

CCR8 Expression Identifies CD4 Memory T Cells Enriched for FOXP3⁺ Regulatory and Th2 Effector Lymphocytes

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CD4⁺ Th2 cells are important regulators of allergic inflammation. CCR8 is thought to play a role in Th2-mediated responses, however, expression of CCR8 in peripheral blood has not been fully characterized. Using a fluorescent form of the ligand selective for CCR8 (F-CCL1), we identified the leukocytes expressing CCR8 in human, monkey, and mouse peripheral blood. CCR8 expression is primarily restricted to a subset of human CD4 memory T lymphocytes (15%). Approximately 40% of CCR8⁺CD4⁺ T cells express Th2 cytokines IL-4 or IL-13 while 13% express the Th1 cytokine IFN- γ . In fact, 50% of all Th2, but only 5% of Th1, cells express CCR8. Upon anti-CD3/anti-CD28 mAb-mediated activation, CCR8⁺CD4⁺ T cells secrete 3- to 7-fold higher levels of IL-4, IL-5, IL-9, and IL-13 and 10- to 20-fold lower levels of IFN- γ or IL-17, compared with CCR8⁻CD4⁺ memory T cells. Two-thirds of CCR8⁺CD4⁺ T cells express cutaneous lymphocyte-associated Ag while the majority lack gut-homing receptors. CCR8⁺CD4⁺ cells express CCR7 and CD62L and are present in spleen and lymph nodes of mice. Approximately 25% of CCR8⁺CD4⁺ T cells express CD25^{high} while 20% of CCR8⁺CD4⁺ express the T regulatory cell transcription factor FOXP3 accounting for 60% of all FOXP3-expressing CD4⁺ T cells. In conclusion, CCR8 marks a diverse subset of CD4 memory T cells enriched for T regulatory and Th2 cells which have the potential for recruitment into sites of allergic inflammation where they could participate in the induction and regulation of the allergic response. *The Journal of Immunology*, 2006, 177: 6940–6951.

The T lymphocyte pool consists of naive T cells and Ag-experienced memory T cells which can be further divided into nonpolarized (T_{NPM}),⁴ also referred to as central memory (T_{CM}), and effector memory (T_{EM}) T cells. T_{NPM} expand and acquire T-effector functions in response to Ag re-encounter while T_{EM} represent a circulating pool of polarized T cells capable of producing immediate effector functions upon restimulation. CD4⁺ T_{EM} cells include Th1 and Th2 cells which migrate to inflamed peripheral tissues where they secrete effector cytokines (IFN- γ by Th1 and IL-4, IL-5, IL-9, and IL-13 by Th2 cells, respectively) involved in amplifying the immune response (1, 2). In recent years, other populations of memory T cells that have the capacity to down-regulate immune responses through either cell-to-cell contact or the release of soluble mediators such as IL-10 and TGF- β , so-called regulatory T (T_{REG}) cells, have gained increased attention (3). A subpopulation of T_{REG} cells, known as naturally occurring T_{REG}, are generated in the thymus during T cell development and are best characterized by high expression levels of the IL-2R α -chain (CD25) and by the expression of the

forkhead family transcription factor FOXP3 which is necessary for their development and function (4, 5).

Migration of diverse T cell subsets during homeostasis and inflammation is controlled by the concerted expression of adhesion molecules, such as integrins and selectins, and chemokine receptors. For example, coexpression of L-selectin (CD62L) and the chemokine receptor CCR7 by T cells is a prerequisite for homing to lymphoid tissues (6–8) while expression of the integrin $\alpha_4\beta_7$ or cutaneous lymphocyte-associated Ag (CLA) are required for lymphocyte migration to the gastrointestinal tract or the skin, respectively (9–11).

Chemokine receptors belong to the class of seven transmembrane G protein-coupled receptors and have been shown to mediate a variety of biological processes upon chemokine binding, including angiogenesis, leukocyte activation, and chemokine-induced transendothelial migration through integrin activation and subsequent transmigration (12). Chemokines are small secreted proteins (~8 kDa) which can be divided into four subfamilies based on the spacing of two conserved cysteine residues (12). They can also be distinguished by their pattern of regulation: 1) lymphoid chemokines such as CCL19, CCL21, and CXCL13 are constitutively expressed in lymphoid tissues and mediate the migration of leukocytes into and within lymphoid tissues by engagement of their respective receptors CCR7 and CXCR5 (6, 13, 14), 2) chemokines constitutively expressed in nonlymphoid tissues, for example, CCL1 in the skin which attracts CCR8⁺ T cells (15), and 3) inducible chemokines such as CCL11 (16), CCL17 (17, 18), and CXCL10 (19) attract effector cells into inflamed tissues through engagement of CCR3, CCR4, and CXCR3, respectively.

We and others have recently reported the expression of the chemokine CCL1 by IgE-activated mast cells in vitro and in vivo, implicating CCL1 in the recruitment of inflammatory cell types involved in allergic inflammation (Refs. 20–22 and J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Villeval, J. Boyce, C.

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⁴ Abbreviations used in this paper: T_{NPM}, nonpolarized memory T cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{REG}, regulatory T cell; CLA, cutaneous lymphoid-associated Ag; F-CCL1, fluorescently labeled CCL-1; DAPI, 4',6'-diamidino-2-phenylindole.

Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication). Indeed, CCR8, the only identified receptor for CCL1, belongs to a small group of chemokine receptors, including CCR3 and CCR4 that have been shown to be preferentially associated with Th2 effector cells (23–25). Th2 cells are recruited to sites of allergic mucosal inflammation where they secrete the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 and orchestrate the hallmarks of allergic lung inflammation such as IgE class switching, mast cell and eosinophil activation, mucus hypersecretion, and airway hyperresponsiveness. The functional involvement of the CCR8/CCL1 axis in the recruitment of Th2 effector cells *in vivo* is supported by an increase in CCR8⁺ CD4⁺ T cell numbers in allergic asthma (Ref. 26 and J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Villeval, J. Boyce, C. Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication) and by a recent study demonstrating CCL1 and CCR8 up-regulation in atopic dermatitis (21). There are also reports suggesting CCR8 expression by CD4⁺CD25⁺ T_{REG} cells, skin-homing CLA⁺ T cells, monocytes, NK cells, and dendritic cells (21, 27–32). Recently, the majority of CD4⁺ and CD8⁺ T cells isolated from normal human skin, as well as small subpopulations of CD4⁺ and CD8⁺ peripheral blood T cells were reported to express CCR8 as well as both, CD45RO⁺ and CD45RO⁺ (15). The authors proposed a mechanism by which CCR8⁺ T cells home to and reside in healthy skin tissue and play a role in immune surveillance.

Intrigued by the increased numbers of CCR8⁺ CD4⁺ T cells in allergic asthma and atopic dermatitis and by the substantial numbers of CCR8-expressing T cells isolated from healthy human skin, we characterized the cell types expressing CCR8 in peripheral blood. The identification and phenotypic characterization of peripheral blood-derived leukocytes expressing the chemokine receptor CCR8 has been hampered by the limited availability of specific and suitable mAbs. Therefore, to identify cells expressing functional CCR8, we used fluorescently labeled CCL1 (F-CCL1), the ligand specific for CCR8. We discovered that CCR8 expression is absent from naive CD4⁺ T cells in the periphery and that it is largely restricted to a small but diverse subset of CD4⁺ T_{NPM} and T_{EM} that preferentially produce Th2 cytokines. The CCR8⁺CD4⁺ T cell compartment includes cells capable of homing to secondary lymphoid tissues and to peripheral tissues such as the skin and the lung, but not to the gut. It also contains a small subset of T_{REG} cells as indicated by the expression of FOXP3. CCR8-expressing cells functionally respond to CCL1-dependent activation with directional migration *in vitro*. Our data support a functional role for the CCR8/CCL1 axis in Th2 effector cell recruitment into sites of allergic mucosal inflammation and suggest that CCR8 participates in the induction, amplification, and/or the resolution phases of inflammatory responses.

Materials and Methods

Proteins and Abs

Recombinant human CCL1 was obtained from R&D Systems and recombinant human CCL1 labeled with Alexa Fluor 647 (F-CCL1) was obtained from Dictygene. Fluorochrome-conjugated Abs to human proteins were purchased from the following sources: CD3 (UCHT1), CD4 (L200), CD8 (RPA-T8), CD19 (HIB19), CD27 (M-T271), CD45RO (UCHL1), CD56 (B159), CD62L (Dreg56), CD94 (HP-3D9), CD16 (3G8), CD29 (MAR4), CRTH2 (BM16), and CLA (HECA-452) were obtained from BD Biosciences; CD25 (4E3) from Miltenyi Biotec; ICOS from eBioscience; CD123 (9F5) from StemCell Technologies; and CD49d from Chemicon International. Unconjugated Abs to CCR3 (7B11), CCR4 (1G1), CCR5 (2D7), CCR7 (7H12), CCR9 (3C3), CCR10 (1B5), CXCR3 (1C6), CXCR6 (7F3), and $\alpha_4\beta_7$ (act-1) were available in-house and were detected using relevant fluorochrome-conjugated secondary Abs. Fluorochrome-conjugated Abs to cynomolgus monkey CD4 (L200), CD8 (RPA-T8), CD56 (B159), CD16 (3G8), CD20 (2H7), CD25 (M-A251), and CD45RO (5H9)

were obtained from BD Biosciences. Fluorochrome-conjugated Abs to mouse CD4 (RM4-5), CD44 (IM7), CD8 (53-6.7), CD3 (145-2C11), and NK-1.1 (PK136) were obtained from BD Biosciences. For immunofluorescence microscopy, a goat polyclonal anti-human FOXP3 Ab and an FITC-conjugated donkey polyclonal to goat IgG H and L chains were obtained from Abcam.

Staining of whole blood, lymph nodes, and spleen with F-CCL1

Wild-type and CCR8^{-/-} C57BL/6 mice were maintained in pathogen-free conditions and used between 6 and 8 wk. CCR8^{-/-} mice were obtained from the laboratory of Dr. C. Martinez (Centro Nacional de Biotecnología Universidad Autónoma, Madrid, Spain). Lymph nodes and spleens from these animals were taken and recovered cells were filtered through a 70- μ m cell strainer to prepare a single-cell suspension. Blood was obtained from cynomolgus monkeys (Charles River Laboratories) and healthy human volunteers after obtaining informed consent.

A total of 200 μ l of whole blood, or 100 μ l of a 5×10^6 /ml cell suspension were stained at 37°C (5% CO₂/95% O₂) for 30 min in a humidified incubator with 5 nM F-CCL1. Staining with F-CCL1 at 4°C or 37°C yielded similar results but fluorescence intensity was greater at 37°C, most likely due to internalization of receptor/ligand complexes. Time course of binding experiments showed that maximum binding was achieved in 30 min at 4°C and in ~3 h at 37°C. To assess CCR8 expression in various cell subsets and to examine costaining with other cellular markers, samples were then placed on ice and stained with relevant mAbs (see *Proteins and Abs*) for 30 min at 4°C. RBC were lysed with ammonium chloride lysis solution (StemCell Technologies) and samples were analyzed by flow cytometry (BD FACSCalibur; BD Biosciences) using CellQuest software. To verify specificity and selectivity, all our phenotypic experiments included control samples with excess unlabeled CCL1 and small molecule antagonists selective for CCR8 (T. Jenkins, B. Guan, M. Dai, G. Li, T. Lightburn, S. Huang, S. Freeze, D. Burdi, S. Jacutin-Porte, R. Bennett, et al., submitted for publication).

Isolation of CCR8⁺ and CCR8⁻CD4⁺ populations

Complete medium consisted of RPMI 1640, 2 mM L-glutamine, 100 μ M nonessential amino acids, 1 mM sodium pyruvate, 10 μ g/ml penicillin/streptomycin (all obtained from Invitrogen Life Technologies), and 10% United States-defined FBS (HyClone).

The isolation of CCR8⁺ and CCR8⁻CD4⁺ memory T cells from human blood was performed as follows. Human PBMCs were isolated from fresh buffy coats (Oklahoma Blood Institute) by centrifugation on Ficoll-Paque PLUS (Amersham Biosciences). Pure (>98%) CD4⁺ memory T cells were obtained by negative selection using a CD4 memory⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. These cells were subsequently stained with 1 nM F-CCL1 overnight in complete medium at 37°C and sorted the following day by FACS into CCR8⁺ and CCR8⁻ subsets using a MOFLO (DakoCytometry) cell sorter. CCR8^{+/+} populations were typically of >97% purity. To examine the coexpression of CD25, CCR8, and FOXP3, CD4⁺ memory T cells were also sorted into CD25⁺ and CD25⁻ subpopulations before CCR8 sorting. Briefly, PBMC were isolated as above, and CD4⁺ T cells isolated using a CD4 T cell isolation kit II (Miltenyi Biotec). From these cells, CD25⁺ T cells were isolated using a CD4⁺CD25⁺ regulatory T cell isolation kit (Miltenyi Biotec) and put to one side. Naive CD4⁺ cells and CD45RO⁺ memory T cells were separated from the remaining CD25⁻CD4⁺ cells using CD45RO⁺ microbeads (Miltenyi Biotec). Both CD4 memory⁺ populations were stained with F-CCL1 (1 nM overnight) and anti-CD45RO-FITC and sorted based on CCR8/CD45RO expression by FACS as above.

Chemotaxis assays

After CD4⁺ memory T cells had been sorted into CCR8⁺ and CCR8⁻ subsets by FACS, they were incubated for 24 h at 37°C in complete medium to allow for washout of F-CCL1 labeling and receptor recycling to the cell surface. Cell migration was subsequently assessed using 3- μ m pore polycarbonate membranes in a 96-well multiscreen-MIC plate format (Millipore). Before performing the experiment, the upper surface of the chemotaxis membrane was precoated with a confluent monolayer of ECV-304 cells. CCL1 was diluted in chemotaxis buffer (CTXB: HBSS with 10 mM HEPES and 0.1% fatty acid-free BSA) to the indicated concentrations and placed in the lower wells of the chemotaxis plate. A total of 100 μ l of cell suspension (2×10^5 cells) was added to the upper wells and the plate was incubated at 37°C for 4 h in a humidified 5% CO₂ incubator. After incubating, the chemotaxis membranes were removed and the migrated cells in the lower chamber were loaded with Calcein AM dye (Invitrogen Life Technologies) before reading with the Discovery-1 cellular imaging platform. The resulting data were analyzed using MetaMorph software (both

Molecular Devices) and expressed as the mean chemotactic index (average number of migrated cells exposed to chemokine divided by the number of cells exposed to CTXB alone) from triplicate samples $\pm$ SD.

Intracellular cytokine staining

For intracellular staining, immediately after sorting, cells were stimulated with 10 ng/ml PMA and 1 μ g/ml ionomycin (both Sigma-Aldrich) for 6 h, the last 5 h in the presence of 10 μ g/ml brefeldin A (BD FastImmune; BD Biosciences) in complete medium at 37°C. Cells were washed with PBS and fixed with 4% paraformaldehyde, before being permeabilized with 0.5% saponin in PBA (5% BSA in PBS; no $\text{Ca}^{2+}/\text{Mg}^{2+}$). Cells were stained with combinations of the following Abs for 30 min at room temperature: mouse Abs to IL-4-PE (3007) and IL-13-PE (32007) (both R&D Systems), a rat Ab to IL-10-PE (JES3-9D7) and a mouse Ab to IFN- γ -FITC (25723.11) (both BD Biosciences) and a rat Ab to IL-4-allophycocyanin (MP4-25D2) (eBioscience). Samples were subsequently washed one time with saponin buffer, one time with PBA, and analyzed by flow cytometry.

Analysis of cytokine mRNA expression and protein secretion

To examine cytokine expression and secretion, sorted cells were resuspended in complete medium containing 50 ng/ml IL-2 (R&D Systems) and 10 μ g/ml anti-CD28 (BD Pharmingen) and plated at 2×10^5 /well in a 96-well EIA high protein-binding plates (Costar) that had been precoated with anti-CD3 (10 μ g/ml; BD Pharmingen). Cells were incubated for 6 and 24 h and supernatants were removed and stored at -80°C . Supernatants were sent to Pierce Endogen for Multiplexed Searchlight ELISA (Pierce) analysis of the following cytokines: IL-4, IL-5, IL-8, IL-9, IL-10, IL-13, IL-17, IFN- γ , and TNF- α . The cell pellets after supernatant removal were also stored at -80°C in preparation for RNA extraction to examine cytokine gene expression, described as follows.

Quantitative RT-qPCR

Total RNA was isolated using RNeasy technology on a Biorobot 8000 workstation and DNase treated according to the manufacturer's protocol (Qiagen). The purity and yield of the RNA were assessed using the Nanodrop ND-1000. Integrity of the RNA was measured with RNA 6000 Nano LabChip on a Agilent 2100 Bioanalyzer (Agilent Technologies) and calculated using the RNA Integrity Number algorithm. First-strand cDNA synthesis was performed with a reverse transcription system (Applied Biosystems) according to the manufacturer's protocol, except that both oligo-dT and random hexamers were used for priming. The integrity of cDNA samples was assessed via qTIAM analysis of 18S and β_2 -microglobulin transcripts by real-time PCR in an ABI PRISM 7700 (Applied Biosystems). Primers and MGB Eclipse probes (Nanogen) for real-time PCR (see below), were designed specifically to U133A&B GeneChip probe sets (Affymetrix) used in initial high-density microarray analysis. Transcripts of interest were assayed in a multiplexed format, using human β_2 -microglobulin RNA, as an endogenous control; analysis of MAS 5.0 data from corresponding U133A&B GeneChip data verified that levels of β_2 -microglobulin transcript remained constant across this set of RNA samples. Transcripts were amplified using *Taq* polymerase (Sigma-Aldrich) and cycling parameters of: an initial 95°C for 2 min, then 40 cycles of 95°C for 20 s, 58°C for 20 s, 76°C for 20 s. Data were analyzed using SDS 1.7 (Applied Biosystems) software. Data were analyzed using the comparative cycle threshold method, with the amount of transcript of interest normalized to β_2 -microglobulin transcripts (user bulletin no. 2 from Applied Biosystems). Forward/reverse/probe primers were: IL-4, ACACAAGTGAAGAAGAAACCTT/CCTGTGCGAGCCGTTTCAGGAAT/TACAGCCACCA TGAGA* Δ ; IL-5, CCCACAAGTGCATTGGTG/TCAGAGTCTCATTG GCTATCAGCAG/GCTTTCTACTCA*TCG; IL-9, GAGACTGTCTCA GATGACCA/GACTCTTCAGAAATGTCAGCGGTT/GCAAACAA*G ATACCC; IL-13, TGTGTCAGCCCTGGAAT/TGCTCGGACATGCAAG TCTGGAAA/CAGTGCCATCGAGA* Δ ; IL-17, ACTTGCTGCTGCTC AAC/GGACCAGGATCTCTTGCTGGAT/GACTACCA*CATGA*AC; T NF- α , GATCATCTCTCGAAGCC/GTTATCTCTCAGTCCACGCCA TT/TAGCAAACCCCTCAAGC; IFN- γ , CATCCAAGTGATGGCTGAAC/ TACTGGGATGCTCTTCGACCTCGAAA/AACAGG*GAAGCCGAAA A; β_2 -microglobulin, GCCTGCCGTGTGAACCATGTGACTTTGTC/CG GCATCTTCAAACCTCCATGA/GTTAAGTGGGATCGAGA; FOXP3, CTGAGCTGCACAAGTGCTTTT/TGGGAACACCTGCTGGGCCCTCT/ TGGACCTGGATGAGC. The base immediately preceding the asterisk is a specially modified base that the vendor (Epoch Biosciences (now Nanogen)) provides to increase the stability of the interaction of the primer. The shills are to indicate the separation between the sequences of the three primers of each gene.

Examination of FOXP3 expression by immunofluorescence microscopy

Sorted cells were resuspended at 1×10^6 /ml in complete medium. A total of 200 μ l of cell suspension was loaded into a Shandon single Cytotunnel, and the cells were spun onto microscope slides using a Shandon Cytospin 3 (both Thermo Electron). Slides were air-dried overnight, fixed in acetone for 5 min, and air-dried for 1 h. Slides were blocked with 10% FBS in PBS for 15 min in a humid chamber, then stained with anti-FOXP3 (1/200 dilution in blocking buffer) for 2 h in a humidified chamber. Slides were then washed for 10 min in PBS with 0.2% gelatin. For detection, slides were stained with tetramethylrhodamine isothiocyanate-conjugated donkey anti-goat IgG (1/200 dilution in 5% donkey serum in PBS) for 30 min in a humidified chamber in the dark. Control slides were prepared and incubated with secondary Ab alone to control for background staining. Slides were washed again with PBS containing 0.2% gelatin and mounted using 4',6'-diamidino-2-phenylindole (DAPI) mounting medium to allow for examination of nuclear colocalization. Slides were then viewed with an immunofluorescence microscope and images captured for analysis.

Results

CD4 memory T cells are the predominant cell type expressing CCR8 in peripheral blood

We recently found that the number of CCR8-expressing cells in lungs of asthmatic individuals is increased 4-fold when compared with healthy controls and that $\sim 70\%$ of all lung CD4^+ T cells express CCR8 (J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Villeval, J. Boyce, C. Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication). Thus, it was important to characterize CCR8 expression in human peripheral blood leukocytes and its potential to orchestrate allergic mucosal inflammation in the lung. CCL1 has been shown to specifically bind to and activate CCR8. To identify CCR8-expressing cells, we used F-CCL1 which exhibits similar chemotactic potency as unlabeled CCL1 (data not shown). For staining peripheral blood leukocytes, F-CCL1 was used at concentrations that induce maximal cell migration (1–5 nM). These concentrations also achieved maximal staining. In human whole blood, F-CCL1 consistently stained a subpopulation of $\text{CD4}^+\text{CD45RO}^+$ memory T cells ($15 \pm 4\%$; $n = 50$ different donors), representing 91 $\pm 7\%$ of all labeled cells identified (Fig. 1A; Table I). Staining specificity and selectivity for CCR8 was thoroughly verified by 1) competition with excess unlabeled CCL1 (Fig. 1, A and B) or TCA-3 for mouse samples, 2) absence of staining in lymphocytes of CCR8-deficient mice (Fig. 1C), and importantly, by 3) competition with several small molecule antagonists shown to be selective for CCR8 vs all other chemokine receptors and a large panel of other G protein-coupled receptors. In addition, MC148-huFc, an engineered fusion protein containing at the N terminus MC148, a selective CCR8 viral chemokine antagonist (33), also gave the same pattern of staining as F-CCL1, and both chemokines were competitive with each other (data not shown). All non-CD4 expression of CCR8 is confined to small subsets of CD8^+ T lymphocytes ($2 \pm 1.6\%$; $n = 12$) and CD56^+ NK cells ($0.7 \pm 0.46\%$; $n = 12$) (Table I). The Th2/T cytotoxic 2 cell-associated PGD_2 receptor, CCR2 (34), is expressed in a subset of $\text{CCR8}^+\text{CD8}^+$ T cells (Fig. 1A). Within the CD56^+ NK cell compartment, CCR8 expression is associated preferentially ($16 \pm 3.8\%$) with a small subset that expresses high CD56 levels ($\text{CD56}^{\text{high}}\text{CD94}^{\text{high}}\text{CD16}^-\text{CD3}^-$) (Fig. 1A) (35, 36). All other subsets of NK cells investigated including NKT cells, $\text{CD8}^+\text{CD56}^+$ and $\text{CD8}^-\text{CD56}^+$ NK cells, had levels of expression similar to the total CD56^+ population. CCR8 expression was not observed in $\text{CD4}^+\text{CD45RO}^-$ or $\text{CD4}^+\text{CD45RA}^+$ naive T cells (Fig. 1A), or $\text{CD4}^+\text{CD45RA}^+\text{CD45RO}^+$ T cells, CD19^+ B lymphocytes, plasmacytoid ($\text{CD123}^{\text{high}}\text{BDCA-2}^+$) or myeloid ($\text{CD123}^{\text{low}}\text{BDCA-1}^+$ or $\text{CD123}^-\text{BDCA-3}^+$) dendritic cells, CD123^+ basophils, CD14^+ monocytes, CD16^+ neutrophils, or

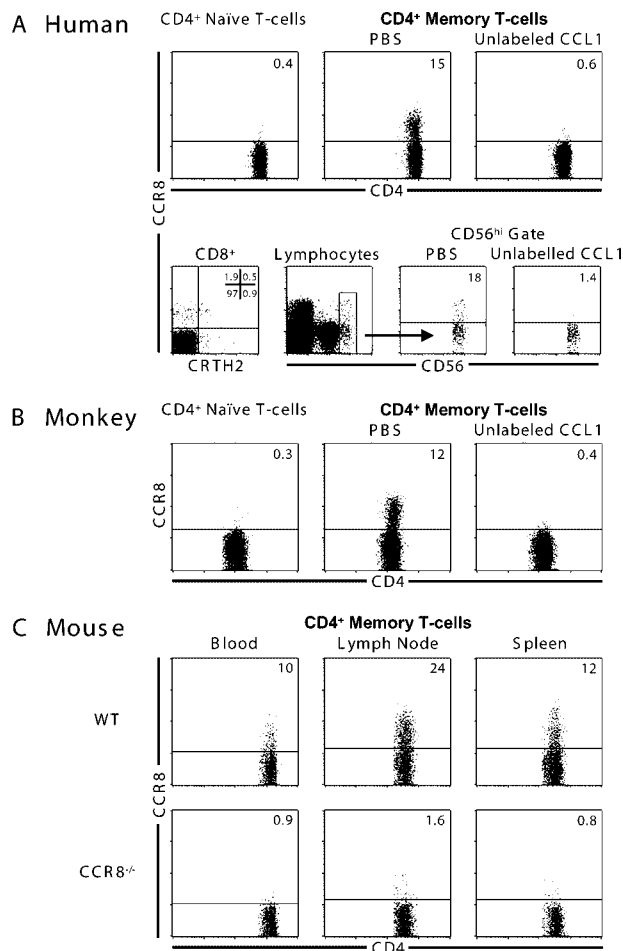


FIGURE 1. CCR8 expression is mainly restricted to a subset of memory CD4⁺ T cells in human, monkey, and mouse peripheral blood. *A*, Flow cytometric analysis of human blood stained with 5 nM F-CCL1 alone (PBS) or with 40 nM unlabeled CCL1 and markers for CD4⁺ memory T cells (CD4, CD45RO; *top panel*), CD8 and CRTH2 T cells (*bottom panel, left*), and CD56⁺ NK cells (*bottom panel, right*). CCR8 expression was examined in the naive CD4⁺ T cell (CD4⁺CD45RO⁺), memory CD4⁺ T cell (CD4⁺CD45RO⁺), CD8⁺ T cell, lymphocyte, and CD56^{high} cell gates, and specificity of staining was demonstrated in all cases by competition with unlabeled CCL1. The percentage of CCR8-expressing cells is indicated in the *top right quadrant* of each plot. For CCR8 vs CRTH2 expression in the CD8 gate, the frequency of all four possible subsets is indicated. *B*, Flow cytometric analysis of cynomolgus monkey whole blood stained with CCL1, CD4, and CD45RA. CCR8 expression was examined in the naive CD4⁺ T cell gate (CD4⁺CD45RA⁺) and the memory CD4⁺ T cell gate (CD4⁺CD45RA⁺) and verified to be specific by competition with unlabeled CCL1. *C*, Flow cytometric analysis of mouse whole blood, as well as spleen and lymph node cell suspensions from wild-type and CCR8-deficient mice stained with CCL1, CD4, and CD44^{high}. CCR8 expression was examined in the gated memory CD4 cell population (CD4⁺CD44^{high}). These plots are representative and complete data set are summarized in Table I.

CCR3⁺CD49d⁺ eosinophils (data not shown). Similarly, CD4 memory T cells were the predominant cell type expressing CCR8 in peripheral blood from cynomolgus monkeys (~10%; Fig. 1*B*; Table I) and naive mice (~10%; Fig. 1*C*, Table I), respectively. We also identified low numbers of CCR8⁺CD8⁺ T cells and NK cells in peripheral blood from cynomolgus monkeys (Table I) and as in humans, the CD56^{high}CD16⁺ NK population was enriched for CCR8⁺ cells (data not shown). In mice, CCR8 was not expressed on CD8⁺ T cells but it was expressed by a small number

of NK (NK1.1; 0.4%) cells and NKT cells (2%) (data not shown). Leukocyte types and numbers expressing CCR8 in peripheral blood of human, monkey, and mouse are summarized in Table I. In addition, we identified CCR8⁺CD4 memory T cells, NK and NKT cell populations in naive mouse spleens (14, 1, and 3%, respectively) and lymph nodes (24, 3, and 4%, respectively) but not in the corresponding tissues derived from CCR8-deficient mice (data not shown).

In summary, our data clearly indicate that CD4⁺ memory T cells are the predominant cell type expressing CCR8 in peripheral blood across species (human, monkey, mouse) and that these cells either have the potential to migrate into secondary lymphoid organs and/or acquire a CCR8⁺ phenotype during activation in these lymphoid tissues as indicated by the presence of CCR8⁺CD4⁺ memory T cells in naive mouse spleens and lymph nodes.

Activated CCR8⁺CD4 memory T cells preferentially produce Th2 cytokines in vitro

Th effector cells are best characterized by the types of cytokines expressed upon activation: Th1 effector cells produce IFN- γ whereas Th2 effector cells produce IL-4, IL-5, IL-9, and IL-13. To investigate the potential of CCR8⁺CD4 memory T cells to express either Th1 or Th2 cytokines, we sorted CD4 memory T cells isolated from human peripheral blood into CCR8⁺ and CCR8⁻ populations (purity >97%) (Fig. 2*A*) and determined the relative cell numbers expressing IL-4, IL-10, IL-13, and IFN- γ by intracellular staining following activation in vitro. To ensure that the sorted populations were representative of their respective counterparts in blood, we compared their relative phenotypic frequencies with respect to expression of CLA, CD25, CCR5, CCR7, CD62L, CD27, and CRTH2 and found no significant differences. As shown in Fig. 2, *B* and *C*, the percentage of activated cells expressing IL-4 was 6-fold higher in the CCR8⁺ than in the CCR8⁻ population (CCR8⁺ 26 $\pm$ 3%; CCR8⁻ 4 $\pm$ 1%) and cells expressing IL-13 were 10-fold more frequent in the CCR8⁺ than in the CCR8⁻ population (CCR8⁺ 20 $\pm$ 2%; CCR8⁻ 2 $\pm$ 0.6%). Conversely, cells expressing IFN- γ were 3-fold more frequent in the CCR8⁻ population (CCR8⁺ 13 $\pm$ 4%; CCR8⁻ 42 $\pm$ 8%). Approximately one-third of all CCR8⁺ IL-4- and IL-13-expressing cells coexpressed both cytokines most likely reflecting a probabilistic distribution of expression of each cytokine as recently demonstrated for in vitro-cultured Th2 cells (37). Within the CCR8⁺ population, there was very little coexpression of IL-4 or IL-13 with IFN- γ , whereas ~50% of IL-4- and IL-13-expressing CCR8⁻ cells coexpressed IFN- γ likely representing still uncommitted Th0 cells. Both cell populations exhibited a similar frequency of IL-10-expressing cells (2%). In summary, ~40% of all CCR8⁺ memory T cells express either IL-4 or IL-13 after activation, consistent with Th2 effector function. When compared with the total number of CD4 memory T cells the CCR8⁺ population contains ~40% of all IL-4, 60% of all IL-13, 15% of all IL-10, but only 5% of all IFN- γ -expressing cells (Fig. 2*D*). Of particular significance is the almost complete inclusion of all IFN- γ -producing Th1 effector cells in the CCR8⁻ population (95%).

The enrichment of Th2 effector cells within the CCR8⁺CD4 memory T cell population was further supported by the higher levels of IL-4 (3 $\pm$ 0.9-fold), IL-5 (5 $\pm$ 1-fold), IL-9 (2 $\pm$ 0.5-fold), and IL-13 (3 $\pm$ 0.8-fold) secreted by CCR8⁺ than by CCR8⁻ CD4 memory T cells 24 h after anti-CD3/anti-CD28 activation (Fig. 2*E*). Levels of the Th1 cytokine IFN- γ (12 $\pm$ 6-fold) and IL-17 (8 $\pm$ 0.1-fold) were greater in the CCR8⁻ population. The differences in IL-9 (6-fold), IL-13 (7-fold), and IL-17 (20-fold) levels were even more pronounced 48 h after activation (data

Table I. CCR8 expression in human, monkey, and mouse peripheral blood^a

	Human			Monkey			Mouse		
	% CCR8 ⁺		% of total CCR8 ⁺ leukocytes	% CCR8 ⁺		% of total CCR8 ⁺ leukocytes	% CCR8 ⁺		% of total CCR8 ⁺ leukocytes
	Mean ± SD	n		Mean ± SD	n		Mean ± SD	n	
CD4 memory T cells	15 ± 4	50	91	11 ± 4	10	77	9 ± 1.4	5	76
CD8 T cells	2.1 ± 1.6	12	8	0.8 ± 0.4	10	22	0	5	0
NK cells	0.7 ± 0.4	12	3	1.2 ± 0.7	5	4	0.4 ± 0.09	5	23

^a The frequencies of CCR8⁺ cells in the indicated cell populations is the average ± SD of multiple donors (n). The percentage of total CCR8⁺ cells contained within each population is shown in the rightmost column for each species.

not shown). Protein levels correlated with mRNA levels as determined by quantitative real-time PCR 6 or 24 h after anti-CD3/anti-CD28 activation (Fig. 2F). The cytokine expression data clearly indicate that CCR8 expression selects for a subset of peripheral blood CD4 memory T cells enriched in Th2 effector cells.

Phenotypic characterization of CCR8⁺CD4 memory T cells

CCR8⁺CD4⁺ T cells have been proposed to express skin-homing receptors and T_{REG} markers but a complete phenotypic characterization in whole blood has not been reported. To further characterize blood-derived CCR8⁺CD4⁺ T cells and their homing potential, we investigated their phenotype in detail.

CCR4 and CCR10, as well as CCR8 and CLA, have been proposed to be critically involved in skin-specific T cell homing during homeostasis and inflammation (15, 38–40). Approximately two-thirds of CCR8⁺CD4 memory T cells express the skin homing receptor CLA (66 ± 12%; n = 20) the majority of which (86 ± 12%; n = 10) also express the chemokine receptor CCR10 (Fig. 3B, Table II). The CCR8⁺CD4 memory T cell subset contains ~36 ± 5% of all CLA⁺ and 65 ± 7% of all CCR10⁺CD4 memory T cells. In addition, while CCR8⁺ CD4 memory T cells contain ~22 ± 6% of all CCR4⁺CD4 memory T cells (Table II), CCR4 is expressed by nearly the entire CCR8⁺ population (97 ± 3%) (Fig. 3B; Table II). It is therefore possible that homing of the CCR8⁺CLA⁺ cells is restricted to the skin under conditions where one or a combination of appropriate chemokines are expressed in the skin.

Interestingly, we observed that within the CCR8⁺ population, IL-4- and IL-13-producing cells were enriched 2-fold in the non-skin homing (CLA[−]CCR8⁺) as compared with the skin-homing (CLA⁺CCR8⁺) subset (30 vs 15% and 21 vs 16% for IL-4 and IL-13, respectively). Conversely, IFN-γ-producing cells were 2-fold enriched in the CLA⁺CCR8⁺ population when compared with the CLA[−]CCR8⁺ subset (12 vs 6%). However, in the CCR8[−] subsets, the frequency of IL-4, IL-13, or IFN-γ-producing cells appeared to be independent of CLA expression. Therefore, Th2 cytokine-producing effector cells are enriched in the CCR8⁺CLA[−] population, presumably a subset capable of homing to the lung.

The integrin α₄β₁ (CD49dCD29) has been implicated in lung homing by virtue of its interaction with VCAM-1 (41). All CCR8⁺CD4⁺ T cells express the β subunit CD29, however, we found that only half of CCR8⁺CD4⁺ T cells express α₄ although the vast majority of CCR8[−]CD4⁺ T cells express this α subunit. Interestingly, the majority of CLA[−]CCR8⁺ cells express high levels of α₄ while the skin-homing cells were mostly negative or intermediate/low for α₄ (Fig. 3D) and expressed α₅ (data not shown). It is thus possible that the CLA[−]CCR8⁺α₄^{high}β₁⁺ subset, enriched in Th2 cells (see above), might preferentially migrate to the lung and participate in allergic inflammation of the airway.

The integrin α₄β₇ and chemokine receptor CCR9 are involved in gut homing of T cells (10, 11, 42, 43). Only 1.5 ± 0.44% of CCR8⁺CD4 T cells are α₄β₇^{high}—accounting for only 0.6% of all α₄β₇^{high} CD4 memory cells. Even less coexpress CCR9 and α₄β₇^{high} (0.7 ± 0.05% representing only 1.5% of all double-positive cells in the CD4 memory population) (Fig. 3C). Therefore, 97–99% of CD4 memory cells expressing gut-homing receptors reside in the CCR8[−] compartment and peripheral CCR8⁺CD4 T cells most likely do not home to the gut. The majority of peripheral blood CCR8⁺CD4 memory T cells express chemokine receptor CCR7 (90%) and CD62L (75%), which colocalize on ~70% of the cells (Fig. 3D, Table II), indicating the ability of these cells to migrate to secondary lymphoid tissues.

High expression levels of the IL-2R α-chain (CD25) have been associated with cell activation and T_{REG} cells. About half (47 ± 6; n = 18 donors) of all CD25^{high}CD4 memory T cells express CCR8 accounting for a quarter (25 ± 6%; n = 22 donors) of all CCR8-expressing CD4 T cells. CD25^{high} cells are enriched 3-fold in the CCR8⁺ subset as compared with the entire CD4 memory T cell population (Fig. 3E, Table II). Interestingly, about two-thirds of all CCR8⁺CD25^{high} T cells belong to the subset of potentially skin-homing T cells (Fig. 3E; Table II). This CCR8⁺CLA⁺CD25^{high} subset accounts for one-quarter of all skin-homing CCR8⁺CD4 memory T cells. CD25^{high} is also expressed in one-quarter of the CLA[−]CCR8⁺ subset, which may be defined as systemic homing, due to the lack of skin and gut-homing properties.

The CD28-related molecule ICOS functions to regulate clonal expansion and more recently has been associated with regulatory functions (44, 45). ICOS is expressed in a quarter of CCR8⁺CD4⁺ cells, representing 40% of all ICOS-expressing CD4 memory T cells (Fig. 3F). Similarly, lack of expression of the TNFR homolog CD27 has been associated with effector-type T cells and with cells recently activated by Ag (46, 47). Approximately one-third of all CD27[−]CD4 memory T cells express CCR8. Both ICOS expression and absence of CD27 are enriched 2-fold in the CCR8⁺ population (Table II) indicating an accumulation of T_{EM} cells.

We also identified small subpopulations of CCR8⁺ cells expressing the Th2-associated receptors CRTH2 (9 ± 4%; n = 14) and CCR3 (10 ± 6%) (Table II). In both cases, these cells represent about one-third of all CRTH2⁺ and CCR3⁺CD4 memory T cells and they are enriched 2-fold in the CCR8 compartment as compared with the total CD4 memory population (Fig. 3F, Table II). CCR4 which is also associated with Th2 function is expressed in virtually all CCR8⁺CD4⁺ cells (Fig. 3B). At the same time, chemokine receptors which are enriched in Th1 effector cells such as CXCR3, CCR2, CCR5, and CXCR6 (25) were expressed by 30, 27, 24, and 11% of the CCR8⁺ cells, respectively. These frequencies are either the same or lower compared with the total CD4 memory population and account for minor fractions of all CD4 memory cells expressing these receptors (Fig. 3G, Table II).

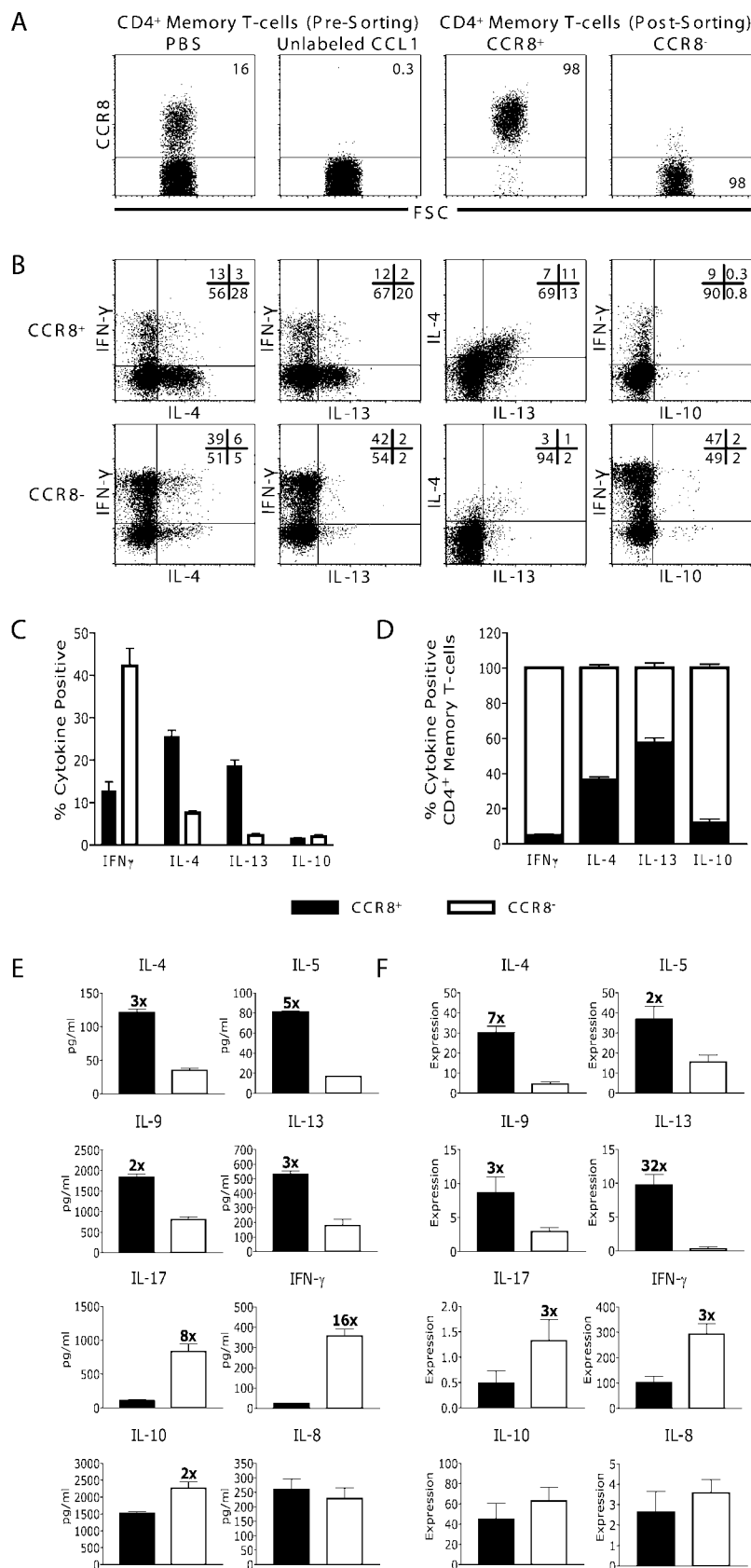


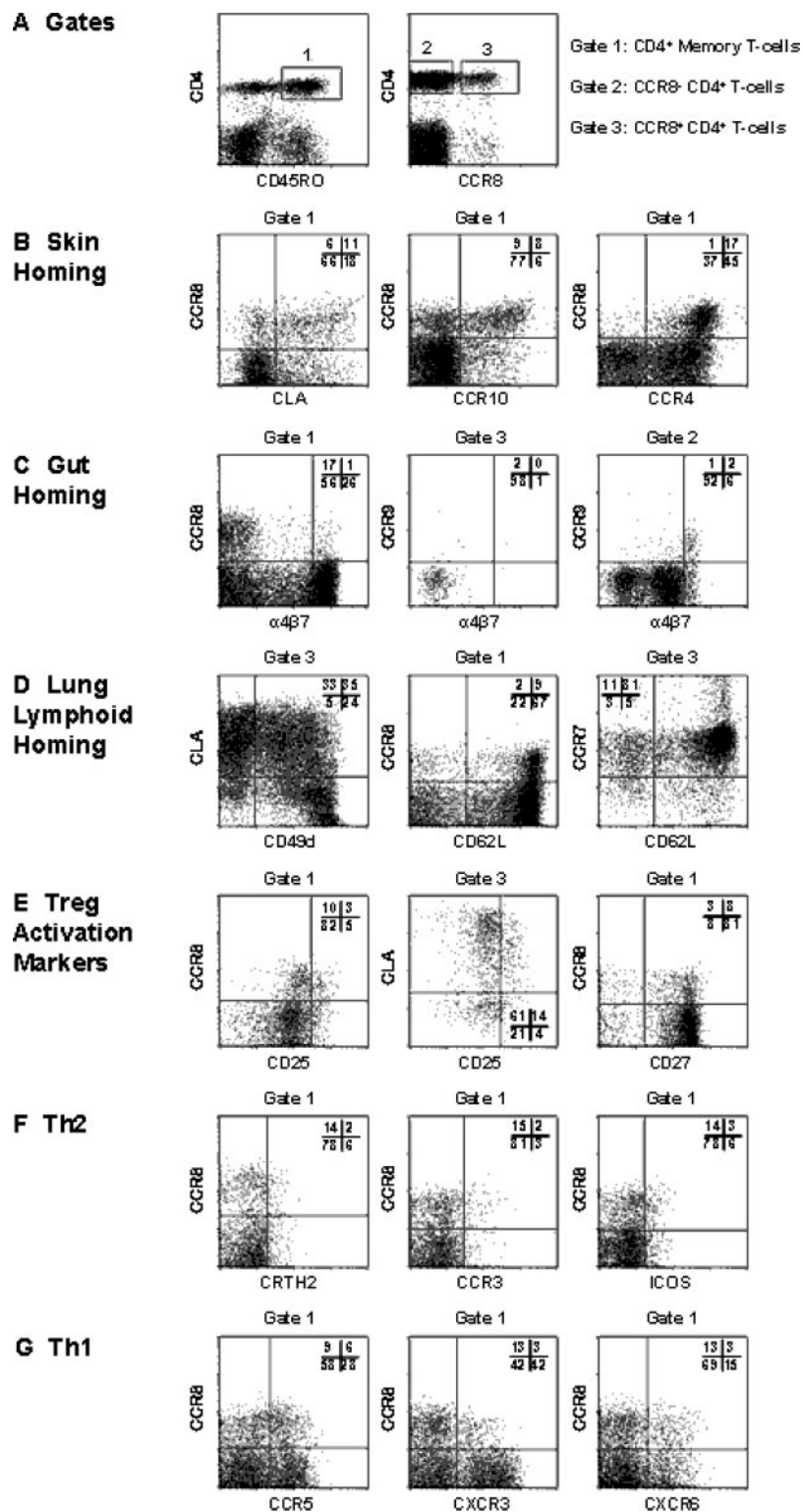
FIGURE 2. CCR8⁺ memory CD4⁺ T cells preferentially produce Th2 cytokines. **A**, Freshly isolated memory CD4⁺ T cells were labeled with F-CCL1 and sorted into the CCR8⁺ and CCR8⁻ subsets. Flow cytometric analysis demonstrating CCR8 expression in isolated memory CD4⁺ cells before sorting (*left panel*) and in sorted CCR8⁺ and CCR8⁻ memory CD4 cells. Staining specificity was monitored using excess unlabeled CCL1 (*second from left*). **B**, A representative example of CCR8⁺ and CCR8⁻ CD4 memory T cells activated for 6 h with ionomycin/PMA, stained with anti-cytokine Abs, and examined by flow cytometry for intracellular cytokine expression. Plots show the relative frequencies of all four possible cell subsets indicated in the *right top corner*. **C**, Percentage of cytokine-producing cells in the CCR8⁺ (■) and CCR8⁻ (□) memory CD4⁺ T cell subsets (average $\pm$ SD of three independent experiments/donors). **D**, Distribution of all memory CD4 T cells expressing a given cytokine between the CCR8⁺ (■) and CCR8⁻ (□) memory CD4 T cell populations (average $\pm$ SD of three independent experiments). **E** and **F**, Levels of (**E**) secreted cytokine and (**F**) cytokine mRNA following activation of CCR8⁺ and CCR8⁻ memory CD4 T cells with anti-CD3/anti-CD28 for 24 h. Shown is a representative experiment of three independent experiments. Numbers indicate fold differences between the two populations.

FOXP3 expression by CCR8⁺ CD4 memory T cells

The forkhead transcription factor family member FOXP3 is the most specific marker for thymic-derived T_{REG} cells and is required for T_{REG} cell development and function (4). CCR8 has recently

been shown to be expressed by CD4⁺CD25⁺ T_{REG} cells and to mediate CCL1-dependent migration of those cells in vitro (27–29). It is however unclear to what degree CCR8 expression is confined to T_{REG} cells. To address this issue, we used a FOXP3-specific Ab

FIGURE 3. Phenotypic analysis of human peripheral blood memory CD4 T cells expressing CCR8. *A*, Flow cytometry plots indicating the gates used in *B–G*. The frequencies of all four possible subsets in each plot are indicated in the *top right quadrant*. *B*, CCR8 coexpression with the skin-homing cell surface markers CLA, CCR10, and CCR4. *C*, CCR8 expression analysis in CD4 memory T cells stained with the gut-homing receptor $\alpha_4\beta_7$ (*left plot*); analysis of CCR9 vs $\alpha_4\beta_7$ expression in CCR8⁺CD4⁺ T cells (*middle plot*) and in CCR8[−]CD4⁺ T cells (*right plot*). The quadrants were established to distinguish the $\alpha_4\beta_7^{\text{high}}$ and $\alpha_4\beta_7^{\text{int}}$ populations rather than according to the isotype control. *D*, Analysis of CLA vs CD49d (α_4) expression in CCR8⁺CD4 T cells (*left plot*); CCR8 expression in CD4 memory T cells stained with CD62L (*middle plot*) and coexpression of lymphoid-homing receptors CCR7 and CD62L in CCR8⁺CD4⁺ T cells (*right plot*). *E*, CCR8 and CD25 coexpression in CD4 memory T cells (*left plot*); expression of CLA vs CD25 in CCR8⁺CD4 T cells (*middle plot*) (quadrants were established to distinguish the CD25^{high} from CD25^{int/low}) and coexpression of CCR8 with CD27 in CD4 memory T cells (*right*). *F*, Coexpression of CCR8 with CRTH2, CCR3, and ICOS in memory CD4 T cells. *G*, Coexpression of CCR8 with CCR5, CXCR3, and CXCR6. Shown are representative examples from multiple donors.



and enumerated CCR8⁺ and CCR8[−]CD4 memory T cells whose nuclei were positive for FOXP3, as determined by costaining with the nuclear dye DAPI. FOXP3 protein localized to the nuclei of ~21% of all CCR8⁺ cells and only 3% of CCR8[−] cells (Fig. 4, *A* and *B*), accounting for ~60 and 40% of all FOXP3⁺ CD4 memory T cells, respectively (Fig. 4*C*). All CCR8⁺FOXP3⁺ cells express CD25 and represent 50–70% of the CCR8⁺CD25⁺ population (Fig. 4*D*). No FOXP3⁺ nuclei could be identified in CD4 naive T cells or in the absence of the FOXP3 Ab indicating staining spec-

ificity (data not shown). Interestingly, cells with high-intensity FOXP3 staining exclusively localized to the CCR8⁺ population. The distribution of FOXP3⁺ cells across the different cell populations investigated was supported by FOXP3 mRNA expression data, showing the highest FOXP3 mRNA levels in CD25⁺CCR8⁺ and CD25⁺CCR8[−] memory T cells (Fig. 4*E*). In addition, activation with anti-CD3/anti-CD28 for 6 h resulted in increased FOXP3 mRNA levels (3- to 5-fold) in the CD25⁺CCR8⁺ and CD25⁺CCR8[−] subsets with no effect on the CD25[−] subsets (data

Table II. CCR8⁺ CD4 memory T cells are enriched in cells expressing CLA, CCR10, CCR4, CD25^{high}, ICOS, CRTH2, CCR3, and CD27[−] as compared to the total CD4 memory T cell population^a

		CD4 Memory	CCR8 CD4 Memory		% of Marker ⁺ CD4 Memory Expressing CCR8	<i>n</i>
	Marker	% expression	% expression	Fold enrichment		
Skin homing	CLA	24 ± 4	66 ± 12	2.8	36 ± 5	18
	CCR10	12 ± 2	65 ± 8	5.4	65 ± 7	15
	CCR4	62 ± 12	97 ± 3	1.6	22 ± 6	5
Gut homing	CCR9	7 ± 3	2 ± 1	0.3	4 ± 2	9
	α ₄ β ₇ ^{high}	26 ± 6	1.5 ± 0.4	0.06	0.6	10
	CCR9/α ₄ β ₇ ^{high}	5 ± 1	0.7 ± 0.05	0.14	1.5	4
Lymphoid homing	CCR7	87 ± 9	90 ± 9	1.1	15 ± 3	6
	CD62L	82 ± 8	75 ± 10	1.1	14 ± 2	8
Treg and/or activation	CD25 ^{high}	8 ± 2	25 ± 6	3.1	47 ± 6	22
	CLA ⁺ CD25 ^{high}	5 ± 0.2	18 ± 6	3.6	36 ± 13	4
	CCR10 ⁺ CD25 ^{high}	3 ± 1.4	19 ± 2	6	59 ± 9	4
	ICOS	12 ± 3	26 ± 6	2	34 ± 5	6
	CD27 [−]	10 ± 5	20 ± 6	2	35 ± 18	7
Th2	CRTH2	4 ± 2	9 ± 2	2.2	33 ± 8	14
	CCR3	4 ± 2	10 ± 6	2.5	35 ± 11	5
Th1	CCR5	25 ± 5	24 ± 5	1.0	13 ± 5	5
	CXCR3	59 ± 12	30 ± 12	0.5	7 ± 2	5
	CXCR6	13 ± 4	11 ± 5	0.8	12 ± 5	5

^a Phenotypic characterization of CCR8⁺ and total CD4 memory T cell populations was performed by flow cytometry. Numbers represent average expression frequencies ± SD of the indicated markers in either population for the number of donors investigated (n). Fold enrichment refers to the ratio between the frequency in the CCR8⁺ subset over that of the total memory CD4⁺ population. The second column from the right shows the percentage of total CD4 memory T cells expressing any given marker that also express CCR8.

not shown). The high overlap of FOXP3, CD25^{high}, CLA, and CCR10 expression (see above and Table II) indicates the existence of CCR8⁺ T_{REG} cells with skin-homing potential. Nevertheless, our data indicate that T_{REG} cells, as identified by FOXP3 expression, comprise a minority of the entire CCR8⁺ CD4 memory T cell population (~20%) (Fig. 4B).

CCR8⁺ CD4 memory T cells migrate in response to CCL1

The main proposed biological function mediated by CCR8 is ligand-induced cell migration. We compared the responsiveness of sorted CCR8⁺ and CCR8[−] memory T cells to CCL1 in transwell chemotaxis assays to address functional expression of CCR8 on CD4 memory T cells. The CCR8⁺ cells specifically migrated to CCL1, which induced a maximum response at 0.3 nM (Fig. 5) whereas the CCR8[−] cells were unresponsive. Both populations responded similarly well to the CCR7 ligand CCL21 (data not shown).

Discussion

CCR8 has been proposed to play a role in Th2-mediated responses, in T_{REG} function, and in skin immunosurveillance but the precise phenotype of CCR8-expressing cells in the periphery is poorly characterized. We describe the phenotype and functional properties of CCR8⁺ cells in peripheral whole blood.

The selectivity, specificity, and high affinity of CCL1 for CCR8 presented the possibility of preparing a fluorescently labeled variant of CCL1 that could be used as a probe for the identification of leukocytes expressing CCR8. A subpopulation of CD4⁺CD45RO⁺ memory T cells (15%) is the most abundant leukocyte type expressing CCR8 in human (>90%), monkey (77%), and mouse (76%) peripheral blood. CCR8 is also expressed by small populations of CD8⁺ T cells and CD56⁺ NK cells. Most intriguingly, we identified preferred association of CCR8 with CD56^{high}CD94^{high}CD16[−]CD3[−] NK cells in some of the donors investigated. These cells might represent a unique population of activated NK cells in vivo (35, 36), as supported by a study reporting CCR8 expression by adherent and IL-2-activated, but not nonactivated, NK cells in vitro (31).

Very limited expression of CCR8 on T cells in peripheral blood has been reported recently by Schaerli et al. (15) using a newly developed anti-CCR8 polyclonal Ab raised against the N-terminal peptide. However, in contrast to their report, we found that all CCR8⁺ CD4 T cells did not coexpress CD45RO and CD45RA but exclusively express high levels of CD45RO, indicative of memory T cells. The reasons for this discrepancy are unclear although post-translational modifications in the N-terminal peptide of chemokine receptors are known to occur and could affect cross-reactivity of the Ab to primary cells. In addition, specificity of the polyclonal anti-CCR8 Ab to primary cells was not demonstrated as conclusively as we demonstrated specificity and selectivity of F-CCL1 binding. The same report suggests that 30–40% of peripheral blood-derived CCR8⁺ T cell clones cultured in vitro express IFN-γ, and only 10% express IL-4. This is in contrast to our results indicating that ~40% of all CD4⁺CCR8⁺ T cells are Th2 cells while 13% are Th1 cells. This difference could be due to the use of T cell clones as compared with freshly isolated peripheral blood T cells. Although ~13% of CCR8⁺ CD4 memory T cells expressed IFN-γ, this population only represents ~5% of all IFN-γ-producing CD4 memory T cells, whereas CCR8⁺ Th2 cells represent ~50% of all Th2 cells. These results are in agreement with reports indicating expression of CCR8 on in vitro differentiated and activated Th2 cells (23, 24, 48). We also found that levels of secreted IL-17 are far greater in the CCR8[−] population. IL-17 has recently been suggested to be a crucial mediator of autoimmune responses thought to be Th1 in nature (49). Most recently, IL-17-producing CD4⁺ memory Th cells (Th17) have been proposed to represent a separate differentiation lineage than Th1 or Th2 cells (50).

The presence of a small population of CCR8⁺ Th1 effector cells was also supported by CCR8 coexpression with the Th1-associated chemokine receptors CXCR3 (30%), CCR2 (27%), CCR5 (24%), and CXCR6 (11%). However, while CD4 memory T cell subsets expressing these receptors are enriched in Th1 effectors, they also contain a significant frequency of nonpolarized cells and Th2 effectors and thus their expression is not restricted to Th1 cells (25). More interestingly, although ~40% of CCR8⁺CD4 cells are Th2 effectors, only 9% expressed CRTH2 which is considered the most

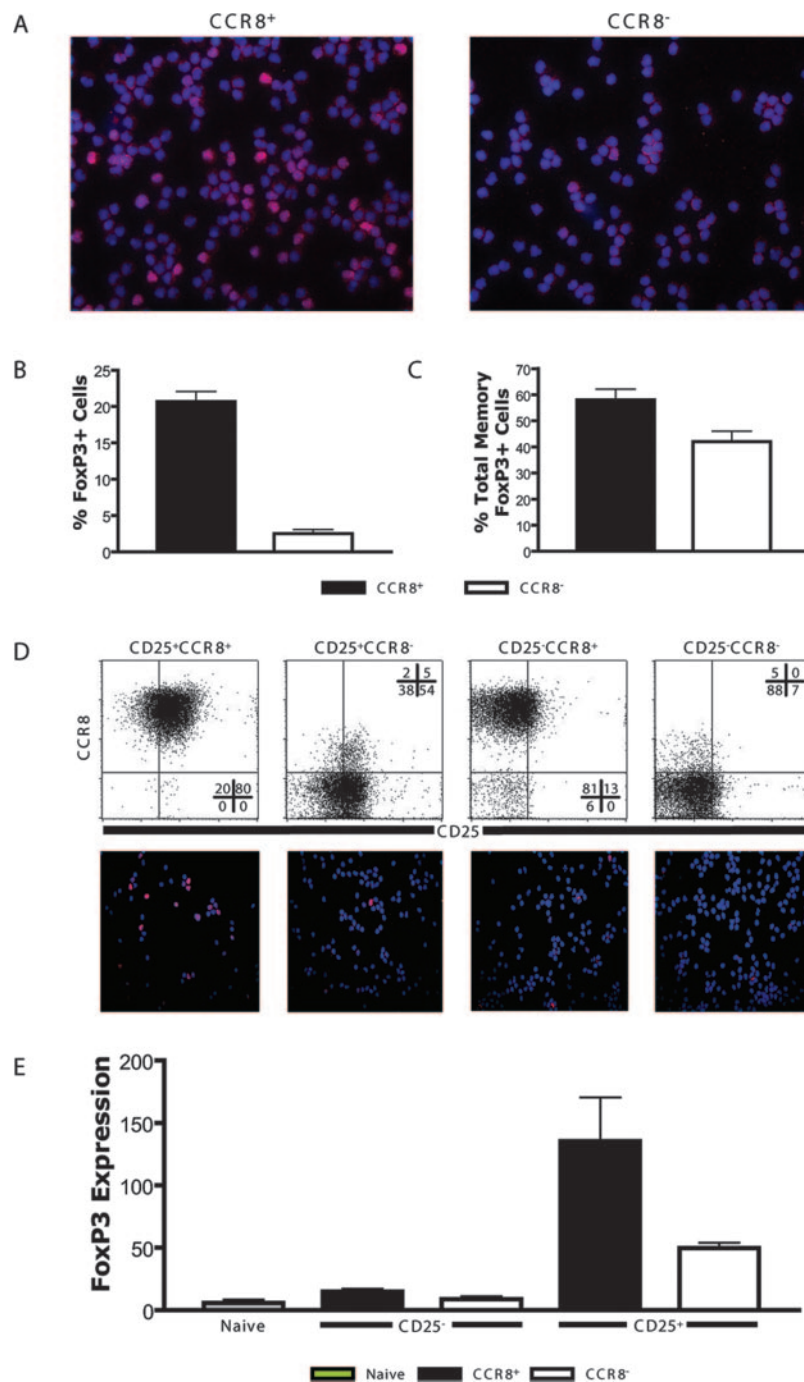


FIGURE 4. FOXP3 expression by 20% of CCR8⁺CD4 memory T cells. *A*, Freshly isolated CCR8⁺ and CCR8⁻CD4 memory T cells were stained with an Ab specific for FOXP3 and the nuclear dye DAPI. The merged images are shown. *B*, Percentage of CCR8⁺ (■) and CCR8⁻ (□) cells expressing nuclear FOXP3 (average $\pm$ SD of three independent experiments). *C*, Percentage of total FOXP3⁺ CD4 memory T cells which are CCR8⁺ (■) or CCR8⁻ (□) (average $\pm$ SD of three independent experiments). *D*, Representative flow cytometry analysis of sorted CD4 memory T cells based on CCR8 and CD25 expression (*upper panel*). Representative images of nuclear FOXP3 staining of the sorted populations (*lower panel*). *E*, Real-time PCR analysis of FOXP3 mRNA expression in the four subsets shown in *D* and in naive CD4 T cells.

reliable marker for peripheral Th2 effector cells (34, 51, 52). Although CRTH2 expression in CD4 T cells is restricted to Th2 effectors, our data clearly indicate that CRTH2 is expressed only on a fraction of all peripheral blood Th2 cells in agreement with the findings of Iwasaki et al. (52) who found CRTH2 expression in about half of Th2 cells in a broad panel of subjects. Other chemoattractant receptors associated with Th2 cells, including CCR8 (Refs. 23 and 24 and data presented herein), CCR3 (53), and CCR4 (23, 54), might be involved in mediating migration of these cells into inflamed tissues. CCR8 is the chemokine receptor most highly enriched in Th2 cells. In fact, we and others have reported increased numbers of CCR8⁺ T cells in asthmatic lung biopsies (Ref. 26 and J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Vallel, J. Boyce, C. Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication) and atopic skin lesions

(21). In addition, we observed a correlation between mRNA levels of CCR8 with those of IL-13, IL13-R α 2, IL-5, eosinophil cationic protein, and mast cell tryptase in human asthmatic lung biopsies. CCR8 mRNA was also detected in cells isolated by laser capture microscopy from bronchial alveolar-associated lymphoid tissue from normal (nonasthmatic) subjects (E. Fedyk, unpublished observation). The presence of CCR8⁺ Th2 effector cells in peripheral blood (data herein), their accumulation at sites of allergic inflammation, and the concomitant identification of activated mast cells as a major source producing CCL1 *in vivo* indicates an important role for the CCL1-CCR8 axis in the orchestration of allergic mucosal inflammation. In support of this notion is the phenotype of CCR8-deficient mice in a mast cell-dependent model of allergic airway inflammation, including reduced lung inflammation, Th2 cytokine levels, airway hyperresponsiveness, and mucus hypersecretion

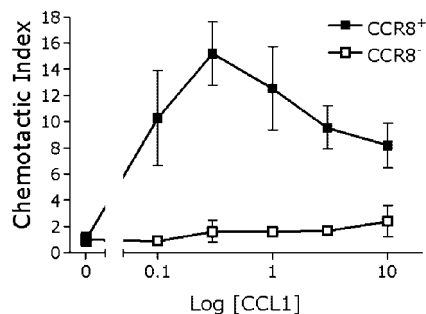


FIGURE 5. CCL1 induces cell migration of sorted CCR8⁺ but not CCR8⁻ CD4 memory T cells. Freshly isolated cells were incubated overnight to allow for CCR8 re-expression before chemotaxis assays. The chemotactic index was calculated as the fold difference in migrated cell numbers between the indicated chemokine concentration and the control without chemokine. Average of triplicate determinations $\pm$ SD are represented.

(J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Villeval, J. Boyce, C. Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication).

Importantly, although CCR8 expression enriches for Th2 effector cells in peripheral blood, its expression is not exclusive to this effector subset. Actually, $\sim 50\%$ of CCR8⁺CD4⁺ memory T cells do not express either IFN- γ , IL-4, or IL-13. We prefer to call these cells T_{NPM} rather than T_{CM} because we find no correlation between CCR7 expression and effector phenotype, or lack thereof, in agreement with other investigators (25, 55). In fact, 90% of CCR8⁺CD4⁺ T cells express CCR7 with no bias toward T_{NPM} or T_{EM} subsets. Although T_{EM} cells were initially described as memory T cells with effector function and lack of CCR7 expression (56), it is now generally accepted that only a small fraction of circulating T_{EM} have lost CCR7 expression and that rather, the term effector memory is more accurately ascribed to cells capable of rapidly producing cytokines upon Ag encounter regardless of their lymphoid tissue-homing capacity. Indeed, this notion is also consistent with our observation that 80% of CRTH2 CD4 T cells, a well-recognized terminally differentiated Th2 subset, express CCR7. It remains to be determined whether the differences in CCR7 expression are due to the properties of the Abs used in the various studies. Effector-dependent modifications of CCR7 may differentially affect Ab reactivity and/or CCR7 function.

We found coexpression of CD62L and CCR7 in $\sim 70\%$ of CCR8⁺ CD4 cells and for the first time we show the presence of CCR8⁺CD4⁺CD44^{high} memory T cells in naive mouse lymph nodes and spleens, further supporting the ability of CCR8⁺ T cells to home to secondary lymphoid tissues. Whether these cells represent previous peripheral blood CCR8⁺ T cells or cells that up-regulate CCR8 while in lymphoid tissue remains to be investigated.

Similarly to CCR7/CD62-L coexpression being required for T cell homing to lymphoid tissues, expression of the integrin $\alpha_4\beta_7$ or CLA is a prerequisite for T cell homing to the gut or the skin, respectively. We found that $\alpha_4\beta_7$ was not expressed by CCR8⁺CD4 memory T cells in agreement with a report indicating the absence of CCR8⁺ cells among T cells isolated from normal small intestine or colon (15). However, two-thirds of the CCR8⁺CD4⁺ memory T cells express the skin homing-associated receptor CLA, suggesting a functional role for CCR8 in mediating skin-specific homing. Consistent with these data, CD4 memory⁺ T cells selected via chemotaxis to CCL1 are enriched in CLA expression (27). In addition, constitutive expression of CCL1 in nor-

mal skin as well as functional expression of CCR8 on CD4⁺ and CD8⁺ T cells isolated from normal skin have been reported (15), suggesting a functional role of the CCL1-CCR8 axis in skin-specific immunosurveillance. In another study, increased levels of CCL1 in atopic dermatitis lesions correlated with increased numbers of CCR8⁺ T cells suggesting an involvement of CCR8 in mediating T cell migration to inflamed skin (21). Nevertheless, functional involvement of CCR8 in skin-specific T cell homing during homeostasis and/or acute inflammation remains to be demonstrated. We report here coexpression of the chemokine receptors CCR10 and CCR4 on CCR8⁺CLA⁺ CD4 memory T cells. Both receptors have been shown to mediate T cell recruitment to inflamed skin (39, 57). The relative contribution of CCR4, CCR8, and CCR10 or their synergistic cooperation in mediating skin-specific T cell migration, as demonstrated for CCR4 and CCR10 (39), still requires more detailed investigation using gene-deficient mice or function blocking Abs. Tissue microlocalization of functionally distinct T cell subsets may involve the sequential action of several chemokine receptors with one receptor mediating migration from blood while others may mediate migration within the tissue.

The expression of CCR8 by thymus-derived T_{REG} cells is supported by several studies showing functional CCR8 expression (cell migration) on human thymic CD4⁺CD25⁺ (29) and peripheral blood-derived CD4⁺CD25⁺ T_{REG} (28). High expression of the IL-2R α subunit CD25 is considered the most reliable cell surface marker for naturally occurring T_{REG} cells, although CD25 is also up-regulated on activated T cells (5). T_{REG} cells have been shown to play an important role in controlling autoimmunity and in the regulation of pathological and physiological immune responses (58). Our data indicate that $\sim 25\%$ of the CCR8⁺CD4⁺ memory T cells express high CD25 levels and that 50% of total CD4⁺CD25^{high} cells are contained within the CCR8 compartment. These results are in good agreement with the fact that $\sim 20\%$ of the CCR8⁺ T cells and 3% of the CCR8⁻ T cells express FOXP3 (with all FOXP3 staining confined to the CD25⁺ sorted subsets). The CCR8⁺ cells expressing FOXP3 represent $\sim 60\%$ of all FOXP3⁺CD4⁺ memory T cells. Of note is the observation that FOXP3 staining intensity was highest in CCR8⁺ T cells, which might correlate with a stronger suppressor phenotype. Virtually all CD4⁺CD25⁺ cells in the thymus express CCR8 (29) suggesting that CCR8 expression might be lost in the periphery by a subset of originally CCR8⁺FOXP3⁺ cells, although we cannot completely exclude the possibility of contamination of the CCR8⁻ subset with a small fraction of CCR8⁺ cells. However, the concordance of CD25^{high} staining in whole blood among the CCR8⁺ and CCR8⁻ subsets with the FOXP3 data in the sorted populations, makes this possibility unlikely. CCL1 is expressed in the thymus and CCR8/CCL1 interactions may play a role in T_{REG} cell development by either directing the localization of these cells to specific thymic structures or by rescuing them from negative selection upon TCR/MHC activation given that CCR8 signaling has been shown to have antiapoptotic effects (59–62). However, it should be noted that CCR8-deficient mice do not exhibit any of the characteristics of the severe autoimmune and lymphoproliferative disorder resulting from FOXP3 deficiency (63, 64). Therefore, either the role of CCR8 in T_{REG} development and function is not essential or chemokine receptor redundancy (e.g., CCR4) compensates for the absence of CCR8 function. This is supported by a recent study that demonstrates involvement of CCR4 in T_{REG} migration into cardiac allografts (65). We conclude that CCR8 expression is not restricted to CD4 T_{REG} cells, but that the CCR8⁺CD4 T cell population is enriched in FOXP3⁺ T_{REG} cells.

In summary, we provide data indicating that the chemokine receptor CCR8 is expressed by a small and heterogeneous population of peripheral blood CD4 memory T cells enriched in Th2 effector and T_{REG} cells. CCR8⁺CD4⁺ memory T cells comprise two major subsets according to their tissue-homing specificity: skin-homing cells, which account for approximately two-thirds of CCR8⁺CD4⁺ cells and nonskin, nongut homing cells which account for about one-third of CCR8⁺CD4⁺ cells. Within each homing subset, the relative frequencies of T_{NPM}, T_{EM}, and CD25^{high} cells are very similar as in the total CCR8 population. The major difference between these two homing subsets is the relative frequencies of Th1 and Th2 cells. In the skin-homing subset, the frequencies of Th1 and Th2 effectors are similar while in the systemic homing subset, the frequency of Th2 effectors is three to five times higher. Altogether, CCR8 may participate in the induction and amplification phase of inflammatory responses to pathogen or allergen by recruiting T_{EM} cells. At the same time it may also participate in the recruitment of T_{REG} cells contributing to the establishment of the appropriate balance of effector/regulatory cells which will influence the final outcome of the inflammatory response. T_{REG} cells would provide the regulatory function required to contain collateral damage that may be caused by an exaggerated immune response and/or also participate in the resolution phase of an ongoing inflammatory response. CCL1/CCR8 interactions may also be important for intratissue migration of T_{REG} to target cells producing CCL1, including dendritic or T cells. In this regard, APCs and Th1 cells have been reported to produce CCL1 (66, 67). CCR8⁺ T_{REG} attraction by Th1 cells might further shift the balance toward a Th2 response. Whereas the functional involvement of CCR8 in skin-specific T cell recruitment still needs to be validated, data in mouse models of allergic airway inflammation (Ref. 68 and J. A. Gonzalo, Y. Qiu, J. M. Lora, A. Al-Garawi, J. L. Villeval, J. Boyce, C. Martinez, G. Marquez, I. Goya, Q. Hamid, et al., submitted for publication) indicate the functional importance of the CCL1-CCR8 axis in Th2 responses. Thus, functional inhibition of the chemokine receptor CCR8 or its ligand CCL1 might prove a valuable strategy for the treatment of allergic diseases such as asthma or atopic dermatitis.

Disclosures

The authors have no financial conflict of interest.

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