γδ T Cell Homing to Skin and Migration to Skin-Draining Lymph Nodes Is CCR7 Independent

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γδ T Cell Homing to Skin and Migration to Skin-Draining Lymph Nodes Is CCR7 Independent

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In most species, γδ T cells preferentially reside in epithelial tissues like the skin. Lymph duct cannulation experiments in cattle revealed that bovine dermal γδ T cells are able to migrate from the skin to the draining lymph nodes via the afferent lymph. For αβ T cells, it is generally accepted that epithelial and mucosal tissue egress is regulated by expression of the CCR7 chemokine receptor. In this study, we tracked the migratory route of bovine lymph-derived γδ T cells and examined their CCR7 cell surface expression in several compartments along this route. Total lymph cells from afferent and efferent origin were labeled with PKH fluorescent dyes and injected into the bloodstream. PKH+ cells already reappeared in the afferent lymph after 4 h. The vast majority of the PKH+ cells retrieved from the afferent lymph were of the WC1+ γδ T cell phenotype, proving that this PKH+ γδ T cell subset is able to home to and subsequently exit the skin. PKH+ γδ T cells from afferent and efferent lymph lack CCR7 surface expression and display high levels of CD62L compared with CD4 T cells, which do express CCR7. Skin homing receptors CCR4 and CCR10 in contrast were transcribed by both CD4 and γδ T cells. Our findings suggest that γδ T cell skin egress and migration into the peripheral lymphatics is CCR7-independent and possibly mediated by CD62L expression. The Journal of Immunology, 2012, 188: 000–000.

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Abbreviations used in this article: DETC, dendritic epidermal T cell; HEV, high endothelial venules; LN, lymph node; PLN, prescapular lymph node.

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Materials and Methods

PCR and cloning of bovine CCR7

Bovine lymphocyte cDNA was used as template material for PCR. PCR reactions were performed in a 20 μl reaction volume containing 5 pmol of each primer using Pfu polymerase (Invitrogen) according to the protocol supplied by the manufacturer.

Primers BovCCR7For+Kpn1, 5'-GGCTACCTCCCAGGCTGGG-GAAGCC-3' and BovCCR7Rev+BstEI, 5'-GCTGATGTTGGCCGGTCGAGAACAGGTGTTG-3' were designed based on a published sequence of bovine CCR7 in GenBank (http://www.ncbi.nlm.nih.gov/nuccore/NM_001024930) and were used under the following cycling conditions: an initial denaturation step of 7 min at 95°C, followed by 35 cycles of 30 s at 45°C, 45 s at 55°C, 1 min at 72°C, followed by a final elongation step of 5 min at 72°C. PCR products were visualized in a 1% TAE agarose gel, and bands of the correct size (1175 bp) were purified using the Zymoclean Gel DNA recovery kit (Baseclear). PCR products were ligated into the pcR4 blunt TOPO vector (Invitrogen) and transformed into TOP10 competent Escherichia coli (Invitrogen). Plasmid DNA was purified with the Zymo Miniprep kit and digested with KpnI and BstEII restriction enzymes (New England Biolabs). Digestions were electrophoresed on a 1% TAE agarose gel, and the desired fragment was purified from gel as described before. Subsequently, the purified fragment was ligated into a pcDNA3.1+ vector (Invitrogen) with compatible overhangs and a C-terminal His6-tag sequence with T4 DNA Quickligase (New England Biolabs) in a 5-min reaction at room temperature. PCR product was cloned in the BstEII and KpnI sites of pcDNA3.1+ (pcDNA-CCR7). The sequence of pcDNA-CCR7 was confirmed by sequencing at Baseclear (Leiden, The Netherlands).

Transfection and selection

CHO-K1 cells were cultured in 6-well plates in Optimem Glutamax medium (Life Technologies) containing 10% FCS. The cells were transfected with pcDNA3-CCR7 at 80% confluence using Fugene 6 transfection reagent (Roche) according to the manufacturer’s protocol. At 24 h posttransfection, Geneticin (G418; Invitrogen) was added in a concentration of 0.5 mg/ml. At 72 h after transfection, the G418 concentration was raised to 1 mg/ml to select for cells containing the pcDNA3-CCR7 vector. The surviving CHO-K1 cells were screened for cross reaction with the Alexa Fluor 647 Rat Anti-Human CD197 (CCR7) Ab (clone 3D12) and the Alexa Fluor 488 Rat IgG2a, κ Isotype Control (BD Pharmingen) by flow cytometry on a FACScalibur (Becton Dickinson). A total of 400 CCR7 (clone 3D12) positive cells were sorted by flow cytometric single-cell sorting (BD Influx, Becton Dickinson) before further phenotyping and analyses.

Animals and surgical procedures

Seven Holstein-Friesian calves (3 mo of age) were used in this study. Calves were housed in a conventional animal house, fed according to requirements, and checked daily for general health. The calves were cannulated bilaterally to collect simultaneously afferent lymph on the left side and efferent lymph on the right side of the animal. The basic technique of prescapular lymph duct cannulation for collecting pseudoafferent lymph from skin has been reported previously (18). The current surgical procedures have been described in detail elsewhere (4), with the modifications that the efferent lymph vessel of the right PLN was cannulated as described earlier (19). After labeling, the cells were injected back into the bloodstream of the donor calves via the jugular vein.

Cell labeling and tracking

Approximately $1.7 \times 10^{10}$ afferent and efferent lymph cells were labeled with PKH26 and PKH67 (Sigma Aldrich), respectively. Labeling of lymph cells was performed as described earlier (19). After labeling, the cells were tested for repeated cross-reactivity with the anti-CCR7 Ab (clone 3D12) by flow cytometric single-cell sorting (BD Influx, Becton Dickinson) before further phenotyping and analyses.

mRNA analysis

Transcripts of CCR4, CCR7, and CCR10 were analyzed using quantitative RT-PCR on sorted CD4 and γδ T cells from PBMCs, efferent lymph cells, and afferent lymph cells. Primer and probe sequences used for quantitative RT-PCR of CCR4, CCR7, and CCR10 and reference gene GAPDH have been described previously (20). CD4 and γδ T cells were separated by flow cytometric cell sorting and counted using an BD Influx (Becton Dickinson). Sorted cells were lysed, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cdNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Subsequently, quantitative RT-PCR was carried out using Bio-Rad IQ supermix (Bio-Rad Laboratories) on a Bio-Rad MyIQ Single-Color Real Time PCR Detection System (Bio-Rad Laboratories) using 50 ng cDNA as starting template. Final primer and probe concentrations were 1 μM and 4 nM, respectively. Amplification consisted of an initial denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final elongation step of 5 min at 72°C. Relative expression ratios were calculated using the Pfaffl method described previously (21).

FIGURE 1. T cell subset distribution. Subset distribution of CD4 and γδ T cells as a percentage of total lymphocytes present in PBMCs, afferent lymph, efferent lymph, and PLNs. Data shown are the means of three independent experiments with PBMCs, afferent and efferent lymph, and of two independent experiments with PLNs of three animals. Error bars indicate SEM. *p < 0.05, **p < 0.005.
Afferent lymph cells were obtained from afferent lymph draining the skin and from different compartments of the bovine peripheral lymphatic system, to gain insight into the lymphocyte subset distribution in the lymph compartment. The same accounts for the efferent lymph, in which presence of PKH-labeled cells. Reappearance of PKH-labeled cells in the afferent lymph was interpreted as a proof for skin homing and subsequent skin egress. PKH-labeled cells reappearing in the efferent lymph could have entered this compartment by directly entering the PLN from the blood across HEVs or by traveling via the skin and the afferent lymph. Results are shown in Fig. 2. At 4 h after reinjection, both PHK26 and PKH67 cells could already be recovered from the afferent lymph, indicating that lymph cells of both afferent and efferent origin only need 4 h to enter the skin from the bloodstream and exit the skin again into the afferent lymphatic system.

Results

Efflux of γδ T cells from the bovine skin into the peripheral lymph compartment

To gain insight into the lymphocyte subset distribution in the different compartments of the bovine peripheral lymphatic system, we tested cells obtained from afferent lymph draining the skin and from efferent lymph draining the PLNs of three calves. The proportion of CD4 T cells and γδ T cells in the total lymphocyte pool of these compartments was analyzed by flow cytometry and compared with proportions found in blood (PBMCs). Results are summarized in Fig. 1. The afferent lymph showed a large presence of γδ T cells, ranging from 48 to 63% of the total lymphocyte population. The same accounts for the efferent lymph, in which γδ T cells made up 45 to 82% of the total lymphocyte population. The γδ T cells in both afferent and efferent lymph were almost exclusively of the WC1 subset (data not shown). When comparing the CD4 and γδ T cell compositions of afferent and efferent lymph with blood, we observed that there is a 2-fold enrichment of γδ T cells in the afferent and efferent lymph implies that there is substantial egress of γδ T cells from the skin trafficking into the draining lymphatic system ultimately draining into the venous circulation. The relatively low proportion of γδ T cells found in the LN under homeostatic conditions has been described earlier in sheep (19) and contributes to the idea that γδ T cells travel through the lymphatic system with minimal interaction in the LN.

γδ T cells represent the main recirculating cell subset in lymph

To qualify and quantify the recirculation of bovine lymphocytes, we conducted in vivo cell tracking experiments in two calves. Afferent and efferent lymph cells were obtained and labeled with a PKH26 and PKH67 fluorescent dye, respectively, and were injected back into the bloodstream of the jugular vein. At several time points after reinjection, lymph cell samples from both the afferent and efferent lymph were taken and analyzed for the presence of PKH-labeled cells. Reappearance of PKH-labeled cells in the afferent lymph was interpreted as a proof for skin homing and subsequent skin egress. PKH-labeled cells reappearing in the efferent lymph could have entered this compartment by directly entering the PLN from the blood across HEVs or by traveling via the skin and the afferent lymph. Results are shown in Fig. 2. At 4 h after reinjection, both PHK26 and PKH67 cells could already be recovered from the afferent lymph, indicating that lymph cells of both afferent and efferent origin only need 4 h to enter the skin from the bloodstream and exit the skin again into the afferent lymphatic system.
lymph (Fig. 2A). In the efferent lymph (Fig. 2B), the first PKH-labeled cells appear at 4 h after reinjection as well. Cellular outflow rates of the afferent lymph (right cannula) and efferent lymph (left cannula) were determined at each time point to calculate the total number of PKH-labeled cells present in each compartment at a certain time. The maximum absolute number of PKH-labeled cells recovered from the afferent lymph was reached at 10 h postinjection after which it declined, whereas the number of PKH-labeled cells recovered in the efferent lymph increased over time postinjection after which it declined, whereas the number of PKH-labeled cells recovered from the afferent lymph was reached at 10 h post reinjection or later. By combining the labeled cells recovered in the efferent lymph increased over time at a certain time. The maximum absolute number of PKH-labeled cells present in each compartment (left cannula) were determined at each time point to calculate the flow rates of the afferent lymph (right cannula) and efferent lymph.

Afferent origin of the lymph with the cellular outflow rate and the total numbers of cells was highest at 28 h post-reinjection or later. By combining the total numbers of PHK-labeled cells recovered from the afferent lymph with the cellular outflow rate and the total numbers of cells originally injected (1.7 × 10^9 of both PKH26- and PKH67-labeled cells), we calculated that over the period of 28 h, 0.6% of the total reinjected afferent labeled cells (PKH26) have recirculated through the efferent lymph in addition to the total reinjected efferent labeled cells (PKH67). Similar calculations were made for the efferent lymph, indicating that 7.8% of the total reinjected afferent labeled cells (PKH26) have migrated through the efferent lymph cannula in 28 h versus 4.6% of the total reinjected efferent labeled cells (PKH67). When comparing the percentages of cells of afferent and efferent origin recovered from afferent lymph (0.6% of afferent origin versus 0.3% of efferent origin) and efferent lymph (7.8% of afferent origin versus 4.6% of efferent origin), the recovery percentage of cells of afferent origin is ~2 fold higher in both compartments under equal lymph flow conditions.

The phenotype of the PKH26- and PKH67-labeled cell subsets recovered from both lymph compartments was up to 95% γδ positive at all four time points, indicating that the recirculating cell populations consisted of almost exclusively γδ T cells until at least 28 h after reinjection. Compared with total afferent and total efferent populations of γδ T cells described earlier (Fig. 1), there is an enrichment for γδ T cells in the recirculating PKH+ populations, and γδ T cells somehow are the preferential cell type to recirculate. Most importantly, from the appearance of PKH+ γδ T cells in the afferent lymph, we can conclude that recirculating γδ T cells are able to home to and subsequently also egress from the skin.

Recirculating γδ T cells lack CCR7 expression contrary to CD4 T cells

Under homeostatic conditions, CD4 T lymphocytes require expression of the LN homing CCR7 chemokine receptor to exit peripheral tissue and enter the afferent lymphatics (16, 17). Because γδ T cells were found to travel from the blood into the afferent and efferent lymph, we examined the expression of CCR7 in the compartments involved in their route of travel. PBMCs, afferent and efferent lymph cells, and cells from the PLNs of three calves were tested for CCR7 expression using the rat anti-human CD197 (CCR7) Ab (clone 3D12) in flow cytometric analysis. Using CHO-K1 cells transfected with bovine CCR7, the anti-CCR7 Ab (clone 3D12) was found to cross-react with recombinant bovine CCR7 (Fig. 3A) and was used in our assays to define CCR7 expression on bovine CD4 and γδ T (Fig. 3B–D). CCR7 is expressed on the majority of the CD4 T cells in blood, efferent lymph, and the PLN, whereas the fraction of CCR7-expressing γδ T is below 10% in all compartments. CD4 T cells in the afferent lymph display less CCR7 than the CD4 cells in the other compartments.

PKH+ recirculating γδ T cell subsets in afferent and efferent lymph were also tested for CCR7 expression. Afferent and efferent lymph samples taken at 28 h after reinjection were enriched

![FIGURE 4. CCR7 expression in recirculating γδ T cell subsets. Flow cytometric analysis of afferent and efferent PKH67+-sorted lymph cells collected at 28 h after reinjection of PHK67-labeled cells (effferent origin). Sorted afferent cells were 86% PKH67+ and sorted efferent cells were 40% PKH67+. γδ TCR+ gated afferent (left panel) and efferent (middle panel) cells and CD4+ gated efferent cells (right panel) were analyzed for PKH67 positivity and expression of CCR7. Data of one experiment with one animal are shown.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
for PKH26<sup>+</sup> and PKH67<sup>+</sup> cells by flow cytometric cell sorting to phenotype these cells. The difference between γδ and CD4 T cell CCR7 expression is shown in Fig. 4. Reinjected PKH67<sup>+</sup> γδ T cells originating from the efferent lymph show minimal CCR7 expression when recovered from either the afferent or the efferent lymph. The same holds for reinjected PKH26<sup>+</sup> γδ T cells originating from the afferent lymph (data not shown). We observed that γδ T cells expressing higher CCR7 (<1% within γδTCR<sup>+</sup>-gated cells) have a larger forward scatter, indicating that these cells might be activated. It is possible that in this activated state, some γδ T cells do express CCR7. However, this expression is marginal in comparison with observed γδ T cell migration and contrasts with the high cell surface expression on CD4 T cells. Because both afferent and efferent lymph γδ T cells lack CCR7 expression compared with CD4 T cells, we conclude that it is unlikely that γδ T cells require CCR7 expression to exit the skin or to pass through the PLNs.

γδ T cells transcribe skin homing receptors CCR4 and CCR10

In addition to cell surface expression, CCR7 gene expression was analyzed using quantitative RT-PCR (Fig. 5A). Quantitative RT-PCR data correlated highly with protein expression data as determined by flow cytometry. Subsequently, gene expression of the skin homing receptors CCR4 and CCR10 was investigated. CCR4 (Fig. 5B) and CCR10 (Fig. 5C) were transcribed by both γδ T cells and CD4 T cells, illustrating that both cell types have the ability to home to the skin. CD4 T cells expressed higher CCR4 whereas γδ T cells expressed higher CCR10 in blood. CCR10 ligands (e.g., CCL27) are constitutively produced by keratinocytes (22, 23), whereas CCR4 ligands (e.g., CCL17) are upregulated under inflammatory conditions (24). Although both cell types have the ability to home from the blood to the skin, it is possible that γδ T cells mainly do so under homeostatic conditions to exert their function, whereas CD4 T cell traffic and function is more related to skin inflammation.

γδ T cells have high levels of CD62L cell surface expression

In addition to the chemokine receptors, we investigated cell surface expression levels of several adhesion molecules by flow cytometry. The β1 (VLA-4) and β2 (CD18) integrin expression was comparable in both CD4 and γδ T cells for all compartments, except for the afferent lymph where expression of both integrins was higher in CD4 T cells (Fig. 6A). CD62L expression was observed to be substantially higher on γδ T cells throughout all compartments as shown in Fig. 6B and 6C. CD4 T cells in the PBMC and efferent lymph compartment consist of a CD62L<sup>hi</sup> and CD62L<sup>lo</sup> population, representing pools of central (CD62L<sup>hi</sup>) and effector (CD62L<sup>lo</sup>) memory T cells in cattle (25). High CD62L expression is a known feature of ruminant γδ T cells and probably contributes to their highly mobile profile, as suggested by previous studies (19, 26).

**Discussion**

In the current study, we describe substantial, CCR7-independent recirculation of bovine γδ T cells through skin. The γδ T cells were the main recirculating subset in both afferent and efferent lymph, able to traffic from the blood through the skin back into the afferent and efferent lymph within 4 h. Both CD4 and CD8 T cells require CCR7 expression for their egress from resting extra- and lymphoid tissues into draining LNs via the afferent lymph (16, 17), and lymphatic endothelial cells of the afferent lymphatics constitutively express the CCR7 ligand CCL21 (13–15). CCL21 responsiveness in chemotaxis assays has been described for a subset of CD8<sup>+</sup> γδ T cells previously (27). CCR7 transcript expression in WC1<sup>+</sup> γδ T cell subsets, however, was found to be low by others (28). In our studies, we found minimal CCR7 cell expression in WC1<sup>+</sup> γδ T cells.
surface expression on recirculating γδ T cells inafferent and efferent lymph, contrasting with CCR7 expression on CD4 T cells. From this finding, we conclude that γδ T cells predominantly use a CCR7-independent pathway for extra-lymphoid tissue egress and subsequent migration into the afferent lymphatics. It is possible that high CD62L expression is involved in the CCR7-independent tissue egress (29) and migratory behavior (30, 31) of γδ T cells, likely in conjunction with other, yet undefined, chemokine receptors. Tissue egress of Th1 cells involving alternative exit receptors has been described in mice with chronic skin inflammation recently (32).

Classic sheep cannulation experiments have demonstrated that the afferent lymph input only accounts for ~10% of all cells found in the efferent lymph (11). This corresponds with the data from our cell tracking experiments. Of the PKH-labeled recirculating cells recovered in efferent lymph, we calculated that ~4.6% of the cells of efferent origin and 7.8% of the cells of afferent origin had previously traveled through the afferent lymph. So, >90% of the recirculating cells found in efferent lymph have entered directly from the blood via the PLN. Because our recirculating subset contained up to 95% γδ T cells, this implies that γδ T cells are capable of entering LNs directly from the blood like naive αβ T cells. Lymphocyte LN entrance from the blood requires passing of the LN’s HEVs. Interactions between peripheral-node addressins displayed on the HEV and CD62L on naive αβ T cells result in initial tethering and rolling of the T cell to the HEV surface. The subsequent firm adhesion of the cell to the vascular endothelium, required for transmigration and LN entrance, is mediated by CCR7-dependent integrin activation and clustering (33–35). The minimal display of CCR7 on the cell surface of blood-derived γδ T cells implies that the mechanism for entering LNs from the blood is presumably different from the mechanism used by naive αβ T cells.

Furthermore, skin homing was investigated by determining the expression of the CCR4 and CCR10 chemokine receptors with quantitative RT-PCR. These CCRs have been implicated in T cell skin homing of both CD4 (36, 37) and γδ (38, 39) T cells. CCR4 and CCR10 and their ligands have been identified as being expressed in cattle (20, 40, 41), and transcripts were found in both CD4 and γδ T cells. γδ T cells expressed higher CCR10 and CD4 T cells higher CCR4. CCR10 ligands are constitutively expressed, whereas CCR4 ligands are upregulated during skin inflammation. This could reflect the steady-state traffic and function of γδ T cells versus the more activation-induced traffic and function of CD4 T cells in the skin. Another possibility is that CCR10 is more important for the skin homing of γδ T cells from the blood than it is for skin homing of CD4 T cells (42).

We found a remarkably high expression of CD62L on γδ T cells in blood compared with that on CD4 T cells that was described earlier in the literature (19). It is possible that this high CD62L expression contributes to LN access like it does in naive CD4 T cells. In the murine literature, high CD62L expression has also been correlated with active trafficking behavior involving LNs (29, 29, 43). Notably, some γδ T cell subsets also have the ability to perform intranodal movements in (mesenteric) LNs. Similar findings have not been reported in cattle to date. In peripheral LNs like the PLN, no signs of accumulation or interaction were found, and bovine γδ T cells are thought to pass through more quickly than other lymphocytes (19). This corresponds to the histological distribution of bovine γδ T cells in an LN, where they are restrictively found in the traffic zones and not in the B and T cell areas (44–46). Considering the intranodal migratory requirements of CD4 T cells (47), lack of CCR7 as found in our current study could be responsible for the short LN transition time of bovine γδ T cells.

In this study, we describe a rapid recirculation of bovine γδ T cells between the skin, the blood, and the PLN during homeostatic conditions. The exact reason for this constant recirculation is unknown. In a previous study (4), we phenotypically analyzed migrating γδ T cells for the expression of MHC class II and specific activation markers [IL-2R or CD25 (48, 49)]. Ag-presenting functions by γδ T cells, including bovine γδ T cells, have been previously reported (48, 50). The γδ T cells from the afferent lymph, however, were all MHC class II negative and did not express markers reflecting recent activation (4). Steady-state T cell trafficking to and from organs with large epithelial surfaces like the skin, as described in our current study, probably has a role in immune surveillance (2).

In the murine and human literature, the identification of new γδ T cell subsets is advancing. Trafficking subpopulations associated with skin, as described in our current study, have also been identified in mice (51) and humans (52) recently. Their phenotype and migratory behavior, however, is dissimilar to that of the migratory γδ T cell subset we found in cattle. For now, it remains unclear if a homologous subpopulation exists in mice and man.

We conclude that γδ T cells may use different trafficking pathways than αβ T cells. Our study indicates that trafficking molecules used by αβ T cells (e.g., CCR7) can be of less importance for γδ T cells and vice versa (e.g., CCR10 and CD62L). Discovering the basis for trafficking of subpopulations of bovine γδ T cells can further address the role of γδ T cells in immune surveillance and may reveal novel mechanisms that may be relevant to other cell populations and other species.

Disclosures

The authors have no financial conflicts of interest.

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