protective as well as harmful cutaneous immune responses.

Antibodies secreted by differentiated B cells, including B-1 cells, are key effector molecules in the defense against invading pathogens; however, they can also be pathogenic in autoimmunity and allergy. Total Ig titers in skin draining lymph were well below that of the blood, suggesting that antibody does not fully penetrate and/or saturate the skin. This finding could be of great consequence to the many disease treatments that utilize depleting antibody if intravenous administration does not effectively reach the skin. We found that B cell and ASC accumulation in the skin during chronic inflammation leads to increased localized Ig titer (Fig. 6A), either directly or as a combination of increased ASCs and of higher antibody production on a per cell basis. This localized antibody production in the skin likely represents a mechanism by which the body clears or contains an ongoing skin infection and ensures protection against reinfection. While an accumulation of pathogen-specific ASCs and B cells would be beneficial, the recruitment of autoreactive B cells to the site of inflammation with subsequent localized antibody production has the potential to exacerbate inflammation in autoimmune diseases that affect the skin such as cutaneous lupus erythematosus or pemphigus.

Young and colleagues previously found that efferent lymph B cells segregate into recirculating and non-recirculating cell pools. The lymph node recirculating pool was marked by expression of CD21 and L-selectin. Conversely, CD21 and L-selectin double-negative B cells were excluded from lymph node recirculation and preferentially migrated into the spleen (184). Consistent with the concept that leukocytes in the afferent lymph are migrating to the lymph node, we find that slightly fewer afferent lymph B cells co-stain for both of these markers (Fig. 7) than what was shown for efferent lymph B cells

(184). It is conceivable that the CD21$^-$ L-selectin$^-$ afferent lymph B cells do not migrate from the blood to lymph nodes via high endothelial venules, and instead enter the lymph node by way of (skin) tissue and afferent lymph. Surprisingly, a lower percentage of B cells isolated from the uninflamed skin, but not the granuloma, were positive for both CD21 and L-selectin. The difference in CD21 and L-selectin expression between B cells residing in skin and B cells exiting the skin suggests that they represent two different populations: one that is sessile in and one that recirculates through skin. Alternatively, these markers could be upregulated once a B cell exits from skin. This difference is only observed in uninflamed skin, implying that the lack of CD21 and L-selectin denotes a skin resident population that is diluted as B cells traffic to and accumulate in the chronically inflamed skin.

While the mechanisms by which T cells migrate into skin are well characterized and critical to cutaneous inflammation and immunosurveillance (29), little is known about B cell migration into skin. Here, we observed that B cells in the skin draining lymph expressed high levels of alpha-4 and beta-1 integrins and a smaller subset bound E-selectin (Fig. 8). Thus, skin B cells are well equipped with adhesion molecules typically associated with T cell homing to the skin. Importantly, CD4 T cells draining the skin were responsive to chemokine ligands for CCR4, CCR10, and CCR8 (Fig. 8D), chemokine receptors key to the recruitment of T cells into skin in mice and humans (203). Ligands for CCR4, CCR10, and CCR8 were also able to attract skin draining CD4 T cells in the sheep, implying that the mechanism of skin homing via these receptors is evolutionary conserved. However, co-isolated ovine skin B cells were unresponsive to ligands for these receptors (Fig. 8), suggesting that B cells utilize alternative chemokine receptors to migrate into the skin.

The CCR6 ligand CCL20 is constitutively expressed in the epidermis and by dermal endothelial cells and presumably mediates the recruitment of Langerhans cell precursors into skin (158). While most blood-borne B cells express surface CCR6, they lack responsiveness to CCL20 but acquire it upon B cell receptor stimulation (204). Our study shows that skin-draining B cells are spontaneously responsive to CCL20; thus, the CCR6-CCL20 axis is a likely candidate for mediating B cell localization to skin. Rituximab, an antibody to human CD20, depletes circulating B cells to effectively treat B cell lymphomas and autoimmune disorders, including skin diseases (180). However, the efficacy of B cell depletion in many extralymphoid sites, including the skin, remains unknown. As an alternative approach, targeting specific trafficking molecules, such as CCR6, to restrict B cell entry into effector sites could reduce disease pathology while not affecting general B cell function.

In conclusion, our data show that innate-like and conventional B cells with the potential to activate T cells or produce antibody reside in and/or continuously recirculate through healthy and inflamed skin. Thus, our study reveals a so far unappreciated role of skin B cells as potential regulators of cutaneous immunity and inflammation.

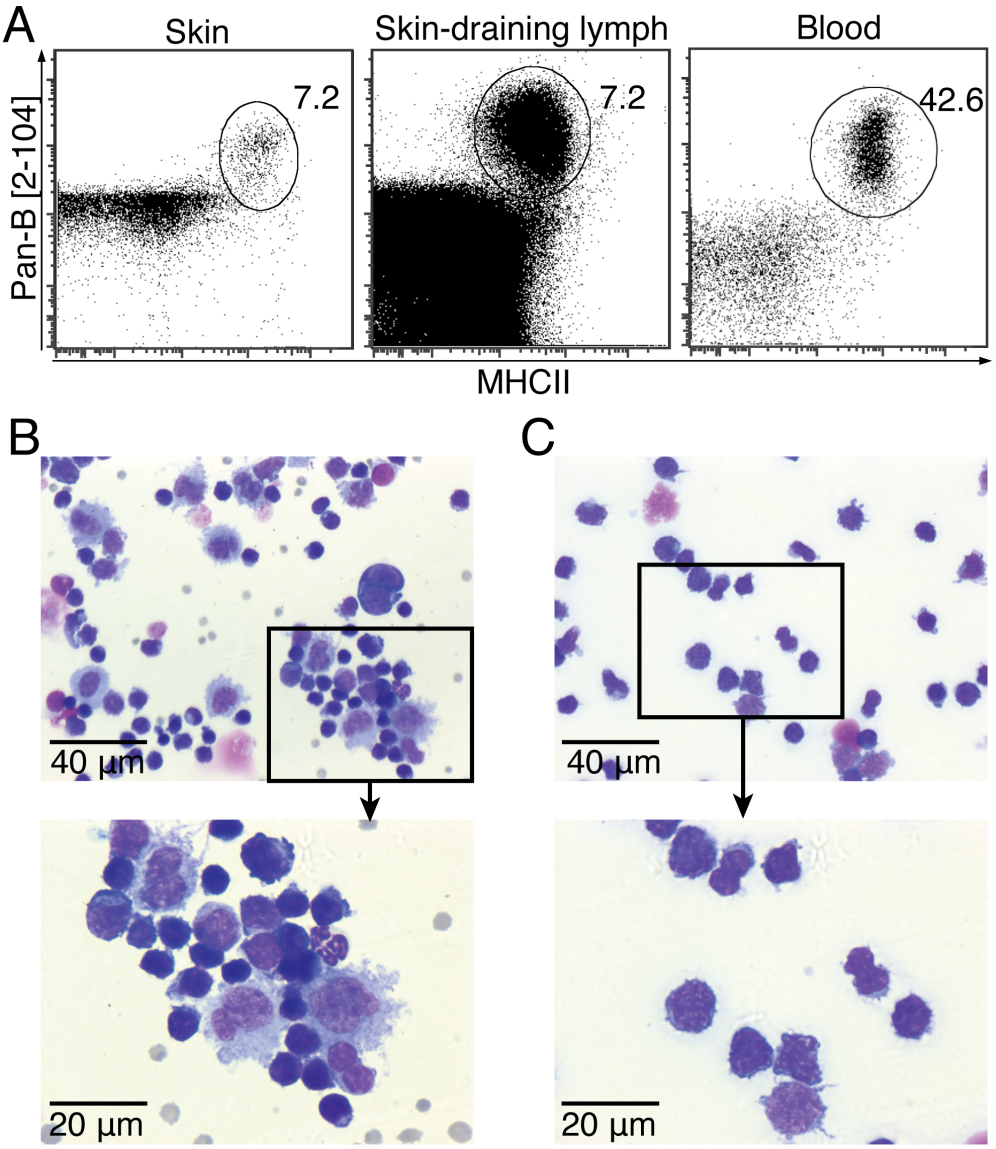


Figure 1. B cells reside in and recirculate through uninflamed skin. Lymphocytes from uninflamed skin, uninflamed skin-draining afferent lymph and peripheral blood from adult sheep were analyzed. (**A**) Flow cytometric analysis of the expression of pan-B-cell marker recognized by clone 2-104 and MHC class II on gated lymphocytes from uninflamed skin, uninflamed skin-draining afferent lymph, and blood. One representative animal of at least 5 (lymph and blood) or 3 (skin) individually analyzed animals is shown. (**B-C**) Pappenheim-stained cytospins of either unfractionated (**B**) or MACS-positively enriched for pan B cell marker 2-104 (**C**) skin-draining lymph.

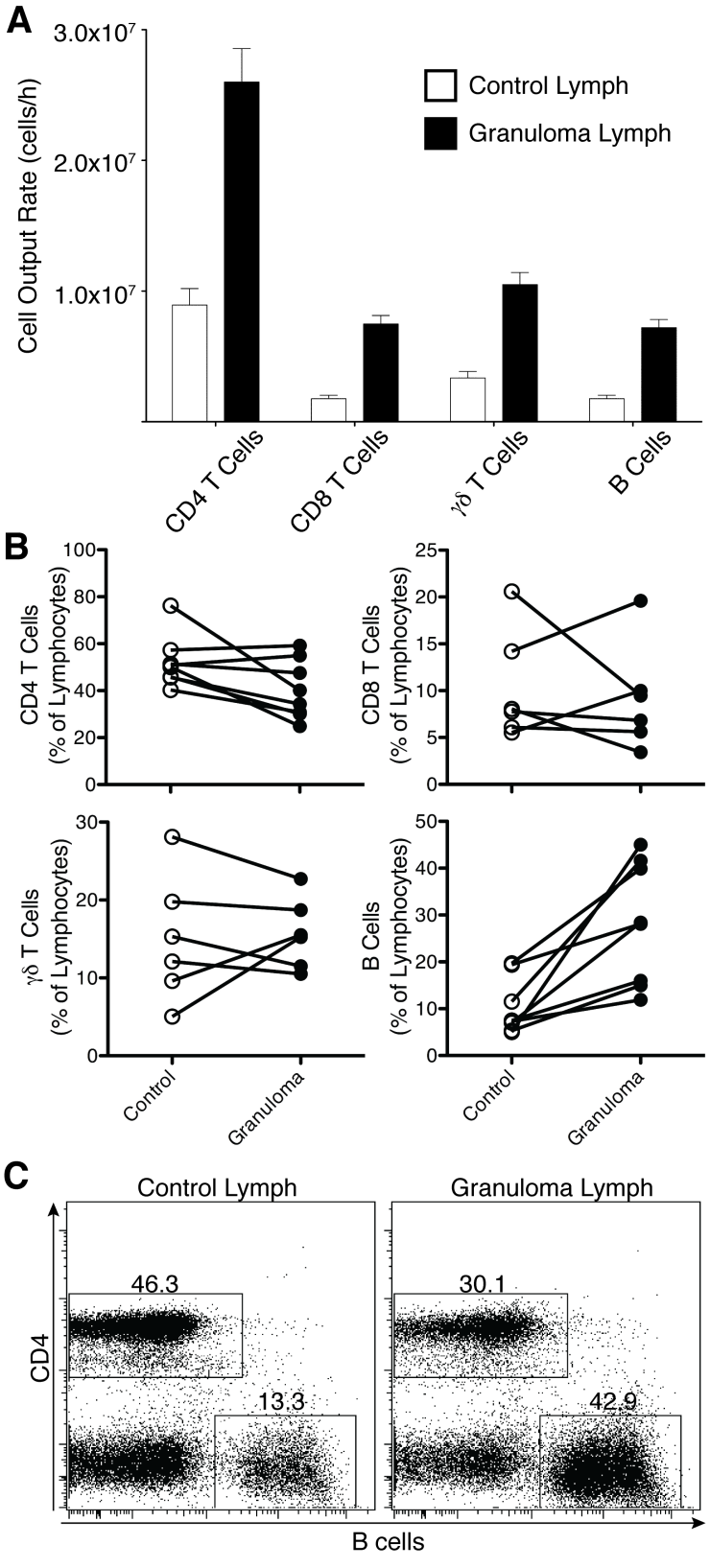

A
3.0x10^7
2.0x10^7
1.0x10^7
Cell Output Rate (cells/h)
Control Lymph
Granuloma Lymph
CD4 T Cells
CD8 T Cells
γδ T Cells
B Cells
B
CD4 T Cells (% of Lymphocytes)
CD8 T Cells (% of Lymphocytes)
γδ T Cells (% of Lymphocytes)
B Cells (% of Lymphocytes)
Control
Granuloma
C
Control Lymph
Granuloma Lymph
46.3
13.3
30.1
42.9
CD4
B cells

Figure 2. B cell traffic increases in chronic skin inflammation. Chronic cutaneous inflammation was induced by subcutaneous injection of CFA into sheep flanks. Ovine lymph was collected after catheterization of draining afferent lymphatics of uninflamed (control) or chronically inflamed (3-5 weeks after induction of inflammation) skin. (**A**) Numbers of cells collected from skin draining afferent lymphatics over time (cell output) were determined for CD4, CD8, and γδ T cells, and B cells. Data points show the mean ± SEM of multiple time points analyzed for cell output from control (uninflamed) and granulomatous skin. One representative animal out of at least 4 individually analyzed animals is shown. (**B**) The percentage of lymph-borne CD4, CD8, and γδ T cells, and B cells draining control and granulomatous skin showing all individually analyzed animals. Connected lines indicate individual animals (**C**) Flow cytometric analysis of CD4 and B cells on gated lymphocytes. Numbers indicate the percentage of positive cells in the specified gates. One example staining of at least 8 individually analyzed sheep is shown.

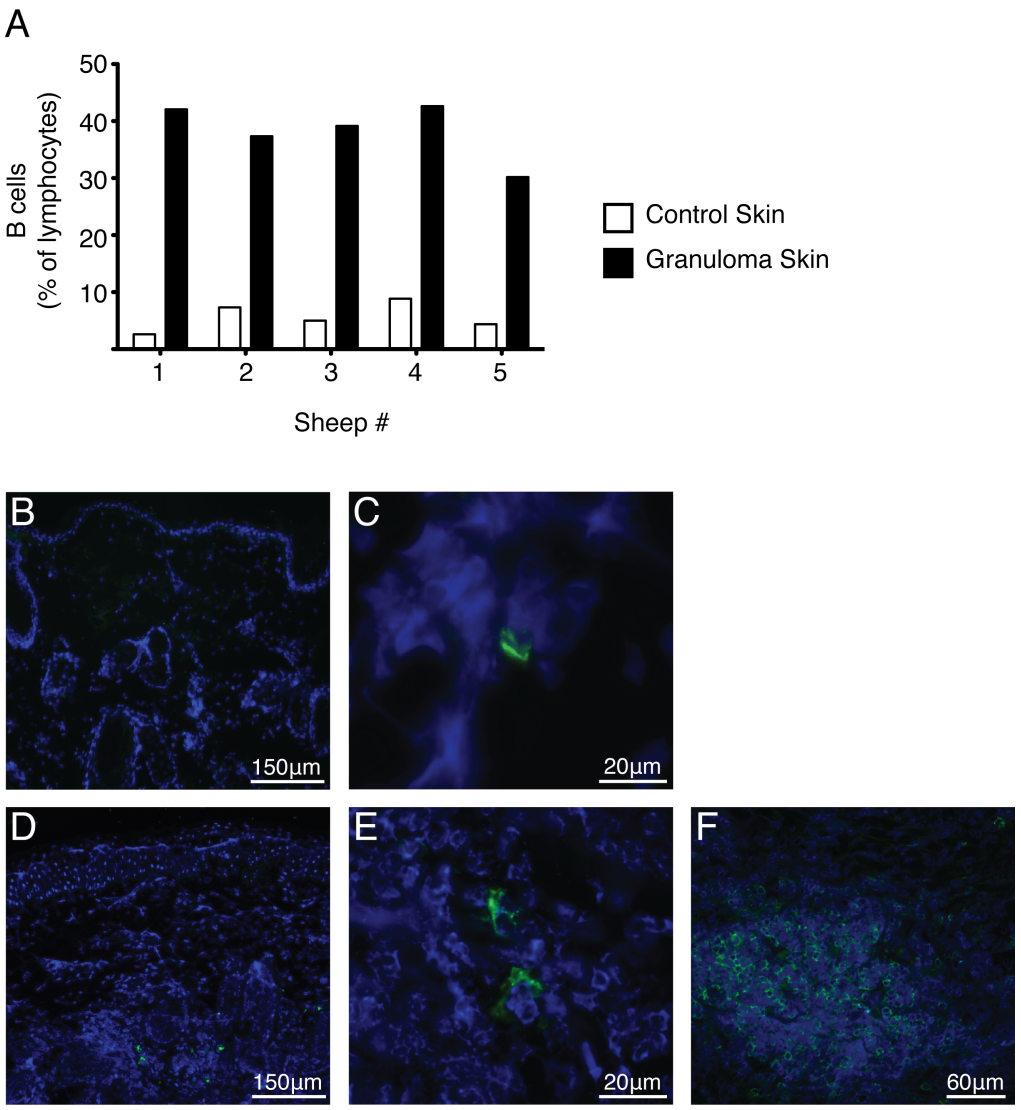


Figure 3. B cells accumulate in chronically inflamed skin. Healthy control skin and CFA-induced skin granulomas were analyzed 3-5 weeks post induction of inflammation. (**A**) The percentage of (pan-B cell marker) 2-104-reactive B cells among total lymphocytes isolated from uninflamed and granulomatous skin was determined by flow cytometry for all animals (N=5). (**B** – **F**) Immunofluorescence staining of 6 µm thick frozen skin sections. One representative staining of IgM^+ (FITC, green) cells and DAPI (blue) in uninflamed (**B** and **C**) or granulomatous (**D-F**) skin of a minimum of 4 animals analyzed each.

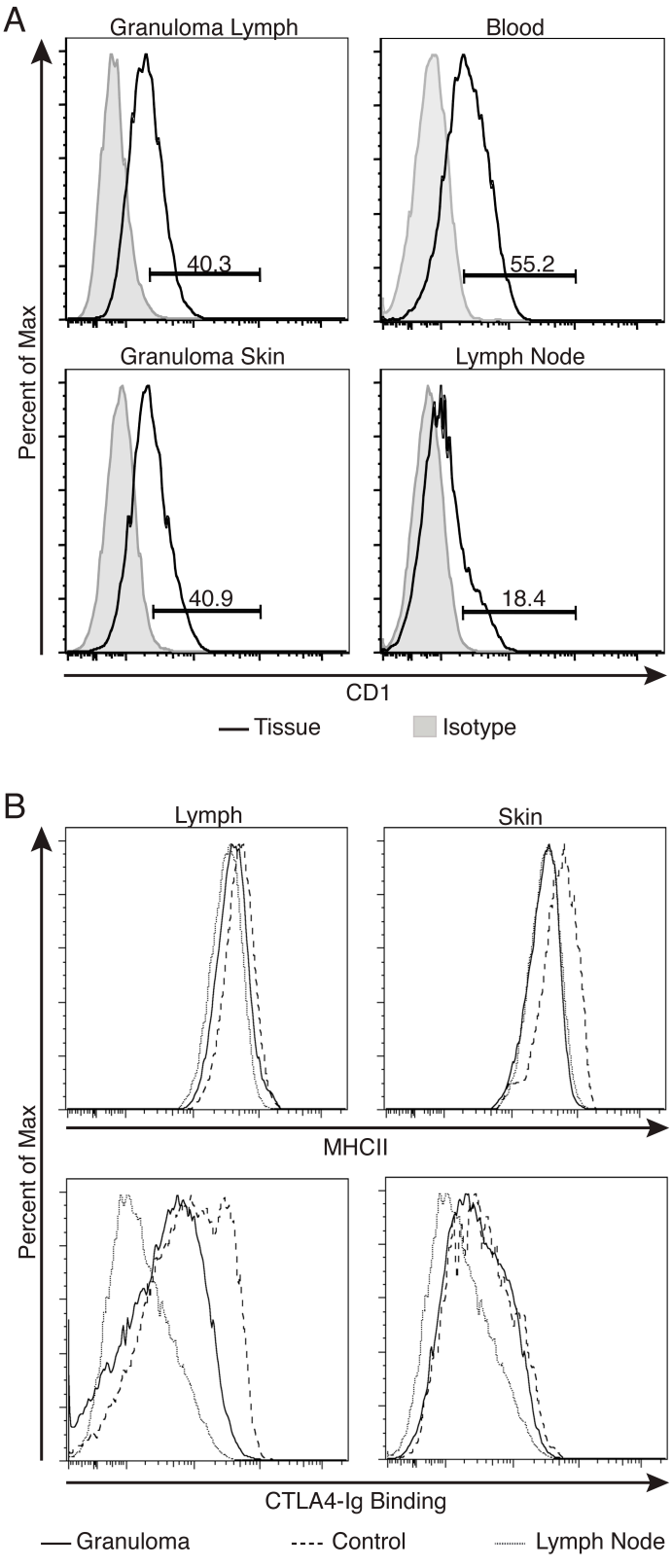

A
Granuloma Lymph
Blood
40.3
55.2
Granuloma Skin
Lymph Node
40.9
18.4
Percent of Max
CD1
Tissue
Isotype
B
Lymph
Skin
MHCII
Percent of Max
CTLA4-Ig Binding
Granuloma
Control
Lymph Node

Figure 4. Skin B cells are well-suited for antigen presentation. Lymphocytes from skin granuloma and control skin, skin-draining afferent lymph and peripheral blood were isolated from sheep (**A**) Flow cytometric analysis of CD1 and isotype control staining on gated B cells (pan-B-cell marker 2-104^{+}, MHCII^{+} lymphocytes) from skin granuloma-draining afferent lymph and blood. Numbers indicate percent positive B cells. (**B**) Flow cytometric analysis of MCHII expression and CTLA4-Ig binding (B7.1/B7.2 expression) on B cells isolated from lymph node and skin granuloma, control skin, and skin-draining lymph. One representative animal of 4 (CD1), 7 (MHCII), or 6 (CTLA4-Ig) individually analyzed animals is shown.

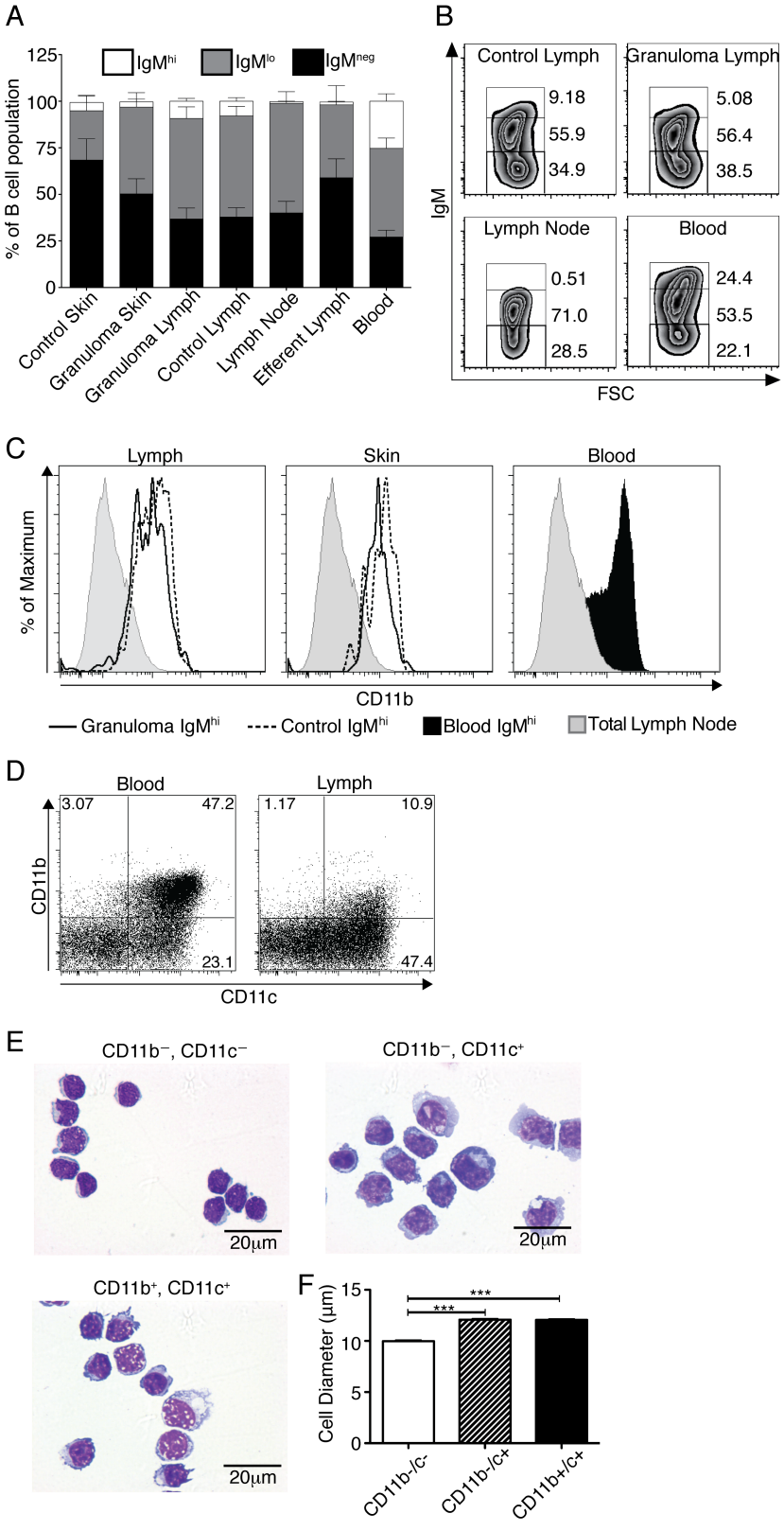

A
IgM^hi
IgM^lo
IgM^neg
% of B cell population
125
100
75
50
25
0
Control Skin
Granuloma Skin
Granuloma Lymph
Control Lymph
Lymph Node
Efferent Lymph
Blood
B
Control Lymph
Granuloma Lymph
9.18
55.9
34.9
5.08
56.4
38.5
Lymph Node
Blood
0.51
71.0
28.5
24.4
53.5
22.1
IgM
FSC
C
Lymph
Skin
Blood
% of Maximum
CD11b
Granuloma IgM^hi
Control IgM^hi
Blood IgM^hi
Total Lymph Node
D
Blood
Lymph
3.07
47.2
23.1
1.17
10.9
47.4
CD11b
CD11c
E
CD11b⁻, CD11c⁻
CD11b⁻, CD11c⁺
CD11b⁺, CD11c⁺
20μm
20μm
20μm
F
Cell Diameter (μm)
15
10
5
0
***
***
CD11b-/c-
CD11b-/c+
CD11b+/c+

Figure 5. Larger B cells and B1-like B cells recirculate through the skin. Lymphocytes from skin- draining afferent lymph, control skin-draining lymph node, blood, and skin were isolated from sheep. (**A**) The relative distribution of IgM^{hi}, IgM^{lo}, and IgM^{neg} among B cells (pan-B-cell marker $2\text{-}104^{+}$, $MHCII^{+}$ lymphocytes) from different tissues based on flow cytometry. Data points represent the mean ± SEM of 4-8 individually analyzed animals per tissue. (**B**) One representative staining of IgM expression on B cells from control (uninflamed) skin- and skin granuloma-draining lymph, control skin lymph node, and blood is shown. (**C**) Flow cytometric analysis of CD11b expression on skin, lymph, and blood IgM^{hi} B cells compared to total lymph node B cells (pan-B-cell marker $2\text{-}104^{+}$, $MHCII^{+}$ lymphocytes). One representative animal of at least 3 (control skin) or 4 (all other tissues) individually analyzed animals is shown. (**D**) Flow cytometric analysis of CD11b and CD11c co-expression on blood and afferent lymph B cells. (**E)** B cells (live, lymphocytes, $MHCII^{+}$, pan B cell $marker^{+}$) were FACS-sorted according to specified CD11b and CD11c expression and cytospun and Pappenheim-stained for cytological evaluation. **(F)** Quantification of cellular diameters from **E.** One representative of a minimum of 3 individually analyzed lymph samples is shown (D-F).

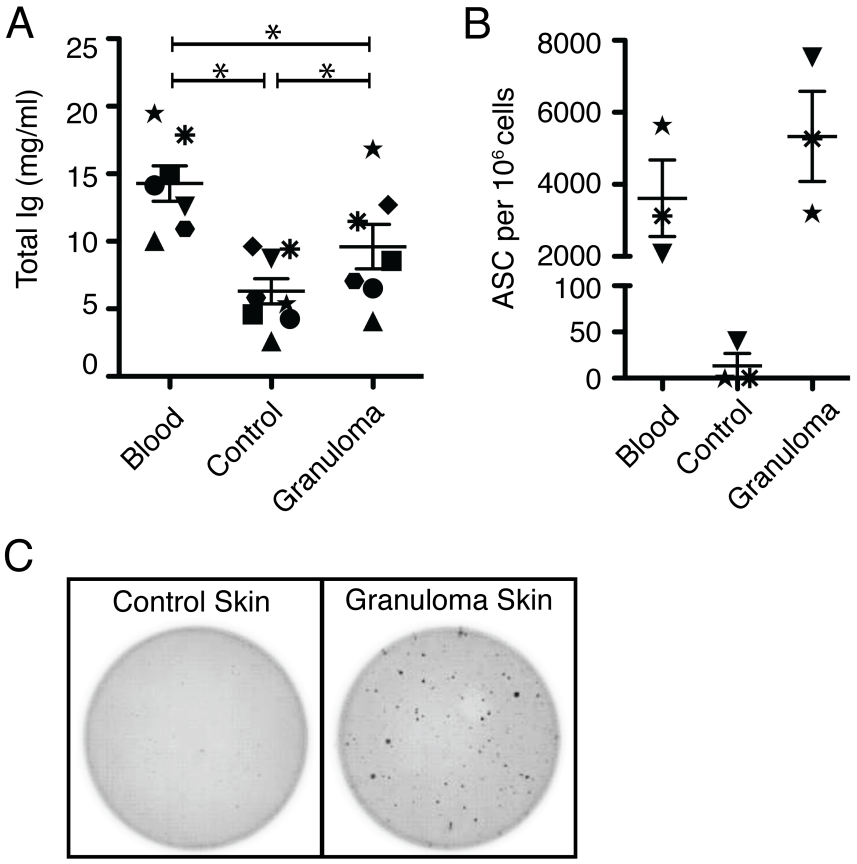


Figure 6. Antibody titers and ASCs increase locally during chronic skin inflammation. (A) ELISA of total Ig antibody titers in plasma from blood and afferent lymph draining control skin or chronically inflamed skin (granuloma). **(B)** ELISPOT assay analyzing total Ig secreting cells in blood, uninflamed control, or granulomatous skin of the same animal. (**A** and **B**) Individual animals are identified by unique symbols; bars represent the mean ± SEM. **(C)** Developed ELISPOT wells analyzing $2x10^5$ and $1x10^4$ cells/well from uninflamed control and granulomatous skin, respectively. One representative animal of 3 is shown. *, $p<0.05$.

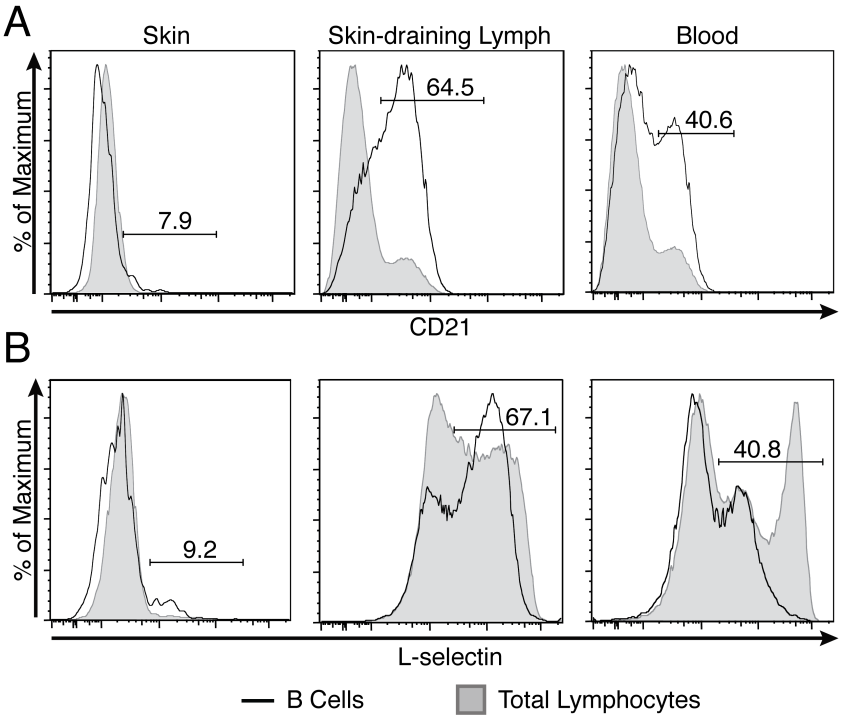


Figure 7. CD21 and L-selectin expression on skin-associated B cells. Flow cytometric analysis of CD21 (top row) or L-selectin (bottom row) on gated B cells (solid line) and total lymphocytes (shaded). Numbers indicate the percentage of positive B cells in the specified gates. One representative animal of at least 5 individually analyzed animals is shown.

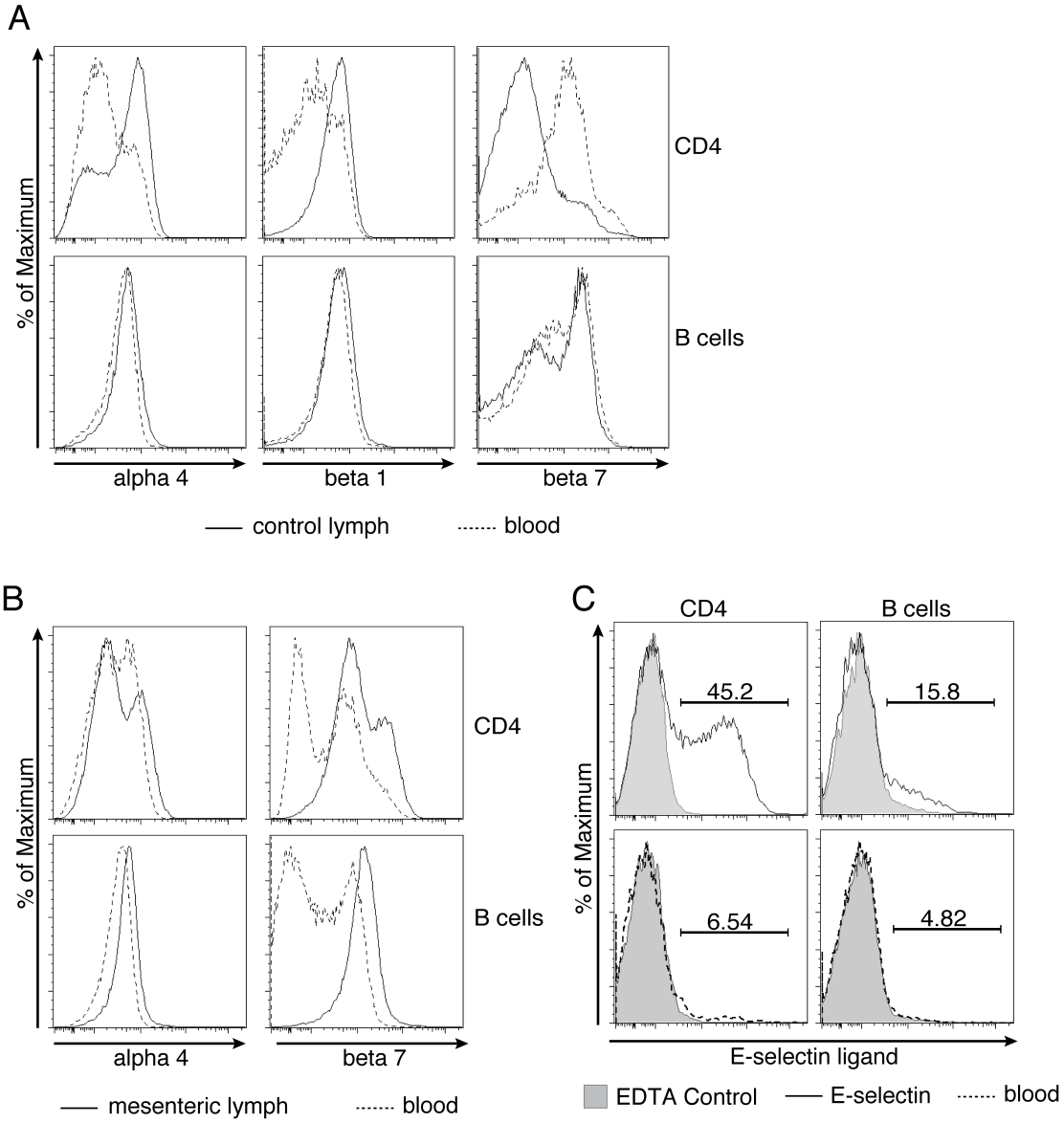

A
% of Maximum
CD4
B cells
alpha 4
beta 1
beta 7
control lymph
blood
B
% of Maximum
CD4
B cells
alpha 4
beta 7
mesenteric lymph
blood
C
CD4
B cells
45.2
15.8
6.54
4.82
% of Maximum
E-selectin ligand
EDTA Control
E-selectin
blood
D
B Cells
CD4 T Cells
Migration (% of input)
40
30
20
10
0
1
10
100 300
Chemokine Concentration (nM)
hCCL1
hCCL28
mCCL17
mCCL20

Figure 8. Homing receptor expression and chemotactic responsiveness of skin B cells. (**A**) Flow cytometric analysis of alpha-4, beta-1, and beta-7 integrins on gated CD4 T cells and B cells from afferent lymph draining uninflamed skin (solid line) or blood (dashed line). One representative animal of at least 6 individually analyzed animals is shown. (**B**) Flow cytometric analysis of alpha-4 and beta-7 integrins on gated CD4 T cells and B cells from mesenteric efferent lymph (solid line) or blood (dashed line). One representative animal of at least 4 individually analyzed animals is shown. (**C**) Flow cytometric analysis of E-selectin ligand expression on gated CD4 T cells and B cells from afferent lymph draining uninflamed skin or blood. Shaded line represents control staining in EDTA. One representative animal of at least 3 individually analyzed animals is shown.

Chapter 4: Skin B-1 B cells can secrete IL-10 and require α4 integrin for cutaneous localization

Abstract

B1 cells are innate-like lymphocytes that provide important effector functions during infection and inflammation by rapidly secreting pro- and anti-inflammatory cytokines and efficiently mounting T cell-independent (TI) antibody responses. Despite their importance, however, the migratory paths, target organs, and trafficking molecules involved in B1 cell migration are poorly-defined. Our previous studies have demonstrated the presence of B-1-like cells in the skin and skin draining lymph of sheep. Here, we extend our studies to mice and humans to reveal that innate-like B cells are a so far uncharacterized part of the normal cutaneous immune system. In addition, we find that in mice, B1 cells, unlike conventional follicular B2 cells, are efficiently deployed into the inflamed skin through differential expression of the trafficking molecule α4β1 integrin (VLA-4). Furthermore, innate B cells are a contributing source of IL-10 in both IL-10-reporter mice and normal human skin. In summary, the data supports a model in which B1 cells, through the constitutive expression of inflammation-seeking homing molecules, are poised to migrate into barrier sites, including the skin, where they rapidly provide requisite effector functions, such as production of IL-10, and fulfill a so far unappreciated role in skin immunity.

Introduction

B-1 B cells are an innate B cell subset critical in homeostasis, as well as during infection and inflammation. B-1 B cells are responsible for the vast majority of circulating IgM (100), and this natural IgM helps maintain tissue homeostasis and control inflammation (205). B-1 B cells are also capable of rapidly secreting IgM in response to inflammation and viral and bacterial infections (91 Itakura, 2005 #205, 104). In addition to antibody production, B-1 B cell effector functions include the production of cytokines and antimicrobial factors, thereby bridging the gap between innate and adaptive immune responses. While the antibody-dependent functions of B-1 B cells are relatively well established, their antibody-independent activities are only beginning to be explored. For example, in a recent study describing "innate response activator B cells," a subset of GM-CSF-producing innate-like B-1 B cells is essential for neutrophil activation, bacterial clearance, and host survival during sepsis (117). Another study reveals the phagocytic and microbicidal potential of mouse peritoneal B-1 B cells. These cells are capable of phagocytosing and killing bacteria with subsequent antigen presentation to CD4 T cells (112). IL-10 production by B-1 cells has also been described in a variety models ((116, 206); reviewed in (207)), and enables B-1 B cells to play a key role in regulating immune responses.

B-1 B cells primarily reside within the peritoneal and pleural cavities (81, 89), but recent work demonstrates that this is not a sessile population. B-1 B cells recirculate through the peritoneal cavity utilizing CXCR5, CCR7, and S1P1 (121-123) with peritoneal B-1 B cells preferentially migrating to the peritoneum compared to B-2 B cells (121). B-1 B cells can rapidly exit the peritoneal cavity and migrate to distant tissues, such as the spleen (98), intestine (118), influenza infected lung (92), and skin-draining

lymph nodes in contact hypersensitivity (105). Inflammation-induced exit from the peritoneal cavity coincides with the down regulation of alpha-4 and beta-1 integrins, which are proposed to retain peritoneal B-1s within the peritoneum (118). Although chemokine requirements for B-1 cell entry and exit from the peritoneum have been described, other trafficking molecules used in peritoneal recirculation, as well as the molecules used in migration to most other tissues, remain unknown.

We recently identified a population of B-1-like B cells in the skin and skin draining lymph of sheep (2). As B-1 B cells have a myriad of functions and have been found in other barrier sites in the mouse (92, 118), we followed up our ovine study by investigating this subset in mouse skin. We have found that B cells, both B-1 and conventional follicular B-2 B cells, are a so far uncharacterized part of the normal cutaneous immune system. In addition, we find that B-1 B cells, unlike B-2 B cells, efficiently home into the inflamed skin, where they are capable of secreting IL-10. Consistent with this migration preference, B-1 cells and follicular B cells exhibit differential expression of the trafficking molecule α4β1 integrin (VLA-4), which mediates B-1 cell entry into the inflamed skin. In summary, the data support a model in which B-1 B cells are poised to migrate into barrier sites, including the skin, where they can provide rapid effector functions, such as IL-10 production, and fulfill a so far unappreciated role in skin immunity.

Materials and methods

Animals and induction of cutaneous inflammation

Sex- and age-matched CD45.1 or CD45.2 wild-type C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) or Vert-x IL-10 reporter mice (116), provided by Christopher Hunter (University of Pennsylvania, Philadelphia, PA) were used for all mouse experiments. All mice were between 8-16 wk old. To induce chronically inflamed skin, 50-100 µL Complete Freund's Adjuvant (CFA) emulsified 1:1 with sterile saline was injected subcutaneously into the flank skin of mice.

Human tissues

Purified human peripheral blood mononuclear cells (PBMCs) were obtained from the Human Immunology Core at the University of Pennsylvania under an Institutional Review Board–approved protocol. Declaration of Helsinki protocols were followed and all donors gave written, informed consent. Fresh normal, healthy human skin was provided by the University of Pennsylvania's Skin Disease Research Center (SDRC) under its Institutional Review Board-approved protocol. Human skin samples were obtained from skin surgery procedures and would have otherwise been discarded. Before use, all samples were de-identified.

Cell isolation

Leukocytes were isolated from shaved human or mouse skin samples by mechanical disruption followed by 2- 30 minute enzymatic digestion steps using 0.1mg/mL DNase I (Roche) and 0.13U/mL Liberase TM (mouse skin, Roche) or 0.26U/ml (human skin, Roche) in HBSS at 37°C. Between digestion steps, released cells were collected and

washed in assay media (RPMI 1640 medium [Invitrogen] with 5% newborn calf serum (NCS, [Hyclone Laboratories]). After the second digest, remaining partially-digested skin was mashed through a 100 μM cell strainer (BD Bioscience), washed with assay media, and passed through a final 70μM cell strainer (BD Bioscience). Leukocytes were isolated from the small intestine, as described (208). Peritoneal cavity cells (PerCs) were isolated by washing the peritoneal cavity with 5-10mL PBS (Invitrogen). Cells were released from lymph nodes and spleens by passage through 70μM cell strainer. PMBCs were isolated from mouse blood by gradient centrifugation with Histopaque-1083 (Sigma-Aldrich) as per manufacturer's instructions.

Cell stimulation and flow cytometry

To reduce non-specific surface staining, mouse cells were preincubated with rat IgG (Jackson ImmunoResearch) and mAb to CD16/ CD32 (2.4G2; UCSF Monoclonal Antibody Core); human cells were preincubated with rat IgG (Jackson ImmunoResearch Laboratories), and human FcR Binding Inhibitor (eBioscience). After blocking, the mouse cells were labeled with the following biotinylated or fluorochrome- conjugated (FITC, Pacific Blue/eFluor450, PE, Alexa Fluor 647, allophycocyanin, APC-AF750, PerCp-eFluor710, PE-cyanin 7) anti-mouse monoclonal Abs from eBioscience: CD45.1 (A20), CD45.1 (104), CD45 (30-F11), CD19 (1D3), CD43 (S7), B220 (RA3-6B2), alpha-4 integrin (R1-2), beta-1 (HMB1-1), CD4 (RM4-5), CD11b (M1/70), CD5 (53-7.3). High affinity beta-1 (9EG7, BD Biosciences) was conjugated using the Alexa Fluor ® 647 Antibody Labeling Kit (Molecular Probes) according to manufacturer's instructions. Human cells were labeled with the anti-human monoclonal antibodies specific for CD3

(SK7), CD19 (HIB19), CD20 (2H7), CD45 (2D1) from eBioscience and CD27 (L128) and CD43 (1G10) from BD Bioscience.

To detect IL-10 producing cells by GFP expression in Vert-x IL-10 reporter mice (116), cells were polyclonally stimulated 10μg/mL LPS (Sigma), 10ng/mL PMA (Sigma), and 500ng/mL ionomycin (Sigma) for 5 h with the addition of 10 μg/mL brefeldin A (Sigma) and 2μM monensin (eBioscience) during the last 2 h. Cells were washed, stained with surface markers and analyzed without fixation. Human cells were similarly stimulated but with only PMA and ionomycin, fixed for 20 minutes in 2% paraformaldehyde, permeabilized in 0.5% saponin, and stained with anti-human IL-10 (Miltenyi Biotec, clone JES3- 9D7).

Dead cells were excluded with LIVE/DEAD® Fixable Aqua Dead Cell marker (Invitrogen) used according to manufacturer's instructions. For cell surface staining with biotinylated Abs, PE-Texas Red conjugated streptavidin (Invitrogen) was used as a secondary reagent. Staining of digested skin was performed in parallel with that of digested and undigested lymph node to verify that Ags were not cleaved during the cell-isolation process. Samples were acquired on a BD LSRII or LSRFortessa using FACSDiva software (BD Biosciences), and analyzed with FlowJo software (Tree Star, Ashland, OR). Gates were set according to appropriate isotype-control staining. Unless otherwise indicated B-1 B cells are pregated on live, $CD45^{+}$, $CD19^{+}$, $CD43^{+}$, $B220^{lo/neg}$ lymphocytes, and B-2 B cells pregated on live, $CD45^{+}$, $CD19^{+}$, $CD43^{neg}$, $B220^{+}$ lymphocytes.

Short-term radioactive homing experiments

PerCs were isolated from donor mice and enriched for B-1 B cells to ≥95% purity by negative selection with biotin microbeads after labeling with anti-CD23 and anti-F4/80 biotin (Miltenyi Biotec) followed by positive selection by CD19 microbeads (Miltenyi Biotec). Cells were labeled with ^{111}Indium, as described (209). 2.6-2.7*10^5 B-1 cells were transferred IV to each recipient animal. 15h after transfer, animals were euthanized, and the following tissues were collected: blood, PerC, skin, spleen, skin-draining LN, mesenteric LN, lung, liver, and the rest of body without the tail (injection site). The accumulation of B-1 cells within specific organ is measured as a percentage of the radioactivity recovered from all tissues from an individual mouse.

Short-term florescent homing experiments and antibody blockade

Leukocytes were isolated from PerCs and spleens of donor animals and differentially labeled with either CFSE (Molecular Probes) or Cell Proliferation Dye eFluor® 670. Labeling was achieved by incubating 10^7 cells/mL HBSS with 25 mM HEPES and 0.4 μM CFSE or 5 μM eFluor670 for 10 min at 37℃. Both the eFluor670 and CFSE labeling reactions were stopped by washing 1x in FBS, 1-2x in assay media, and 1x in PBS. 1.5-4*10^6 PerCs and splenocytes adding up to 10^7 cells (for a total of 10^7 cells) were injected IV into recipient mice. 12-15h after transfer single cell suspensions were analyzed for transferred cells (identified by fluorescent labels) and total number of cells were either counted by hemocytometer with trypan blue exclusion (skin and small intestine) or determined by flow cytometry with a fixed number of polystyrene beads (Polybead; Polysciences). In order to compare the relative migration efficiency of PerC B-1 cells to splenic B-2 cells, the ratio of homed CFSE+ to eFluor670+ cells was determined by flow cytometry and normalized to the input population. To control for the effects of the cell labeling, the dye used on each population was switched and no effect on migration

(absolute or relative to the two populations) was observed. In homing experiments testing the effect of alpha-4 integrin blockade, the mixed population of differentially labeled PerCs and splenocytes were divided in two input populations. One group was resuspended in 50 µg anti-alpha-4 (PS/2) and transferred into mice that also received 300 µg PS/2 IP, and the other was resuspended in 50 µg rIgG2$_b$ (LTF-2) and transferred into mice that also received 300 µg LTF-2 IP. PS/2 and LTF-2 were obtained from the UCSF Monoclonal Antibody Core.

Statistical analysis

All statistical analyses were calculated using GraphPad Prism software. Unless otherwise indicated, all values are reported as mean ± SEM. Analyses used include the paired, non-parametric Wilcoxon signed-rank test, the non-parametric one way anova followed by Dunns multiple comparison test, or the student's t test, and the appropriate statistical analysis is indicated in the figure legend. The *p* values < 0.05 were considered statistically significant.

Results and Conclusions

B-1 B cells are part of the cutaneous immune system.

We recently demonstrated that B-1-like B cells recirculate through the skin of sheep (2). As B-1 B cells are more fully characterized in mice and their multi-functionality enables them to be key mediators in immune responses, we followed up our ovine studies in mice. Examination of uninflamed (control) and chronically inflamed skin revealed that B cells are a component of the cutaneous immune system, constituting 11.62 ± 1.36% (control) or 17.92 ± 3.88% (inflamed) of $CD45^+$ lymphocytes (Figure 1A). This is consistent with our observations in the ovine model, although murine B cells comprise a larger proportion of lymphocytes in the uninflamed skin and a smaller proportion of lymphocytes in the same model of cutaneous inflammation. This suggests that although the findings are similar, differences in the populations and/or functions of skin B cells may exist between species. Phenotyping the cutaneous B cells identified a population of $CD19^+$, $CD43^+$, $B220^{lo/neg}$ B-1 B cells in both tissues ([23.13 ± 2.7% (control) and 12.7 ± 2.8% (inflamed) of skin B cells], Figure 1B) that significantly increases in the chronically inflamed skin ($6.0*10^3 \pm 1.4*10^3$ (control) vs $2.5*10^4 \pm 6.1*10^3$ B-1 B cells per gram skin; Figure 1D). These B-1s were a heterogeneous population with 62.62±4.4% (control) and 53.42±2.15% (inflamed) expressing CD5 and 42.0±46% (control) and 31.45±3.0% expressing CD11b (Figure 1C). Interestingly, skin B-1 B cells more closely resemble peritoneal B-1 cells in their expression of CD11b, although others have shown that B-1 B cells lose expression of CD11b after leaving the peritoneum (81, 119). CD11b can pair with CD18 to form complement receptor 3/Mac-1, and its expression on B-1 B cells has been correlated with their ability to form doublets (210). However, the functional relevance of B-1 B cell doublets and the importance of CD11b in survival or migration

outside the peritoneum have not been addressed. Having identified B and B-1 cells in the skin of mice, we investigated whether cutaneous B cells were more a more broadly conserved component of the skin immune system by examining B cells in human skin samples. B cells are much less frequent in human PBMCs (211) than in sheep or mice. Similarly, we were able to find a smaller, but consistent population of B cells in the human skin (Figure 1E and G). The existence of a B-1 B cells in currently under debate (129-132) as the described B-1 B cells are phenotypically similar to other B cell subsets. However, innate functions have been described for the human B-1 subset (129, 133, 134), and as such, we investigated whether any of the cutaneous B cells expressed markers indicative of innate B cells. Human B-1 B cells are described as $CD3^{-}$, $CD20^{+}$, $CD19^{+}$, $CD27^{+}$, $CD43^{+}$, $CD70^{-}$. The digestion process required to isolate skin lymphocytes causes cleavage of the CD70 epitope, but the other markers of this subset were used in the analysis. Using this scheme, we were able to detect a population of B-1-like B cells in human skin (Figure 1F-G). Having established B-1 and B-1-like B cells were present in the skin of mice and humans, we assessed the function potential of murine skin B-1 B cells. Using the Vert-X IL-10 reporter mice (116), we found that like peritoneal B-1 B cells (114), skin B cells are capable of secreting IL-10 upon stimulation (Figure IH-I). Furthermore, analysis of stimulated B cells isolated from human skin showed that some skin B cells are also capable of producing IL-10.

The function of B cells in skin diseases and autoimmune disorders is not well understood. Depletion of systemic B cells in humans with Rituximab has improved clinical manifestations of many autoimmune diseases, such as bullous skin diseases, systemic lupus erythematosus, and psoriasis (180). The exact mechanism of this improvement is as yet unclear. Although autoantibody indicative of many autoimmune

disorders and can play a role in disease manifestation, a decline in autoantibody titers does not always coorelate with disease remission (212, 213). Furthermore, other groups have found that after B cell depletion, there is also a decline in self-reactive CD4 T cells. As such, it is likely that B cell depletion simultaneously intervenes at many points in the disease pathway, possibly by depleting multiple subsets of B cells, including innate B cells. Intriguingly, psoriasis, a cutaneous autoimmune disorder, is characterized by low IL-10 levels, and topical IL-10 treatment improves disease (214). Furthermore, recent cases have been reported where B cell depletion either induced or worsened psoriasis (215, 216). Taken together, these studies suggest that proper regulation of B cell derived IL-10 is critical to human skin disease.

Peritoneal B-1 B cells migrate into uninflamed and inflamed skin.

Since B-1 function may be important in cutaneous disease, and the full impact of a cell in an immune response lies at the intersection of functional capacity and localization, we examined B-1 cell homing from the peritoneal cavity to the skin. Several groups have shown that after LPS or TLR4 ligand stimulation, B-1 B cells rapidly exit the peritoneal cavity (118-120), but Cyster and colleagues demonstrated that without stimulation the rate of peritoneal B-1 cell recirculation is relatively low (121). As such, we adopted the LPS-induced exit approach to produce a synchronized population of exiting B-1 B cells in order to examine their ability to enter inflamed skin. Twenty hours after transferring fluorescently labeled B-1 B cells IP and inducing their exit with LPS (Figure 2A, left), we were able to detect transferred cells in peripheral tissues (Figure 2A, right). Interestingly, the transferred B-1s represented a similar proportion of B-1 B cells in the inflamed skin and the small intestine, a site they've been demonstrated to enter (118), though whether this is due to a B-1 B cell preference to enter the skin or potentially an

inflamed site is unclear. To assess if B-1 entry into the skin was a result of either the LPS inflammatory signals that induced exit or from the cutaneous inflammation, we examined the capacity of B-1 B cells to enter uninflamed skin from the blood. Due to the low cell numbers recovered from uninflamed skin, we employed radioactively labeled B-1 B cells to analyze in vivo trafficking, as radiolabeling is more sensitive than its fluorescent counterparts (217). ^{111}Indium-labeled peritoneal B-1 B cells were allowed to migrate for 15 h, and target tissues were then analyzed by γ-counter for transferred cells (Figure 2B, left). Importantly, we were able to detect transferred cells in the uninflamed skin and other organs (Figure 2B, right). Although most of the ^{111}Indium signal was recovered in the spleen and liver [data not shown], significantly more ^{111}Indium signal was recovered in the skin than in peripheral skin-draining lymph nodes ($p \leq 0.001$), blood ($p \leq 0.001$), or even the peritoneal cavity ($p \leq 0.01$). This demonstrates that B-1 B cells are capable of entering even uninflamed skin from the blood. B-1 B cells preferentially recirculate through the peritoneum compared to B-2 B cells (121). To determine if B-1 B cells display a similar tropism for the skin, we differentially labeled splenic B-2 B cells and peritoneal B-1 cells and allowed them to home into the inflamed skin. As reported, peritoneal B-1 B cells exhibited a strong preference for migration into the peritoneal cavity over B-2 B cells (homing index range 7.3-72.0 [peritoneal B-1/splenic B-2]). Peritoneal B-1 cells also exhibited a strong skin homing preference compared to splenic B-2 B cells (homing index range 2.0-98.2 [peritoneal B-1/splenic B-2]). Despite the large range in migration preference between mice and experiments, B-1 B cells consistently demonstrated a preference to enter the peritoneum or the inflamed skin compared to uninflamed or inflamed lymph nodes or the spleen. These data clearly illustrate the peritoneal B-1 B cells are capable of exiting the peritoneal cavity and

migrating from the blood into the skin. However, whether all B-1 B cells in the skin are constantly recirculating or whether there are distinct populations of migratory versus resident cells has yet to be determined.

Integrin $\alpha4\beta1$ mediates B-1 cell migration into the skin

We next asked what trafficking receptors could mediate B-1 B cell migration into the skin. We compared expression of homing receptors expressed by peritoneal B-1 versus splenic B-2 B for insights as to what could mediate the observed strong preference for B-1 entry in the skin, and among the most differentially express receptors were the alpha-4 and beta-1 integrins (Figure 3A, left). Analysis of B-1 and B-2 populations in the skin revealed a similar enrichment of a $\alpha4,\beta1$-integrin bright within the skin B-1 B cell population (Figure 3A, right). To test if this integrin could mediate B-1 B cell migration into the skin, we performed homing experiments as before (Figure 2C) with a blocking antibody to alpha-4 integrin or a control antibody. B-1 B cell migration to the spleen, uninflamed or inflamed skin-draining lymph nodes was not significantly affected (Figure 3B). In contrast, we revealed that migration into the inflamed skin and peritoneal cavity were almost completely inhibited (p = 0.005 and p = 0.007, respectively) when blocking alpha-4 integrin. Interestingly, there was a strong, although not significant (p = 0.63), trend for reduced migration into the inflamed skin-draining lymph node, which suggests that B-1 B cells can utilize $\alpha4\beta1$ to mediate migration inflamed lymph nodes. Alternatively, the reduction of B-1 cells in the draining lymph node could be a result of decreased traffic through the inflamed skin, but given the magnitude of the number of cells recovered from lymph nodes versus skin and the relatively short time period, that conclusion is unlikely. Importantly, alpha-4 integrin blockade selectively inhibited B-1 B cell migration but not B-2 B cell migration into the

inflamed skin. This is in contrast to the integrin requirement for peritoneal cavity entry. It has been shown that B-2 B cells require this α4 integrin to enter the peritoneum (125), and has been suggested, but not shown, that B-1 B cells also require it. This dichotomy of trafficking receptor requirements illustrates a B cell subset selective mechanism for migration into an extralymphoid site with potential therapeutic implications.

Cutaneous B-1 B cells express high affinity beta-1 integrin

B-1 B cells utilize α4β1 integrin to enter the skin, but it has also been shown that downregulation of these integrins mediates release from the peritoneal cavity after TLR4 ligand stimulation (118). How is it, then, that the same trafficking receptor could simultaneously be downregulated to exit the peritoneum but then also be required to enter a subsequent site? To address this question we considered the affinity state of the α4β1 integrin. Low affinity integrin interactions are capable of mediating tissue retention, but high affinity interactions are required for tissue entry (218). We utilized an antibody for beta-1 integrin that only recognizes the high-affinity state (219), and examined high affinity beta-1 on peritoneal B-1 B cells and splenic B-2 B cells (Figure 4A). Like the expression pattern of α4β1, a larger proportion of peritoneal B-1 B cells expressed high affinity beta-1 than splenic B-2 B cells. Furthermore, B-1 versus B-2 B cells in the inflamed skin exhibited a similar staining pattern (Figure 4B). In order to address if the high affinity confirmation of beta-1 is regulated similarly to alpha-4 and total beta-1 integrins, we stimulated peritoneal B-1 B cells in vivo with LPS. As previously published by Fagarasan and colleagues, LPS treatment induced a significant downregulation of alpha-4 (p = 0.0009) and total beta-1 integrin (p = 0.0006) ((118), Figure 4C-D). Surprisingly, high affinity beta-1 was significantly upregulated (p = 0.002) after LPS stimulation. These findings suggest that whereas downregulation of low affinity α4β1 is

required for release from the peritoneum, high affinity $\alpha 4\beta 1$ can then subsequently mediate entry into distant tissues. Whether the high affinity beta-1 can be further triggered or if there is a selective enrichment for B-1 cells that already express high affinity integrin upon peritoneal exit is not clear and, further experiments will be required to determine the regulation of $\alpha 4\beta 1$ in B-1 B cells.

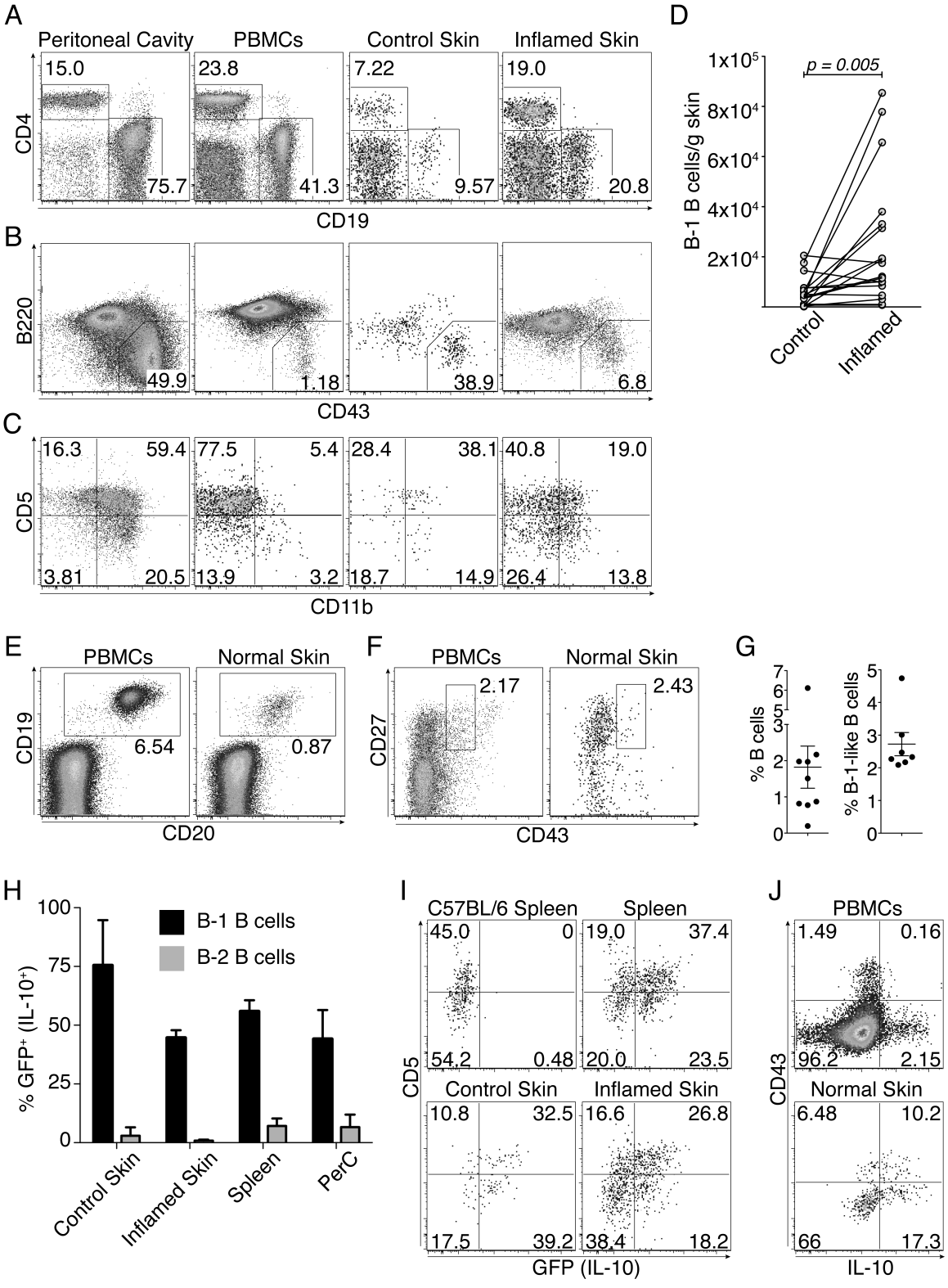

A
Peritoneal Cavity
PBMCs
Control Skin
Inflamed Skin
15.0
75.7
23.8
41.3
7.22
9.57
19.0
20.8
CD4
CD19
B
B220
49.9
1.18
38.9
6.8
CD43
C
16.3
59.4
3.81
20.5
77.5
5.4
13.9
3.2
28.4
38.1
18.7
14.9
40.8
19.0
26.4
13.8
CD5
CD11b
D
1x10^5
8x10^4
6x10^4
4x10^4
2x10^4
p = 0.005
B-1 B cells/g skin
Control
Inflamed
E
PBMCs
Normal Skin
6.54
0.87
CD19
CD20
F
PBMCs
Normal Skin
2.17
2.43
CD27
CD43
G
% B cells
% B-1-like B cells
H
100
75
50
25
0
B-1 B cells
B-2 B cells
% GFP+ (IL-10+)
Control Skin
Inflamed Skin
Spleen
PerC
I
C57BL/6 Spleen
Spleen
45.0
0
54.2
0.48
19.0
37.4
20.0
23.5
Control Skin
Inflamed Skin
10.8
32.5
17.5
39.2
16.6
26.8
38.4
18.2
CD5
GFP (IL-10)
J
PBMCs
1.49
0.16
96.2
2.15
Normal Skin
6.48
10.2
66
17.3
CD43
IL-10

Figure 1. B-1 B cells are part of the cutaneous immune system. Lymphocytes from the peritoneal cavity, PBMCs, uninflamed (control) skin, and 3-week CFA-inflamed skin were analyzed. (A) Flow cytometric analysis of CD4 T cells and B cells pre-gated on live, CD45+ lymphocytes, (B) $CD43^{+}$, $B220^{lo/neg}$ B-1 B cells gated on the above CD19+ B cells, and (C) CD5 and CD11b expression on above gated B-1 B cells. Representative staining from at 3-5 independent experiments with 3-5 mice each are shown. (D) Enumeration of B-1 cells per gram uninflamed vs inflamed skin from each mouse analyzed in (A). Significance was determined with the via Wilcoxon signed-rank test. (E-G) Lymphocytes from human peripheral blood and normal skin were analyzed for (E) B cells gated on live, CD45+ lymphocytes and (F) $CD43^{+}$, $CD27^{+}$ B-1-like cells from the B cell gate of (E). Representative plots from 5 (PBMCs) or 7 (skin) human donors. (G) The frequency of B cells (left) and B-1-like cells (right) as a percentage of lymphocytes and B cells, respectively, in human skin for all samples analyzed. Bars represent the means ± SEM. (H) Percentage of IL-10 producing (GFP+) B-1 and B-2 B cells of total B cells from specified tissues of Vert-X IL-10-eGFP reporter mice after stimulation with PMA, ionomycin, and LPS. One experiment of 3 independent experiments of at least 4 mice with similar results is shown. (I) Representative plots showing flow cytometric analysis of GFP expression by B-1 B cells (gated on LIVE/DEAD Fixable Aqua Dead Cell $marker^{-}$, $CD45^{+}$, $CD19^{+}$, $CD43^{+}$, $B220^{lo/neg}$ lymphocytes). (J) Representative flow plots showing flow cytometric analysis IL-10 expression by B cells in normal human skin and PBMCs after stimulation with PMA and ionomycin (gated on LIVE/DEAD Fixable Aqua Dead Cell $marker^{-}$, $CD45^{+}$, $CD19^{+}$ lymphocytes). One of 4 individuals analyzed is shown.

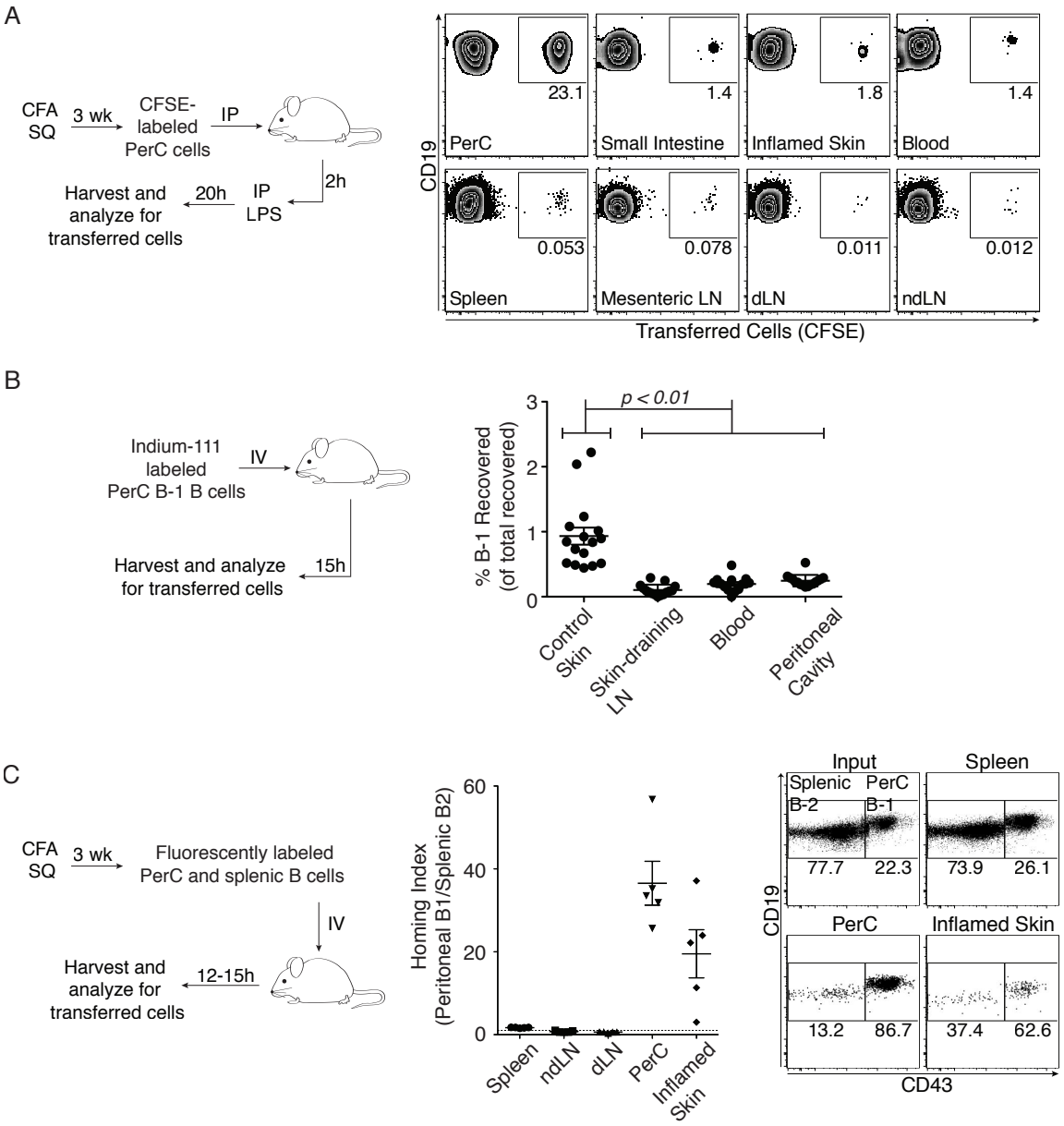


Figure 2. Peritoneal B-1 B cells migrate into uninflamed and inflamed skin. (A) Experimental scheme [left] and flow cytometric analysis of CFSE-labeled donor peritoneal B-1 B cells after IP transfer and LPS-induced exit from the peritoneal cavity [right]. Plots are pre-gated on B-1 B cells (live, $CD45^{+}$, $CD19^{+}$, $CD43^{+}$, $B220^{lo/neg}$ lymphocytes) and gates represent the percentage of transferred B-1 B cells in each organ. Compiled plots from one of two independent experiments with similar results are

shown. (B) Experimental scheme [right] and homing of peritoneal B-1 B cells into uninflamed skin [left]. Peritoneal cavity cells were MACS-enriched for B-1 B cells, labeled with ^{111}Indium, and IV transferred into recipient mice. 15 h post-transfer, organs were harvested and the redistribution of radioactivity (homing into specified tissues) was quantified with a γ-counter. Combined analysis of 2 individual experiments with 8 mice each. (C) Experimental scheme [left] and homing of peritoneal B-1 vs splenic B-2 B cells into inflamed skin [middle, right]. Peritoneal and splenic B cells were differentially labeled with fluorescent dyes and transferred IV into recipient mice that had 3-week cutaneous CFA inflammation. The homing index of peritoneal B-1/splenic B2 (peritoneal dye^{+}, live, CD45^{+}, CD19^{+}, CD43^{+}, B220$^{lo/neg}$ lymphocytes/splenic dye^{+}, CD45^{+}, CD19^{+}, CD43^{neg}, B220^{+} lymphocytes) for specified organs is shown [middle]. Representative plots of the gated input populations and those populations recovered in specified organs from one of 5 individual experiments with similar results. (B-C) Bars represent the means ± SEM; significance was determined using an nonparametric one-way ANOVA followed by Dunns multiple comparison test (B).

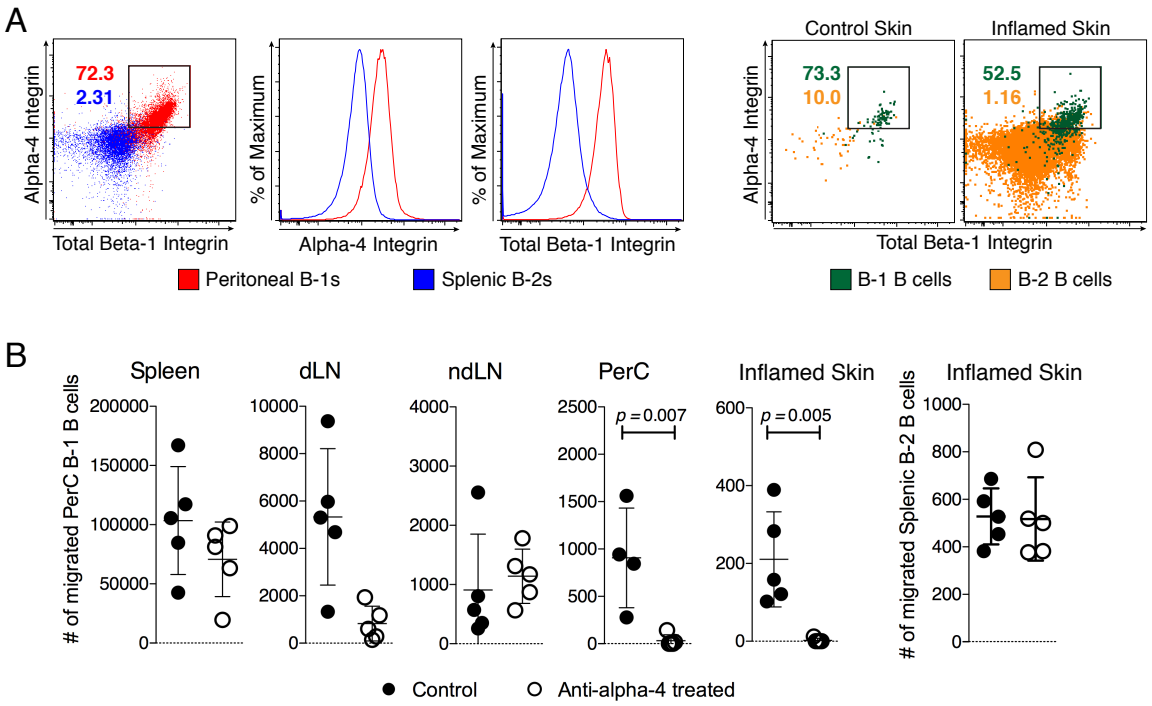


Figure 3. Integrin α4β1 mediates B-1 cell migration into the skin. (A) Flow cytometric analysis of alpha-4 and beta-1 integrin expression on peritoneal B-1 cells vs splenic B-2 cells [left] and by control (uninflamed) and inflamed skin [right]. Representative plots from 2 (control skin) or 5 (all other tissues) independent experiments with 3-5 mice each are shown. Gates represent high alpha-4, beta-1 expression. (B) Homing of peritoneal B-1 B cells (as in Figure 2C) in mice that were treated with α4-integrin blocking antibody or isotype control. 12 h after transfer, lymphocytes were isolated from specified organs, and the number of transferred cells was enumerated by flow cytometry. One of two experiments with similar results is shown. Bars represent mean ± SD, and significance was determined using student's t test.

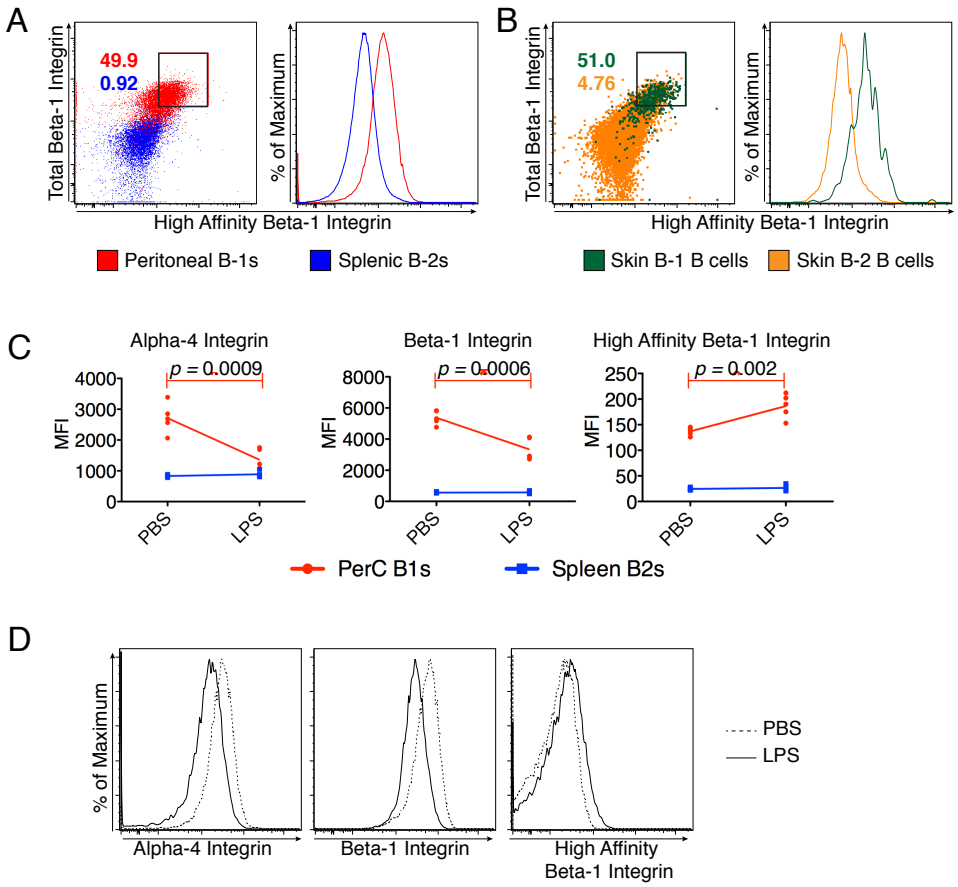


Figure 4. Cutaneous B-1 B cells express high affinity beta-1 integrin. (A-B) Flow cytometric analysis of high affinity beta-1 integrin expression by peritoneal B-1 cells vs splenic B-2 cells (A) and on B-1 vs B-2 B cells from inflamed skin (B). Gates represent high high-affinity beta-1 integrin expression. (C) Mean fluorescence intensity from flow cytometric analysis of alpha-4, beta-1, and high-affinity beta-1 integrins on peritoneal B-1 and splenic B-2 B cells from mice given 20 μg LPS or PBS IP. 6 h after treatment, spleens and peritoneal cavity B cells were analyzed for integrin expression. One of two experiments with similar results is shown, and significance was determined using student's t test.. (D) Representative expression of integrins by peritoneal B-1 B cells from (C) treated with either PBS or LPS.

Chapter 5: Discussion

Recirculation of γδ T cells

The recirculation and migration of innate lymphocytes is critical to their contribution to immune responses. γδ T cells are rapidly responding T lymphocytes that secrete cytokines, such as IL-17, earlier than their αβ T cell counterparts (167, 168) to support the activation and mobilization of the immune response (52, 73, 166, 167). In Chapter 1 we demonstrated that cytokine-secreting γδ T cells migrate through the skin. In contrast to αβ T cells that utilize CCR7 to exit the skin (75, 76), γδ T cell migration into the afferent lymph was independent of CCR7. This is in line with findings in cows (220) and mice (73), and supports that skin exit receptor requirements of γδ T cells are conserved across mammalian species. Although studies have started ruling out potential exit receptors for γδ T cells, e.g., γδ T cells do not use CCR7, mechanisms by which skin-recirculating γδ T cells enter the skin are still unknown. CCR4 and CCR10 are important for dendritic epidermal T cell (DETC) homing into the skin (62, 63), but these resident cells are restricted to mice, and it is unclear if epidermal γδ T cells in other species represent a homologous cell type (44, 69). CCR4 and CCR10, as well as CCR8, also mediate CD4 T cell migration into skin (20, 30). Thus far, ovine γδ T cells appear unique from both DETCs and αβ T cells in their skin entry requirements, as skin-draining γδ T cells are unresponsive to CCR4, CCR8 and CCR10 ligands. Further studies in mice are necessary to not only elucidate the trafficking receptor requirements for γδ T cells but also to delineate the function of skin sessile versus recirculating γδ T cells.

Many studies have investigated the role of cutaneous γδ T cells in wound healing.

In mice, DETCs promote wound healing by the production of keratinocyte growth factors (68, 221). In humans, epidermal Vδ1 T cells can produce insulin-like growth factor 1, which enhances wound healing in vitro (71). Intriguingly, Vδ1 T cells are also predominantly found in the dermis with similar levels of skin-homing molecule, CLA. A small population of Vδ1 T cells was also detected in the blood with a subset of those expressing CLA (71). Though *in vivo* functional studies in humans are exceptionally difficult, it would be interesting to know if the small population of blood-borne Vδ1, CLA^+ γδ T cells were skin-recirculating dermal γδ T cells and what the contribution of dermal versus epidermal Vδ1 T cells is to wound healing.

It is tempting to speculate that ovine skin-recirculating γδ T cells we describe are homologous to the $IL\text{-}17^+$ dermal γδ T cells in the mouse. Dermal γδ T cells of mice and skin-recirculating ovine γδ T cells not only share the ability to produce IL-17 and express CCR6, they also both express variant TCRs (73, 170, 171). Similarly, data suggest that mouse dermal γδ T cells can recirculate because they also localize to the skin-draining lymph node in the subcapsular sinus (48), where afferent lymph enters the lymph node. Although the potential for murine dermal γδ T cells to recirculate has yet to be assessed, the ability to divide the workload of continuous immunosurveillance between resident and recirculating subsets of γδ T cells would be advantageous to achieving consistent and robust protection from exogenous threats. While sessile epidermal γδ 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. Furthermore, though it is well established that memory αβ T cells exhibit tissue-specific tropisms in their recirculation (22, 187, 222), whether the γδ T cells described in Chapter 2 are a specific skin-recirculating subset or whether they exhibit a more ubiquitous recirculation pattern is unclear. Preliminary data [Geherin, Debes, unpublished results] suggests that unlike αβ T cells, γδ T cells from the skin do not preferentially return; however, additional experiments are necessary to confirm this finding.

Recirculation of B-1 B cells

The data presented in Chapters 2 and 3 is the first description of B-1 B cells as a part of the normal skin immune system. It furthermore demonstrated that peritoneal B-1 B cells can rapidly enter into this barrier site by relocating from the peritoneal cavity or by migrating directly from the blood under both homeostatic and inflammatory conditions. B-1 B cells can rapidly secrete antibody and relocate from the peritoneal cavity to the omentum, spleen, small intestines and draining lymph nodes within hours of an inflammatory signal (98, 105, 118-120). Itakura and colleagues demonstrated that in a hapten model of contact hypersensitivity, within an hour of cutaneous challenge, B-1 B cells are able to migrate from the peritoneal cavity to the spleen. They subsequently migrate to the skin draining lymph nodes where B-1 derived hapten-specific IgM contributes to the inflammatory response (105, 223). As we have found that peritoneal B-1 cells readily migrate to inflamed skin, it seems likely that in the contact hypersensitivity model, B-1 B cells could also home to the CHS-inflamed skin and secrete local antibody or cytokines that were not assessed in previous studies. Choi and colleagues demonstrated that B-1 cell derived natural IgM binds the influenza virus and is, at least partially, produced in the lung. Furthermore, this pre-existing natural antibody

is required for full protection from lethal dose infection and is not compensated for by B-2 B cells (92, 104). The circumstances that predicate pre-existing local B-1 cell responses versus the ability to be recruited when needed are currently unknown. However, one could imagine that as the full characterization of the anatomic localization of B-1 cells in extralymphoid tissues has only just begun, local responses of endogenous cells may be more frequent than what is currently believed. It is also conceivable that differences in the homeostatic populations within tissues and the type of inflammation may determine if B-1 B cell subsets within the tissue can respond or require additional B-1 B cell recruitment to fulfill their role in the immune response.

In Chapter 3 we investigated the trafficking molecule requirements of B-1 B cell migration into the skin. Although few studies have examined B-1 B cell migration to extralymphoid cites, several groups have reported on the requirements of B-1 B cell recirculation into and out of the peritoneal cavity. Peritoneal B-1 B cells utilize CXCR5 and CCR7 to enter the peritoneal cavity from the blood (121-123, 224). In contrast, integrin requirements for B-1 cell entry into the peritoneal cavity or other sites were previously unknown. Alpha-4, beta-1 integrin, which can bind VCAM-1 and fibronectin, has been suggested to be important for B-1 cell homeostasis in the peritoneal cavity as $MADCAM^{-/-}/VCAM^{-/-}$ double knockout mice exhibit significantly fewer B-1 B cells in the peritoneal cavity compared to $MADCAM^{-/-}$ single knock-outs (125). However, whether this decrease in peritoneal B-1 B cells is because of entry versus exit requirements or due to survival has not been investigated. Conversely, upon exit from the peritoneal cavity after LPS stimulation, it has been shown that alpha-4 and beta-1 integrins are downregulated (118), and this allows B-1 B cell release from the peritoneal cavity. These studies, as well as our findings, are summarized in Figure 1, left.

As peritoneal B-1 B cells, which we show preferentially migrate into the skin compared to follicular splenic B-2 B cells, express high levels of alpha-4 and beta-1 integrins compared to B-2 B cells, we investigated the role of this integrin in homing to the inflamed skin. Not only did the majority of cutaneous B-1 cells highly express alpha-4 and beta-1 integrins, similar to their peritoneal counterparts, but treatment with a blocking antibody to alpha-4 integrin significantly abrogated not only migration to the inflamed skin, but it also abrogated homing to the peritoneal cavity. VCAM-1, the binding partner to $\alpha 4\beta 1$ integrin, is constitutively expressed in the skin and upregulated in inflammation (225). As B-1 B cells from both the uninflamed and inflamed skin expressed similar levels of $\alpha 4\beta 1$ integrin, it is likely that B-1 cell homing to skin in general is mediated by this interaction, similar to homing to the peritoneal cavity, which was not inflamed in these experiments. As these high levels of $\alpha 4\beta 1$ integrin are suggestive of an inflammation-seeking cell, it is interesting to hypothesize that B-1 B cells home to various inflamed tissues. Evidence for this can be found within our own homing experiments. Alpha-4 integrin blockade caused B-1 B cell migration to the inflamed skin-draining lymph node to be reduced to the level of the uninflamed skin-draining lymph node. If this hypothesis was true, it would position B-1 B cells as a sentinel population capable of rapid responses in all tissues, not just those considered mucosal and/or barrier sites. Also interesting are the differential requirements of B-1 versus B-2 cell homing in the skin. Whereas B-1 B cells are acutely sensitive to alpha-4 integrin blockade, B-2 B cells were unaffected in their homing capacity to the skin. Similarly, effector T cells are also not dependent on alpha-4 integrin to migrate into the skin (226), suggesting a dichotomy in adhesion molecule usage for innate versus conventional lymphocyte subsets. Further experiments should expand upon both

uninflamed and inflamed skin-associated adhesion molecules, such as E- and P-selectin ligand (20), to address whether 1) B-1 B cells also have some dependency on these receptors and 2) what receptors are needed for B-2 B cell entry into the skin.

Prior to the studies conducted in Chapter 3 of this thesis, the B-1 B cell usage of alpha-4 integrin seemed contradictory. Data suggested that it might be used by B-1 B cells to enter the peritoneal cavity (125), but it had conversely been shown to be down-regulated upon exit from the peritoneal cavity (118). Ha and colleagues also transferred peritoneal B cells IP and gave an alpha-4 integrin blocking antibody and observed fewer B-1 B cells in the peritoneum, concluding that alpha-4 is used to retain B-1 B cells in the peritoneal cavity (118). Alternative interpretations of these results are that peritoneal B-1s were unable to re-enter the site after exiting and that there is selective exit of alpha-4^{hi} B-1 B cells. That B-1s were shown to downregulate alpha-4 and beta-1 integrins in response to LPS in culture negates the second alternative conclusion (118), but the contribution of blocked re-entry to the peritoneal cavity in the decreased cellularity of that site after alpha-4 blockade cannot be disregarded.

To reconcile our findings that B-1 B cells utilize $\alpha 4\beta 1$ integrin to enter the skin but these integrins are down-regulated to exit the peritoneal cavity (118), we investigated the affinity state of the integrin utilizing an antibody that recognizes only high-affinity beta 1 (219). We hypothesized that down-regulation of low-affinity $\alpha 4\beta 1$ could potentially mediate release from the peritoneum but that high affinity $\alpha 4\beta 1$ would be required for entry into the skin because only the high affinity state mediates firm adhesion required for cell entry from the blood. In vivo stimulation of peritoneal B-1 B cells with LPS led to a down-regulation of the levels of total alpha-4 and beta-1 integrins, as described (118). In contrast, the expression level of high-affinity beta-1 integrin increased on peritoneal B-1

B cells, supporting divergent roles for low- versus high-affinity beta-1 integrin. Further investigation of high-affinity beta-1 integrin on cutaneous B cells showed that the majority of B-1 cells in the skin also express high-affinity beta-1, supporting a role in cutaneous migration. It appears that B-1s utilize high-affinity $\alpha4\beta1$ to enter the skin, but the regulation of integrin affinity in this subset is not yet defined.

The finding that peritoneal B-1 B cells basally express high affinity beta-1, which is then upregulated after LPS, leads to the hypothesis that B-1 B cells broadly recirculate through extralymphoid sites, especially inflamed sites (Figure 1, right). However, this also raises several questions regarding the regulation of B-1 B cells $\alpha4\beta1$. Examination of B-1 B cells that had exited from the peritoneal cavity would clarify if all released B-1s express high levels of high-affinity $\alpha4\beta1$, and as such may represent a distinct population that is readily capable of migration into the skin or inflamed/effector sites. Alternatively, B-1 B cells may also utilize an additional chemokine or TLR signal to further upregulate high-affinity $\alpha4\beta1$ expression so that any recirculating B-1 could be induced to enter the skin or sites of inflammation. Although high-affinity integrins are triggered by chemokine signals on the vascular endothelium in the canonical model of lymphocyte migration (reviewed in (227)), some subsets of effector T cells can firmly adhere to the endothelium without activation by chemokine signals (228). If the expression of high-affinity $\alpha4\beta1$ on B-1s is independent of chemokine signaling, it would strongly support B-1 B cells as a rapidly tissue-surveying migratory population, well suited to trafficking to extralymphoid sites and sites of inflammation.

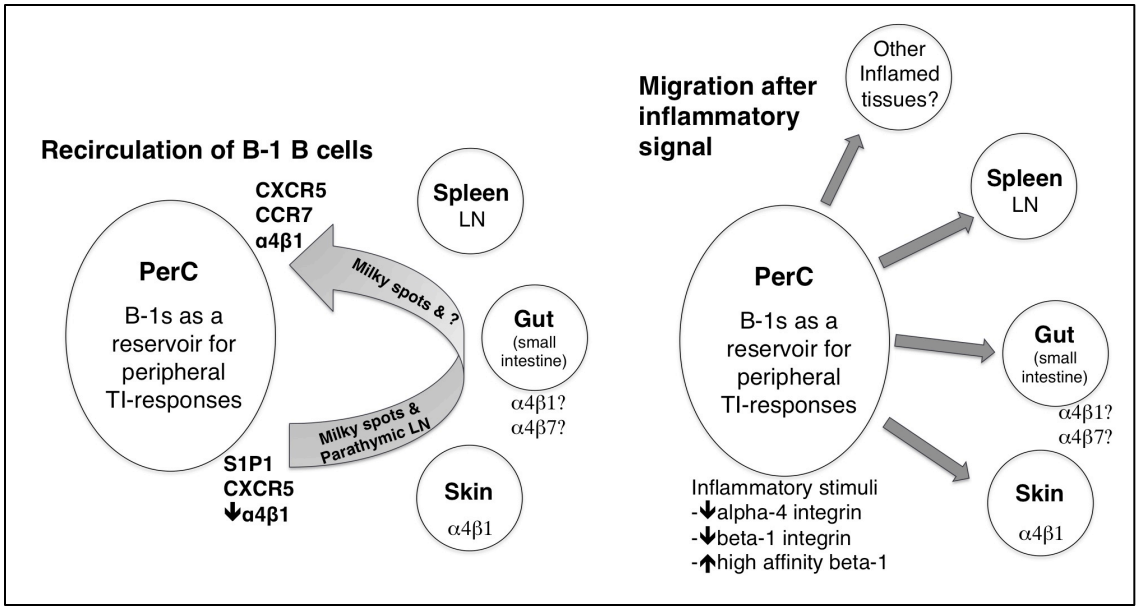


Figure 1. Recirculation of peritoneal B cells. (Left) The peritoneal cavity serves as a reservoir for B-1 B cells. To survey tissues, B-1 cells leave the peritoneal cavity by downregulating $\alpha 4\beta 1$ integrin and exit through the omentum to the parathymic lymph node using CXCR5 and S1P1. In order to return to the peritoneal cavity, B-1 B cells require CXCR5, CCR7, and alpha-4 integrin. (Right) After an inflammatory signal, B-1 B cells express more high affinity $\alpha 4\beta 1$ and rapidly exit the peritoneum to home to the spleen, gut, skin, and potentially other inflamed tissues. Migration into the inflamed skin also requires alpha-4 integrin.

Do B-1 B cells and γδ T cell interact in the skin?

γδ T cells can recognize antigen presented by CD1 (229, 230). As we have shown that ovine cutaneous B cells express CD1, it is possible that cutaneous γδ T cells interact with B cells directly in the skin. Lipid antigens from bacteria, viruses, and fungi can be presented by CD1 (231), and antigen presentation to γδ T cells, which are found in much higher abundance in the skin than in the draining lymph nodes, could be immunologically advantageous as γδ T cells can rapidly secrete cytokine. However, there is little evidence in the literature regarding the interaction of B-1 B cells and γδ T

cells. One study utilized a double transgenic mouse in which γδ T cells recognize a self-thymus leukemia antigen, and the B-1 cells recognize red blood cells (232). Only in mice with activated autoreactive γδ T cells was there the development of B-1 antibody mediated anemia, suggesting that non-cognate interactions, possibly through CD1, between γδ T cells and B-1 B cells are possible. Studies coculturing various B-1 and γδ subsets, as well as utilizing intravital microscopy in the skin, could more clearly elucidate any potential interaction.

Innate-like lymphocytes share many characteristics between species

Our studies and others of innate lymphocytes in the skin highlight that at least some aspects of the cutaneous immune system are conserved across mice, sheep, cows, and humans. For example, although there are differences in the proportions of various lymphocyte subsets, populations of γδ T cell, B-1(like), and B-2 cells can be found in sheep, mouse, and human skin. Also similar across species, CCR7 is dispensable to the cutaneous exit of murine, human, bovine, and ovine γδ T cells (1, 73, 78, 233). Despite apparent functional homology in trafficking receptor usage, some distinctions in γδ populations across species do exist, e.g. we were unable to detect an ovine population of γδ T cells similar to the human subset of γδ T cells that upregulates CCR7 and antigen presenting machinery (80). However, it is too early to completely preclude the existence of this population as it is restricted to a specific subset of human Vδ2 γδ T cells, and it is possible that a homolog has just not yet been identified in other species.

Similarly, our studies show parallel populations of B cells in the skin of sheep, mice, and humans. B cells in the skin and skin-draining lymph of sheep express alpha-4

and beta-1 integrins, and we have shown that the mouse B-1 subset utilizes alpha-4 integrin to home to the skin. We have demonstrated that B-1 B cells in mouse skin are capable of producing IL-10 after in vitro stimulation. Preliminary results from our lab also show that cutaneous B cells in sheep and humans are capable of producing IL-10 (Geherin, unpublished observations), but further investigation is required to determine which B cell subsets produce IL-10 in sheep and humans.

Cutaneous B cells and B cell-depleting therapies

The elucidation and preliminary characterization of B cells subsets in the skin may change the paradigm of cutaneous immunity. Previously disregarded in this extralymphoid site in the absence of inflammation (29, 38, 39), realizing that B cells may play an active role in the manifestation of autoimmunity and in response to infection *in situ* could remodel our comprehension of many cutaneous diseases. Primarily due to the advent of Rituximab; a humanized antibody to CD20 that is used to deplete B cells; the past decade has shown significant advances in our understanding of cutaneous autoimmune diseases that are at least partially mediated by B cells (180). Autoantibodies are known to play a role in cutaneous autoimmune disorders, and especially for skin-restricted diseases, such as pemphigus vulgaris and pemphigus foliaceus, the decline in autoantibody titers after Rituximab treatment correlates well with a decrease in clinical severity ((234-236). However, for some systemic autoimmune disorders with cutaneous symptoms, such as rheumatoid arthritis or systemic lupus erythematosus, improvements in clinical severity do not always correlate with decreases in autoantibodies (212, 213). Furthermore, autoantibodies can be present in the circulation well before onset of autoimmune disease (237, 238), which implies that mechanisms beyond the presence of self-reactive antibodies are required for disease

progression. The ability of B cell depletion to improve disorders that are not solely based on autoantibodies suggests that alternative functions of B cells, such as antigen presentation to autoreactive CD4 T cells, play a role in these diseases. This indeed was suggested by Eming and colleagues (236) who observed that after Rituximab treatment, pemphigus vulgaris patients also exhibited a significant reduction in desmoglein-3 (the auto-antigen in pemphigus vulgaris) specific CD4 T cells. This mechanism was more fully examined in mouse models of collagen-induced arthritis and autoimmune diabetes where Bouaziz and colleagues utilized draining lymph node populations to determine that B cells are needed for optimal CD4 T cell priming (239), but analysis of antigen presentation in the skin has not been addressed. Interestingly, Rituximab treatment of individuals with ectopic eczema has shown that despite near complete depletion of B cells in the blood, B cells in the eczema lesions were only depleted by 50% (201). The efficacy of B cell depletion by anti-CD20 treatment in a broad range of tissues has been more thoroughly examined in mice. Similar to humans, anti-CD20 is highly effective at depleting B cells from the blood and lymph nodes, and nearly as well as, the spleen (240). Interestingly, despite CD20 expression, B-1a B cells in both the peritoneal cavity and the spleen are significantly more resistant to depletion than their B-2 counterparts (240). Whether the partial depletion of B cells in human skin lesions is due to selective B cell subset depletion or the presence of a protective cutaneous niche remains to be seen but may have therapeutic indications.

IL-10 in cutaneous disease

We have only begun the investigation into the function of B-1 B cells in the skin, and proposed functions are summarized in Figure 2. Upon stimulation, the B-1 B cells are able to upregulate IL-10 production as demonstrated by the Vert-x IL-10 reporter

mice (116). In contrast, the B-2 B cells expressed comparatively little IL-10, suggesting that cutaneous B-1 and B-2 B cells may exhibit differential effects in vivo. Our studies in sheep in Chapter 2 demonstrated that B-2 B cells in the skin and skin-draining lymph expressed CD1 and higher levels MHCII and CD80/86 than in skin-draining lymph nodes, suggesting that these cells play a pro-inflammatory role in the skin itself and/or in the draining lymph node (2). In contrast, the murine B-1 B cells can secrete IL-10, and preliminary data shows a subset of cutaneous B-1 B cells expresses Blimp-1, a transcription factor required for antibody production in B cells (reviewed in (241)), Chapter 3; Wilson RP and Geherin SA, unpublished observation). Our lab has also detected IgM-secreting cells in the skin, as well as increased apoptotic debris by tunnel staining in the skin of secretory-IgM-deficient mice ((110); Wilson RP, unpublished observations). Secreted IgM is critical for the proper clearance of apoptotic cells in vivo (242), and subsets of B-1 B cells have been shown to have BCR specificity for such dying cells (243). Taken together, this data eludes to a model by which cutaneous B-1 B cells exert an anti-inflammatory roles through the secretion of IL-10 and anti-apoptotic IgM whereas B-2 B cells provide the pro-inflammatory functions of antigen presentation and potentially cytokine secretion.

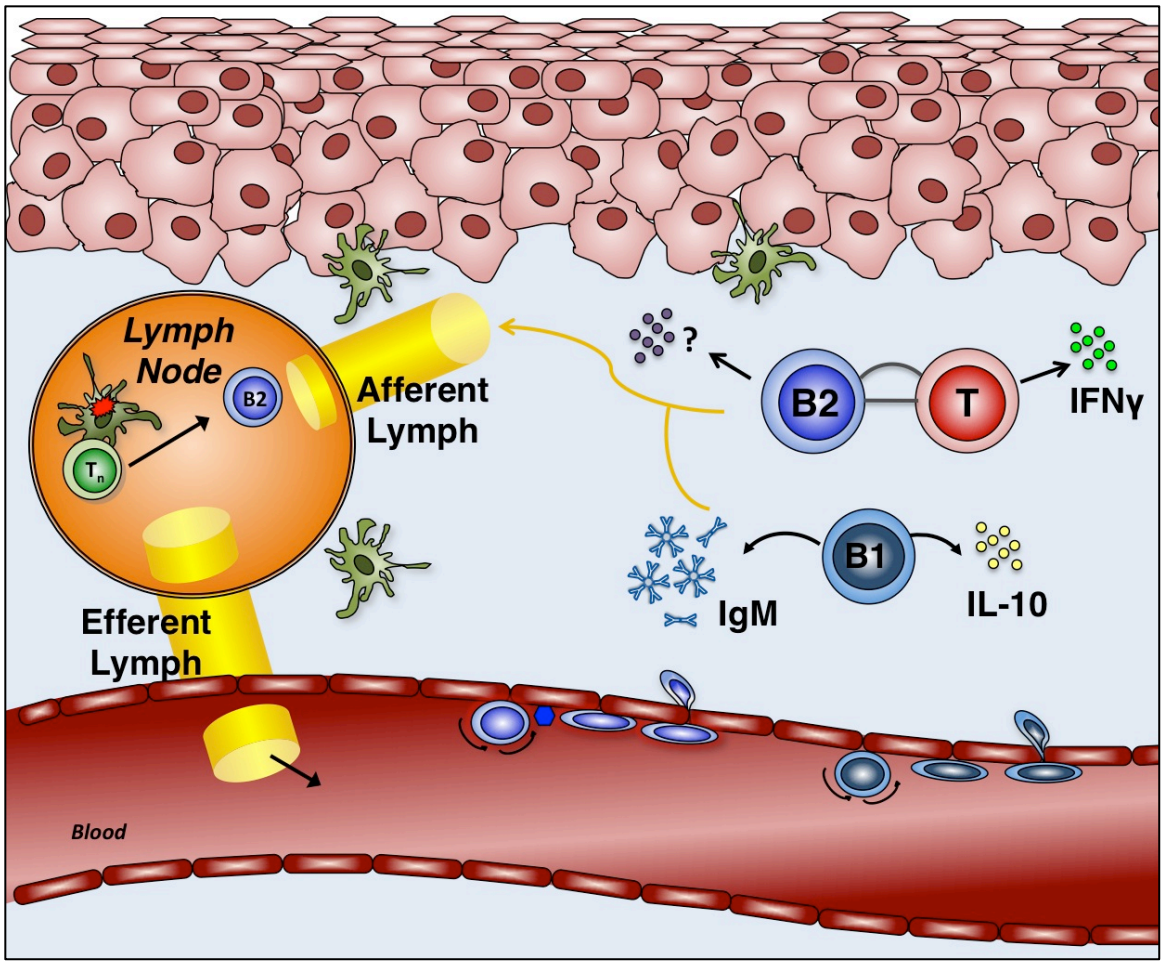


Figure 2. Proposed functions of B cells in the skin. Both B-1 and B-2 B cells are found in uninflamed and inflamed skin and may contribute to opposing roles in cutaneous immunity. Skin B-1 B cells can secrete IL-10, and possibly IgM, supplying anti-inflammatory mediators in skin maintenance and immune responses. Conversely, B-2 B cells have higher expression of costimulatory molecules and may provide the pro-inflammatory functions, such antigen presentation and cytokine production. Both of these subsets are capable of exiting to the skin and migrating to the draining lymph nodes, where they may also contribute to systemic immune response.

It is possible that cutaneous B-1 cells may subset into functionally distinct groups or that waves of immigrants versus resident cells may have unique functions. Precedence for the temporal regulation of distinct functional B cells subsets has been described in a murine model of experimental autoimmune encephalomyelitis (244). Prior

to disease onset, depletion of B cells leads to exacerbated disease due to the deletion of IL-10 producing B regulatory cells. In contrast, B cell depletion after disease onset improves disease symptoms by reducing B-cell auto-antigen presentation to CD4 T cells (244). Future experiments analyzing the production of IL-10 by B-1 B cells over time, either in the CFA model from the acute to chronic phase or in cutaneous inflammation model where IL-10 is known to play a role, will allow for a closer examination of cell type versus temporal regulation of cutaneous B cell subsets. A more detail analysis of B-1/innate B cells subsets in the skin could also elucidate their functional capacity in the skin. Both B regulatory B10 cells and innate response activator B cells phenotypically resemble B-1a cells (117, 206). Thus additional functional analyses will reveal other potential innate B cell functions, such as production of GMCSF or phagocytosis.

The role of IL-10 in cutaneous disease is complex and varied. In mice overexpressing IL-10 in the epidermis, there is reduced Langerhans cell migration to the draining lymph node after hapten challenge along with a decreased ability to stimulate allogeneic T cell responses (245), both of which could cause dysregulation of the immune response in infection. However, when these mice are exposed to UVB radiation, they develop fewer skin tumors (245), suggesting that high levels of cutaneous IL-10 can have a protective effect. Similarly, in a model of cutaneous Francisella tularenis infection, IL-$10^{-/-}$ are more protected from lethal challenge than wildtype mice (246).

The role of IL-10 in cutaneous human autoimmune disorders is equally unclear. In some disorders, such as systemic lupus erythematosus and bullous pemphigoid disease, systemic IL-10 levels are elevated compared to healthy individuals (247, 248). Conversely, in other autoimmune disorders, such as psoriasis and allergic contact dermatitis, serum and/or local cutaneous IL-10 levels are lower than normal, and both

diseases have been successfully treated with topical IL-10 therapy (214, 249). Although studies of cutaneous autoimmune disorders and infections have not considered the role of B-cell derived IL-10 in disease, case reports from B cell depletion provide insight into the importance of the source of IL-10. A patient being treated for rheumatoid arthritis received a B-cell depleting therapy and subsequently developed psoriasis (215). Similarly, another individual completed treatment for B cell lymphoma that included B cell depletion in conjunction with strong immunosuppressives that coincided with remission of his psoriasis. When the treatment wore off, another round of B cell depleting therapy was administered for treatment of the psoriasis; however, the psoriatic lesions grew worse, suggesting it was the strong immunosuppressives rather than B cell depletion that caused clinical symptoms of psoriasis to improve (216). In contrast, there is a report of the successful use of B cell depletion for the treatment psoriatic arthritis (250). Although interpretation of these cases is difficult due to the systemic effects of B cell depletion, they support an active role of B cells in the cutaneous immune system. Furthermore, as IL-10 is a critical mediator in psoriasis, these studies may allude to the importance of proper regulation of IL-10 production by cutaneous B cells.

Understanding the homing receptors B-1 B cells use to migrate to the skin would enable the specific targeting of cutaneous B-1 cell infiltration as a therapeutic intervention. By inhibiting access of a certain cell subset to a particular tissue in a disease state, the tissue microenvironment can be changed to improve clinical manifestations of disease. Several drugs that target trafficking molecules primarily on T cells have been developed, and they have been effective at improving several autoimmune disorders (251, 252). One such drug, Natalizumab, targets α4 integrin and can dramatically improve multiple sclerosis and Crohn's disease, athough it also

increases susceptibility to JC polyomavirus. Interestingly, there is also a reported case of worsening psoriasis after treatment for multiple sclerosis with Natalizumab (253). From these studies it is intriguing to speculate that human IL-10-producing B cells play a role in psoriasis and that they may also use alpha-4 integrin to home to the skin.

Future Directions

Our studies demonstrating B-1 B cells in the skin of sheep, mice, and humans present many questions. Although we show a unique dependence on $\alpha 4\beta 1$ integrins for migration into the skin, additional trafficking molecules must work in tandem to effectively guide B-1 B cells into the skin. Even if B-1 B cells stably express high affinity integrin and therefore do not need a chemokine trigger, at least one unknown chemokine mediates chemotaxis into and through the tissue. According to the Immunological Genome Project, B-1 B cells express mRNA for several chemokine receptors, including CCR1, CCR6, CCR7, CCR9, CXCR4, and CXCR5. However, B-1 B cells do not express CCR1 protein by flow cytometry, can home into the skin in the absence of CCR6, and do not migrate to ligands for either receptor in an ex vivo chemotaxis assay (data not shown). Additional studies should attempt to elucidate what chemokine-chemokine receptor pair drives B-1 cell migration so that we may more fully understand the homing capacity of this subset.

We show that peritoneal B-1 B cells migrate into the skin; however, like most homing experiments, we recover a relatively small number of cells in the skin compared to the total transferred. This may simply be the nature of trafficking experiments, but it may also be due to a selective preference of a particular type of B-1 B cell. To try to discern if a particular B-1 B cell subset may exhibit skin tropism, the B cell receptor usage of skin B-1 B cells could be investigated. This would tell us if there is a particular

enrichment of any VJD usage and may tell us how cutaneous B-1 B cells compare to peritoneal and splenic B-1 B cells.

In addition to discerning trafficking and B cell receptor usage, the function of cutaneous B-1 B cells should also be investigated. The production of IL-10 and observation that some B-1 B cells can express Blimp-1 suggests that these B cells may play a regulatory role in skin immunity, but this remains to be demonstrated. Furthermore, although the CFA model is especially useful for studies in multiple species, it may not be the best model to study B-1 B cell function. One particular model that could be used is TLR-7-induced psoriasis-like disease in mice. It has been previously shown that B-1 B cell produced IL-10 is critical for controlling inflammation in this model, but the presence of B-1 B cells in the skin was not analyzed (254). Analysis of B-1 B cells in the psoriatic skin, and if present, subsequent alpha-4 integrin blockade would demonstrate the regulatory capacity of cutaneous B-1 B cells.

Our studies also revealed B-2 B cells in the uninflamed skin with a dramatic increase in the chronically inflamed skin. These cells likely also fulfill a currently unappreciated role in skin immunity, and investigation into how they migrate into the skin as well as their function in the tissue would enhance our understanding of cutaneous disease and inflammation.

Summary and concluding remarks

Innate lymphocytes are important in infection and autoimmune disorders; however, their characterization in extralymphoid tissues is still underway. The results presented in this thesis provide new insights into the recirculation and function of cutaneous γδ T cells and B-1 B cells. In Chapter 1, we demonstrated that ovine γδ T cells in the uninflamed and

inflamed skin and skin-draining lymph migrate through the skin independent of CCR7 with the capacity to make proinflammatory cytokines IL-17 and IFNγ. This is in line with studies in other species, demonstrating a strength of the ovine cannulation model to observe not only tissue-residing, but actively recirculating lymphocytes. In Chapter 2, we showed that B cells are a component of the cutaneous immune system. This was a heterogeneous population with both conventional and innate-like B cells. In chapter 3, we followed up on our ovine studies in the mouse and determined that B-1 B cells readily traffic to the skin in an alpha-4 integrin dependent mechanism. We furthermore revealed the ability of cutaneous B-1 B cells to secrete IL-10. Collectively, the body of work presented in this thesis highlights the diversity of lymphocyte subsets in the skin and provides novel targets for the modulation of cutaneous immune responses.

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