The Skin, a Novel Niche for Recirculating B Cells

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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 coexpress high levels of IgM and CD11b. Skin B cells have increased MHC class II, 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 Ab-secreting cells during inflammation increases local Ab titers, which could augment host defense and autoimmunity. Although skin B cells express typical skin-homing receptors, such as E-selectin ligand and α-4 and β-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. ***The Journal of Immunology*****, 2012, 188: 6027–6035.**

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 (1). Although B cells are found in the afferent lymph draining uninflamed skin of both sheep and humans (2, 3), the widely accepted view is that B cells do not enter the skin during homeostasis (4). In contrast, B cells accumulate in the dermis during infection and autoimmunity (5–7), 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 (MZ) and follicular B cells. In contrast, B-1 B cells are an innate-like subset that resides in the peritoneal and pleural cavities and responds to T-independent Ags, bridging innate and adaptive immune responses (8, 9). Although their primary residence is within the coelomic cavities, B-1 B cells are capable of exiting the body cavities in response to infection (10, 11); however, they have not been described to enter the skin.

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Abbreviations used in this article: ASC, Ab-secreting cell; MHCII, MHC class II; MZ, marginal zone; PBS-Tween, PBS containing 0.05% Tween-20.

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 Ref. 12). Primarily, blood-borne lymphocytes enter lymph nodes through high endothelial venules. Alternatively, lymphocytes recirculate through extralymphoid tissues, such as skin; 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. Although two distinct blood-borne B cell subsets differentially recirculate through lymph node or spleen (13), and IgA^+ B cells preferentially recirculate through mucosal sites (14), little is known about B cell recirculation through nonmucosal extralymphoid tissues. To home to the skin, CD4 T cells rely on the coordinated expression of E-selectin and α-4 and β-1 integrins and use the chemokine receptors CCR4, CCR8, and/or CCR10 (reviewed in Refs. 15–17). In contrast, the molecules involved in B cell migration to the skin remain uncharacterized.

To investigate B cells in the skin, we used a model of lymph cannulation (18) to 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 Ag presentation to T cells in situ, and Ab-secreting cells (ASCs), the effector stage of B cells, accumulate in the chronically inflamed skin, leading to increased local Ab titers. Although skin B cells express α-4 and β-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 use 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 mo of age, were purchased from 3/D Livestock (Woodland, CA), the University of Cal-

ifornia, 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 (19). Briefly, following lymphectomy, the afferent and efferent lymph vessels anastomose, forming pseudoafferent vessels that carry afferent (prenodal) lymph (19). 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. The cannulated lymphatics drained the skin and muscles of the rear flank (20). Every 1–12 h, lymph collection bottles were changed, and the composition and numbers of lymph-borne leukocytes were determined by flow cytometry to calculate the hourly output of different lymphocyte subsets: B cells and CD4, CD8, and γδ T cells. A total of 0.3–0.5 ml CFA, emulsified 1:1 with sterile saline, was injected s.c. into the drainage area of the prefemoral node to induce acute (<24 h) or chronic (>21 d) inflammation, as described (21). Mesenteric efferent lymph vessels were cannulated, as described (22), either in parallel to cutaneous afferent lymph vessel cannulation or as an independent nonsurvival 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 a French press coffee filter (Bodum) and washed. Blood was collected by venipuncture and mixed with heparin. Blood leukocytes were separated from RBCs by either gradient centrifugation with Histopaque-1077 (Sigma-Aldrich) or lysis with RBC 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 (FITC, PE, Alexa Fluor 647, allophycocyanin, PE-cyanin 7; Alexa Fluor 700, Pacific Blue) mAbs. The following mouse anti-sheep Abs were used: CD1 (20.27; Serotec), CD4 (44.38; Serotec), CD8 (38.65; Serotec), CD45 (1.11.32; Serotec), γδ TCR (86D; VMRD), MHC class II (MHCII; TH14B; VMRD), L-selectin (DU1-29; VMRD), and IgM (25.69; Serotec). The following mouse anti-human mAbs that also recognize sheep integrins (23, 24) were used: α-4 integrin (HP2/1; Serotec), β-1 integrin (TS2/16; eBioscience), and β-7 integrin (fib27; eBioscience). Supernatants for the following ovine Ags were produced from hybridomas: pan-B cell marker (2-104) (13, 25), CD21 (2-87-6) (26, 27), CD11b (12-5-4) (28), and CD11c (17-196) (17). Some mouse mAbs 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 Abs, allophycocyanin- (BD Bioscience), PerCP-Cy5.5- (BD Biosciences), Pacific Orange- (Invitrogen), 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 to verify that Ags 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 100 mM Tris-HCl (Teknova) and blocked with immunofluorescence buffer: 10% rat serum (Equitech-Bio) in 20 mM Tris with 0.9% NaCl and 0.05% Tween-20 (Sigma-Aldrich). Sections were incubated with anti-sheep IgM (25.69; Serotec), washed in immunofluorescence buffer, subsequently stained with anti-mouse IgG1 FITC (RMG1-1; BioLegend), and embedded with ProLong Gold Antifade (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 the manufacturer's 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. Bright-field images were acquired 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/population and animal was 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 (29, 30). Recombinant mouse CCL17 and recombinant human CCL1, CCL20, and CCL28 (R&D Systems) were titrated in triplicates between 1 and 100 nM or between 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 (Life Technologies), 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-inactivated 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 OD_{490} using an EMax Endpoint ELISA microplate reader (Molecular Devices). A standard curve derived from whole-ovine IgG (Jackson ImmunoResearch) on the same plate was used to quantify Ab 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-inactivated rabbit serum (Life Technologies). Cells were plated in RPMI 1640 containing 10% heat-inactivated rabbit serum (Invitrogen) and incubated for 12–14 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 the manufacturer's instructions, and allowed to dry overnight, and the spots were enumerated using an Olympus SZ51 dissecting microscope (Olympus). The limit of detection was 5 ASCs/10^6 cells. Values below this threshold are displayed as 0. ELISPOT wells were photographed using an ImmunoSpot Reader and Image Acquisition software (both from Cellular Technology).

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. The p values < 0.05 were 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 (3, 4), 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 used a lymph-cannulation model established by Lascelles and Morris (18). By cannulating the skin-draining afferent lymphatics, the model allows for the analysis of lymphocytes during their physiological recirculation through uninflamed and inflamed skin (31). Because of 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. (2), 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 consisted of both small and slightly larger lymphocytes (Fig. 1C). The small B cells averaged <10 μm in diameter with a scant rim of basophilic cytoplasm, whereas larger B cells >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 (32).

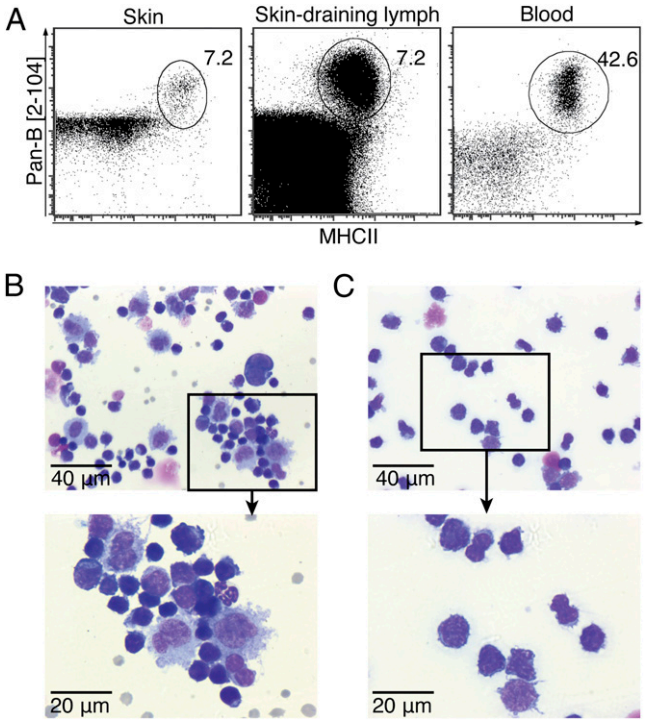

FIGURE 1. B cells reside in and recirculate through uninflamed skin. Lymphocytes from uninflamed skin, uninflamed skin-draining afferent lymph, and peripheral blood from adult sheep were analyzed. (**A**) Flow cytometric analysis of the expression of pan-B cell marker recognized by clone 2-104 and MHCII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

We used an established model of granulomatous skin inflammation, in which inflammation is induced by s.c. injection of CFA (21, 22). Draining lymph vessels were surgically cannulated 3-4 wk later when the typical skin granulomas had formed at the injection sites. As previously shown for total lymphocytes and T cells (21), 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 γδ 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-wk-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% versus 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). Although only very few IgM^+ B cells were visible in the deep dermis of the uninflamed skin (Fig. 3B, 3C), the dermal and s.c. granuloma harbored numerous IgM^+ B cells that were dispersed throughout the tissue (Fig. 3D, 3E) or clustered 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 contribution of B cells 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 colocalizing skin T cells. To explore whether skin B cells are equipped for efficient T cell activation, we examined Ag-presenting and costimulatory molecule expression on skin B cells. CD1 is a key Ag-presenting and regulatory molecule related to MHCI that presents lipid molecules to CD1-restricted T cells, such as NKT cells and some γδ T cells, both of which are known to play important roles in the immunosurveillance and immunoregulation of the skin (33, 34). 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 with 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 γδ T cells, generating an effective cutaneous immune response.

Compared with 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 Ag presentation to both canonical and CD1-restricted 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 into the capacity to modulate cutaneous immune responses. IgM^{hi} ex-

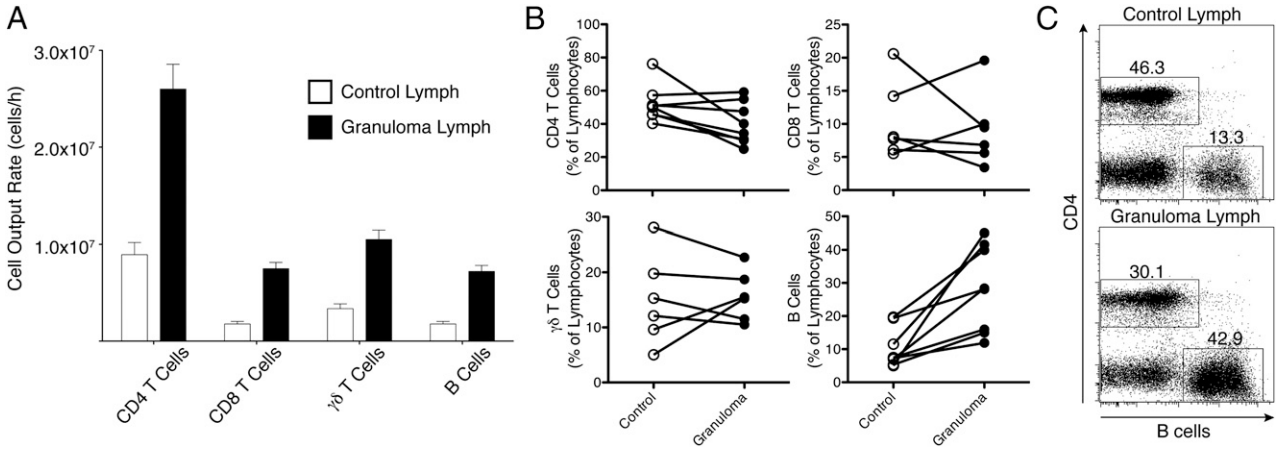

FIGURE 2. B cell traffic increases in chronic skin inflammation. Chronic cutaneous inflammation was induced by s.c. injection of CFA into sheep flanks. Ovine lymph was collected after catheterization of draining afferent lymphatics of uninflamed (control) or chronically inflamed (3–5 wk after induction 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 represent the mean ± SEM of multiple time points analyzed for cell output from control (uninflamed) and granulomatous skin. One representative animal of at least four 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 eight individually analyzed sheep is shown.

pression marks more innate-like B cells, such as 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, 5B), 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 (35) 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, 5B). Data suggest that MZ B cells recirculate in humans (36), and MZ B cells are characterized by high expression of CD1 (8). 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

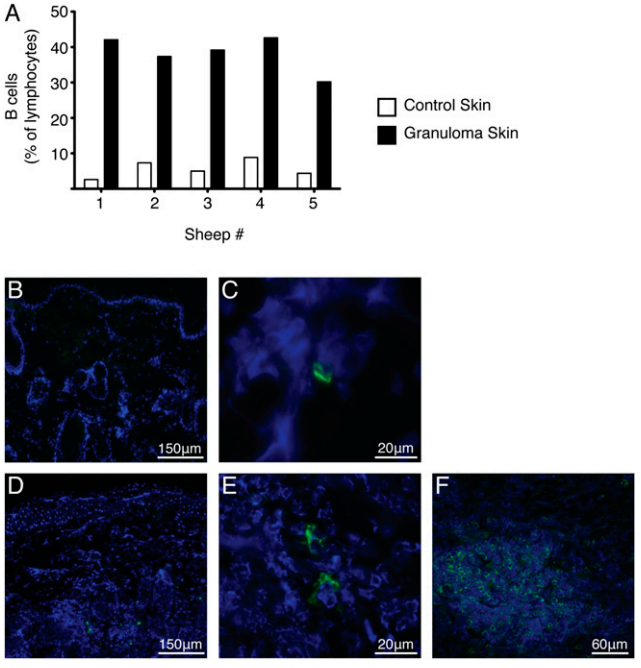

FIGURE 3. B cells accumulate in chronically inflamed skin. Healthy control skin and CFA-induced skin granulomas were analyzed 3–5 wk post-induction of inflammation. (**A**) The percentage of (pan-B cell marker) 2-104–reactive B cells among total lymphocytes isolated from uninflamed and granulomatous skin was determined by flow cytometry for all animals (n = 5). (**B**–**F**) Immunofluorescence staining of IgM (FITC, green) and DAPI (blue) on 6 μm-thick frozen sections of uninflamed (B, C) or granulomatous (D–F) skin. One representative staining of at least four animals is shown.

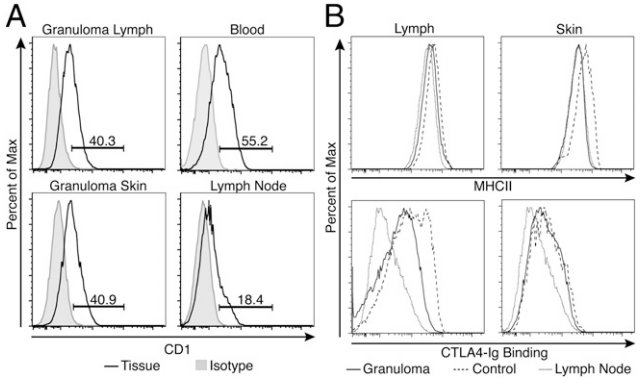

FIGURE 4. Skin B cells are well suited for Ag presentation. Lymphocytes from skin granuloma and control skin, skin-draining afferent lymph, and peripheral blood were isolated from sheep. (**A**) Flow cytometric analysis of CD1 and isotype-control staining on gated B cells (pan-B cell marker 2-104$^+$, MHCII$^+$ lymphocytes) from skin granuloma-draining afferent lymph and blood. Numbers indicate the percentage of positive B cells. (**B**) Flow cytometric analysis of MHCII 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 four (CD1), seven (MHCII), or six (CTLA4-Ig) individually analyzed animals is shown.

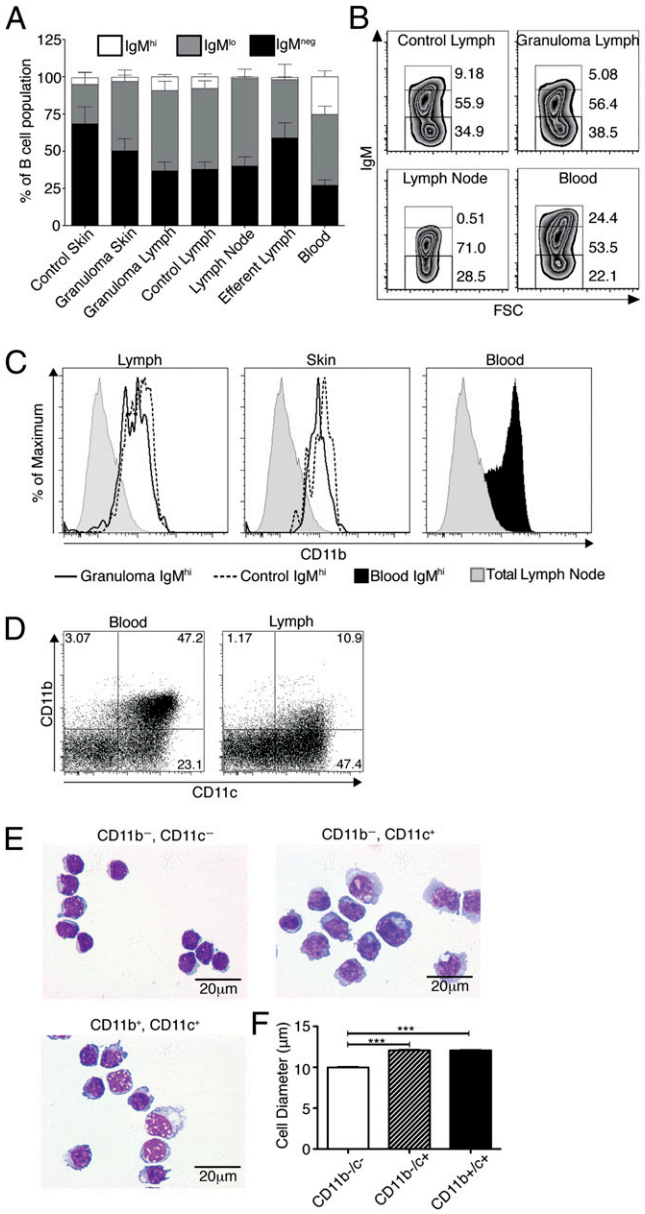

FIGURE 5. Larger B cells and B-1–like B cells recirculate through the skin. Lymphocytes from skin-draining afferent lymph, control skin-draining lymph node, blood, and skin were isolated from sheep. (**A**) The relative distribution of IgM^{hi}, IgM^{lo}, and IgM^{neg} among B cells (pan-B cell marker $2\text{-}104^+$, $MHCII^+$ lymphocytes) from different tissues based on flow cytometry. Data represent the mean ± SEM of four to eight 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 with total lymph node B cells (pan-B cell marker $2\text{-}104^+$, $MHCII^+$ lymphocytes). One representative animal of at least three (control skin) or four (all other tissues) individually analyzed animals is shown. (**D**) Flow cytometric analysis of CD11b and CD11c coexpression 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, cytospun, and stained with Pappenheim stain for cytological evaluation. (**F**) Quantification of cellular diameters from (E). One representative of a minimum of three individually analyzed lymph samples is shown in (D–F). $^{***}p < 0.0001$.

$CD1^+$ B cells in the granuloma and granuloma lymph are not MZ B cells (data not shown). Because B-1 cells express CD1 (37), we wondered whether skin-associated B cells belong to this subset. In sheep, CD11b is a marker of B-1–like cells (35); 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 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 (35), we determined CD11c expression by skin B cells and found that CD11c was expressed at higher frequencies than was 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 consisted of mature lymphocyte ~10 μm in 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). 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 $CD11c^+$ cells are more activated lymphoid cells in contrast to the more quiescent morphology of the dual-negative cells.

Ab titers and ASCs increase locally during chronic inflammation

Abs secreted by effector stage B cells, ASCs, are critical to host defense, as well as to autoimmunity and allergy. Having found that B cells accumulate in the chronically inflamed skin, we wondered whether an increase in localized Ab titers was a functional consequence. To address this, we compared total Ig levels in the plasma of blood and lymph and found that, although blood titers were always highest, Ab 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

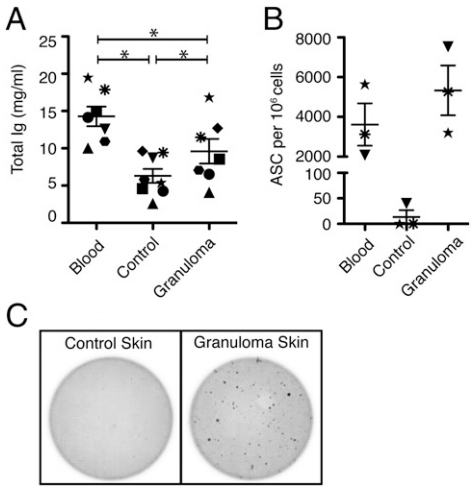

FIGURE 6. Ab titers and ASCs increase locally during chronic skin inflammation. (**A**) ELISA of total Ig Ab titers in plasma from blood and afferent lymph draining control skin or chronically inflamed skin (granuloma). (**B**) ELISPOT assay analyzing total Ig-secreting cells in blood, uninflamed control, or granulomatous skin of the same animal. In (A) and (B), individual animals are identified by unique symbols; bars represent the mean ± SEM. (**C**) Developed ELISPOT wells analyzing 2×10^5 and 1×10^4 cells/well from uninflamed control and granulomatous skin, respectively. One representative animal of three is shown. $^*p < 0.05$.

correlated with Ab 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, 6C). Ab titers in the draining lymph did not increase to the same extent as did the ASCs in the tissue. This is not unexpected, because the cannulated lymphatics drain a larger site than just the granuloma region, causing the Ab titers to be a diluted average of the entire drainage site.

Although chronic inflammation is generally not associated with increased vascular leakage, we excluded that the increased Ab 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 (<24 h), which is characterized by drastic blood vascular leakage and edema, and found that Ab 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 Ab 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 (13). 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 (13). We compared B cells in the afferent lymph draining the skin with 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 of 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 nonrecirculating B cell pools.

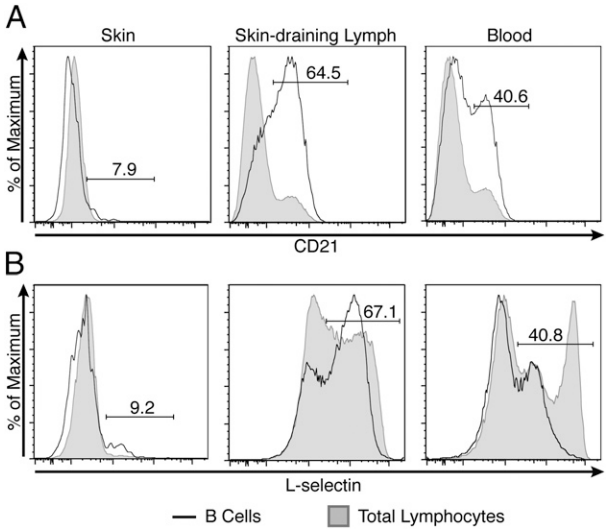

FIGURE 7. CD21 and L-selectin expression on skin-associated B cells. Flow cytometric analysis of CD21 (**A**) or L-selectin (**B**) on gated B cells (open graphs) and total lymphocytes (filled graphs). Numbers indicate the percentage of positive B cells in the specified gates. One representative animal of at least five individually analyzed animals is shown.

Skin B cells use 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 (24), CD4 T cells in skin-draining lymph expressed high levels of both α-4 and β-1 integrins, as well as low levels of β-7 integrin (Fig. 8A, *top panels*). As expected (24), CD4 T cells in the blood contained a population of cells that expressed high levels of α-4 and β-7, consistent with the presence of gut-homing T cells (Fig. 8A). Unexpectedly, lymph-borne B cells showed equal or lower expression of α-4 and β-1 integrins, but higher levels of β-7 integrin, relative to coisolated 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

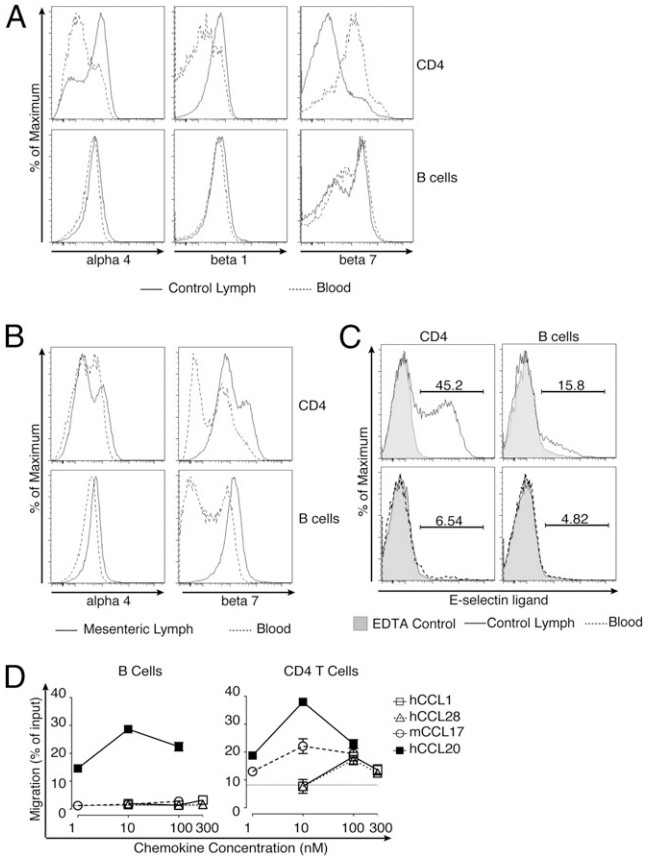

FIGURE 8. Homing receptor expression and chemotactic responsiveness of skin B cells. (**A**) Flow cytometric analysis of α-4, β-1, and β-7 integrins on gated CD4 T cells and B cells from afferent lymph draining uninflamed skin (solid line) or blood (dashed line). One representative animal of at least six individually analyzed animals is shown. (**B**) Flow cytometric analysis of α-4 and β-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 four individually analyzed animals is shown. (**C**) Flow cytometric analysis of E-selectin binding by gated CD4 T cells and B cells from afferent lymph draining uninflamed skin or blood. Shaded graphs represent control staining in EDTA. One representative animal of at least three individually analyzed animals is shown. (**D**) Chemotaxis of lymph-borne B cells and CD4 T cells toward human (h) CCL1, CCL20, and CCL28, as well as mouse (m) CCL17, was tested ex vivo in a Transwell chemotaxis assay. Data are expressed as the percentage of cells of the respective subset that migrated to the lower chamber, and data represent the mean ± SD of triplicate wells at each concentration. Horizontal lines indicate migration to media alone. One representative animal of at least four individually analyzed animals is shown.

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 β-7 integrin (Fig. 8A, 8B), which, when paired with α-4 integrin, 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, although 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 (15–17). Data imply that CCR6 mediates the migration of Langerhans cell precursors into skin (38, 39). Therefore, we tested whether skin-associated B cells migrate in response to ligands for these skin-associated chemokine receptors in an in vitro chemotaxis assay. In contrast to coisolated 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 Ab 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 (6, 7, 40). 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 (4). 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 other investigators showed that B cells travel in the afferent lymph draining from uninflamed human skin (3), 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 (B cells and CD4, CD8, and γδ T 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 *Mycobacterium tuberculosis* granulomas of the lung, in which they play a protective role by aiding in the recruitment of other immune cells (41). B cells may fulfill a similar role in chronic skin inflammation. We found increased expression of Ag-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 naive 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 Ag presentation could be of critical importance, because B cells are able to present their cognate Ag efficiently at very low concentrations relative to other APCs (42).

B cells are key players in many autoimmune diseases, even in those that were primarily considered T cell mediated, such as multiple sclerosis (6, 43). Furthermore, B cell depletion in humans revealed a role for B cells in cutaneous disorders, including bullous skin diseases (6) and atopic eczema, a condition not typically associated with autoantibody (44). 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 Ab production (i.e., Ag presentation and cytokine production) are critical in cutaneous immune responses (6).

B-1 cells are typically located within the peritoneal and pleural cavities, sites of constant microbial exposure. By producing natural Ab 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 (8). 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 (35). Ninety percent of efferent lymph lymphocytes are blood derived, and only 10% originate from the afferent lymph (12, 45). Therefore, the appearance of 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 (46) and to migrate into the lung and draining lymph nodes during pulmonary infection (10, 47). Additionally, B-1 cells migrate from the peritoneal cavity to the skin-draining lymph nodes during the course of cutaneous contact hypersensitivity (48). Our study extends these findings by showing that B-1–like cells recirculate through the skin itself; thus, they are well positioned to participate in protective, as well as harmful, cutaneous immune responses.

Abs 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 Ab does not fully penetrate and/or saturate the skin. This finding could be of great consequence to the many disease treatments that use depleting Ab if i.v. 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). This localized Ab production in the skin likely represents a mechanism by which the body clears or contains an ongoing skin infection and ensures protection against reinfection. Although 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 Ab production has the potential to exacerbate inflammation in autoimmune diseases that affect the skin, such as cutaneous lupus erythematosus or pemphigus.

Young et al. (13) found that efferent lymph B cells segregate into recirculating and nonrecirculating 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. 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 costain for both of these markers (Fig. 7) than what was shown for efferent lymph B cells (13). 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.

Although the mechanisms by which T cells migrate into skin are well characterized and critical to cutaneous inflammation and immunosurveillance (16), little is known about B cell migration into skin. In this study, we observed that B cells in the skin-draining lymph expressed high levels of α-4 and β-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 (49). 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 evolutionarily conserved. However, coisolated ovine skin B cells were unresponsive to ligands for these receptors (Fig. 8), suggesting that B cells use alternative chemokine receptors to migrate into 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 (38). Although most blood-borne B cells express surface CCR6, they lack responsiveness to CCL20 but acquire it upon BCR stimulation (50). 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 Ab to human CD20, depletes circulating B cells to effectively treat B cell lymphomas and autoimmune disorders, including skin diseases (6). 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 Ab reside in and/or continuously recirculate through healthy and inflamed skin. Thus, our study reveals a so-far unappreciated role for skin B cells as potential regulators of cutaneous immunity and inflammation.

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Disclosures

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