

A role for CCR4 in development of mature circulating cutaneous T helper memory cell populations

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Expression of the chemokine receptor CCR4 is strongly associated with trafficking of specialized cutaneous memory T helper (Th) lymphocytes to the skin. However, it is unknown whether CCR4 itself participates in the development of cutaneous Th populations. We have addressed this issue via competitive bone marrow (BM) reconstitution assays; equal numbers of BM cells from CCR4^{+/+} and CCR4^{-/-} donors were allowed to develop side-by-side within RAG-1^{-/-} hosts. Cells from both donor types developed equally well into B cells, naive CD8 T cells, naive CD4 T cells, interferon- γ ⁺ Th1 cells, and interleukin-4⁺ Th2 cells. In marked contrast, circulating cutaneous memory Th cells (i.e., E-selectin ligand⁺ [E-lig⁺]) were more than fourfold more likely to be derived from CCR4^{+/+} donors than from CCR4^{-/-} donors. Most of this effect resides within the CD103⁺ subset of the E-lig⁺ Th population, in which donor CCR4^{+/+} cells can outnumber CCR4^{-/-} cells by >12-fold. No similar effect was observed for α 4 β 7⁺ intestinal memory Th cells or CD103⁺/E-lig⁻ Th cells. We conclude that CCR4 expression provides a competitive advantage to cutaneous Th cells, either by participating in their development from naive Th cells, or by preferentially maintaining them within the memory population over time.

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Each member of the Th lymphocyte population expresses only a subset of the total adhesion molecule and chemoattractant receptor repertoires. The specific "fingerprint" of these molecules on a given lymphocyte determines the tissues through which it can travel (1, 2). The two best understood tissue-specific memory Th subsets are distinguished by mutually exclusive expression of the carbohydrate ligand for E-selectin (E-lig; known as cutaneous lymphocyte-associated antigen in humans), which identifies cutaneous T cells, or the integrin α 4 β 7, which identifies intestinal memory Th cells (1, 2).

Interaction of E-lig with endothelial E-selectin plays an important role in the skin-homing cascade (3, 4), whereas interaction of α 4 β 7 with endothelial mucosal addressin cell adhesion molecule 1 plays a similar role in the gut

(2, 5, 6). Memory lymphocytes with receptors for cutaneous antigens reside within the E-lig⁺ population (4), whereas those with receptors for intestinal antigens reside within the α 4 β 7⁺ population (6). Adoptive transfer models confirm that E-lig⁺ memory lymphocytes home specifically to cutaneous sites and α 4 β 7^{hi} memory lymphocytes home to intestinal sites (2, 5, 6).

The chemokine receptor CCR4 is expressed by essentially all cutaneous Th cells in human blood and skin, but is rarely found on intestinal Th cells (7–9). Equally important, one of the chemokine ligands for CCR4 (i.e., CCL17) is presented lumenally by human cutaneous venules, but not by intestinal venules (7, 10). It is likely that CCR4–CCL17 interaction contributes to skin-specific homing by triggering adhesion to endothelium (7). CCR4 is also associated with cutaneous lymphocytes in the mouse, as murine E-lig⁺ but not E-lig⁻ memory Th cells respond to CCR4 ligands in chemo-

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taxis assays (11). The evolutionary pressure implied by maintenance of this association since the time when humans and mice shared a common ancestor hints strongly at its biological relevance.

Memory Th cells derive from naive Th cells that have recognized antigen (for review see reference 2). Naive Th cells express a limited, uniform fingerprint of trafficking molecules, contrasting sharply with the diverse repertoires of memory populations. The naive repertoire includes CD62L, CCR7, and LFA-1 (for review see references 1, 2) and restricts homing to lymphoid organs (1). Antigen is presented to naive T cells by dendritic cells within lymphoid organs, where dendritic cells orchestrate an “imprinting” process (12). This entails that dendritic cells convey information regarding the tissue-specific origins of the antigens they carry (12). Naive T cells receiving such information differentiate into memory cells capable of homing to the type of tissue from which the antigen originated (2). This scenario predicts that naive Th cells recognizing antigen on cutaneous-derived dendritic cells would differentiate into E-lig⁺/CCR4⁺ memory Th cells.

In the present report, we explore the possibility that CCR4 might itself play a role in cutaneous Th development. This notion originates from the finding that Langerhans (cutaneous dendritic) cells, after acquiring cutaneous antigens *in vivo*, secrete functional CCR4 ligands upon entering the draining lymph node (13). However, initial studies suggested that CCR4^{-/-} mice generate normal numbers of E-lig⁺ memory Th cells (11). Despite this finding, there are two hypotheses that might continue to support a role for CCR4 in this process. First, CCR4 might play a redundant role with other molecules compensating for its absence. Second, which we explore in this report, CCR4^{-/-} mice may lack an important feature present in normal animals: developmental competition between cells lacking CCR4 and cells expressing CCR4.

Cyster et al. proposed that competition between individual lymphocytes controls their access to limited microenvironments *in vivo* (14). Such competition is a matter of life or death if the microenvironment provides required signals for continued survival and development. By creating *in vivo* conditions where WT and CCR4^{-/-} cells compete during memory Th differentiation, we ask whether the presence and/or function of CCR4 might play a role in the development of cutaneous memory Th populations.

RESULTS AND DISCUSSION

Competitive bone marrow reconstitution assay

Equal numbers of mixed BM cells (10⁶ each) from CCR4^{+/+} and CCR4^{-/-} donors were injected *i.v.* into lethally irradiated RAG-1^{-/-} hosts to create “experimental” BM chimeras (BMCs). CCR4^{+/+} (i.e., WT) donors expressed the CD45.1 congenic marker, whereas CCR4^{-/-} donors expressed CD45.2.

To control for potential differences in reconstitution efficiency between CD45.1 and CD45.2 donor stem cells, “control BMCs” were created in which both CD45.1 and

CD45.2 donors were WT. Pairs of control and experimental BMCs were always created and harvested in parallel. Transferred BM cells were allowed to engraft the host for 6–12 wk before harvesting each BMC pair.

To assess the “engraftment efficiency” of each donor type, we determined the CD45.1:CD45.2 ratio for a population of BM-derived cells that would not be affected by CCR4. B cells do not express CCR4 during their development (7), so the CD45.1:CD45.2 ratio was determined for splenic B cells in each BMC. This ratio ranged from 0.50 to 1.53 for 16 BMCs and was not significantly different between control and experimental BMCs. This confirms that CCR4 deficiency does not compromise donor cell engraftment, nor does it compromise B cell development. Thus, for each mouse, the CD45.1:CD45.2 ratio for each T cell population studied were corrected for engraftment efficiency to yield *R* by the formula:

$$R = \frac{(\text{CD45.1:CD45.2})_{\text{specific T cell pop.}}}{(\text{CD45.1:CD45.2})_{\text{B cell}}}$$

CCR4 deficiency does not influence development of naive, Th1, or Th2 T subsets

R was next determined for naive CD4 and CD8 splenic T cells (Fig. 1 a). There were no significant differences in *R* between control (Fig. 1 b, white bars) and experimental BMCs (Fig. 1 b, black bars). The findings were identical for naive T cells from PLNs, MLNs, and PBLs (unpublished data). These results suggest that CCR4 function is not involved in the development of naive T cells. In addition, no significant differences were seen in *R* between experimental and control BMCs for neutrophils, NK cells, or any of the major thymic subsets (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20041059/DC1>).

CCR4 has been proposed as a marker for cultured T cells polarized to produce Th2 cytokines (15). We used the competitive assay to assess the influence of CCR4 on Th1 and Th2 development *in vivo*. PLNs and spleen cells from BMCs were treated briefly with PMA, ionomycin, and brefeldin A to induce intracellular cytokine accumulation. Treated cells were stained for CD4 and congenic CD45 markers, plus intracellular IFN- γ and IL-4 (Fig. 1 c). There were no significant differences in *R* between control BMCs (Fig. 1 d, white bars) and experimental BMC (Fig. 1 d, black bars), suggesting that CCR4 function does not influence the development of Th1 or Th2 populations *in vivo*.

A role for CCR4 in development of tissue-specific Th memory populations

We found that memory Th cells expressing E-lig or $\alpha 4\beta 7$ could be clearly identified in the PBLs of our BMCs (Fig. 2 a) as previously reported for humans (2). This suggests that normal tissue-specific memory Th development can occur in our BMCs. Interestingly, we found a dramatic (greater than fourfold) increase in *R* for E-lig⁺ memory Th cells between experimental and control BMCs (Fig. 2 b, top; *P* <

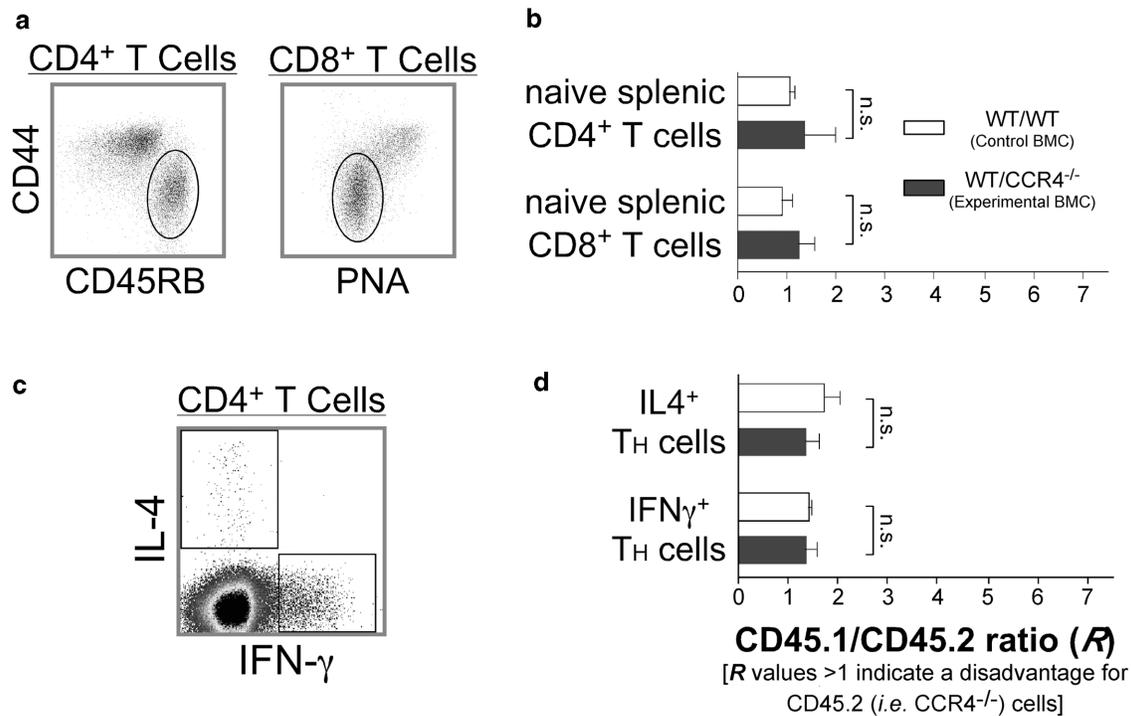


Figure 1. CCR4 deficiency does not influence development of naive, Th1, or Th2 T cells. (a) Identification of naive CD4⁺ (CD45RB^{hi}/CD44^{lo}) and naive CD8⁺ (CD44^{lo}/PNA^{lo}) T cell populations. (b) Transplanted CCR4^{-/-} bone marrow develops into naive T cells with the same efficiency as competing WT cells in BMCs, as there is no significant difference in the

0.009). In contrast, we found only a small (but significant) 1.5-fold increase in *R* for $\alpha 4\beta 7^+$ Th cells between experimental and control BMCs. The difference in *R* between E-lig⁺ and $\alpha 4\beta 7^+$ Th cells from experimental, but not control, BMCs was highly significant ($P < 0.04$). E-lig⁺ Th memory cells from PLNs, where $\alpha 4\beta 7^+$ Th memory cells were too rare for analysis, showed a similarly dramatic difference in *R* between experimental and control BMCs (Fig. 2 b, bottom). These findings suggest a role for CCR4 function in development of the mature circulating cutaneous Th population.

The CCR4 effect resides primarily within the E-lig⁺/CD103⁺ Th population

A large percentage of intradermal T cells in humans (like intraepithelial T cells in many organs) express the integrin αE (CD103), as a heterodimer with $\beta 7$. The $\alpha E\beta 7$ dimer is thought to participate in lymphocyte retention within epithelium via its ligand E-cadherin (16). Therefore, CD103 expression by E-lig⁺ mouse T cells may indicate their recent residence within the skin (17).

We detected a large number of E-lig⁺/CD103⁺ Th cells within the PLNs of our BMCs (Fig. 2 c, bottom) as described previously (17). We also observed a significant population of E-lig⁺/CD103⁺ Th cells within the PBLs (Fig. 2 c, top). The difference in *R* between control and experimental BMCs was significantly larger for the E-lig⁺/CD103⁺ Th

R for naive T splenocytes. Data from spleens of eight BMC pairs (mean \pm SEM). (c) Intracellular staining for IL-4 and IFN- γ CD4⁺ lymphocytes used for identification of IL-4⁺ Th2 and IFN- γ ⁺ Th1 cells in the BMCs. (d) CD4⁺ cells in experimental BMCs show no significant difference in the *R* compared with control BMCs. Data from five BMC pairs (mean \pm SEM).

population than for the E-lig⁺/CD103⁻ Th population (Fig. 2 d), reaching a 5:1 ratio. There was no effect on the E-lig⁻/CD103⁺ population, showing this not to be a general effect on CD103⁺ memory Th cells (Fig. 2 d). Results were similar for both PBLs Th cells (Fig. 2 d, top) and PLN Th cells (Fig. 2 d, bottom).

Other subsets of E-lig⁺ Th cells

Next, we examined E-lig⁺ Th subsets defined by markers other than CD103 (Fig. 3). We distinguished subsets by the Treg marker CD25 (Fig. 3, top center), or by the presence of intracellular IFN γ (i.e., cutaneous Th1 cells; Fig. 3, top right). E-lig⁺ cells producing IL-4 (i.e., cutaneous Th2) were not present in sufficient quantities to assess *R* (unpublished data). The *R*s were significantly greater for experimental versus control chimeras in all E-lig⁺ populations, but not in any of the E-lig⁻ populations. CD103-defined subsets were included for comparison (Fig. 3, top left). The *R*s for E-lig⁺/CD103⁺, E-lig⁺/CD103⁻, and E-lig⁻/CD103⁺ cells were similar to that seen previously in Fig. 2 d. In contrast, the *R* was not significantly different between E-lig⁺/CD25⁺ and E-lig⁺/CD25⁻ populations (Fig. 3, top middle), showing that absence of CCR4 affected both populations equally. The difference in *R* was also insignificant between E-lig⁺/IFN γ ⁺ and E-lig⁺/IFN γ ⁻ populations (Fig. 3, top right). Thus, the effect of CCR4 on cutaneous Th development resides mostly within

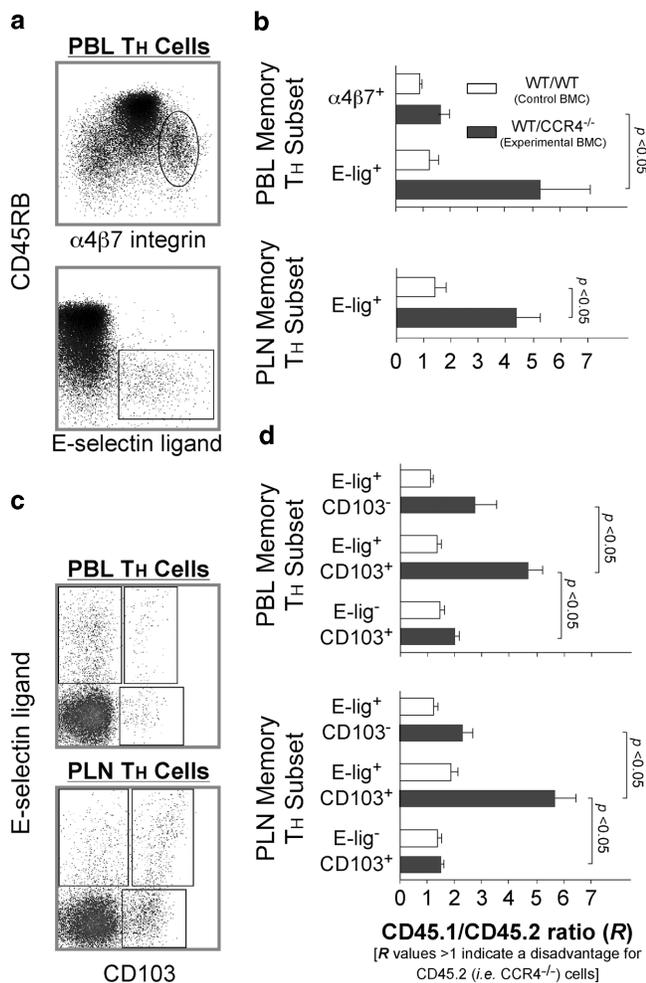


Figure 2. CCR4 participates in generation of cutaneous CD4⁺ memory Th populations. (a) Identification of tissue-tropic PBL Th cell populations. CD4⁺ T cells analyzed by FACS for E-lig (skin memory cells) or $\alpha 4\beta 7$ integrin (intestinal memory cells) together with the naive Th marker CD45RB. (b) In PBLs, experimental chimeras show a highly increased R compared with control chimeras. This was significantly higher than that of $\alpha 4\beta 7^+$ cells. Data from 6 and 10 BMC pairs are shown for PBLs and PLNs, respectively (mean \pm SEM). (c) FACS analysis of PBLs and PLNs show that E-lig⁺ memory cells are separated into two subpopulations based on CD103 expression. (d) Experimental chimeras show a significantly larger difference in R for the E-lig⁺/CD103⁺ Th population than for the E-lig⁺/CD103⁻ population. However, R within the E-lig⁻/CD103⁺ Th population shows no significant alteration between experimental and control chimeras. Data from 15 BMC pairs (mean \pm SEM).

the CD103⁺ subset and is evenly distributed among CD25⁺, CD25⁻, IFN γ ⁺, and IFN γ ⁻ Th subsets.

E-lig⁺ Th subsets during cutaneous inflammation

Next, we asked whether CCR4 influenced development of the aforementioned populations during cutaneous inflammation. We induced contact hypersensitivity by challenging the ears of presensitized mice with OVA₃₂₃₋₃₃₉ peptide and harvesting the cervical PLNs on day 5. The R for E-lig⁺/

CD103⁺ was dramatically greater for challenged versus unchallenged experimental BMCs (13 vs. 6; $P = 0.05$; compare Fig. 3, left, top and bottom). There was no significant difference for subsets defined by CD25 or IFN γ . Thus, R was dramatically increased in the E-lig⁺/CD103⁺ population during cutaneous inflammatory responses, but unchanged in those populations defined by CD25 and IFN γ . This inflammatory model did not produce significant numbers of E-lig⁺ Th2 cells (unpublished data).

Expression of functional CCR4 by Th subsets

One testable explanation for the differences between E-lig⁺/CD103⁻ and E-lig⁺/CD103⁺ populations might be that CCR4 is only expressed by the CD103⁺ cutaneous Th subset. Therefore, we assessed the ability of each population (from WT) to respond to a CCR4 ligand in chemotaxis assays (7, 11, 18). Naive Th cells from PBLs (Fig. 4 a, left) or PLN (right) had no appreciable increase in migration to CCL22 (black bars) over medium alone (gray bars). Both E-lig⁺/CD103⁻ and E-lig⁺/CD103⁺ Th cells responded robustly to CCL22, suggesting that both populations are likely to express high levels of CCR4. All three Th subsets responded equally well to the more universal chemoattractant CXCL12, a ligand for CXCR4 (Fig. 4 a, white bars).

Expression of E-lig and CD103 by Th cells from unmanipulated WT and CCR4^{-/-} mice

As reported previously, CCR4^{-/-} mice possess normal proportions of E-lig⁺ memory Th cells (11). We have confirmed this finding for PLNs (Fig. 4 b, bottom left) and shown that it is also true for PBLs (Fig. 4 b, top right). Furthermore, we show that CCR4^{-/-} mice have identical proportions of CD103⁺ cells within their E-lig⁺ Th populations in both PLNs (Fig. 4 b, bottom right) and PBLs (top right).

The fact that normal numbers of E-lig⁺ Th cells are found within unmanipulated CCR4^{-/-} mice indicates that their naive cells are fully capable of differentiating into E-lig⁺/CD103⁻ and E-lig⁺/CD103⁺ Th cells. However, in the presence of competing WT cells, the CCR4^{-/-} cells are less likely to be found within these populations. Thus, although clearly capable of differentiating into E-lig⁺ Th subsets, CCR4^{-/-} cells are less efficient at doing so than WT cells. The effect is not observable in the unmanipulated CCR4^{-/-} mice because we are seeing only a snapshot of a developmental process that has already reached a steady state; although less efficient at becoming E-lig⁺ Th cells than WT cells, CCR4^{-/-} cells, when given enough time and protected from competition with normal cells, can form an E-lig⁺ Th pool that appears normal. Unlike the unmanipulated CCR4^{-/-} mice, the chimeric mice provide an environment in which such competition can occur, mimicking the normal developmental environment of cutaneous Th cells.

The role of CCR4 in generating cutaneous Th populations

Tang and Cyster demonstrated that Langerhans cells that have recently acquired cutaneous antigen secrete functional

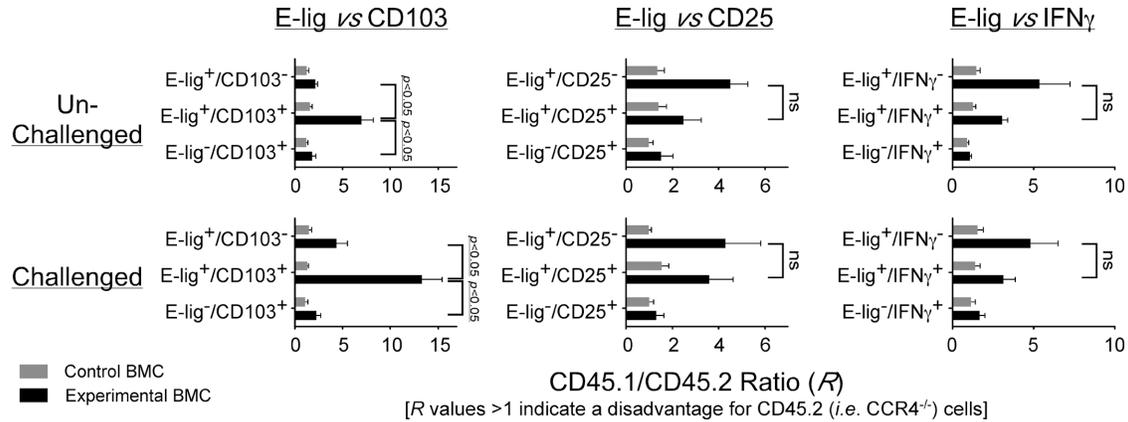


Figure 3. Comparison of E-lig⁺ Th subsets defined by CD103, CD25, and IFN γ before and after cutaneous inflammation. BMCs were sensitized to OVA₃₂₃₋₃₃₉. PLNs were isolated from "unchallenged

mice" (top row) or from "challenged mice" (bottom row) were harvested on day 5 after challenge. Data represent mean and SD from five unchallenged (top row) and four challenged BMC pairs (bottom row).

CCR4 ligands upon entry into draining LNs (13). This could suggest that CCR4 ligands are part of the dendritic cell signaling event that "imprints" naive Th cells to become cutaneous memory Th cells. Although CCR4 is not observed on naive Th cells (Fig. 4 a and references 2, 7–9, 11), it is certainly possible that naive Th cells express CCR4 at levels below detectability that can nonetheless transduce an effective differentiation signal.

Alternatively, CCR4 may influence proliferation or continued viability of E-lig⁺ cells after differentiation. Memory cell clonal lineages that repeatedly encounter antigen are likely to proliferate over time. In contrast, memory cells that never again encounter antigen after initial differentiation

would remain only at low numbers, if maintained at all. If, as we have previously proposed (7), CCR4 plays a role in entry of Th cells into cutaneous sites from the blood, E-lig⁺ Th cells lacking CCR4 may have difficulty entering cutaneous sites, especially in the presence of competing WT cells. Thus, E-lig⁺/CCR4⁺ Th cells would be more likely to reencounter cutaneous antigens than E-lig⁺/CCR4⁻ Th cells. Memory Th cells recognizing cutaneous antigens but incapable of homing to skin would eventually be outnumbered by fully functional WT cutaneous Th cells. We feel that this latter interpretation is the most likely because the effects of CCR4 deficiency are most pronounced in the CD103⁺ population and are greatly amplified during inflammation. As CD103 is a

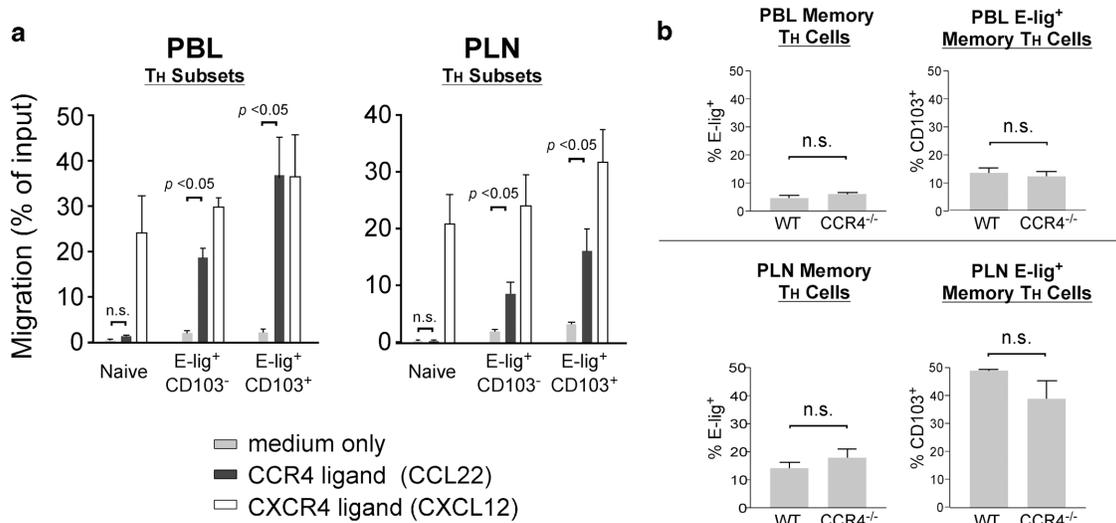


Figure 4. Comparison of memory Th populations from unmanipulated WT and CCR4^{-/-} mice. (a) Both E-lig⁺/CD103⁺ and E-lig⁺/CD103⁻ populations express functional CCR4. Lymphocytes were isolated from PBLs and PLNs, and migrated to medium alone (gray bars), the CCR4 ligand CCL22 (black bars), or the CXCR4 ligand CXCL12 (white bars) in

transwells. The data (mean \pm SEM) are from four independent experiments with four to eight transwells per data point per experiment. (b) Both WT and CCR4^{-/-} mice have similar numbers of E-lig⁺ cells in their PBL and PLN Th populations (left). Furthermore, there is no difference in the proportion of cutaneous Th cells expressing CD103 between the two genotypes (right).

marker of intradermal T cells, it is possible that its expression requires a further differentiation signal to cells that have already been committed to the cutaneous Th lineage. In light of our findings, it is possible that this further signal might require actual trafficking of E-lig⁺ Th cells to the skin.

E-lig⁺/CD103⁺ Th cells are known to be greatly enriched in Treg activity when compared with E-lig⁺/CD103⁻ Th cells (17). However, we did not see a similar effect when assessing E-lig⁺/CD25⁺ Th cells in parallel.

It is noteworthy that E-lig⁺ Th1 cells and other E-lig⁺ Th cells are similarly affected by CCR4 deficiency. In vitro studies of polarized Th cultures suggested that CCR4 was a marker of Th2 rather than Th1 cells (15). According to the data presented here, the influence of CCR4 on Th development depends more on the cutaneous homing profile of a given Th cell, rather than its Th1 or Th2 status.

Conclusions

We have tested the ability of CCR4^{-/-} BM cells to differentiate in vivo into various lymphocyte subtypes when developing in the presence of equal numbers of WT BM cells. We found that CCR4^{-/-} and WT donor cells develop equally well into B cells, naive CD4⁺ T cells, naive CD8⁺ T cells, Th1 cells, and Th2 cells. In contrast, CCR4^{-/-} cells do not develop equally well into E-lig⁺ memory Th cells, with WT cells outnumbering CCR4^{-/-} cells 13:1 within the CD103⁺/E-lig⁺ population during cutaneous inflammation. Our findings suggest that chemokine receptor function can strongly influence the development of mature tissue-specific memory lymphocyte populations.

MATERIALS AND METHODS

Mice. C57Bl/6N (Charles River Laboratories); RAG-1^{-/-} and B6.SJL-ptprc(a)Pep3(b)BoyJ (WT, CD45.1^{+/+}; The Jackson Laboratory); CCR4^{-/-} (11) maintained in our own facility on C57Bl/6N background.

Chimeric mice. WT, CD45.1^{+/+}, WT, CD45.2^{+/+}, and CCR4^{-/-}, CD45.2^{+/+} mice were killed at 4–7 wk, and BM was prepared as described previously (19) and mixed at a 1:1 ratio: WT, CD45.1^{+/+} + WT, CD45.2^{+/+} = “control BMC”; and WT, CD45.1^{+/+} + CCR4^{-/-}, CD45.2^{+/+} = “experimental BMCs.” 2 × 10⁶ BM cells were injected retro-orbitally to anesthetized RAG-1^{-/-} recipients after 2 × 600 rad X-irradiation. Drinking water contained antibiotics for the first 2 wk. Animals were killed 6–12 wk after reconstitution, and suspensions were prepared from PLNs, MLNs, and spleen by gentle disruption through nylon mesh. Blood was drawn by cardiac puncture, and PBLs were purified on Lympholyte Mammal (Cedar Lane). Animal experiments were approved by the Animal Care and Use Committee of Children’s Hospital, Boston.

Flow cytometry. The following conjugated MAbs were used: CD45.1-FITC or -PE, CD45RB-PE, CD19-PE, CD4-PECy7, CD8-PECy7, CD44-allophycocyanin, CD62L-allophycocyanin, CD103-PE, and CD25-PE (all obtained from BD Biosciences). αβ7 was detected with DATK32 (BD Biosciences), followed by biotinylated goat anti-rat IgG (Caltag), and streptavidin-Cy5 (Jackson ImmunoResearch Laboratories). Recombinant murine E-selectin IgG chimeric molecules (R&D Systems) was followed by Cy2 goat anti-human IgG (Jackson ImmunoResearch Laboratories).

Cytokine single cell analysis was performed as described previously (20). Cells were activated 5 h with 50 ng/ml PMA and 1 μM ionomycin with 10 μg/ml brefeldin-A (all obtained from Sigma-Aldrich). Cells were

fixed and permeabilized with Fix & Perm kit (Caltag) and stained with anti-IL4-PE and anti-IFNγ-allophycocyanin together with anti-CD4 PECy7 and anti-CD45.1-FITC (all from BD Biosciences). Cells were analyzed on a dual-laser MoFlo cytometer (DakoCytomation) configured for six colors with Summit 3.1 software.

Chemotaxis. PLNs and PBLs were obtained from ten 4–6 wk C57Bl/6N mice as described before. Migration was performed as described previously (7, 11, 18) with four to eight replicates for each chemokine or control. Optimal chemokine concentrations were used as determined previously: 100 nM for murine CCL22 (R&D Systems) and 30 nM for CXCL12 (Gryphon).

Induction of contact hypersensitivity. Stratum corneum was gently stripped from mouse ears with adhesive tape, and cutaneous lipids were removed with acetone as described previously (21). Each prepared ear was painted with an aqueous mixture of 25 μg cholera toxin (List Biologicals) and 200 μg OVA_{323–339} (AnaSpec). This procedure was repeated three times at 10-d intervals for all mice presented in Fig. 3. PLNs from unchallenged mice were harvested 15–25 d after the third topical immunization. Challenged mice were given a fourth topical immunization 15–25 d after the third, and cervical LNs were harvested on day 5. Cervical LNs were appreciably larger in challenged versus unchallenged mice (unpublished data).

Statistical analysis. For statistical comparison of two samples, a two-tailed Student’s *t* test was used when applicable.

Online supplemental material. Additional experiments demonstrate that there are no significant differences in *R* between experimental and control BMCs for several more leukocyte populations. These include peripheral blood neutrophils, spleen NK cells, and each of the four major thymic subsets. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20041059/DC1>.

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