Brief Definitive Report

Unique Chemotactic Response Profile and Specific Expression of Chemokine Receptors CCR4 and CCR8 by $CD4^+CD25^+$ Regulatory T Cells

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Abstract

Chemokines dictate regional trafficking of functionally distinct T cell subsets. In rodents and humans, a unique subset of $CD4^+CD25^+$ cytotoxic T lymphocyte antigen (CTLA)-4^+ regulatory T cells (Treg) has been proposed to control peripheral tolerance. However, the molecular basis of immune suppression and the trafficking properties of Treg cells are still unknown. Here, we determined the chemotactic response profile and chemokine receptor expression of human blood-borne $CD4^+CD25^+$ Treg cells. These Treg cells were found to vigorously respond to several inflammatory and lymphoid chemokines. Treg cells specifically express the chemokine receptors CCR4 and CCR8 and represent a major subset of circulating $CD4^+$ T cells responding to the chemokines macrophage-derived chemokine (MDC)/CCL22, thymus and activation-regulated chemokine (TARC)/CCL17, I-309/CCL1, and to the virokine vMIP-I (ligands of CCR4 and CCR8). Blood-borne $CD4^+$ T cells that migrate in response to CCL1 and CCL22 exhibit a reduced alloproliferative response, dependent on the increased frequency of Treg cells in the migrated population. Importantly, mature dendritic cells preferentially attract Treg cells among circulating $CD4^+$ T cells, by secretion of CCR4 ligands CCL17 and CCL22. Overall, these results suggest that CCR4 and/or CCR8 may guide Treg cells to sites of antigen presentation in secondary lymphoid tissues and inflamed areas to attenuate T cell activation.

Key words: chemokines • lymphocyte homing • cytokines • T lymphocyte subsets • immunosuppression

Introduction

The efficient operation of the immune system is critically dependent on a complex series of cellular interactions and movements to specific locations. Chemokines are small chemotactic cytokines characterized by critically positioned cysteine residues, which recruit distinct leukocyte subsets to sites of inflammation and specific microenvironments within secondary lymphoid tissues (1, 2). Chemokine receptor expression is exquisitely regulated depending on the stage of activation and differentiation of T cells and coordinates tissue localization and encounters with APCs (3–5).

One of the fundamental features of the immune system is its ability to preserve a delicate balance between effector responses and mechanisms of immunoregulation. Given the central role of chemokines in the regulation of immunity, we postulated that some of these molecules could participate in turning off adaptive immune responses by recruiting cells with immunoregulatory functions. Various subpopulations of $CD4^+$ T cells that can mediate immunosuppression both in vitro and in vivo have been described (6–8). Recent studies demonstrated that $CD4^+CD25^+CD45RB^{low}$ memory T cells expressing cytotoxic T lymphocyte antigen (CTLA)-4 are regulatory T cells (Treg) that mediate tolerance to organ-specific self-antigens and can prevent autoimmunity and intestinal inflammation in mice (9, 10). Recently, human peripheral blood $CD4^+CD25^+$ T cells have also been characterized as suppressor T cells (11). We now report the unique chemotactic response profile of human blood-borne $CD4^+CD25^+$ Treg cells. Treg cells specifically express the chemokine receptors CCR4 and CCR8 and respond to the chemokines macrophage-derived chemokine (MDC/CCL22), thymus and activation-regulated chemokine (TARC/CCL17), I-309/CCL1,

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and the virokine vMIP-I, which are agonistic ligands of these receptors. Mature DCs producing CCL17 and CCL22 were found to preferentially attract Treg cells, suggesting involvement of these chemokines in Treg cell function.

Materials and Methods

Chemokines and Abs. CCL1, CCL22, CCL17, vMIP-I, CCL11, CXCL11, and CXCL12 were purchased from Dictagene, whereas CXCL13, CCL19, CCL2, CCL3, CCL4, CCL20, and CCL5 were purchased from R&D Systems. Abs for various surface molecules were from BD PharMingen unless indicated otherwise. Goat anti–human CCR4 Ab was from Research Diagnostics Inc. Goat anti–human CCR8 Ab was from Alexis Corporation. Mouse anti–human CD4 Ab was from Novocastra Laboratories Ltd. All the conjugated and unconjugated secondary Abs for immunofluorescent stainings were from Jackson ImmunoResearch Laboratories.

Cell Isolation. Human $CD4^+$ T cells were purified from healthy donor peripheral blood by Ficoll-Paque (Amersham Pharmacia Biotech) density gradient centrifugation followed by isolation with immunomagnetic beads ($CD4^+$ T cell isolation kit; Miltenyi Biotech). $CD4^+$ T cells were stained with PE-labeled anti-CD4 and FITC-labeled anti-CD25 mAbs and sorted using a FACStar™ (Becton Dickinson). Purity was routinely >95%. Human monocytes were purified from PBMCs by isolation with immunomagnetic beads (Monocyte isolation kit; Miltenyi Biotech) and cultured in RPMI 1640 with 5% FetalClone I (HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin (Sigma-Aldrich; complete medium). Dendritic cells (DCs) were derived from human monocytes by culturing them in complete medium in the presence of 10 ng/ml of IL-4 (BD PharMingen) and 800 U/ml of GM-CSF (Myelogen; Schering-Plough) for 6 d. Maturation of DCs was induced by stimulation with 1 μg/ml of LPS in fresh complete medium. After 16 h of stimulation, DCs were washed and cultured for an additional 24 h in complete medium. Finally, the supernatants were harvested and tested in chemotaxis.

Chemotaxis Assays. Chemotaxis assays were performed using 5-μm pore polycarbonate filters in a Transwell chamber (Corning Costar Corporation) as described (12). After migration, 1.35×10^4 polystyrene beads (Polysciences Inc.) were added in the lower compartment of the Transwell. Migrated $CD4^+$ T cells were recovered and stained with anti-CD4, anti-CD25, and anti-CD45RO mAbs. The number of migrated T cells was measured by flow cytometer acquisition of a fixed number of beads. For the CCL17 and CCL22 preclearing experiment, 2 ml of DC supernatants were incubated 2 h at 4°C with 4 μg of mAbs, anti-CCL17, and/or anti-CCL22 (R&D Systems) or an isotype-matched control Ab coupled to 25 μl of protein G sepharose beads (Amersham Pharmacia Biotech), and after filtration to remove beads, the supernatants were used for chemotaxis. To calculate specific migration, the number of cells in each subpopulation in the absence of chemokine was subtracted from the number of the corresponding cell subpopulation migrated in the presence of chemokines. To evaluate the percentage of specific migration, the number of specifically migrated $CD4^+CD25^+$ T cells was divided by the total number of specifically migrated cells. To calculate the chemotactic index, the number of cells migrated in response to chemokines was divided by the number of spontaneously migrated cells.

Proliferation Assay. To assess the proliferative response of human $CD4^+$ T cells, we purified allogeneic PBMCs and depleted T cells with anti-CD3 microbeads (Miltenyi Biotech). Then, 2.5×10^4 PBMCs were irradiated (3,000 rad) and mixed to 2.5×10^4 of migrated $CD4^+$ T cells that were depleted or not of $CD25^+$ T cells by FITC-labeled anti-CD25 mAb followed by anti-FITC microbeads (FITC multisorting kit; Miltenyi Biotech). $[^3H]$thymidine incorporation was measured by scintillation counting on day 3 after a 6-h pulse.

Confocal Microscopy Analysis. Slides for double immunofluorescent stainings were postfixed with 4% paraformaldehyde, blocked with 15% FCS/PBS. After washing, the slides were incubated with the primary Ab. The slides were washed again and incubated with the fluorocrome (Rhodamine Red-X, Cy2)-conjugated secondary Abs. After washing, slides were mounted with 90% glycerol/PBS and analyzed with a confocal microscope (MRC-1024; Bio-Rad Laboratories) equipped with a 15-mW Kr/Ar laser.

Results

Cell surface staining of purified human $CD4^+$ peripheral blood T cells revealed that ~8–10% of these cells expressed the IL-2Rα chain CD25. Human $CD25^+CD4^+$ T cells were virtually all $CD45RO^+$, lacked expression of CD69, were mostly $CD45RB^{low}$ and $CD62L^+$, and showed constitutive intracellular expression of CTLA-4 (data not shown).

As the mechanism of immunosuppression requires contact between target and Treg cells (13), we reasoned that Treg cells could use specific chemokine receptors to localize in the vicinity of effector T cells and/or APCs. We initially analyzed the migration of purified populations of $CD4^+$ T cells to a large panel of chemokines (Fig. 1). Analysis of the percentage of specific migration revealed that ~60% of $CD4^+$ T cells specifically migrating in response to CCL1 expressed CD25. Human herpesvirus 8 (HHV8)-encoded vMIP-I, CCL22, and CCL17 also showed preferential chemotactic activity on $CD25^+$ Treg cells (Fig. 1 a). Given the fact that $CD25^+$ T cells are $CD45RO^+$ and receptors for inflammatory chemokines are expressed on effector/memory T cells, we compared the chemotactic responsiveness of $CD25^+$ Treg cells with that of bulk $CD25^-CD4^+$ T cells or only with $CD45RO^+CD25^-$ $CD4^+$ T cells. This analysis revealed that CCL17 and CCL22 were more potent and efficacious but less selective than CCL1 for Treg cells. The chemotactic index in response to CCL17 and CCL22 was ~15–18 for $CD25^+$ T cells, ~5–8 for $CD45RO^+CD25^-$ T cells (Fig. 1 b), and ~40% of specifically migrated $CD4^+$ T cells expressed CD25 (Fig. 1 a). By contrast, the chemotactic index in response to CCL1 was only ~4 for $CD25^+$ T cells (Fig. 1 b), but >50% of specifically migrated $CD4^+$ T cells were $CD25^+$. It should be noted that Treg cells also responded to several chemokines including interferon-inducible T cell α chemoattractant (I-TAC/CXCL11) and Epstein-Barr virus–induced molecule 1 ligand chemokine (ELC/CCL19) (Fig. 1 b). CCL19 exhibited powerful chemotactic activity with a chemotactic index close to ~100 (Fig. 1 b), but $CD25^+$ Treg cells were not specifically hyperresponsive to CCL19 when compared

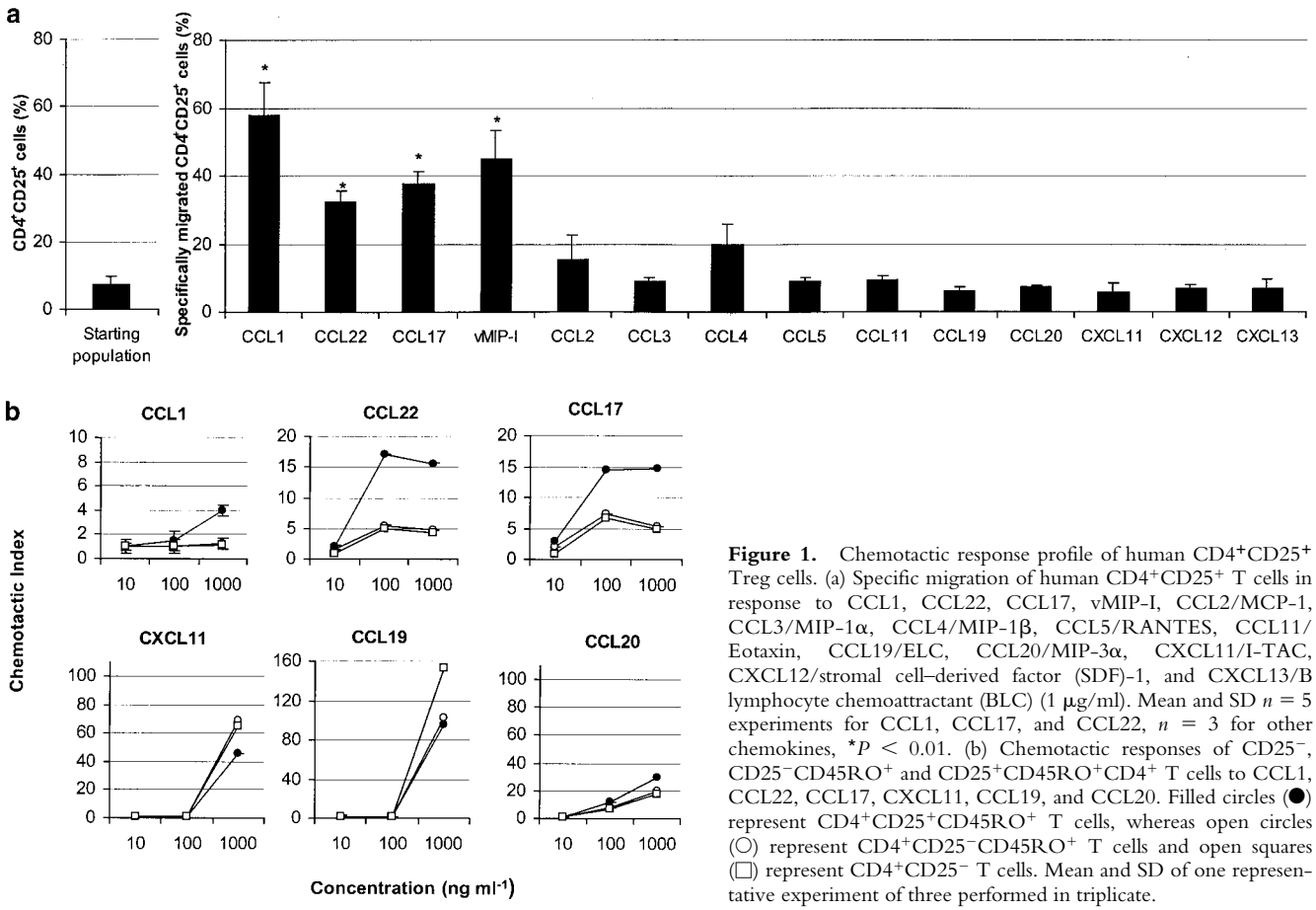

Figure 1. Chemotactic response profile of human $CD4^+CD25^+$ Treg cells. (a) Specific migration of human $CD4^+CD25^+$ T cells in response to CCL1, CCL22, CCL17, vMIP-I, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL11/Eotaxin, CCL19/ELC, CCL20/MIP-3α, CXCL11/I-TAC, CXCL12/stromal cell–derived factor (SDF)-1, and CXCL13/B lymphocyte chemoattractant (BLC) (1 μg/ml). Mean and SD $n = 5$ experiments for CCL1, CCL17, and CCL22, $n = 3$ for other chemokines, $^*P < 0.01$. (b) Chemotactic responses of $CD25^-$, $CD25^-CD45RO^+$ and $CD25^+CD45RO^+CD4^+$ T cells to CCL1, CCL22, CCL17, CXCL11, CCL19, and CCL20. Filled circles (●) represent $CD4^+CD25^+CD45RO^+$ T cells, whereas open circles (○) represent $CD4^+CD25^-CD45RO^+$ T cells and open squares (□) represent $CD4^+CD25^-$ T cells. Mean and SD of one representative experiment of three performed in triplicate.

with $CD4^+CD45RO^+$ T cells (Fig. 1 a). By contrast, the bulk $CD25^-$ T cell population, which included $CD45RA^+$ naive T cells, showed a greater response to the CCR7 ligand CCL19 (Fig. 1 b). Next, we investigated the ability of $CD4^+$ T cells that migrated in response to CCL1 or CCL22 to proliferate. In preliminary experiments we confirmed the anergic and immunosuppressive phenotype of purified $CD4^+CD25^+$ T cells (data not shown). As expected, the allogeneic proliferative response of $CD4^+$ T cells that migrated to CCL1 and CCL22 was markedly reduced in comparison to the response of nonmigrated cells or cells that migrated to CCL19 (Fig. 2 a). Depletion of

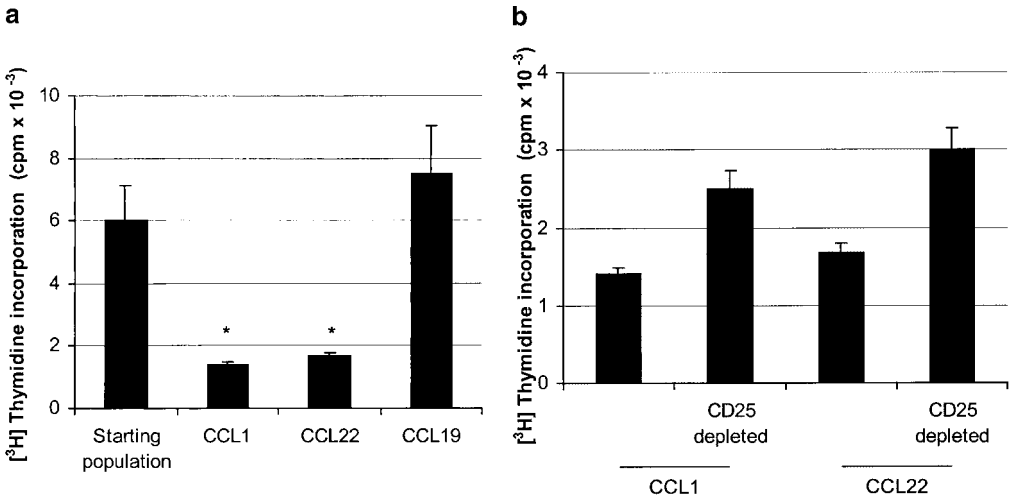

Figure 2. Immunoregulatory activity of $CD25^+$ T cells migrated in response to CCL1 or CCL22. (a) Enrichment of immunosuppressive activity amongst $CD4^+$ T cells migrated in response to CCL1 or CCL22. Alloantigen specific proliferative responses of $CD4^+$ T cells that migrated in response to CCL1, CCL22, and CCL19 were analyzed in triplicate as described in Materials and Methods. $^*P = 0.05$. (b) Reduction of immunosuppressive activity by depletion of $CD25^+$ T cells from $CD4^+$ T cells migrated in response to CCL1 or CCL22. After migration of $CD4^+$ T cells in response to CCL1 or CCL22, the cells were split and one part was depleted of $CD25^+$ T cells by immunomagnetic bead sorting. $CD25^+$-depleted (CD25 depleted) and nondepleted populations were then analyzed for their alloantigen specific proliferative response.

$CD25^+$ cells from $CD4^+$ T cells that migrated to CCL1 or CCL22 resulted in enhanced T cell proliferation (Fig. 2 b).

Based on these findings, we analyzed CCR4 and CCR8 expression on purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Staining with anti-CCR4 and anti-CCR8 Abs and analysis by confocal microscopy showed that the great majority of $CD4^+CD25^+$ T cells expressed chemokine CCR4 and CCR8 (Fig. 3, a and b). Staining of $CD4^+CD25^-$ T cells revealed that a relatively large fraction of these cells stained positive for CCR4 expression, whereas few cells expressed CCR8 (Fig. 3, a and b). These findings suggested coexpression of CCR4 and CCR8 on Treg cells and, consistent with the chemotaxis data, indicated that expression of CCR8 is more restricted to the Treg cell population than CCR4. In agreement with coexpression of these receptors on Treg cells, migration in response to a combination of suboptimal doses of CCL1 and CCL22 resulted in a synergistic rather than additive chemotactic effect (Fig. 3 c). Finally, to prove that these receptors were indeed responsible for the observed migration of Treg cells we took advantage of the recently described selective CCR8 antagonist, MC148, encoded by the virus of *Molluscum contagiosum* (14). In the presence of MC148, specific migration of Treg cells in response to CCL1 but not CCL22 was completely abolished, confirming the specific involvement of CCR8 in CCL1-mediated migration of Treg cells (Fig. 3 d). These findings document that blood-borne human Treg cells possess a unique chemotactic response profile and preferentially express the chemokine receptors CCR4 and CCR8.

Overall, our results suggest that CCR4 and CCR8 ligand chemokines CCL1, CCL17, and CCL22 might participate in downregulating inflammatory T cell–mediated responses. Previous work identified specific requirements for production of these chemokines by T cells and APCs (15, 16). Mature DCs have been reported to produce large quantities of CCL17 and CCL22 (17). Thus, we tested the possibility that Treg cells could be attracted by mature DCs. Maturation of monocyte-derived DCs was induced by stimulation with LPS and the supernatant from fully mature DCs was harvested and tested for the ability to attract Treg cells. In a first series of experiments, we found that the supernatants obtained from mature DCs, containing high levels of CCL17 and CCL22 and small amounts of CCL1, attracted very efficiently CCR4-expressing, but not CCR8-expressing L1.2 cells (data not shown). We then

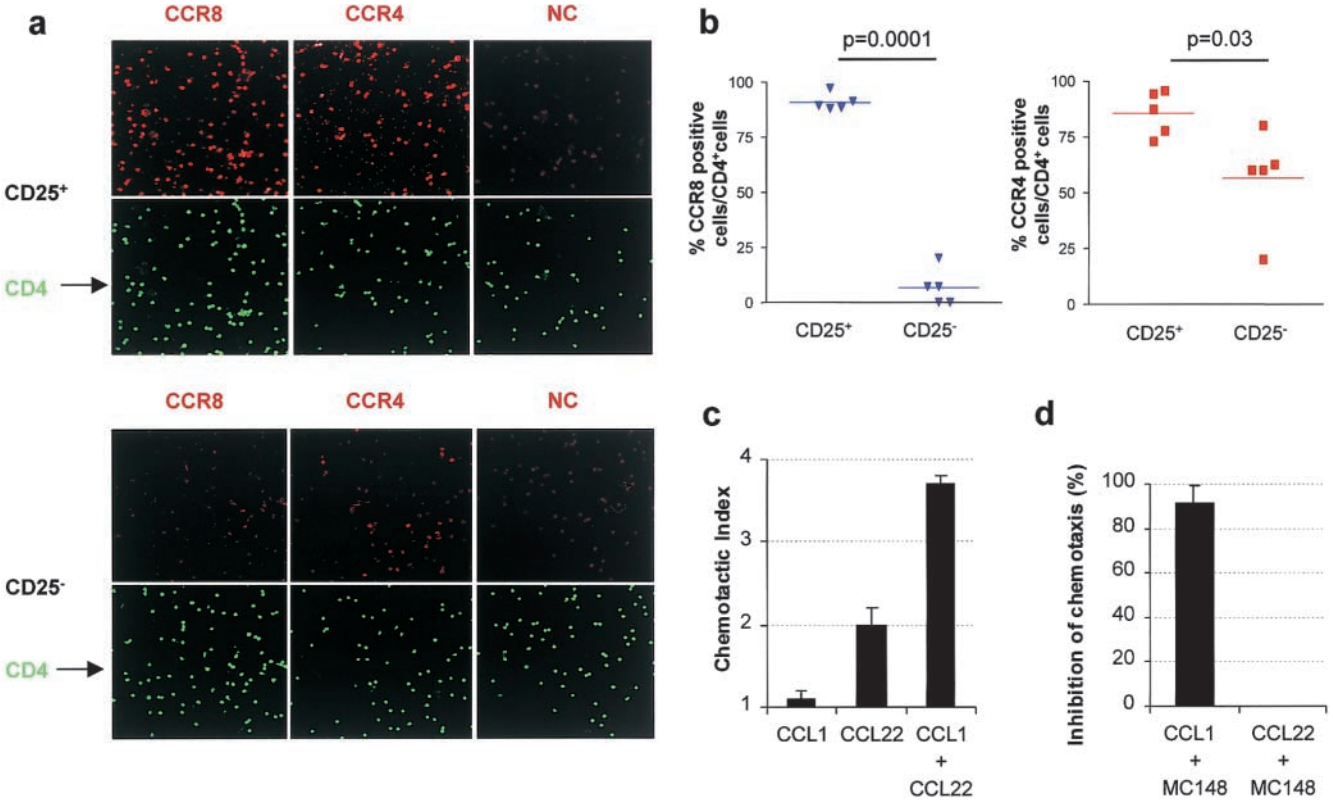

Figure 3. Expression of chemokine receptors CCR4 and CCR8 on $CD25^+$ Treg cells. (a) Cell surface expression of CCR4 and CCR8 on $CD25^+$ Treg cells. Purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were fixed, incubated with anti-CD4, anti-CCR4, or anti-CCR8 Abs, stained with the appropriate fluorochrome-conjugated secondary Abs, and analyzed by confocal microscopy. NC, not stained control. (b) Graphs illustrate the percentage of $CCR4^+$ or $CCR8^+CD4^+$ T cells counted in each subpopulation in five different fields. *P* values are indicated. (c) Synergistic chemotactic response to CCL1 and CCL22 of Treg cells. CCL1 (100 ng/ml) and CCL22 (10 ng/ml) were used separately or in combination. Results are shown as chemotactic index for Treg cells. (d) CCR8 mediates migration of Treg cells in response to CCL1. The percentage of inhibition of specific migration of Treg cells in response to CCL1 and CCL22 (0.5 μg/ml) in the presence of MC148 (2 μg/ml) is shown.

compared the migration of $CD25^-$ and $CD25^+$ Treg cells in response to supernatants of mature DCs and investigated the role of CCL17 and CCL22 present in the supernatants. Our data show that supernatant obtained from mature DCs preferentially attracted $CD25^+$ Treg cells among blood-borne $CD4^+$ T cells (Fig. 4). Neutralization of CCL22 significantly reduced the migration of Treg cells, but neutralization of both CCL17 and CCL22 was required to completely abolish the migration of Treg cells (Fig. 4).

Discussion

In this report, we provide the first evidence that blood-borne human $CD4^+CD25^+$ Treg cells exhibit a distinctive chemotactic response profile and chemokine receptor expression. Treg cells exhibit chemotactic responsiveness to several inflammatory and lymphoid chemokines, but they are specifically hyperresponsive to chemokines that engage the chemokine receptors CCR4 and CCR8 (Fig. 1).

Our investigation documents a broad spectrum of responsiveness of Treg cells to inflammatory chemokines that could potentially allow access to inflamed tissues and contact responding T cells and APCs. However, the specificity of action of chemokines such as CCL17, CCL22, and CCL1 on Treg cells suggests a unique role for these chemokines and their receptors in the physiology of Treg cells. Activated T cells and professional APCs such as DCs and monocytes/macrophages can produce CCL1, CCL17, and CCL22 (15, 16, 18). CCL17 and CCL22 secreted by activated DCs have been shown to attract activated T cells expressing CCR4 (17). Recent data suggest that T cells compete for access to antigen-bearing APCs (19, 20), a phenomenon that may potentially be regulated by chemokines secreted by APCs and chemokine receptors expressed on T cells. Thus, chemokine receptors, such as CCR4 and CCR8, that are upregulated upon TCR-mediated activation and are associated to critical steps of Th cell differentiation (21), may regulate the access of T cells to antigen-bearing APCs and control the dynamics of their interactions. In light of our results, we speculate that constitutive expression of CCR4 and CCR8 may endow Treg cells with a competitive advantage over other T cells for interacting with APCs that secrete chemokines acting on those receptors. Consistent with this hypothesis, we found that mature DCs preferentially attract Treg cells among circulating $CD4^+$ T cells by virtue of their secretion of CCR4 agonistic chemokines. Another possible scenario, not mutually exclusive, envisions that the production of CCL1, CCL17, and CCL22 by different types of cells in inflamed tissues may recruit Treg cells to downregulate an ongoing inflammatory response. In line with this proposal, the previously reported pattern of CCL1 secretion by Fcγ receptor triggered monocytes is consistent with chemokines produced in an inflammatory environment in response to opsonizing Abs and bacterial products (16). Interestingly, FcγRI triggering has been implicated in downregulation of inflammatory responses by modulating cytokine production by monocytes (22). Furthermore, we have recently shown that IL-12 inhibits CCL1 and CCL22 production by activated T cells (18). Conceivably, production of CCL1 and CCL22 by activated monocytes and T cells (15, 18, 23) might promote the recruitment of Treg cells to downregulate inflammatory Th1 responses (24).

Specific expression of CCR4 and CCR8 on Treg cells may allow their migration toward APCs and activated T cells leading to inhibition of APC function or suppression of responding T cells. Although Treg cells specifically express both CCR4 and CCR8, there are important distinctions between these receptors. First, CCR8 appears more selectively expressed on Treg cells than CCR4. CCR4 is expressed on the majority of circulating $CD4^+$ memory T cells, including skin-homing T cells and central memory $CCR7^+$ T cells (12, 25, 26; Fig. 3 a). By contrast, CCR8 expression and responsiveness to CCL1 seems confined to Treg cells and to another small subset of circulating $CD4^+$ T cells (unpublished data). Second, CCR4 agonistic chemokines are consistently more potent and efficacious than CCR8 agonistic chemokines in promoting migration

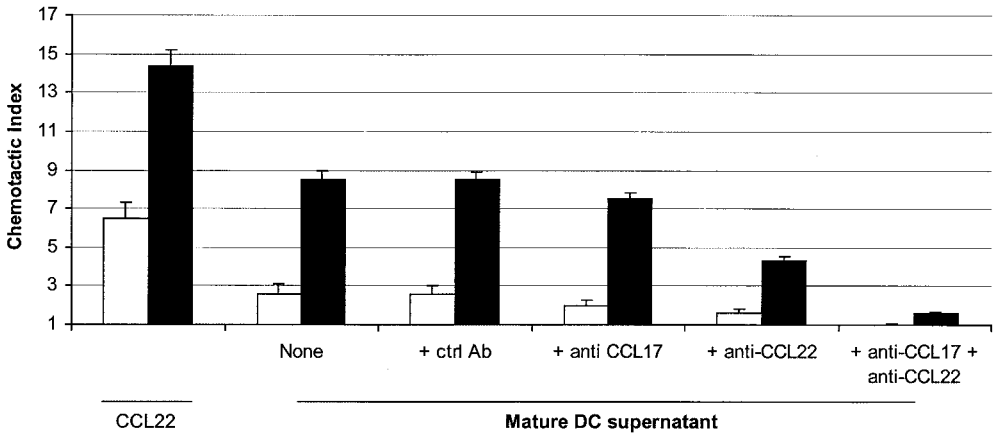

Figure 4. Production of CCL17 and CCL22 by mature human DCs leading to preferential attraction of $CD25^+$ Treg cells. Human monocyte-derived DCs were washed and stimulated with LPS. DCs were washed again after 16 h and cultured for additional 24 h before harvesting the supernatants. Supernatants were tested for their chemotactic activity on $CD4^+$ T cells. Chemotactic index of $CD4^+CD25^+$ (black bars) and $CD4^+CD25^-$ (white bars) T cells is indicated. Where indicated, isotype-matched control (ctrl) or anti-CCL17 and/or anti-CCL22 mAbs (2 μg/ml) were added to the supernatants to remove the investigated chemokine as described in Materials and Methods. Migration to CCL22 is shown for comparison. Results are from one representative experiment of two performed.

of Treg cells. This difference may be due to different levels of chemokine receptor's expression and could imply a distinct involvement for CCR4 and CCR8 in the function of Treg cells.

Based on our findings, regulation of CCL1, CCL17, and CCL22 production during the course of inflammatory responses could dictate the extent, severity, and duration of the response by modulating recruitment of Treg cells expressing CCR4 and CCR8. Interestingly, HHV8, which is found associated with Kaposi's sarcoma and Castelman's disease, encodes for three viral chemokines (vMIP-I, II, and III), which are believed to play a role in subverting the host's immune response (27). vMIP-I and vMIP-II are selective agonists of CCR8 (28, 29), while vMIP-III is an agonist of CCR4 (30). Based on our findings, we can speculate that production of vMIPs by HHV8 may recruit Treg cells in infected tissues and help virally infected cells to evade the host's immune response. Taken together, our data suggest that certain "regulatory" chemokines may control the termination of inflammatory responses by recruiting a specific subset of Treg cells that can limit tissue damage and prevent autoimmunity. Manipulating the recruitment of Treg cells may be useful in a variety of pathological conditions to either achieve tolerance in autoimmunity and transplantation or boost immune responses against tumors and viral infections.

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