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Chemokine Receptor Expression and Function in CD4+ T Lymphocytes with Regulatory Activity

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We have investigated the chemokine receptor expression and migratory behavior of a new subset of nickel-specific skin-homing regulatory CD4+ T cells (ThIL-10) releasing high levels of IL-10, low IFN-γ, and undetectable IL-4. These cells inhibit in an IL-10-dependent manner the capacity of dendritic cells to activate nickel-specific Tc1 and Th1 lymphocytes. RNase protection assay and FACS analysis revealed the expression of a vast repertoire of chemokine receptors on resting ThIL-10, including the Th1-associated CXCR3 and CCR5, and the Th2-associated CCR3, CCR4, and CCR8, the latter at higher levels compared with Th2 cells. The most active chemokines for resting ThIL-10, in terms of calcium mobilization and in vitro migration, were in order of potency: CCL2 (monocyte chemoattractant protein-1, CCR2 ligand), CCL4 (macrophage-inflammatory protein-1β, CCR5 ligand), CCL3 (macrophage-inflammatory protein-1α, CCR1/5 ligand), CCL17 (thymus and activation-regulated chemokine, CCR4 ligand), CCL1 (I-309, CCR8 ligand), CXCL12 (stromal-derived factor-1, CXCR4), and CCL11 (eotaxin, CCR3 ligand). Consistent with receptor expression down-regulation, activated ThIL-10 exhibited a reduced or absent response to most chemokines, but retained a significant migratory capacity to I-309, monocyte chemoattractant protein-1, and thymus and activation-regulated chemokine. I-309, which was ineffective on Th1 lymphocytes, attracted more efficiently ThIL-10 than Th2 cells. I-309 and CCR8 mRNAs were not detected in unaffected skin and were up-regulated at the skin site of nickel-allergic reaction, with an earlier expression kinetics compared with IL-10 and IL-4. Results indicate that skin-homing regulatory ThIL-10 lymphocytes coexpress functional Th1- and Th2-associated chemokine receptors, and that CCR8/I-309-driven recruitment of both resting and activated ThIL-10 cells may be critically involved in the regulation of Th1-mediated skin allergic disorders. The Journal of Immunology, 2001, 166: 996–1002.

Compelling evidence indicates the existence of specialized CD4+ T lymphocytes with the potential to regulate a variety of immune responses through the release of suppressive cytokines (1–3). In particular, IL-10-producing CD4+ T cells appear to be profoundly involved in the modulation of autoimmune diseases as well as in the induction of transplantation tolerance (4–6). Allergic diseases are the consequence of exaggerated immune responses against innocuous non-self Ags. Allergic contact dermatitis to nickel is a prototypic type 1-mediated immune response and depends upon the recruitment of Th1 and Tc1 lymphocytes at the skin site of nickel application in sensitized individuals (7–10). We have recently found that the skin and peripher al blood of nickel-allergic patients as well as the blood of healthy donors bear a substantial proportion of nickel-specific CD4+ T clones which produce high amounts of IL-10, low or undetectable IFN-γ, and no IL-4 (11). These nickel-specific IL-10-producing Th cells (ThIL-10) express the skin-homing receptor, cutaneous lymphocyte-associated Ag (CLA), and block in a IL-10-dependent manner the differentiation and maturation of dendritic cells, thus impairing their capacity to activate nickel-specific Tc1 and Th1 effector lymphocytes (12).

Many recent findings indicate that chemokine receptors are differentially expressed on memory T cells depending on their polarization (13–18), with Th1 lymphocytes expressing CCR5 and CXCR3, and Th2 lymphocytes CCR3, CCR4, and CCR8. As a consequence, selective chemotactic stimuli contribute to the differential positioning of Th1 and Th2 cells within tissues (19–21). Here, we analyzed the chemokine receptor profile and migratory properties of skin-homing nickel-specific ThIL-10 lymphocytes. Our results demonstrate that resting ThIL-10 cells coexpress functional Th1 and Th2 chemokine receptors and migrate in response to a variety of chemokines. Upon activation, ThIL-10 cells are attracted exclusively by CCL1 (I-309), CCL2 (monocyte chemoattractant protein-1, MCP-1), CCL17 (thymus and activation-regulated chemokine, TARC), and CXCL12 (stromal derived factor 1, SDF-1). I-309, which is produced in the skin affected with allergic contact dermatitis to nickel, attracts ThIL-10 and Th2, but not Th1 cells, and may thus contribute to the regulation of the Th1-mediated skin inflammation.

Materials and Methods

Culture medium and Abs

T lymphocytes were cultured in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies) supplemented with 10% FCS. The culture medium contained recombinant human IL-2 (100 U/ml) and recombinant human IL-7 (20 ng/ml).

Abbreviations used in this paper: ThIL-10, IL-10-producing Th cells; CLA, cutaneous lymphocyte-associated Ag; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokine; SDF, stromal-derived factor; MIP, macrophage-inflammatory protein; IP-10, IFN-induced protein of 10 kDa; Tr1, T regulatory cell 1.

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3 Abbreviations used in this paper: ThIL-10, IL-10-producing Th cells; CLA, cutaneous lymphocyte-associated Ag; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokine; SDF, stromal-derived factor; MIP, macrophage-inflammatory protein; IP-10, IFN-induced protein of 10 kDa; Tr1, T regulatory cell 1.

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Technologies, Chagrin Falls, OH) (complete RPMI) plus 10% heat-inactivated FCS (HyClone Logan, UT) or 5% autologous plasma. The anti-CD28 mAb (Leu-28, IgG1) was purchased from Becton Dickinson (San Jose, CA). Mouse mAbs anti-human CCR1 (53504.111, IgG2a), CCR2 (48607.121, IgG2a), CCR5 (45531.111, IgG2a), and CCRX3 (49801.111, IgG1) were obtained from R&D Systems (Minneapolis, MN); anti-CCR3 (LS63 7B11, IgG2a) and anti-CCR4 (328B, IgG) mAbs were kindly provided by Leukosite (Cambridge, MA) and ICOS (Bothell, WA), respectively; anti-CCXCR1 (5A12, IgG2B), CCXCR2 (6C6, IgG1) and CCXCR4 (12G5, IgG2a) were purchased from PharMingen (San Diego, CA). Anti-CD3 (UCHT-1, IgG1) was obtained from Immunotech (Marseille, France); the FITC-conjugated goat anti-mouse IgG were obtained from Dako (Glostrup, Denmark).

T cells

T cell lines were derived from the blood and skin of nonatopic patients affected by allergic contact dermatitis to nickel, as previously described (12). Briefly, >95% pure CD4+ T cells were negatively selected from the nonadherent fraction of PBMC using immunomagnetic beads coated with anti-CD19, anti-CD45-DR, and anti-CD8 mAbs (Dynal, Oslo, Norway). Blood-derived CD4+ T cells were expanded with autologous PBMC and 10 µg/ml NiSO4 (Sigma-Aldrich, Milan, Italy) to enrich for nickel-specific T cells. Skin-derived T cells were obtained from 4-mm punch biopsies performed 48 h after the application of 5% NiSO4 in petrolatum on the back of allergic patients. After extensive washing in PBS, biopsies were placed in culture at 37°C with 5% CO2 in complete RPMI along with 5% autologous plasma and 30 U/ml rIL-2 (generously provided by Chiron Italia, Milan, Italy). Medium was replaced every third day, and T cells emigrating from the tissue were collected at day 10. Blood- and skin-derived T cell lines were cloned by limiting dilution (0.5 cells/well) in the presence of 2×10^5 PBMC, 30 U/ml IL-2, and 1% PMA in complete RPMI plus 10% FCS. Clones were grown in the presence of IL-2 and periodically stimulated with 1% PHA and feeder cells or plate-coated anti-CD3 (1 µg/ml) and soluble anti-CD28 (1 µg/ml) mAbs. Ag specificity of T cell lines and clones was tested using microwell plates, as described (13). CCR1, CCR2, CCR3, and CCR5 mAbs were added at 10 µg/ml NiSO4, in complete RPMI supplemented with 5% autologous plasma for 48 h and pulsed with 5 µCi/ml [3H]ThdR (Amersham, Little Chalfont, U.K.) for the last 12 h of culture. Cultures were harvested onto fiber-coated 96-well plates, and thymidine incorporation was measured in a beta counter (Topcount; Packard, Groningen, The Netherlands). Supernatants from T cells (10^5 cells/ml) stimulated in 24-well plates with immobilized anti-CD3 and soluble anti-CD28 mAbs were collected after 48 h, filtered, and tested for Th IL-10 lymphocytes in resting condition or activated 48 h with immobilized anti-CD3 plus anti-CD28 were added to the top of transwells suspended in complete RPMI plus 0.5% BSA at 1×10^6 cells/ml.

Various concentrations of the different chemokines were added to the bottom chamber of the transwell (0.6 ml). After 1 h of incubation at 37°C with 5% CO2, cells transmigrated into the lower chamber were recovered and counted with a FACSscan for 60 s at a flow rate of 60 µl/min, as previously described (23). Results are shown as migration index, which represents the ratio between T cells migrated to the lower chamber in the presence of the agonistic chemokine and cells migrated in response to the medium alone.

RT-PCR analysis

Total cellular RNA was extracted from 4-mm punch skin biopsies obtained at time 0 and 16, 48 and 60 h after the application of 5% NiSO4 on the back of allergic individuals (n = 2) using the acid guanidinium thiocyanate-phenol-chloroform method. Total RNA (0.5–1 µg) was reverse transcribed using oligo(dT) primers and then subjected to amplification with a GeneAmp RNA PCR kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ). The following synthetic oligonucleotides were used: for I-309, 5‘-ATG CAG ATC ATC ACC ACA GCC CTG and 3‘-ACA GAA AAA TGC TGA GGC ACT GCC (274-bp template) for IFN-1gara; for IL-10, 5‘-TGC AGG TCA TTG GCT TCC (24-bp template) for IL-10; 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-ACA GAA AAA TGC TGA GGC ACT GCC (274-bp template) for CCR8, 5‘-CAT CAC CCT CAT GAG TGT G and 3‘-CAC GTT GAA TGG CAC CCA (410-bp template) for IFN-1gara; for IL-10, 5‘-GAA GGA TCA GCT GGA CAA CTT GTT G and 3‘-GCT CCA AAT GTA GGG CCA GC (306-bp template) for IL-10; 5‘-GGC ATA TCA TCA TAC AGG AG and 3‘-TGC GTT TCC TTC ACA GGA CA (308-bp template) for IFN-1gara; 5‘-TGC AGC TCA TTC AGT TAG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) for IFN-1gara; 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) for IFN-1gara; 5‘-ATG CAG ATC ATC ACC ACA GCC CTG and 3‘-ACA GAA AAA TGC TGA GGC ACT GCC (274-bp template) for IFN-1gara; 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) for IFN-1gara. The actin housekeeping gene was amplified with 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) (24). The β-actin housekeeping gene was amplified with 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) (24). The β-actin housekeeping gene was amplified with 5‘-TGG GAG ATG CAG ATC ACC ACA GCC CTG and 3‘-AGC CAT CAC TGT GAG GTT (306-bp template) (24).

Results

Chemokine receptor mRNA in resting and activated Th1, Th2, and Th11-10 nickel-specific T cells

Th1-10 lymphocytes in resting condition or activated 48 h with immobilized anti-CD3 were washed in PBS added with 1% PBS and stained with specific mAb or isotype control Ig, followed by secondary anti-mouse FITC-conjugated Ab. For CCR1 detection, cells were preincubated for 15 min with 5% FBS and then stained and fixed with 10% paraformaldehyde and 0.5% glutaraldehyde for 20 min before staining with the mAb or the isotype control. Cells were analyzed with a FACSscan equipped with CellQuest software (Becton Dickinson).

Intracellular Ca2+ concentration measurements

T cell clones were either left untreated or activated with anti-CD3 plus anti-CD28 for 48 h in the presence of 30 µM IL-2. Thereafter, cells (5×10^6/ml) were washed and loaded with 8 µM Fluo-3/acetoxymethyl ester in the presence of 1 µM pluronic F-127 (Molecular Probes, Eugene, OR) in complete RPMI with 1% FBS for 40 min at 37°C with frequent gentle agitation. T Cells were then washed twice, stained with TRL (a phage inflammatory protein) (MP1-α, 200 ng/ml), MCP-1 (200 ng/ml), CCL11 (eotaxin, 400 ng/ml), TARC (100 ng/ml), CCL4 (MIP-1B, 100 ng/ml), CCL19 (MIP-3B, 30 ng/ml), I-309 (200 ng/ml), CXCL8 (IL-8, 100 ng/ml), CXCL10 (IFN-induced protein of 10 kDa (IP-10), 100 ng/ml), and SDF-1 (100 ng/ml) (all from R&D Systems), and finally analyzed on a FACSscan. Cells and chemokines were maintained at 37°C during the assay.

Migration assay

Chemoattractive activity of each chemokine was evaluated measuring the lymphocyte migration through a 5-µm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge MA) as previously described (24). Briefly Th1, Th2, and Th11-10 either in resting or stimulated 48 h with immobilized anti-CD3 plus anti-CD28 were added to the top of transwells suspended in complete RPMI plus 0.5% BSA at 1×10^6 cells/ml. Migration of each of the test chemokines was determined in the presence of each chemokine alone.

Reference

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TCR engagement strongly affected chemokine receptor expression. After a transient burst at 6 h in CCR1, CCR2, CCR4, CCR5, and CCR8 mRNA, ThIL-10 clones down-regulated CXC (CXCR1, CXCR3, and CXCR4) receptors, CCR2, and, to a lesser extent, CCR3 and CCR8. In contrast, CCR1, CCR4, CCR5, and CCR7 were up-regulated. When compared with Th1 and Th2, ThIL-10 lymphocytes displayed a broader array of chemokine receptors, with significant expression of both the Th1-associated CCR5 and CXCR3 and the Th2-associated CCR3, CCR4, and CCR8 (13–18, 25). Interestingly, the mRNA signal for CCR8 was

**FIGURE 1.** CC and CXC chemokine receptor mRNA expressed by nickel-specific ThIL-10, Th1, and Th2 clones. Receptor mRNAs were examined by RNase protection assay in resting T cell clones and 6–48 h after stimulation with plate-bound anti-CD3 and soluble anti-CD28 in the presence of exogenous IL-2 (30 U/ml). Similar results were observed in four ThIL-10, two Th1, and two Th2 clones.

**FIGURE 2.** Densitometric analysis of chemokine receptor mRNAs expressed by resting and activated T cell clones. Densitometric values were calculated from RNase protection assay by laser densitometry and normalized against the housekeeping gene L32. Results are expressed as the mean ± SD of experiments performed on four ThIL-10, two Th1, and two Th2 clones. Open columns, resting T cells; hatched columns, 6-h activated T cells; and filled columns, 48-h activated T cells.
2-fold stronger in resting ThIL-10 lymphocytes than in Th2 clones, and the difference was even more pronounced upon activation, being CCR8 markedly down-regulated in TCR-engaged Th2 lymphocytes, but only slightly in ThIL-10 cells. ThIL-10, but not Th1 and Th2, clones expressed low levels of CCR7 mRNA, which was up-regulated upon activation. Finally, both resting and stimulated ThIL-10 clones showed 1.5- to 3.5-fold higher CXCR4 mRNA levels than Th1 and Th2 cells.

Chemokine receptor expression in resting and activated nickel-specific ThIL-10 clones

FACS analysis confirmed the expression of the CCRs 1–5 and the CXCRs 1, 3, and 4 on resting ThIL-10 lymphocytes, whereas CXCR2 was negative (Fig. 3). Activated ThIL-10 lymphocytes strongly reduced the surface expression of CCR1, CCR3, CCR5, CXCR1, and CXCR3. Although CCR1 and CCR5 protein expression decreased, the corresponding mRNA signals were up-regulated following TCR triggering. The expression of CXCR2 and CCRL4, albeit reduced, was maintained at significant levels and that of CXCR4 was unmodified after ThIL-10 activation.

Chemokine receptors signaling in resting and activated nickel-specific ThIL-10 clones

To evaluate the functional relevance of chemokine receptor expression, we measured calcium mobilization in ThIL-10 lymphocytes after treatment with agonistic chemokines. Consistent with the vast repertoire of receptors expressed, all of the chemokines tested induced significant responses in resting ThIL-10 cells (Fig. 4). The most active chemokines were MCP-1 (CCR2 ligand), I-309 (CCR8 ligand), MIP-1α (CCR1–CCR5 ligand), and MIP-1β (CCR5 ligand), followed by TARC (CCR4 ligand), IL-8 (CXCR1 ligand) and eotaxin (CXCR3 ligand), SDF-1 (CXCR4 ligand), and finally MIP-3β (CCR7 ligand). After activation, significant calcium fluxes were measured in response to I-309, MCP-1, TARC, and SDF-1, whereas the responses to the other chemokines were strongly reduced or completely lost. Consistent with the up-regulation of CCR7 mRNA, MIP-3β retained a slight stimulatory effect on activated ThIL-10 clones. Notably, activated ThIL-10 cells became unresponsive to MIP-1α and MIP-1β, in line with the decreased expression of CCR1 and CCR5 receptors. This finding has been previously observed in mature dendritic cells and activated monocytes (26, 27).

Chemotaxis of resting and activated nickel-specific Th1, Th2, and ThIL-10 clones

The migratory behavior of Th1, Th2, and ThIL-10 lymphocytes was compared by measuring their transmigration through transwell filters in response to different concentrations of agonistic chemokines (Fig. 5). Resting ThIL-10 revealed an extraordinary capacity to migrate in response to a broad array of chemotactic stimuli, including both Th1- and Th2-associated chemokines. The highest migration was achieved with MCP-1, MIP-1β, and MIP-1α, followed by TARC, I-309, SDF-1, and eotaxin, whereas more limited responses were observed to IL-8, IP-10, and MIP-3β. ThIL-10 migration to MIP-1β, IP-10 (CCR5 and CXCR3 ligands), and to eotaxin (CXCR3 ligand) was lower compared with that of Th1 and
Th2 clones, respectively. In contrast, I-309 induced a stronger migration in Th IL-10 than in Th2 cells. Responses to MIP-1α, MCP-1, and TARC were comparable among the different Th cell subsets, whereas Th IL-10 and Th2 migrated to a higher extent in response to IL-8 compared with Th1 clones. IL-8 responsiveness was likely due to CXCR1, but not CXCR2 triggering, since the latter receptor was undetectable on Th IL-10 both at mRNA and protein levels. Additionally, neutrophil-activating protein-2, a specific agonist of CXCR2 but not CXCR1, failed to induce calcium mobilization and migration of Th IL-10 (data not shown). Finally, only Th IL-10 cells showed a slight migration to MIP-3β. After stimulation with anti-CD3 and anti-CD28 mAbs, Th IL-10 cells markedly decreased their migratory response to most of the chemokines, and, consistent with the calcium fluxes, retained a significant migration to MCP-1, I-309, TARC, and SDF-1. MCP-1 and TARC were also effective in attracting activated Th1 and Th2 lymphocytes, whereas I-309 attracted Th2, but not Th1 cells. Activated Th1 lymphocytes maintained a significant response to MIP-1α, MIP-1β, and IP-10.

I-309, CCR8, and cytokine mRNA expression in allergic contact dermatitis skin

Since I-309 appeared to selectively attract Th IL-10 and Th2 lymphocytes and was effective on both resting and activated Th IL-10 cells, its expression was investigated during allergic contact dermatitis reaction, in combination with CCR8, IL-10, IL-4, and IFN-γ. RT-PCR was performed on RNA extracted from unaffected (time 0) skin and at 16 – 60 h after the application of 5% NiSO4 in petroleum. B, Densitometric analysis was performed by normalizing the levels of RNA against β-actin. Similar results were obtained in biopsies from two patients.

Discussion

We have analyzed the pattern of chemokine receptors and the migratory behavior of nickel-specific skin-homing IL-10-producing
CD4⁺ lymphocytes compared with polarized Th1 and Th2 cells. Resting ThIL-10⁺ regulatory cells coexpressed the Th1 (CXCR3 and CCR5)- and Th2 (CCR3, CCR4, and CCR8)-associated receptors as well as significant levels of CCR1, CCR2, CCR7, and CXCRI4. In vitro migration to TARC, MCP-1, and MIP-1α was similar in all subsets. In contrast, resting ThIL-10⁺ cells showed a lower response to MIP-1β and IP-10 compared with Th1, and to eotaxin compared with Th2 clones. Noteworthy, CCR8, considered a very specific Th2 marker (17, 18), was highly expressed in ThIL-10⁺ cells, with I-309 attracting more efficiently ThIL-10⁺ than Th2 cells. TCR-engaged ThIL-10⁺ lymphocytes down-regulated most chemokine receptors, both at the mRNA and protein levels. As a consequence, activated ThIL-10⁺ became unresponsive to many chemotactic stimuli, still maintaining a significant response to I-309, MCP-1, TARC, and SDF-1. Interestingly, although CCR1 and CCR5 mRNA were up-regulated, FACS analysis revealed a strong down-regulation of the receptors in activated ThIL-10⁺, which became unresponsive to MIP-1α and MIP-1β. This finding may indicate a posttranscriptional regulation, as suggested to occur for CCR5 in activated dendritic cells and monocytes (26, 27), CCR4, which has been associated with Th2 polarization (15, 16, 18), was expressed by all of the skin-homing T cell subsets, although at higher levels in resting Th2 cells compared with Th1 and ThIL-10⁺ lymphocytes (10- and 2-fold higher, respectively). This finding is consistent with the recent report by Campbell et al. (28), indicating that I-309 attracting more efficiently Th IL-10 than Th2 cells. TCR-activated ThIL-10 became unresponsive to many chemotactic stimuli, still maintaining a significant response to I-309, MCP-1, TARC, and SDF-1. Additionally, the CD26 and the lymphocyte activating receptor CD107a, and G. Girolomoni, manuscript in preparation), which may therefore contribute relevantly to the recruitment of regulatory ThIL-10⁺ cells in the skin. In aggregate, our results demonstrate that ThIL-10⁺ display a broad array of functional chemokine receptors, which allow their comigration along with Th1 and Th2 cells. The expression of high levels of CCR8 in regulatory ThIL-10⁺ lymphocytes may be advantageously exploited to control Th1-mediated allergic disorders.

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