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Differing Requirements for CCR4, E-Selectin, and $\alpha_4\beta_1$ for the Migration of Memory CD4 and Activated T Cells to Dermal Inflammation

Ahmed Gehad,* Nadia A. Al-Banna,[†] Maria Vaci,* Andrew C. Issekutz,*
Karkada Mohan,* Markus Latta,* and Thomas B. Issekutz*,[†]

CCR4 on T cells is suggested to mediate skin homing in mice. Our objective was to determine the interaction of CCR4, E-selectin ligand (ESL), and $\alpha_4\beta_1$ on memory and activated T cells in recruitment to dermal inflammation. mAbs to rat CCR4 were developed. CCR4 was on 5–21% of memory CD4 cells, and 20% were also ESL⁺. Anti-TCR-activated CD4 and CD8 cells were 40–55% CCR4⁺, and ~75% of both CCR4⁺ and CCR4[−] cells were ESL⁺. CCR4⁺ memory CD4 cells migrated 4- to 7-fold more to dermal inflammation induced by IFN- γ , TNF, TLR agonists, and delayed-type hypersensitivity than CCR4[−] cells. CCR4⁺ activated CD4 cells migrated only 5–50% more than CCR4[−] cells to these sites. E-selectin blockade inhibited ~60% of CCR4⁺ activated CD4 cell migration but was less effective on memory cells where $\alpha_4\beta_1$ was more important. Anti- $\alpha_4\beta_1$ also inhibited CCR4[−] activated CD4 cells more than CCR4⁺ cells. Anti-E-selectin reduced activated CD8 more than CD4 cell migration. These findings modify our understanding of CCR4, ESL, $\alpha_4\beta_1$, and dermal tropism. There is no strict relationship between CCR4 and ESL for skin homing of CD4 cells, because the activation state and inflammatory stimulus are critical determinants. Dermal homing memory CD4 cells express CCR4 and depend more on $\alpha_4\beta_1$ than ESL. Activated CD4 cells do not require CCR4, but CCR4⁺ cells are more dependent on ESL than on $\alpha_4\beta_1$, and CCR4[−] cells preferentially use $\alpha_4\beta_1$. The differentiation from activated to memory CD4 cells increases the dependence on CCR4 for skin homing and decreases the requirement for ESL. *The Journal of Immunology*, 2012, 189: 337–346.

Lymphocyte recruitment from blood to inflammatory sites is a multistep process that involves lymphocyte rolling on the endothelium, chemokine (CK)-induced activation, firm adhesion, and transendothelial migration into the tissue (1). Numerous CKs are produced during inflammation, and CK receptors (CKRs) are highly expressed on T cells in these inflamed tissues (2, 3). At least 11 of the ~20 CKRs are associated with T cells in inflamed tissues, including CCR1–6, CCR8–10, CXCR3, and CXCR6 (3, 4). T cells can also coexpress several CKRs, such as CXCR3 and CCR4, and CXCR3 and CCR5 (5, 6).

CCR4 is found on skin-infiltrating CD4 T cells in atopic dermatitis lesions (7, 8) and in contact hypersensitivity (CHS) in mice (9). It is also detected on T cells in psoriatic lesions and induced blisters (10, 11). The production of CCR4 ligands in atopic dermatitis lesions (7) is thought to attract CCR4⁺ CD4 T cells (12), but the relationship between CCR4 and the migration of multiple CCR4⁺ T cell subsets is unknown. Other CKRs also contribute to skin homing of T cells. Ligands of CXCR3 and CCR10 are

produced in inflamed skin, and both CXCR3 and CCR10 are expressed on skin-infiltrating T cells in humans (11, 13). Blockade of CXCR3 was shown to inhibit 90% of the migration of T cell lymphoblasts to dermal inflammation (14), even though CCR4 is coexpressed on about half of the CXCR3⁺ CD4 T cells (6). Thus, the migration of activated CCR4⁺ T cells may also depend on other CKRs. In addition, memory and activated T cells may differ in their requirement for CCR4 in this migration. Only 50% of the migration of memory CD4 T cells to dermal inflammation is inhibited by CXCR3 blockade (14), suggesting that CCR4 on memory CD4 cells may account for the remaining CXCR3-independent migration. The relationship between CCR4 expression and migration to dermal inflammation has only examined CHS and delayed-type hypersensitivity (DTH) in mice and atopic and psoriatic lesions using histology in patients. Thus, the relationship between the expression of CCR4 on memory and activated CD4 T cells and their migration to dermal inflammation is not clear. Similarly, the association between CCR4 expression and migration to inflammation induced by various stimuli such as the cytokines IFN- γ and TNF, TLR agonists, and DTH has not been compared previously.

In addition to CKRs, adhesion molecules such as E-selectin ligand (ESL) are required for some T cells to migrate to inflammatory sites. Human T cells express cutaneous lymphocyte Ag (CLA), a ligand for E-selectin (15), and they upregulate CLA after activation in vitro (16, 17). In skin biopsies of patients with atopic dermatitis and psoriasis, 80–90% of CD4 and CD8 T cells express CLA (10, 18). CCR4 is expressed on ~40% of the circulating CLA⁺ T cells (7), and the proportion of CCR4⁺ cells is increased within the CLA⁺ memory (CD45RA[−]) CD4 subsets of T cells (12). CLA⁺ T cells from human dermal lesions migrate to human skin grafts in SCID mice in response to CCL22, and this was reduced after anti-E-selectin blockade (19). This suggests that

*Department of Pediatrics, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia B3K 6R8, Canada; and [†]Department of Microbiology and Immunology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia B3K 6R8, Canada

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Address correspondence and reprint requests to Dr. Thomas B. Issekutz, Department of Pediatrics, Dalhousie University, Izaak Walton Killam Health Centre, 5850 University Avenue, Halifax, Nova Scotia B3K 6R8, Canada. E-mail address: thomas.issekutz@dal.ca

Abbreviations used in this article: CHO, Chinese hamster ovary; CHS, contact hypersensitivity; CK, chemokine; CKR, chemokine receptor; DTH, delayed-type hypersensitivity; ESL, E-selectin ligand; i.d., intradermal(ly); LN, lymph node; poly I:C, polyinosinic polycytidylic acid; PSL, P-selectin ligand.

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T cell homing to inflamed skin may be partly governed by tissue-specific interactions mediated by CLA and CCR4 on T cells interacting with E-selectin and CCL17 or CCL22 on the endothelium and in the tissues (12, 19).

In addition to E-selectin, other adhesion molecules contribute to T cell migration. CD4 T cells that can bind to both E-selectin and P-selectin have been shown to migrate in mice to CHS-induced inflammation (20). E-selectin and P-selectin blockade or absence of their ligands (FucTVII^{-/-} T cells) reduced the migration of activated T cells to CHS and DTH in mice (21–23). E-selectin and P-selectin blockade also reduced the migration of spleen T cells to dermal sites injected with IFN- γ , TNF, and TLR agonists in rodents (24, 25). CD49d/CD29 ($\alpha_4\beta_1$) can also mediate the migration of T cells to these dermal sites in rats (26, 27), and its blockade was shown to reduce the inflammatory response in other tissues, such as gut (28) and CNS in humans (29). The blockade of α_4 , in addition to ESL and P-selectin ligand (PSL), was shown to reduce the number of Thy1.2⁺ cells in 2,4-dinitro-1-fluorobenzene-induced CHS in mice (30) and significantly reduced the migration of spleen T cells to skin sites injected with cytokines and TLR agonists in the rat (24). However, it is not known whether selectins and $\alpha_4\beta_1$ exert similar effects on the migration of memory and activated CD4 T cells to dermal inflammation and whether they are equally required for migration of CCR4⁺ and CCR4⁻ T cells.

The adhesion molecules mediating the migration of CD8 T cells to dermal inflammation are not well studied. Selectin-deficient mice had reduced Tc1 cell infiltration to CHS (31), but it is unclear whether selectin ligands and $\alpha_4\beta_1$ exert similar effects on the migration of activated CD4 and CD8 T cells to dermal inflammation.

To investigate the role of CCR4 in T cell migration in vivo, mAbs to rat CCR4 were generated and used to determine the relationship between CCR4 expression and the migration of memory and activated T cells to dermal inflammatory reactions. The role of the adhesion molecules (E-selectin, P-selectin, and $\alpha_4\beta_1$) in the migration of CCR4⁺ and CCR4⁻ memory and activated T cells was also examined.

Our results demonstrated low expression of ESL and PSL on unstimulated memory CD4 T cells that is almost entirely restricted to CCR4⁺ T cells. Expression of CCR4, ESL, and PSL was increased after activation in vitro. However, CCR4 was expressed on less than half of anti-TCR-activated CD4 cells, but on substantially more CD8 cells, and most of the cells expressed ESL. CCR4⁺ memory CD4 cells migrated more than CCR4⁻ cells to dermal inflammation but with considerable variation, based on the stimulus (IFN- γ , TNF, TLR agonists, and DTH) used to induce inflammation. T cell activation markedly reduced the CCR4 dermal-specific tropism of CD4 cells and altered the importance of ESL compared with $\alpha_4\beta_1$ in migration to various inflammatory sites. In addition, activated CD8 T cells were highly dependent on E-selectin, whereas P-selectin contributed little to migration of these cells.

Materials and Methods

Reagents

Recombinant CCL22 was purchased from PeproTech (Rocky Hill, NJ), and TNF was obtained from R&D Systems (Minneapolis, MN). IFN- γ was a gift from Dr. P. van der Meide (TNO Primate Center, Rijswijk, The Netherlands). Purity of these cytokines was >98% and they contained <0.01 ng/ μ g endotoxin.

Mouse mAb to rat Ag included the following: W3/25 (anti-CD4), MRC OX-8 (anti-CD8), MRC OX-22 (anti-CD45RC), and R7.3 (anti- $\alpha\beta$ TCR) obtained from Serotec (Raleigh, NC). R7.3-PE was from BD Biosciences (San Diego, CA). Other mAbs used included TA-2, a mAb to rat α_4 (anti-

CD49d), RME-1 mAb, which binds to rat E-selectin, and RMP-1 mAb, which binds to rat P-selectin. These are mouse IgG mAbs developed in our laboratories, and each of these blocks the adhesion function of its respective Ags, based on in vitro and in vivo studies (26, 32–34). The hamster mAb 145.2C11 (anti-mouse CD3) was from the American Type Culture Collection (Manassas, VA) and was used as an isotype control mAb.

Production of stable Chinese hamster ovary-CCR4 transfectants

Total RNA was isolated from Con A-stimulated rat lymph nodes (LNs) and reverse transcribed. CCR4 was amplified by PCR using primers containing HindIII and XbaI restriction sites: 5'-primer, 5'-CCAAAGCTTAATGC-CACAGAGATACGACAGATAC-3'; and 3'-primer, 5'-CACTCTAGACTAT-TACAAAGCGTCATGAAGGTC-3'. The PCR product was ligated into pFLAG-CMV3 (Sigma-Aldrich, St. Louis, MO) and cloned in DH5a. The FLAG-tagged CCR4 was transfected into Chinese hamster ovary (CHO)-K1 cells and grown in G418. Transfectants expressing CCR4 were identified with anti-FLAG mAb M2 (Sigma-Aldrich), sorted on a FACScalibur flow cytometer (BD Biosciences, Mountain View, CA), and cloned by limiting dilution.

Generation of mAb to rat CCR4

Armenian hamsters (Cytogen, West Roxbury, MA) were immunized i.p. at least four times at 2-wk intervals with 2–3 $\times 10^7$ CCR4-expressing CHO cells and boosted 4 d before fusion. Splenocytes were fused with P3U1 myeloma cells at a 1:1 ratio using 50% polyethylene glycol. Hybridomas were grown in 96-well plates, selected in HAT medium, and screened by ELISA on control and CCR4-transfected CHO cells. Bound Ab was detected with rabbit anti-hamster Ig, followed by goat anti-rabbit Ig HRP and the appropriate substrate. Hybridomas producing Ab to CHO-CCR4, but not control cells, were cloned by limiting dilution, and their specificity was determined by ELISA and immunofluorescence on CHO transfectants stably expressing other rat CKRs.

Leukocyte isolation

Blood leukocytes were obtained as described previously (35). Blood was collected in acid citrate dextrose, RBC were sedimented, and the T cells were isolated using Percoll gradient centrifugation, followed by passing the mononuclear cells through a nylon wool column. Spleen T cells were prepared from a suspension of splenocytes after RBC were lysed, and cells were passed through nylon wool. For Ag-activated T lymphoblasts, rats were immunized with 10⁷ PFU vaccinia virus in footpads. Low-density T lymphoblasts were isolated from the draining LN 4 d later using Percoll (26). For exudate T cells, animals were injected with 5 $\times 10^7$ PFU vaccinia virus i.p., and 5 d later, the peritoneal cavity was lavaged with PBS (26). Macrophages were depleted by incubation at 37°C for 1 h, and the non-adherent T cells were passed through nylon wool to obtain T cells.

Memory CD4 T cells were isolated by passage of splenocytes through a nylon wool column, followed by negative selection with anti-CD8, anti-NKR1.1, and anti-CD45RC by panning on goat anti-mouse Ig-coated dishes. For activation of T cells, CD4 T cells were obtained from normal LNs by passage through nylon wool and by negative selection using anti-CD8 and anti-NKR1.1 mAbs by panning. To obtain activated CD8 T cells, the same procedure was followed as for CD4 T cells, except that anti-CD4 was used instead for negative selection (36). To obtain CCR4⁺ and CCR4⁻ cells, the CD4 T cells were incubated with CR4.1 mAb, followed by biotinylated mouse anti-hamster mAb (BD Biosciences), treated with streptavidin magnetic beads for 15 min at 10°C, and passed through a MACS separation column (Miltenyi Biotec, Bergisch Gladbach, Germany).

In vitro activation of T cells

To induce polyclonal T cell activation, LN CD4⁺ or CD8⁺ T cells were incubated with 2 μ g/ml immobilized anti-TCR mAb (R7.3), 10 U/ml IL-2, 10 ng/ml IL-12, and 0.4 μ g/ml anti-CD28 in RPMI 1640 medium plus 10% FBS for 3 d, followed by 20 U/ml IL-2 for another 2 d. For in vitro Ag-stimulated T cells, Lewis rats (150–225 g, male; Charles River Laboratories) were s.c. immunized with 1 mg *Mycobacterium butyricum* in mineral oil at the base of the tail. LN T cells were isolated from draining LNs and restimulated in vitro with 12 μ g/ml *M. butyricum* for 4 d.

Immunofluorescence staining

Cells were suspended in immunofluorescence buffer incubated with a primary hamster mAb at 4°C, washed twice, and incubated with Alexa 488-conjugated goat anti-hamster IgG (Molecular Probes, Eugene, OR). For

three-color staining, cells were stained sequentially with mouse mAb against appropriate CD markers, followed by anti-CCR4. Mouse mAbs were detected using goat anti-mouse-Ig-Alexa 647 and analyzed on a FACSCalibur. In some experiments, cells were incubated with CK at 37°C for 30 min and stained with anti-CCR4 mAb at 4°C as above.

The expression of ligands for E-selectin and P-selectin on T cells was determined as previously described (24) using mouse E-selectin and mouse P-selectin chimera constructs fused to human μ -chain (a gift from Dr. J. Lowe, University of Michigan, Ann Arbor, MI) as reported previously (37). Briefly, cells were incubated (45 min at 4°C) with either E- or P-selectin chimera constructs in immunofluorescence buffer. Binding was detected by using sequential incubation with biotin-labeled mouse anti-human μ -chain (BD Biosciences), followed by washing and incubation with streptavidin-conjugated PE (BD Biosciences).

In vivo T cell migration

Lymphocyte migration was measured as previously described using syngeneic radioisotope-labeled T cells as tracers (14, 26). Briefly, T cells were isolated as above, labeled with $\text{Na}_2^{51}\text{CrO}_4$ or $[^{111}\text{In}]\text{joxine}$ (Amersham Biosciences, Piscataway, NJ), and $4\text{--}10 \times 10^6$ cells having $0.1\text{--}0.5 \times 10^6$ cpm were injected i.v. per rat. Immediately afterward, the skin on the back of each animal was shaved, and 50 μl vehicle alone and the inflammatory agents indicated in the figures were injected intradermally into duplicate sites. In some experiments, 1.5–2 mg anti-P-selectin Ab (RMP-1), anti-E-selectin Ab (RME-1), and/or anti- α_4 integrin (TA-2) mAb was given i.v. just prior to labeled T cells. After 20 h, animals were sacrificed, the injected skin was removed, and dermal sites were punched out with a 12-mm leather punch. Blood, lymphoid tissues, liver, and lung were also collected, and together with skin lesions, their radioisotope contents were determined by gamma counting.

For DTH reactions, animals were sensitized 11 d previously by s.c. injection with an emulsion of 75 μg OVA and 25 μg *M. butyricum* and homogenized in mineral oil at the base of the tail. To elicit DTH reactions, 10 μg OVA was injected intradermally (i.d.).

Statistics

Data were expressed as mean \pm SEM of multiple assays, and Student *t* test was used for analysis. A *p* value < 0.05 was considered significant.

Results

Development of anti-rat CCR4 mAbs

Rat CCR4 was cloned by RT-PCR and stably transfected into CHO cells, and hamsters were repeatedly immunized with the CCR4-expressing CHO cells. Hamster splenocytes were used to generate hybridomas, which were screened by ELISA for Ab to CCR4. Four mAbs, CR4.1–CR4.4, specifically reacting with CCR4 transfectants were isolated. Characterization of the CR4.1 mAb showed that it strongly stained CHO-CCR4 cells and did not react with CHO cells transfected with four other rat CKRs (Fig. 1A). The CR4.1 mAb also stained T cell lymphoblasts. CCL22, a CCR4 ligand, which should induce CCR4 internalization, completely abolished CR4.1 staining of both CHO-CCR4 transfectant T cells (Fig. 1B) and T cell lymphoblasts (Fig. 1C).

Expression of CCR4 on spleen, LN, blood, and Ag-activated T cells

In rats, CCR4 was expressed on 2–7% of CD4 T cells in the blood, the spleen, and LNs and was almost absent on CD8 T cells (Fig. 2). The expression on CD4 T cells is restricted to the memory CD45RC⁺ T cells with 5, 10, and 20% of these cells being CCR4⁺ in LNs, the blood, and the spleen, respectively (Fig. 2).

After s.c. Ag injection, ~12% of LN CD4 CD45RC⁺ T lymphoblasts from the draining LN expressed CCR4 and 8% of the CD8 cells expressed CCR4. In contrast, in vitro culture of LN cells with Ag markedly increased the proportion of CCR4⁺ CD4 T cells to 70%. CD4 cells from the inflamed peritoneal cavity after virus injection were mostly (75%) CCR4⁺, suggesting that either CD4 T cells rapidly migrated out of the LN after expressing CCR4 or upregulated CCR4 after reaching an inflammatory site such as the infected peritoneal cavity (Fig. 3).

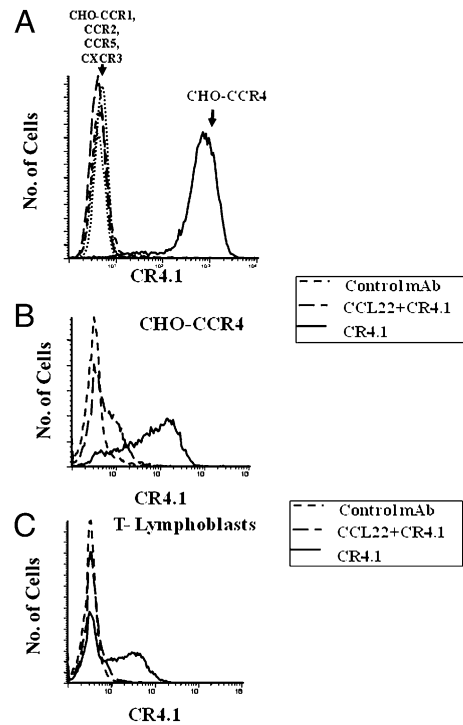


FIGURE 1. Characterization of anti-CCR4 mAb CR4.1. Immunofluorescence staining of CKR-transfected and control CHO cells with mAb CR4.1 (A). CCR4-transfected CHO cells (B) and T lymphocytes (C) were stained with control (dotted line) or mAb CR4.1 after preincubation with (solid line) or without (dashed line) CCL22.

Expression of CCR4, ESL, and PSL on normal and activated CD4 and CD8 T cells

ESL was present on 1–6% of unstimulated CD4 LN T cells, almost all of which expressed CCR4. Similarly, most of the PSL-expressing CD4 T cells (1–4%) also expressed CCR4. However, most of the CCR4⁺ CD4 cells lacked ESL or PSL. In fact, of the CCR4⁺ CD4 T cells, only ~12% expressed ESL and ~14% expressed PSL (Fig. 4A, 4B).

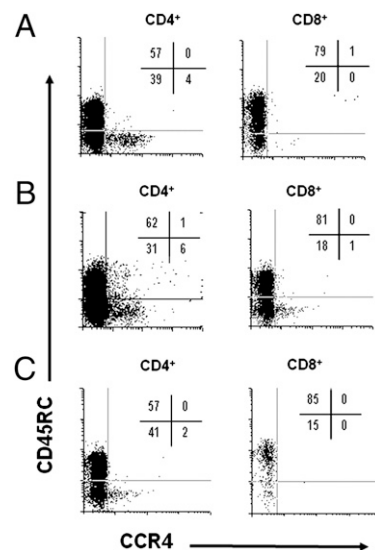


FIGURE 2. Expression of CCR4 on T cells from peripheral blood, spleen and normal LNs. T cells from peripheral blood (A), splenocytes (B), and normal LNs (C) were stained with mAb to CD4, CD8, CD45RC, and CCR4 and a fluorescent-conjugated secondary Ab. Each dot plot is representative of four to five stainings.

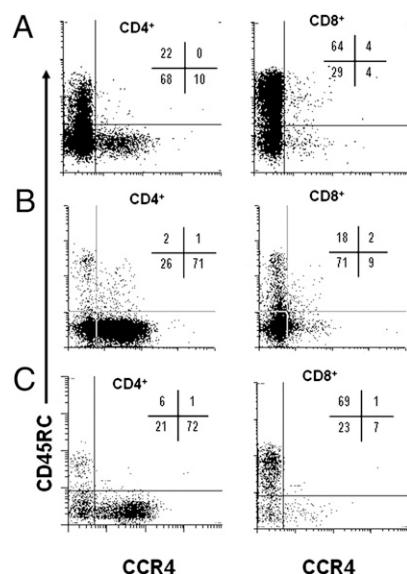


FIGURE 3. Expression of CCR4 on LN lymphoblasts, Ag-stimulated T cells, and T cells from viral peritoneal exudates. LN T lymphoblasts (**A**), in vitro Ag-stimulated T cells (**B**), and T cells from viral peritoneal exudates (**C**) were stained with mAbs to CD4, CD8, CD45RC, and CCR4 and a fluorescent-conjugated secondary Ab. Each dot plot is representative of four to five stainings.

As shown in Fig. 4C, there was a steady increase in the expression of CCR4 on CD4 T cells during in vitro activation, and ~40% of the activated CD4 T cells expressed CCR4 after 5 d. The expression of ESL and PSL increased rapidly after activation (Fig. 4A, 4B). After 5 d, ESL was present on ~80% of the activated CD4 cells and was expressed by a similar proportion of CCR4⁺ (87%) and CCR4⁻ (78%) cells. Moreover, PSL was present on 44% of CCR4⁺ and 22% of CCR4⁻ CD4 cells. Therefore, even though most of the ESL⁺ and PSL⁺ unstimulated CD4 cells express CCR4, after activation, both CCR4⁺ and CCR4⁻ were mainly ESL and PSL positive.

There was a steady increase in the expression of CCR4 on CD8 T cells during in vitro activation, and ~55% of the activated CD8 T cells expressed CCR4 after 5 d. The expression of ESL and PSL also increased rapidly after activation (Fig. 5). ESL was present on 70% of the activated CD8 T cells and was present on both CCR4⁺ and CCR4⁻ cells to the same extent (Fig. 5B). PSL was present on 33% of CD8 T cells (Fig. 5B). Therefore, on unstimulated CD8 T cells, most of the ESL and PSL are expressed on CCR4⁺ cells, whereas both CCR4⁺ and CCR4⁻ activated CD8 T cells express ESL and PSL (Fig. 5).

Migration of memory CCR4⁺ and CCR4⁻ CD4 T cells to dermal inflammation

The migration of unstimulated CCR4⁺ CD4 cells to dermal inflammatory sites was compared with that of CCR4⁻ CD4 cells (Fig. 6A). Memory CCR4⁺ CD4 cells were extensively recruited to the inflamed skin sites. CCR4⁺ CD4 cells migrated 4- to 7-fold more than CCR4⁻ cells to IFN- γ , TNF, and IFN- γ plus TNF. The TLR agonists, polyinosinic polycytidylic acid (poly I:C) and LPS, recruited 5-fold more CCR4⁺ than CCR4⁻ memory cells, whereas DTH sites recruited 7-fold more CCR4⁺ than CCR4⁻ cells.

There was no difference in the circulation of CCR4⁺ and CCR4⁻ cells in the blood. CCR4⁻ T cells accumulated three times as much in the peripheral cervical and axillary LNs than CCR4⁺ T cells but accumulated similarly in the spleen (Fig. 6B). Thus, even though 85–90% of memory CD4 T cells were CCR4⁻ (Fig.

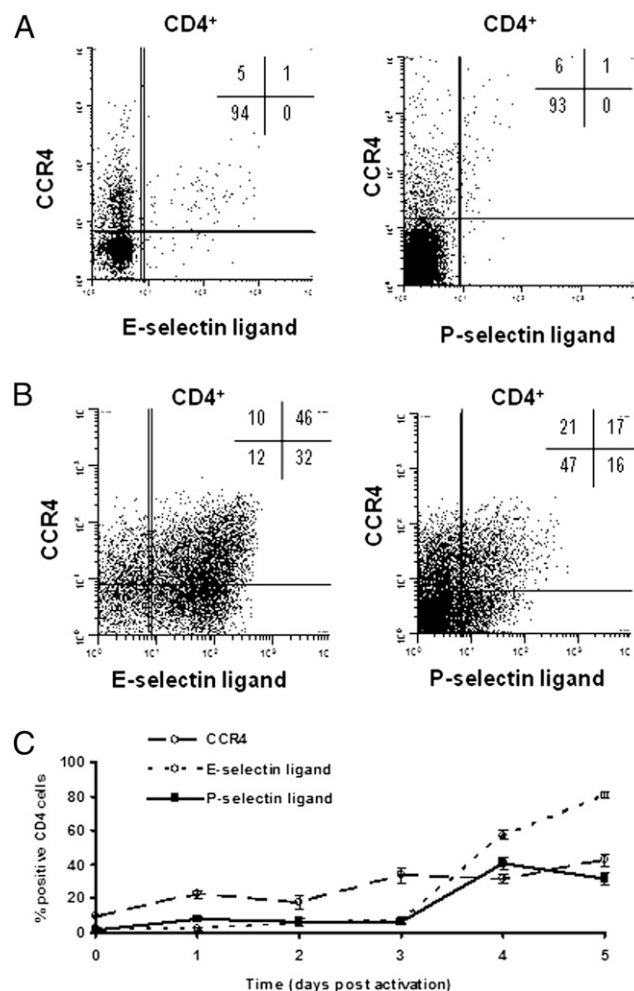


FIGURE 4. Expression of CCR4, ESL, and PSL on CD4 T cells during in vitro activation. Freshly isolated T cells (**A**) and CD4 T cells activated for 5 d (**B**) were stained with a mAb to CCR4 (CR4.1), and for ESL and PSL, and a secondary fluorescent Ab ($n = 3-8$). (**C**) Expression of CCR4, ESL, and PSL on CD4 T cells determined daily during in vitro activation with anti-TCR plus anti-CD28 mAbs ($n = 3-8$).

2A, 2B), CCR4⁺ T cells accounted for most of the migration to dermal inflammation induced by each of the inflammatory stimuli and significantly less of the recruitment to LNs.

Effect of E-selectin and $\alpha_4\beta_1$ blockade on memory CCR4⁺ CD4 T cell migration

Because memory CD4 T cells expressed ESL (Fig. 4A) and $\alpha_4\beta_1$ (data not shown) (24), the effects of E-selectin and $\alpha_4\beta_1$ blockade on the migration of CCR4⁺CD4⁺ memory T cells to dermal inflammation were determined (Fig. 7). Memory CCR4⁺ CD4 T cells were radiolabeled and injected i.v. into animals treated with anti-E-selectin or anti- $\alpha_4\beta_1$ mAb. E-selectin blockade significantly inhibited 25–60% of the migration of memory CCR4⁺ CD4 T cells to TNF and DTH sites ($p < 0.05$) but had no significant effect on the migration to other inflammatory stimuli. In contrast, $\alpha_4\beta_1$ blockade inhibited 60–80% of the CCR4⁺ cell migration to almost all of the different types of inflammatory sites in the skin (Fig. 7A).

Anti-E-selectin and anti- α_4 mAb treatment had no effect on the accumulation of labeled T cells in the blood, the spleen, and cervical and axillary LNs (Fig. 7B). Similarly, it had no effect on the free radioisotope content in plasma or on the level of radioactivity in the liver and lung (data not shown), indicating that the mAbs did not cause the clearance of the labeled lymphocytes.

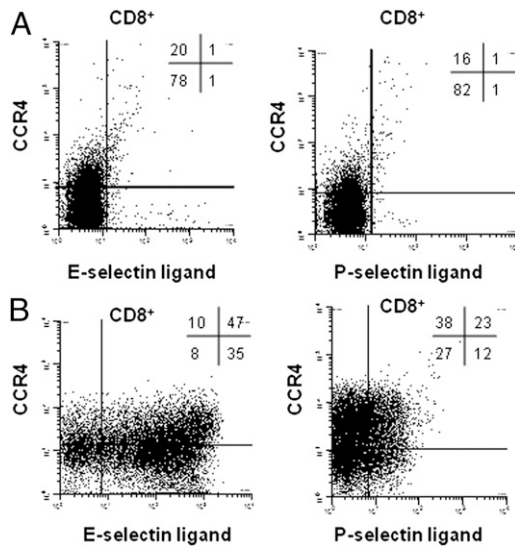


FIGURE 5. Expression of CCR4, ESL, and PSL on CD8 T cells during *in vitro* activation. Freshly isolated T cells (**A**) and CD8 T cells activated *in vitro* with anti-TCR and anti-CD28 mAbs (**B**) were stained with a mAb to CCR4 (CR4.1) and for ESL and PSL and a fluorescent-conjugated secondary Ab. Each dot plot is representative of three to six stainings.

Migration of activated CCR4⁺ and CCR4⁻ CD4 T cells to dermal inflammation

The ability of anti-TCR-activated CCR4⁺ CD4 T cells to migrate to dermal inflammatory sites was compared with activated CCR4⁻ T cells (Fig. 8). Unlike memory CCR4⁺ and CCR4⁻ CD4 T cells, activated CCR4⁺ and CCR4⁻ cells migrated relatively similarly to

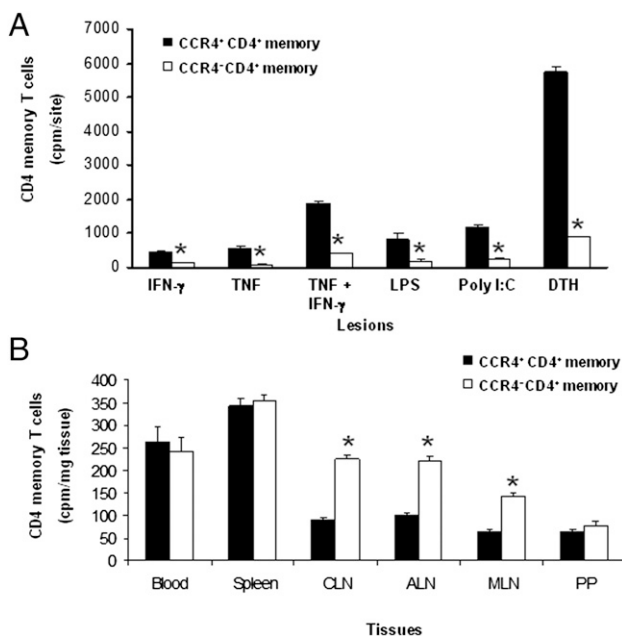


FIGURE 6. Migration of CCR4⁺ and CCR4⁻ memory CD4 T cells to dermal inflammation and lymphoid tissues. Spleen CD4 T cells were separated into CCR4⁺ and CCR4⁻ CD45RC⁺ T cells, radiolabeled, and injected i.v. into animals that received i.d. injections of cytokines, TLR agonists, and OVA to induce a DTH reaction and to control diluent. Accumulation of radiolabeled CD4 T cells at injected sites (**A**) and in the blood, spleen, cervical LN (CLN), axillary LN (ALN), mesenteric LN (MLN), and Peyer's patches (PP) (**B**) was determined after 20 h. Each bar shows the increase \pm SEM over control sites (117 ± 25 for CCR4⁺; 61 ± 15 for CCR4⁻) or the mean \pm SEM in tissues of 10–13 animals. * $p < 0.005$.

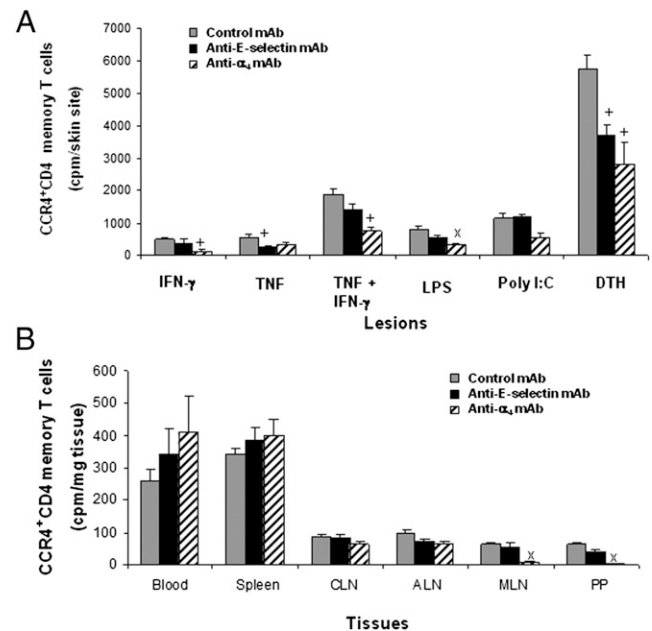


FIGURE 7. Effect of E-selectin and $\alpha_4\beta_1$ blockade on the migration of CCR4⁺ memory CD4 T cells to dermal inflammation. Spleen CD4 T cells were separated into CCR4⁺CD45RC⁺ T cells, radiolabeled, and injected i.v. into animals that also received mAbs to E-selectin or α_4 integrin or an isotype-matched control mAb i.v. Each animal received i.d. injections of inflammatory stimuli as in Fig. 6. Accumulation of labeled CD4 T cells at the injected sites (**A**) and in blood and lymphoid tissues (**B**) was determined after 20 h. Each bar shows the increase \pm SEM over control sites (107 ± 17) or the mean \pm SEM in tissues of 4–11 animals. * $p < 0.05$, ** $p < 0.01$.

most of the dermal inflammatory stimuli. There was no difference in the migration of CCR4⁺ and CCR4⁻ cells to IFN- γ and/or TNF and only ~35% more migration of CCR4⁺ cells to the TLR agonists than CCR4⁻ CD4 cells. Migration to the DTH reaction was twice as great by CCR4⁺ than CCR4⁻ cells (Fig. 8A), but even this is a much smaller difference than observed with memory cells, which differed by 7-fold (Fig. 6A). There was no difference in the circulation of CCR4⁺ and CCR4⁻ T cells in the blood, but CCR4⁻ T cells accumulated in significantly greater numbers in the spleen and mesenteric LN than CCR4⁺ T cells. CCR4⁻ T cells and CCR4⁺ T cells accumulated similarly in the peripheral cervical and axillary LNs (Fig. 8B).

Effects of E-selectin, P-selectin, and $\alpha_4\beta_1$ blockade on activated CCR4⁺ CD4 T cell migration

Blockade of E- and P-selectin was shown to partially inhibit activated CD4 T cell migration to DTH and CHS sites in mouse models (20, 22, 23). To determine the contribution of E-selectin, P-selectin, and $\alpha_4\beta_1$ to the migration of activated CCR4⁺ and CCR4⁻ CD4 cells and recruitment to other types of inflammatory reactions, these cells were radiolabeled and injected i.v. into animals treated with anti-E-selectin, anti-P-selectin, and/or anti- α_4 mAbs.

E-selectin blockade significantly inhibited T cell migration to all of the inflammatory stimuli (Fig. 9A). It inhibited ~30% of the T cell migration to poly I:C, ~60% of the migration to IFN- γ , TNF, IFN- γ plus TNF and LPS, and ~70% of the migration to DTH. P-selectin blockade alone inhibited 30–40% of the migration to IFN- γ plus TNF, LPS, and poly I:C, which was similar in effect to E-selectin blockade. In other inflamed sites, such as IFN- γ , TNF, and DTH, blockade of P-selectin did not significantly inhibit migration. When animals were treated with both anti-E-

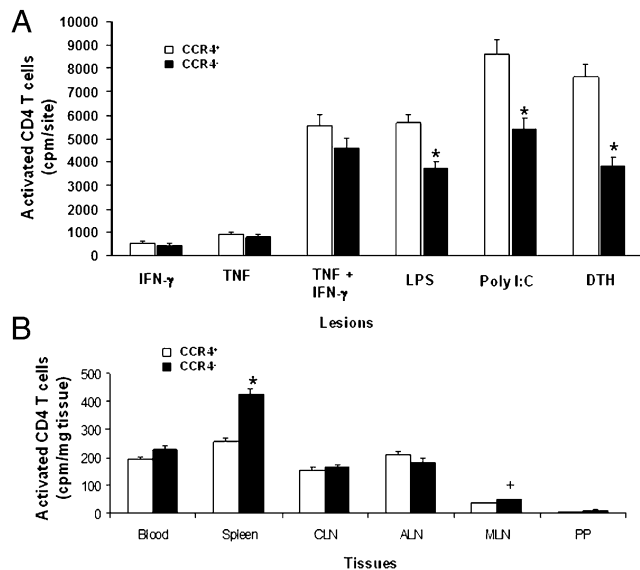


FIGURE 8. Migration of CCR4⁺ and CCR4⁻ activated CD4 T cells to dermal inflammation and lymphoid tissues. Anti-TCR-activated CD4 T cells were separated into CCR4⁺ and CCR4⁻ cells, radiolabeled, and injected i.v. into animals receiving i.d. injections of inflammatory stimuli as in Fig. 6. Accumulation of labeled CD4 T cells at the injected sites (**A**) and in blood and lymphoid tissues (**B**) was determined after 20 h. Each bar shows the increase \pm SEM over control sites (118 ± 15 for CCR4⁺; 87 ± 14 for CCR4⁻) or the mean \pm SEM in tissues of 18–19 animals. * $p < 0.05$, * $p < 0.005$.

and anti-P-selectin mAbs, the migration of activated CCR4⁺ CD4 cells was inhibited by 80–90% to most of the dermal inflammatory sites, except to poly I:C (40% reduction). Thus, the addition of P-selectin blockade to that of E-selectin further inhibited the migration observed with anti-E-selectin treatment (Fig. 9A).

Blockade of $\alpha_4\beta_1$ significantly inhibited the migration of activated CCR4⁺ CD4 cells to most of the inflammatory stimuli (Fig. 9B). It inhibited ~40% of the T cell migration to TNF, IFN- γ plus TNF, LPS, and poly I:C but inhibited ~20% of the T cell migration to DTH and had no effect on recruitment to IFN- γ (Fig. 9B). $\alpha_4\beta_1$ blockade was not as effective as ESL blockade in inhibiting the recruitment of activated CCR4⁺ CD4 cells to inflamed sites such as DTH and IFN- γ . When animals were treated with both anti-E-selectin and anti- $\alpha_4\beta_1$ mAbs, there was an 80–90% reduction of T cell migration to most inflammatory lesions, including poly I:C ($p < 0.005$) (Fig. 9B). When E-selectin, $\alpha_4\beta_1$, and P-selectin were simultaneously blocked, T cell migration to nearly all lesions was almost completely inhibited. Therefore, all three adhesion molecules are involved in the migration of activated CCR4⁺ CD4 cells to dermal inflammation, and all three must be blocked to virtually abolish cell recruitment. E-selectin appears to be especially important in the migration of activated CCR4⁺ CD4 cells to all inflamed skin sites, except poly I:C. However, when E-selectin is blocked, anti-P-selectin and anti- $\alpha_4\beta_1$ seem to each reduce the migration of CCR4⁺ CD4 cells further.

Effects of E-selectin, P-selectin, and $\alpha_4\beta_1$ blockade on CCR4⁻ CD4-activated T cell migration

Activated CCR4⁺ and CCR4⁻ CD4 cells have similar increases in the expression of ESL and PSL (Fig. 4B). However, the effect of E-selectin, P-selectin, and $\alpha_4\beta_1$ blockade on the migration of activated CCR4⁻ CD4 T cells has not been examined. E-selectin blockade significantly inhibited T cell migration to all of the inflammatory stimuli and to a similar or greater extent as for CCR4⁺ cells. It

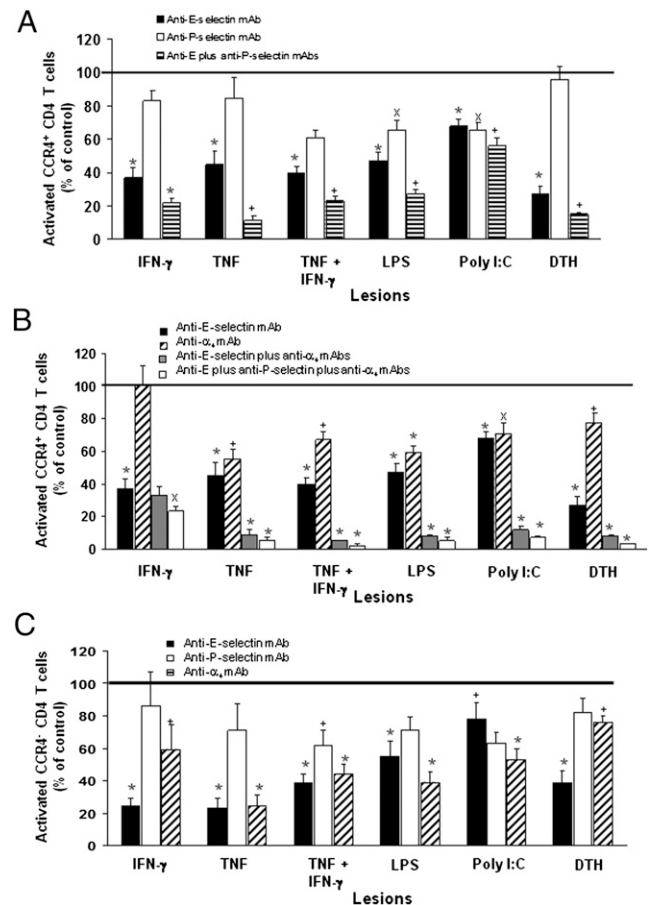


FIGURE 9. Effect of $\alpha_4\beta_1$, E- and P-selectin blockade on CCR4⁺ and CCR4⁻ activated CD4 T cell migration to dermal inflammation. Animals were treated with anti-E- or anti-P-selectin, anti- α_4 integrin mAb, or combinations of these mAbs. Accumulation of activated CCR4⁺ (**A**, **B**) and CCR4⁻ activated CD4 T cells (**C**) in dermal inflammatory sites was determined as in Fig. 6. Results are expressed as percentage of migration in response to the stimuli shown in the test animals as compared with controls injected with isotype-matched mAb. Each bar shows mean \pm SEM of 5–14 animals. * $p < 0.05$, * $p < 0.01$, * $p < 0.005$.

inhibited 20–40% of the T cell migration to poly I:C and LPS and 60–80% of the T cell migration to IFN- γ , TNF, IFN- γ plus TNF, and DTH (Fig. 9C). Therefore, E-selectin blockade similarly inhibits the migration of activated CCR4⁻ and CCR4⁺ CD4 T cells to dermal inflammation. The effect of P-selectin blockade on CCR4⁻ cells was also similar to its effect on CCR4⁺ CD4 T cells.

Blockade of $\alpha_4\beta_1$ significantly inhibited the migration of activated CCR4⁻ CD4 T cells to all of the inflammatory stimuli, and to most of the stimuli, this effect was greater than its inhibition of CCR4⁺ cell migration (Fig. 8C). Blockade of $\alpha_4\beta_1$ inhibited 50–60% of the CCR4⁻ CD4 T cell migration to IFN- γ plus TNF, LPS, and poly I:C and inhibited ~80% of the T cell migration to TNF (Fig. 9C), whereas CCR4⁺ CD4 T cell migration was inhibited to a lesser extent (Fig. 9B). Therefore, all three adhesion molecules are required for the migration of activated CCR4⁻ CD4 T cells to dermal inflammation. E-selectin and $\alpha_4\beta_1$ appear to be especially important in the migration of activated CCR4⁻ CD4 T cells to all of the inflamed skin sites.

Effects of E-selectin, P-selectin, and $\alpha_4\beta_1$ blockade on activated CD8 T cell migration

ESL and PSL is present on ~70 and ~33% of the activated CD8 T cells, respectively (Fig. 5B), but the effect of E-selectin, P-

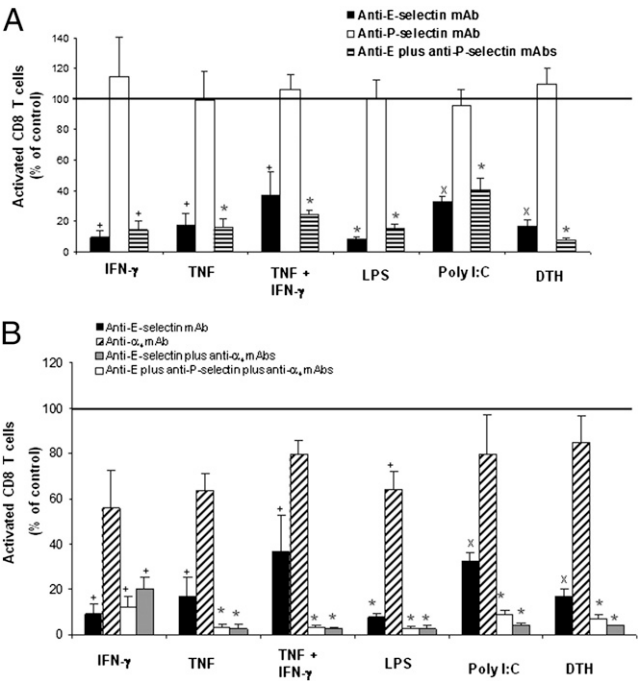


FIGURE 10. Effect of $\alpha_4\beta_1$, E- and P-selectin blockade on activated CD8 T cell migration to dermal inflammation. Animals were treated with anti-E- and/or anti-P-selectin mAbs (A) or anti- α_4 integrin mAb or combinations of these mAbs (B) or control mAb as indicated. The accumulation of activated CD8 T cells in dermal inflammatory sites is expressed as a percentage of migration in control animals. Each bar shows mean \pm SEM of five to eight animals. * $p < 0.05$, $\times p < 0.01$, $\ast p < 0.005$.

selectin, and $\alpha_4\beta_1$ blockade on the migration of activated CD8 T cells has not been examined. E-selectin blockade significantly inhibited T cell migration to all of the inflammatory stimuli ($p < 0.05$). It inhibited $\sim 60\%$ of the T cell migration to IFN- γ plus TNF and poly I:C and $\sim 80\%$ of the T cell migration to IFN- γ , TNF, LPS, and DTH (Fig. 10A). In nearly all of the lesions, anti-E-selectin was substantially more effective at inhibiting the migration of activated CD8 T cells than activated CD4 T cells (Figs. 9, 10A). P-selectin blockade had no effect on the migration of CD8 cells to dermal inflammation, and migration was not reduced further when P-selectin was blocked in the presence of anti-E-selectin. The blockade of $\alpha_4\beta_1$ significantly inhibited $\sim 30\%$ of the activated CD8 T cell migration to LPS ($p < 0.05$) but did not inhibit the migration to IFN- γ plus TNF, poly I:C, and DTH (Fig.

10B), whereas it inhibited $\sim 40\text{--}60\%$ of the migration of activated CD4 T cells to these lesions (Fig. 9) (see also Table I).

The absolute requirement for ESL and $\alpha_4\beta_1$ on CD8 T cell migration was demonstrated when both adhesion molecules were blocked. This blockade inhibited $\sim 90\%$ of the CD8 T cell migration to TNF, IFN- γ plus TNF, LPS, poly I:C, and DTH ($p < 0.005$).

Discussion

Our results describe, to our knowledge, 1) the development of the first mAb to rat CCR4 and the expression of CCR4, ESL, and PSL on normal and activated CD4 and CD8 cells. We also 2) demonstrate quantitatively the greatly increased ability of memory CD4 T cells expressing CCR4 to migrate to dermal inflammation in vivo and the difference in the relative migration of CCR4 $^+$ and CCR4 $^-$ cells to various inflammatory stimuli. We also show 3) that most activated CD4 T cells do not require CCR4 for migration to dermal inflammation in vivo and that 4) the role of E-selectin and $\alpha_4\beta_1$ differ between memory and activated CD4 cell migration. ESL is required for skin homing of activated CCR4 $^+$ and CCR4 $^-$ CD4 cells, whereas there is a greater dependence on $\alpha_4\beta_1$ for the migration of memory CCR4 $^+$ CD4 T cells. Surprisingly, activated CD8 T cells also are highly dependent on E-selectin for their migration to all dermal inflammatory stimuli studied (Table I).

An anti-rat CCR4 mAb, which we developed, was used to determine the expression of CCR4 on memory CD4 cells in various tissues. Although CCR4 $^+$ memory cells were found in the circulation (Fig. 2) (6, 12) and in inflamed tissues (Fig. 3) (8, 10, 11), the relationship between CCR4 expression and migration to dermal inflammation induced by various inflammatory stimuli, such as specific cytokines, TLR agonists (LPS and poly I:C), and DTH, has not been previously determined, except for CHS in mice.

As shown in this study, most memory cells are not CCR4 $^+$, yet CCR4 $^+$ memory CD4 cells accounted for much of the recruitment to inflamed skin in vivo. Virtually all of the unstimulated CD4 cells migrating to dermal inflammation express CXCR3 and have a memory phenotype (14). Therefore, all of the skin-infiltrating memory CCR4 $^+$ CD4 cells also express CXCR3. Previously, it was shown that only $\sim 50\%$ of their migration was inhibited by CXCR3 blockade (14). This suggests an important role for CXCR3 and a CCR4-associated, CXCR3-independent component to the memory cell migration to inflamed skin in vivo.

In contrast with memory CD4 cells, activated CD4 cell migration to inflamed skin did not depend on the expression of CCR4. Activated CCR4 $^+$ CD4 cells migrated to a similar extent to the

Table I. Dependence of T cells on CCR4, ESL, PSL, and $\alpha_4\beta_1$ for migration to dermal inflammation

		Inflammatory Stimulus					
		IFN- γ	TNF	TNF+ IFN- γ	LPS	Poly I:C	DTH
CCR4	Memory CD4	+++	+++	+++	+++	+++	+++
	Activated CD4	—	—	+	+	+	++
ESL	Memory CD4	—	+	—	+	—	+
	Activated CD4	++	++	++	++	+	+++
$\alpha_4\beta_1$	Activated CD8	+++	+++	++	+++	++	+++
	Memory CD4	+++	+	++	++	++	++
ESL + PSL	Activated CD4	+++	++	+	++	+	+
	Activated CD8	++	+	+	+	+	+
ESL + $\alpha_4\beta_1$	Activated CD4	+++	+++	+++	+++	++	+++
	Activated CD8	+++	+++	+++	+++	++	+++
	Activated CD4	++	+++	+++	+++	+++	+++
	Activated CD8	+++	+++	+++	+++	+++	+++

—, No effect; +, 10–40%; ++, 40–70%; +++, >70% inhibition.

cytokines and only 30–50% more to the TLR agonists and to DTH as CCR4[−] cells (Fig. 8A). These activated CCR4⁺ CD4 cells did not follow the same pattern of enhanced migration observed with memory CCR4⁺ CD4 cells (Fig. 6A). Even though CCR4 expression is upregulated on activated CD4 cells in rats (Fig. 4C), mice (38), and humans (6, 38, 39), our studies indicate it is not a selective marker for skin homing of activated CD4 cells. This has not been specifically studied previously *in vivo*. Reports have found that the migration of cells from Ag-stimulated LNs, where the proportion of activated and memory cells is not well defined, is partially CCR4 dependent (40).

CCR4⁺ T cells have been associated with the set of type 2 cytokine-secreting T cells and with Th2-mediated inflammatory responses, mainly because of the production of IL-4 from freshly isolated CCR4⁺ CD4 cells (6) and the presence of CCR4⁺ T cells in the skin and blood of patients with atopic dermatitis (7, 8, 41). However, CCR4 is not restricted to type 2 responses. Activation of CCR4⁺ CD4 T cells increases production of both IL-4 and IFN- γ (6), suggesting some Th1 and Th2 cells express CCR4 (42). CCR4⁺ T cells have been identified in Th1/Th17-mediated inflammatory responses, such as in psoriatic lesions (10). Our work suggests that the association of CCR4 expression on skin-infiltrating T cells is likely dependent on the inflammatory stimulus. Our studies did not specifically examine Th2-mediated inflammation, but we found that even in type 1 reactions, CCR4 on memory cells was strongly associated with migration to these reactions.

In addition, many studies correlate the expression of CCR4, chemotaxis to CCR4 ligands, and/or production of CCR4 ligands to CLA⁺ memory cells (12) or to skin-infiltrating CD4 cells (7), but this does not directly link CCR4 to skin homing of activated cells. Activated CD4 cells express several other CKRs, such as CXCR3 (14, 43); therefore, CCR4[−] cells may use CXCR3 to migrate to inflammation in which CXCR3 ligands are produced. Likewise, the production of CCR10 ligands by activated epidermal keratinocytes (44) may attract CCR10⁺CCR4[−] T cells (45). *In vivo*-activated CD4 T cells from CCR4^{−/−} mice are recruited normally to CHS, but their migration was inhibited by CCR10 blockade (46). In contrast, it was recently shown that cells from CCR4-deficient chimeric mice have fewer memory CD4 cells in ova peptide-induced DTH reactions (47). However, skin CD4 cells were measured after repeated immunization for ≥ 48 d and reflects the effect of CCR4 deficiency on long-term accumulation, whereas our studies have investigated the short-term migration of T cells out of the blood that occurs during an acute inflammatory reaction. Thus, our results clarify the relationship between CCR4 and memory and activated skin-infiltrating CD4 cells, because they suggest that activated CD4 cells do not rely on CCR4 for migration to inflamed skin, whereas CCR4 is a marker for most memory cells migrating to dermal inflammation.

More than 50% of the migration of memory CCR4⁺ CD4 cells was inhibited by $\alpha_4\beta_1$ blockade, whereas E-selectin blockade had little effect on recruitment to most stimuli tested and only a modest effect on TNF and DTH lesions (Fig. 7A). This may be because only 1–6% of the memory cells expressed ESL (Fig. 4), whereas virtually all T cells express $\alpha_4\beta_1$ (26). Nevertheless, E-selectin blockade can potentiate the inhibitory effect of α_4 blockade, as shown previously on unstimulated T cells (24, 48), suggesting that ESL does contribute to the migration of a small subset of memory CCR4⁺ CD4 cells. In the absence of $\alpha_4\beta_1$, it is likely that other integrins, such as CD11a/CD18 and CD103, participate in the T cell migration (49, 50). The blockade of CD11a/CD18 inhibits 40–80% of lymphoblast T cell migration to dermal inflammatory sites.

Compared with memory cells, the migration of activated CD4 cells was more dependent on E-selectin and less dependent on $\alpha_4\beta_1$ (see Table I). E-selectin blockade reduced the migration of activated CCR4⁺ and CCR4[−] CD4 cells by 60–80% to IFN- γ and DTH, whereas anti- α_4 mAb inhibited $\leq 20\%$ of the migration (Fig. 9B, 9C). In response to other stimuli, E-selectin and $\alpha_4\beta_1$ both inhibited migration of the activated CD4 cells, but memory cells were more consistently inhibited by blocking $\alpha_4\beta_1$ rather than E-selectin. Blocking E-selectin markedly potentiated the inhibitory effect of $\alpha_4\beta_1$ blockade on activated CCR4⁺ CD4 cell migration (Fig. 8B). Our results therefore demonstrate that E-selectin contributes to a significant portion of the activated CCR4⁺ CD4 cell migration, whereas the contribution of $\alpha_4\beta_1$ is more moderate and variable depending on the inflammatory stimuli (Table I). The difference in the migration of these two CCR4⁺ CD4 cells may be related to the increased ESL expression on CD4 cells after activation (Fig. 4C) as a result of the induction of $\alpha 1,3$ -fucosyl transferases in the activated cells (51, 52).

Our results showing that E-selectin blockade partially inhibits the migration of activated CD4 cells extend results showing that the migration of human Th2 cells to human skin grafts injected with CCL22 in SCID mice was inhibited by E-selectin blockade (19). *In vivo*-activated ESL⁺ T cells were also shown to accumulate in 2,4-dinitro-1-fluorobenzene-induced CHS in mice (20). This has suggested that ESL expressed by CCR4⁺ cells was required for migration of activated CD4 cells to dermal inflammation. However, activated CCR4⁺ and CCR4[−] CD4 cells express similar levels of ESL (Fig. 4). ESL mediates part of the migration of both activated CCR4⁺ and CCR4[−] CD4 cells, and $\alpha_4\beta_1$ is also critical to the recruitment of CD4 cells to dermal inflammation (Fig. 9). In fact, migration to some inflammatory stimuli such as the viral analog poly I:C is almost independent of E-selectin. Thus, neither ESL nor CCR4 expression nor even the combination of these two can identify activated CD4 cells with dermal tropism.

P-selectin blockade reduced part of the migration of activated CCR4⁺ CD4 cells to TNF and IFN- γ , LPS, and poly I:C but had no effect on migration to other sites such as DTH (Fig. 9A). The migration of activated CD4 cells to CHS in mice was inhibited by blocking P-selectin (23) and/or E-selectin (20). In our study, P-selectin blockade potentiated the inhibitory effect of E-selectin blockade on activated CCR4⁺ CD4 cell migration, even when P-selectin blockade alone had no significant effect (Fig. 9A). However, it should be noted that only $\sim 40\%$ of the migration of activated CCR4⁺ CD4 cells to poly I:C was inhibited after blocking both E-selectin and P-selectin (Fig. 9A). This may be due to differences in adhesion molecule expression with poly I:C versus other stimuli on the endothelium. These findings underscore the importance of considering multiple types of inflammatory reactions in developing a model of CKR and CAM usage in defining dermal tropism.

Thus, our results suggest a revised model for the skin homing of CD4 cells, whereby most skin-infiltrating memory CD4 cells require $\alpha_4\beta_1$ and express CCR4, and activated CD4 cells require ESL but are not dependent on CCR4 expression, with a considerable variation of the adhesion molecules involved, depending on the nature of the inflammatory stimulus.

Finally, our results also demonstrate that the migration of activated CD8 cells to inflamed skin is strongly ESL dependent and PSL independent. E-selectin blockade inhibited up to 90% of the activated CD8 cell migration to inflamed skin (Fig. 10). This is substantially greater than its effect on activated CD4 cells (Fig. 8). In contrast, PSL blockade, which reduced migration of activated CD4 cells to some sites (Fig. 8A, 8C), did not affect the migration of activated CD8 cells to inflamed skin (Fig. 10A). This contrasts

with Tc1 cell migration to mouse CHS, which was reported to be partially dependent on both ESL and PSL (31). Blocking $\alpha_4\beta_1$ alone, in contrast to its effect on CD4 cells, had minimal effect on activated CD8 migration but potentiated the inhibitory effect of E-selectin blockade (Fig. 10B), and the combination abolished the migration of activated CD8 cells to inflamed skin. Approximately 60% of those activated CD8 cells expressed CCR4 (Fig. 9B).

In conclusion, our findings contrast the expression of CCR4 on memory CD4 cells with dermal tropism and that on activated CD4 T cells. They also demonstrate the greater role of $\alpha_4\beta_1$ on memory cell migration and the major contribution of ESL on migration to dermal sites by activated CD4 and CD8 cells. In addition, they show the considerable variation in CAM usage in CD4 and CD8 cell migration to different stimuli especially between DTH reactions and a TLR4 versus a TLR3 agonist.

Disclosures

The authors have no financial conflicts of interest.

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