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Chemokine Receptor Expression and Function in CD4$^+$ T Lymphocytes with Regulatory Activity[1]

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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 (Th^{IL-10}) releasing high levels of IL-10, low IFN-γ, and undetectable IL-4. These cells inhibit in a 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 Th^{IL-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 Th^{IL-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 Th^{IL-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 Th^{IL-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 Th^{IL-10} lymphocytes coexpress functional Th1- and Th2-associated chemokine receptors, and that CCR8/I-309-driven recruitment of both resting and activated Th^{IL-10} cells may be critically involved in the regulation of Th1-mediated skin allergic disorders.

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 peripheral 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 (Th^{IL-10})[3] 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 Th^{IL-10} lymphocytes. Our results demonstrate that resting Th^{IL-10} cells coexpress functional Th1 and Th2 chemokine receptors and migrate in response to a variety of chemokines. Upon activation, Th^{IL-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 Th^{IL-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

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[3] Abbreviations used in this paper: Th^{IL-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.

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, $IgG2_B$), CCR2 (48607.121, $IgG2_B$), CCR5 (45531.111, $IgG2_B$), and CXCR3 (49801.111, IgG1) were obtained from R&D Systems (Minneapolis, MN); anti-CCR3 (LS63 7B11, $IgG2_A$) and anti-CCR4 (328B, IgG) mAbs were kindly provided by Leukosite (Cambridge, MA) and ICOS (Bothell, WA), respectively; anti-CXCR1 (5A12, $IgG2_B$), CXCR2 (6C6, IgG1) and CXCR4 (12G5, $IgG2_A$) were purchased from PharMingen (San Diego, CA). Anti-CD3 (UCHT-1, IgG1) was obtained from Immunotech (Marseille, France); the FITC-conjugated anti-CLA mAb (HECA-452, rat IgM), the control isotype FITC-conjugated rat IgM, and the control mouse IgG were purchased from PharMingen. FITC-conjugated goat anti-mouse IgG were obtained from Dako (Glostrup, Denmark).

T cell clones

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-HLA-DR, and anti-CD8 mAbs (Dynal, Oslo, Norway). Blood-derived $CD4^+$ T cells were expanded with autologous PBMC and 10 μg/ml $NiSO_4$ (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% $NiSO_4$ in petrolatum on the back of allergic patients. After extensive washing in PBS, biopsies were placed in culture at 37°C with 5% CO_2 in complete RPMI along with 5% autologous plasma and 30 U/ml rhIL-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% PHA 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 irradiated PBMC and 10 μg/ml $NiSO_4$ in complete RPMI supplemented with 5% autologous plasma for 48 h and pulsed with 5 μCi/ml [^{3}H]TdR (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 (10^6 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 IL-10, IL-4, IFN-γ, and TGF-β content by ELISA (R&D Systems).

RNase protection assay

Total RNA was extracted from resting and 6-to 48-h activated T cell clones using TRIzol (Life Technologies) according to the manufacturer's instructions. Two multiprobe template sets, hCR5 and hCR6 (RiboQuant; PharMingen), were used for in vitro transcription reactions in the presence of a GACU pool and a T7 RNA polymerase to synthesize [^{32}P]UTP-labeled antisense probes. RNase protection analysis of 2.5 μg of total RNA was performed after overnight hybridization at 60°C with 2.5×10^6 cpm of hCR5 or hCR6, followed by digestion with RNase A and T1 according to standard protocols. Protected fragments were treated with proteinase K, extracted with phenol-chloroform plus isoamyl alcohol (50:1), and finally precipitated in ethanol in the presence of ammonium acetate. The samples were electrophoresed on 5% denaturing sequencing gels and then exposed on film (Kodak, Rochester, NY). The levels of mRNA expression were quantified by densitometric analysis with an imaging densitometer (model GS-670; Bio-Rad, Hercules, CA), supported by the Molecular Analyst Image software, and the values were normalized against the housekeeping gene L32.

FACS analysis

$Th^{IL\text{-}10}$ lymphocytes in resting condition or activated 48 h with immobilized anti-CD3 were washed in PBS added with 1% FBS 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 2% paraformaldehyde and then permeabilized with 0.5% saponin for 20 min before staining with the mAb or the isotype control. Cells were analyzed with a FACScan equipped with CellQuest software (Becton Dickinson).

Intracellular Ca^{2+} 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 U/ml 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. Cells were then washed twice, stimulated with CCL3 (macrophage inflammatory protein (MIP)-1α, 200 ng/ml), MCP-1 (200 ng/ml), CCL11 (eotaxin, 400 ng/ml), TARC (100 ng/ml), CCL4 (MIP-1β, 100 ng/ml), CCL19 (MIP-3β, 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 FACScan. Cells and chemokines were maintained at 37°C during the assay.

Migration assay

Chemotactic property 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 (22). Briefly Th1, Th2, and $Th^{IL\text{-}10}$ either in resting or stimulated 48 h with immobilized anti-CD3 plus anti-CD28 were added to the top chamber 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% CO_2, cells transmigrated into the lower chamber were recovered and counted with a FACScan 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% $NiSO_4$ 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 amplificate); for CCR8, 5′-CAT CAC CCT CAT GAG TGT GG and 3′-CAC GTT GAA TGG GAC CCA GA (410-bp amplificate); for IL-10, 5′-GAA GGA TCA GCT GGA CAA CTT GTT G and 3′-GCT CCA AAT GTA GGG GCA GG (306-bp amplificate); for IL-4, 5′-GCG ATA TCA CCT TAC AGG AG and 3′-TTG GCT TCC TTC ACA GGA CA (308-bp amplificate); for IFN-γ, 5′-TGC AGG TCA TTC AGA TGT AG and 3′-AGC CAT CAC TTG GAT GAG TT (306-bp amplificate) (24). The β-actin housekeeping gene was amplified with 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA and 3′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG primers (660-bp amplificate). To eliminate genomic DNA contamination, reverse transcription was performed after digestion with RNase-free DNase (PharMingen) for 30 min at 37°C. As a control, reactions were also performed in the absence of reverse transcriptase. For semiquantitative analysis, RNA concentrations, primers, and PCR cycles were titrated to obtain standard curves to verify linearity and to permit analysis of signal strength. The levels of mRNA expression were quantified by densitometric analysis as described above, with the values normalized against β-actin.

Results

Chemokine receptor mRNA in resting and activated Th1, Th2, and $Th^{IL\text{-}10}$ nickel-specific T cells clones

T cell clones were isolated from either the peripheral blood or lesional skin of patients affected with allergic contact dermatitis to nickel. $Th^{IL\text{-}10}$ clones released high amounts of IL-10, low levels of IFN-γ, and very limited amounts or no IL-4, and expressed the CLA receptor which allows the recruitment of memory T cells in the skin (8) (Table I). To determine the chemokine receptor expression, T cell clones were collected in resting phase and 6–48 h after anti-CD3/anti-CD28-induced activation, and the mRNA content was evaluated by RNase protection assay using CC and CXC chemokine receptor multiprobe template sets. In resting conditions, $Th^{IL\text{-}10}$ cells expressed a wide range of CC and CXC receptors (Figs. 1 and 2). As previously reported for other T cell subsets

Table I. *T cell clones used in the study*

Clone	Source	[^{3}H]TdR Uptake[a] $NiSO_4$ −	[^{3}H]TdR Uptake[a] $NiSO_4$ +	CLA[b] (ΔMFI)	IFN-γ[c] (pg/ml)	IL-4[c] (pg/ml)	IL-10[c] (pg/ml)	Pattern
AR2.77	Skin	2.2 ± 0.1	48.7 ± 0.4	470	49	10	4,560	Th^{IL-10}
AC5.53	Skin	1.9 ± 0.2	22.1 ± 3	195	980	0[d]	11,600	Th^{IL-10}
CS1.12	Skin	0.9 ± 0.03	11.9 ± 0.8	247	440	10	4,390	Th^{IL-10}
AR2.33	Blood	2.8 ± 0.2	7.7 ± 1.4	735	30	0	3,450	Th^{IL-10}
AC5.52	Blood	0.6 ± 0.05	7.4 ± 1.1	210	300	0	1,650	Th^{IL-10}
AR2.2	Skin	2 ± 0.1	82.2 ± 3.5	1067	0	11,930	1,670	Th2
AC6.14	Blood	2.1 ± 0.4	32.2 ± 0.7	189	140	21,700	4,890	Th2
CS1.19	Blood	0.5 ± 0.02	19.8 ± 0.2	342	28,420	140	50	Th1
AC5.41	Blood	1.9 ± 0.3	41.6 ± 3.1	561	2,780	250	85	Th1

[a] T cells (5×10^4 cells/well) were cocultured with irradiated PBMCs (10^5 cells/well) in the absence or presence of 10 μg/ml $NiSO_4$ for 3 days. Results are given as mean cpm $\times 10^3$ ± SD of triplicate cultures.
[b] CLA expression was determined by flow cytometry analysis using the FITC-conjugated HECA-452 rat mAb. Data are expressed as ΔMFI which represents the mean fluorescence intensity of anti-CLA-stained cells subtracted from the fluorescence of FITC-labeled rat IgM.
[c] T cell clones were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs (both at 1 μg/ml) for 48 h, and supernatants were examined for cytokine content by ELISA. Values are expressed as pg/ml/10^6 cells.
[d] Below the detection limits of the ELISA kit (pg/ml): IL-4, ≤4, IFN-γ, ≤3.

(25), TCR engagement strongly affected chemokine receptor expression. After a transient burst at 6 h in CCR1, CCR2, CCR4, CCR5, and CCR8 mRNA, Th^{IL-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, Th^{IL-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

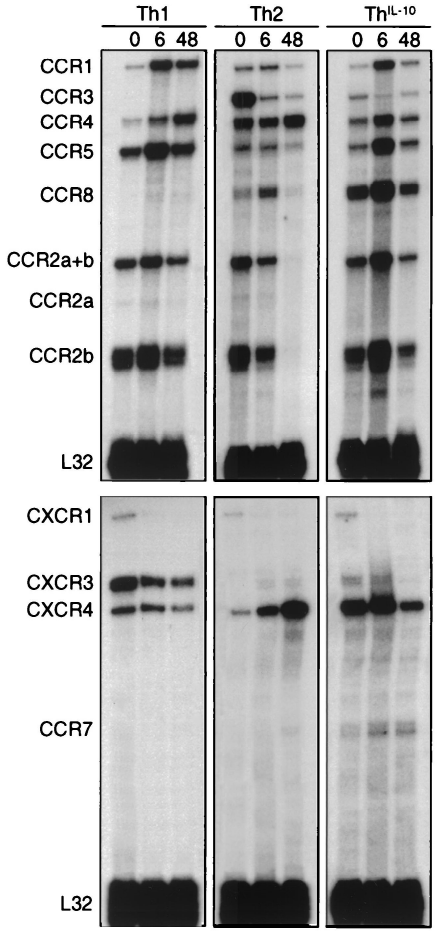

FIGURE 1. CC and CXC chemokine receptor mRNA expressed by nickel-specific Th^{IL-10}, Th1, and Th2 clones. Receptor mRNAs were examined by RNase protection assay in resting T cell clones and 6–48 h after stimulation with plate-bound anti-CD3 and soluble anti-CD28 in the presence of exogenous IL-2 (30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

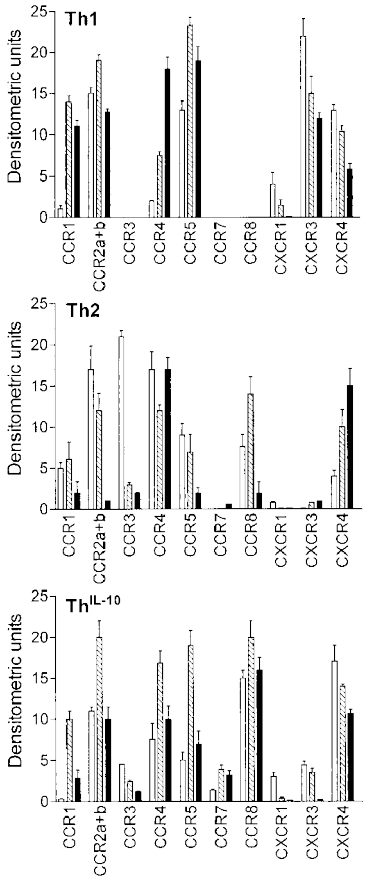

FIGURE 2. Densitometric analysis of chemokine receptor mRNAs expressed by resting and activated T cell clones. Densitometric values were calculated from RNase protection assay by laser densitometry and normalized against the housekeeping gene L32. Results are expressed as the mean ± SD of experiments performed on four Th^{IL-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.

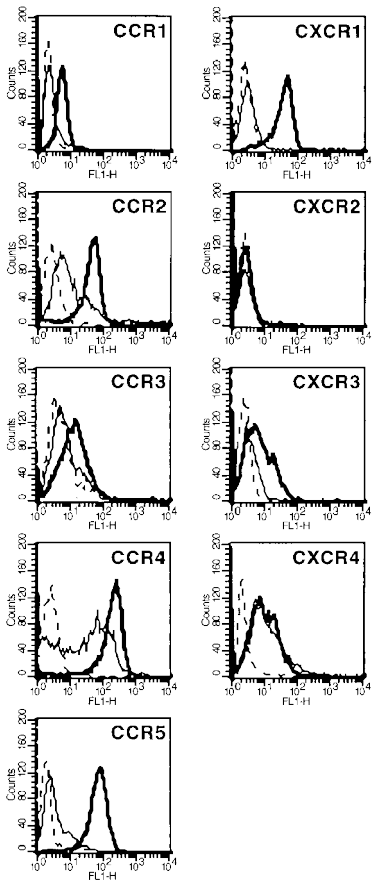

FIGURE 3. Chemokine receptors expressed by resting and activated Th^{IL-10} lymphocytes. Cells in resting state (bold lines) or 48-h activated with immobilized anti-CD3 (thin lines) were stained with the specific mAbs or with the isotype control Ig (dashed lines), followed by FITC-conjugated secondary Ab, and then examined with a FACScan. Results are representative of four Th^{IL-10} clones.

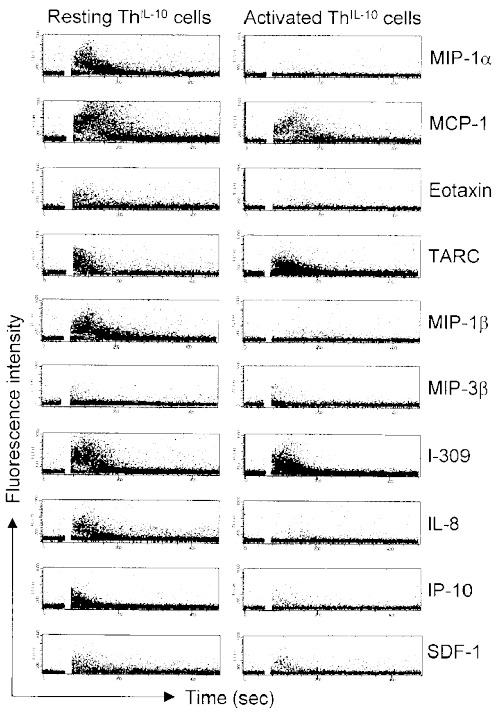

FIGURE 4. Intracellular calcium fluxes induced in Th^{IL-10} clones by chemokines. Th^{IL-10} cells were loaded with 8 μM fluo-3/acetoxymethyl ester in the presence of 1 μM pluronic F-127 and tested for intracellular calcium mobilization in response to different concentrations of agonistic chemokines. Calcium fluxes were measured in the resting state and 48 h after activation with anti-CD3/anti-CD28 in the presence of 30 U/ml IL-2. Shown is one experiment of four performed with different Th^{IL-10} clones.

2-fold stronger in resting Th^{IL-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 Th^{IL-10} cells. Th^{IL-10}, but not Th1 and Th2, clones expressed low levels of CCR7 mRNA, which was up-regulated upon activation. Finally, both resting and stimulated Th^{IL-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 Th^{IL-10} clones

FACS analysis confirmed the expression of the CCRs 1–5 and the CXCRs 1, 3, and 4 on resting Th^{IL-10} lymphocytes, whereas CXCR2 was negative (Fig. 3). Activated Th^{IL-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 CCR2 and CCR4, albeit reduced, was maintained at significant levels and that of CXCR4 was unmodified after Th^{IL-10} activation.

Chemokine receptors signaling in resting and activated nickel-specific Th^{IL-10} clones

To evaluate the functional relevance of chemokine receptor expression, we measured calcium mobilization in Th^{IL-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 Th^{IL-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 (CCR3 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 Th^{IL-10} clones. Notably, activated Th^{IL-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 Th^{IL-10} clones

The migratory behavior of Th1, Th2, and Th^{IL-10} lymphocytes was compared by measuring their transmigration through transwell filters in response to different concentrations of agonistic chemokines (Fig. 5). Resting Th^{IL-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. Th^{IL-10} migration to MIP-1β, IP-10 (CCR5 and CXCR3 ligands), and to eotaxin (CCR3 ligand) was lower compared with that of Th1 and

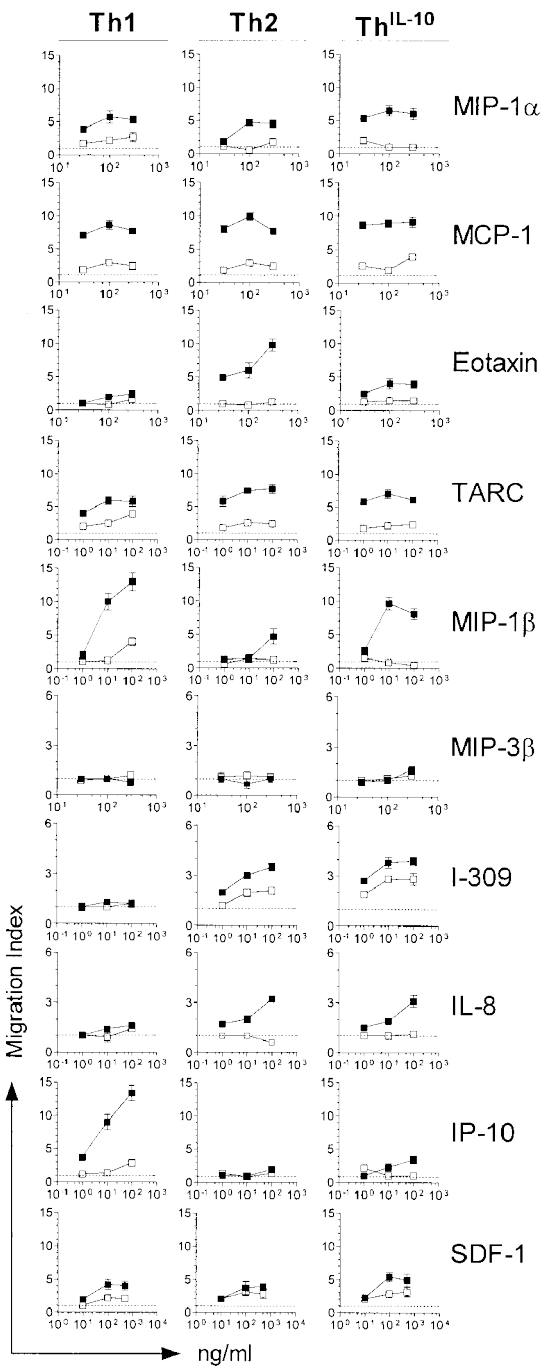

FIGURE 5. Migratory response of Th^{IL-10} cells to chemokines. Migratory responses to various concentrations of CC and CXC chemokines was examined in resting (■) and activated (□) Th^{IL-10} cells. Data are expressed as migration index (number of cells migrated with the agonistic chemokine/number of cells migrated with medium alone; mean ± SD). Similar results were obtained in three separate experiments using different Th^{IL-10} clones.

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

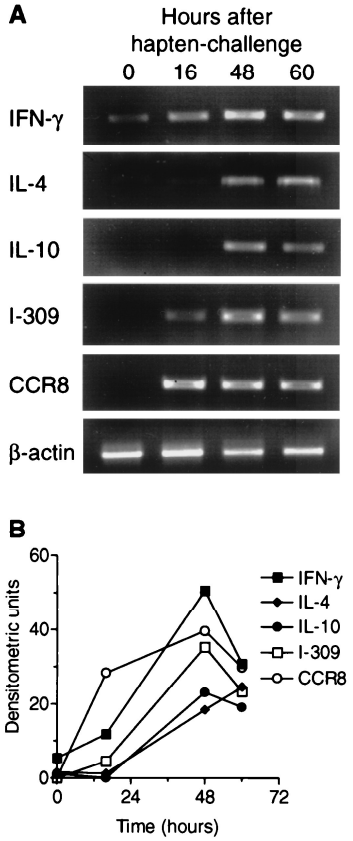

FIGURE 6. I-309, CCR8, and cytokine mRNA expression during allergic contact dermatitis to nickel. *A,* RT-PCR analysis was performed on RNA extracted from skin biopsies obtained from nickel-allergic patients at time 0 and at 16–60 h after the application of 5% $NiSO_4$ in petroleum. *B,* Densitometric analysis was performed by normalizing the levels of RNA against β-actin. Similar results were obtained in biopsies from two patients.

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, 48, and 60 h after nickel challenge. Fig. 6 demonstrates that I-309, CCR8, IL-10, and IL-4 mRNAs were undetectable in normal skin. Both CCR8 and I-309 were already expressed at 16 h after nickel application and reached a peak at 48 h, whereas IL-4 and IL-10 mRNAs became detectable only after 48 h. In contrast, a faint signal for IFN-γ mRNA was already present in unaffected skin, augmented at 16–48 h, and decreased thereafter.

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 $Th^{IL\text{-}10}$ 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 CXCR4. In vitro migration to TARC, MCP-1, and MIP-1α was similar in all subsets. In contrast, resting $Th^{IL\text{-}10}$ 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 $Th^{IL\text{-}10}$ cells, with I-309 attracting more efficiently $Th^{IL\text{-}10}$ than Th2 cells. TCR-engaged $Th^{IL\text{-}10}$ lymphocytes down-regulated most chemokine receptors, both at the mRNA and protein levels. As a consequence, activated $Th^{IL\text{-}10}$ 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 $Th^{IL\text{-}10}$, 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 $Th^{IL\text{-}10}$ lymphocytes (10- and 2-fold higher, respectively). This finding is consistent with the recent report by Campbell et al. (28), indicating that CCR4 is involved in the skin homing of memory T lymphocytes, being coexpressed on the majority of CLA^+ T cells independently of the cytokine profile.

IL-10-releasing T lymphocytes with regulatory function have been obtained in vivo after repeated intranasal stimulation with peptide Ag (29) or repeated superantigen challenge (30), and following immunization through UVB-irradiated skin (31). In addition, Groux et al. (32) have shown that murine and human lymphocytes cultured in the presence of IL-10 develop a phenotype characterized by high release of IL-10, IL-5, and TGF-β, variable amounts of IFN-γ, and no IL-4. These lymphocytes, called T regulatory cells 1 (Tr1), inhibit immune responses both in vitro and in vivo in a IL-10-dependent manner. More recently, a Tr1 polarization was induced in vitro by using dendritic cells treated with corticosteroids (33). Our skin-homing $Th^{IL\text{-}10}$ cells resemble Tr1 lymphocytes, but release lower levels of IFN-γ and display variable TGF-β production. Moreover, nickel-specific $Th^{IL\text{-}10}$ lymphocytes display the IL-12R β2 chain, the CD26 and the lymphocyte activation Ag-3 (12), markers related to a Th1 polarization. Whether these suppressive $Th^{IL\text{-}10}$ cells represent a distinct Th subset or a further maturation step of Th1 or Th2 cells has to be determined.

An interesting observation of our study was that I-309 was capable of attracting more efficiently $Th^{IL\text{-}10}$ than Th2 cells, both in resting and activated conditions, whereas it was ineffective on Th1. We also found that I-309 mRNA was significantly expressed in the skin after Ag challenge. I-309 expression paralleled that of CCR8 and preceded IL-4 and IL-10 expression. I-309-mediated recruitment of $Th^{IL\text{-}10}$ cells may thus represent an important mechanism for terminating Th1-mediated allergic reactions and limiting excessive tissue damage. In line with this hypothesis, it has been shown that administration of IL-10 strongly reduces murine contact hypersensitivity (34, 35), and IL-10-deficient mice show enhanced and more sustained cutaneous inflammatory responses to haptens (36). Activated Th1 and Th2 lymphocytes are the major source of I-309 (25, 37), but we have recently found a significant production of I-309 by IFN-γ-stimulated keratinocytes (C. Albanesi, C. Scarponi, S. Sebastiani, A. Cavani, M. Federici, S. Sozzani, and G. Girolomoni, manuscript in preparation), which may therefore contribute relevantly to the recruitment of regulatory $Th^{IL\text{-}10}$ cells in the skin. In aggregate, our results demonstrate that $Th^{IL\text{-}10}$ 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 $Th^{IL\text{-}10}$ lymphocytes may be advantageously exploited to control Th1-mediated allergic disorders.

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