STCP-1 (MDC) CC Chemokine Acts Specifically on Chronically Activated Th2 Lymphocytes and Is Produced by Monocytes on Stimulation with Th2 Cytokines IL-4 and IL-13

David P. Andrew, Ming-shi Chang, Jennifer McNinch, Scott T. Wathen, Marynette Rihanek, Julia Tseng, Jason P. Spellberg and Chester G. Elias III

*J Immunol* 1998; 161:5027-5038;
http://www.jimmunol.org/content/161/9/5027

References
This article cites 43 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/161/9/5027.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
STCP-1 (MDC) CC Chemokine Acts Specifically on Chronically Activated Th2 Lymphocytes and Is Produced by Monocytes on Stimulation with Th2 Cytokines IL-4 and IL-13

David P. Andrew,1 Ming-shi Chang, Jennifer McNinch, Scott T. Wathen, Marynette Rihanek, Julia Tseng, Jason P. Spellberg, and Chester G. Elias III

STCP-1 stimulated T cell chemoattractant protein-1 (STCP-1) (macrophage-derived chemokine; MDC), a recently described CC chemokine for chronically activated T lymphocytes, was found to act specifically on a subset of memory CD4 lymphocytes that displayed a Th2 cytokine profile. Also, STCP-1, thymus and activation regulated chemokine (TARC), eotaxin, and eotaxin-2 acted specifically on in vitro derived Th2 lymphocytes, while IP-10 (IFN-γ-inducible 10-kDa protein) showed some preference for Th1 lymphocytes. The corresponding receptors for eotaxin, TARC, and IP-10 are also differentially expressed on Th1 and Th2 lymphocytes. In desensitization Ca flux experiments, TARC and STCP-1 bound to a common receptor and therefore at least one chemokine receptor for STCP-1 is CCR4. STCP-1 expression is restricted to immune cells. Dendritic cells, B cells, and macrophages produce STCP-1 constitutively, while NK cells, monocytes, and CD4 lymphocytes produce STCP-1 upon appropriate stimulation. Production of STCP-1 is positively modulated by Th2 cytokines IL-4 and IL-13 but inhibited by IL-10. The Journal of Immunology, 1998, 161: 5027–5038.

Chemokines are a large and growing family of more than 30 6- to 14-kDa (nonglycosylated) heparin-binding proteins that mediate a wide range of biologic functions (1). The chemokines are divided into four families based on the position of four cysteine residues that form two disulfide bonds (2–4), while there are presently two chemokine receptor families (5). Chemokines play a vital role in leukocyte adhesion and extravasation. In various in vitro assays, chemokines support the chemotaxis or transendothelial migration of leukocytes (1), while in vivo injection (6) or overexpression of chemokines (7) results in leukocyte accumulation at the site of chemokine expression. Antagonists of chemokines prevent leukocyte trafficking (8) and have beneficial effects in several acute and chronic inflammatory models (9, 10). Chemokines also modulate angiogenesis (11) and hemopoiesis (1), as well as T lymphocyte activation (12, 13), and several act as coreceptors with CD4 for entry of M tropic and T tropic HIV-1 (14, 15).

We have previously described a chemokine, STCP-1,2 which acts specifically on chronically activated T lymphocytes (16), also reported as macrophage-derived chemokine (MDC) (17). On chronic stimulation, T lymphocytes develop into Th1 or Th2 lymphocytes, depending on the cytokines to which they are exposed (18). Recently, Th1 lymphocytes, but not Th2 lymphocytes, were demonstrated to adhere to CD62E and CD62P selectins (19), while only Th2 lymphocytes express the chemokine receptor CCR3 (20). It is thought that trafficking of Th1 and Th2 lymphocytes is differentially controlled through the selective expression of adhesion molecules and chemokine receptors. We describe here two CC chemokines, STCP-1 and TARC, which act specifically on chronically activated Th2 lymphocytes. STCP-1 and TARC may play a role in Th2 lymphocyte trafficking or, alternatively, in the development and effector action of Th2 lymphocytes.

Materials and Methods

Purification of cell populations

Human peripheral blood was collected in 10% (v/v) 0.1 M EDTA, layered onto 1-Step Polymorphs gradient (Accurate Chemical, Westbury, NY) and centrifuged for 400 × g for 30 min at room temperature. Neutrophils and mononuclear cell layers were collected, resuspended in DPBS without calcium and magnesium (Life Technologies, Grand Island, NY), and centrifuged for 15 min at −750 × g. RBC were lysed in the neutrophil fraction by resuspending the pellet in E-Lyse (Cardinal Associates, Santa Fe, NM) for 5 min on ice. Both cell fractions were washed twice with ice-cold DPBS. The mononuclear cells were allowed to adhere to protein-coated plastic for 2 to 3 h and then nonadherent cells were gently washed off the plate. After a further 12 h, the nonadherent dendritic cells were washed off the plate and depleted of B lymphocytes and T lymphocytes with anti-CD19 and anti-CD2 Dynabeads (Dynal, Great Neck, NY) (five beads per cell). The remaining cells were cultured in 50 ng/ml granulocyte/macrophage-CSF (GM-CSF) and 40 ng/ml IL-4 DMEM, 10% FCS, plus additives for 7 days (21). CD4, CD8, CD14, CD56, and CD19 populations were purified from mononuclear cells with the relevant Miltenyi Beads (Miltenyi Biotech, Auburn, CA) using 20 µl of beads for 107 mononuclear cells in PBS, 1% BSA, 5 mM EDTA at 5 × 105 cells/ml for 30 min at 4°C. They were then spun down, resuspended in PBS, 1% BSA, 5 mM EDTA at 5 × 107 cells/ml and passed over a VS column (Miltenyi Biotech) in a magnetic field to remove nontagged cells. Cells were removed by forcing 20 ml of PBS, 1% BSA, 5 mM EDTA over the VS column, outside the magnetic field.

Mouse T lymphocytes were isolated by Gey’s treatment of splenocytes to remove RBCs, followed by nylon wool depletion to remove B lymphocytes and macrophages. CD4 lymphocytes were obtained by depletion of the purified T lymphocytes with anti-CD8 Dynabeads to greater than 95% purity. OVA lines were made by footpad injection of 100 µl of a 1:1 mix of OVA (1 mg/ml):CFA into C57/BL mice and BALB/c mice. Seven days

Received for publication January 29, 1998. Accepted for publication July 1, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. David P. Andrew, LeukoSite Inc., 215 First Street, Cambridge, MA 02142. E-mail address: dandrew@hotmail.com.

2 Abbreviations used in this paper: STCP-1, stimulated T cell chemoattractant protein-1; bEND3, brain-derived endothelial cells; MIP, macrophage chemokine receptor protein; TARC, thymus and activation-regulated chemokine; KLH, keyhole limpet hemocyanin; HIV-1, human immunodeficiency virus type 1; HUVEC, human umbilical vein endothelial cells; HSA, human serum albumin; GM-CSF, granulocyte/macrophage-CSF.
later the lymph nodes were removed and the lymphocytes isolated. BALB/c lymphocytes were stimulated with OVA (50 μg/ml), IL-4 (5 ng/ml), and anti-IFN-γ (1 μg/ml), while C57/BL lymphocytes were stimulated with OVA (50 μg/ml), IL-12 (5 ng/ml), and anti-IL-4 (1 μg/ml) in the presence of mitomycin C-treated splenocytes for 5 to 7 days. This was followed by 4 to 5 days in IL-2-containing medium. The OVA-specific T lymphocytes were then either used or restimulated for 5 days and expanded in IL-2 before use in assays.

Human umbilical vein endothelial cells (HUVEC), astrocytes, bronchial smooth muscle cells, and fibroblasts were all obtained from Clonetics (San Diego, CA), while synovioocytes were obtained from synovial biopsies of rheumatoid arthritis patients.

Abs and reagents

The following mAbs were used in this study: OKT3; an anti-human CD3 mAb; American Type Culture Collection (ATCC, Manassas, VA) 145-2C11; anti-mouse CD3 mAb (ATCC), anti-human CD28 mAb (Becton Dickinson, Mountain View, CA), and anti-mouse CD28 mAb (PharMingen, San Diego, CA). Anti-CD4 TC, anti-CD58 FITC, anti-CD45RA FITC, anti-CD29 FITC, and anti-CD11a FITC mAbs for immunofluorescence studies were all obtained from PharMingen. All chemokines with the exception of STCP-1 were purchased from R&D Systems (Minneapolis, MN). The human endothelial cell line EC304 was purchased from ATCC, while the bEND3 cell line was kindly provided by Dr. W. Risau (Max-Planck Institute, Bad Neuheim, Germany). All cytokines were obtained from R & D Systems. In the activation assays, we used the stimulants at the following concentrations: IL-1α (5 ng/ml), IL-1β (5 ng/ml), IL-2 (5 ng/ml), IL-3 (5 ng/ml), IL-4 (5 ng/ml), IL-5 (5 ng/ml), IL-6 (5 ng/ml), IL-7 (5 ng/ml), IL-9 (5 ng/ml), IL-10 (20 ng/ml), IL-11 (10 ng/ml), IL-12 (5 ng/ml), IL-13 (20 ng/ml), IL-15 (20 ng/ml), IL-16 (40 ng/ml), IL-17 (40 ng/ml), IFN-γ (20 ng/ml), granulocyte-CSF (5 ng/ml), GM-CSF (5 ng/ml), IL-1Rα (1 μg/ml), leptin (2 μg/ml), TNF-α (5 ng/ml), TNF-β (5 ng/ml), and LPS (50 ng/ml).

Generation of anti-STCP-1 mAbs

BALB/c mice were immunized i.p. with 10 μg each of STCP-1/Keyhole limpet hemocyanin (KLH) conjugated in TFA at day 1, IFAt at day 20, and PBS at day 40. At day 60, the mice were boosted with 10 μg of STCP-1/KLH in PBS, and after 4 days, the spleens were removed and fused to SP20 myeloma cells (ATCC). Fusions were screened by ELISA, using plates coated with STCP-1. Hybridomas producing anti-STCP-1 mAbs were subcloned for further characterization. An ELISA was set up using one of the anti-STCP-1 mAbs, 7D7, as a capture mAb and a biotinylated rabbit polyclonal to STCP-1 to reveal captured STCP-1. The assay proved specific for STCP-1 with a sensitivity of 10 pg/ml.

Preparation of chronically activated Th1 and Th2 lymphocytes

As previously described (18), six-well Falcon plates were coated overnight with 10 μg/ml anti-CD28 and 2 μg/ml OKT3, and then washed twice with PBS. Peripheral blood CD4 lymphocytes (Poietics Systems, German- town, MD) were cultured at 10^5 to 10^6 cells/ml in DMEM with 10% FCS and IL-2 (4 ng/ml). IL-12 (5 ng/ml) and anti-IL-4 (1 μg/ml) were used to direct to Th1, while IL-4 (5 ng/ml) and anti-IFN-γ (1 μg/ml) were used to direct to Th2. After 4 to 5 days, the activated Th1 and Th2 lymphocytes were washed once in DMEM and cultured for 4 to 7 days in DMEM with 10% FCS and IL-2 (4 ng/ml). Following this, the activated Th1 and Th2 lymphocytes were restimulated for 5 days with anti-CD28/OKT3 and cytokines as described above, but with the addition of anti-CD95L (1 μg/ml) to prevent apoptosis. After 4 to 5 days, the Th1 and Th2 lymphocytes were washed and then cultured again with IL-2 for 4 days. Activated Th1 and Th2 lymphocytes were maintained in this way for a maximum of three cycles. Activated mouse T lymphocytes were prepared in a similar manner but using anti-mouse CD3 and anti-mouse CD28 mAbs, as well as mouse cytokines and anti-mouse IL-4 or anti-mouse IFN-γ.

ECV304/bEND3 transmigration and chemotaxis assays

Transwell inserts (5 μM pore diameter) were coated with 2% gelatin for 2 h. Then 0.45 ml of DMEM with 5% FCS was placed in the lower wells of the chambers and 2 × 10^6 ECV304 cells or bEND3 cells were added to each gelatin-coated insert in 0.2 ml of DMEM 5% FCS. After 2 days, the wells and inserts were washed twice with RPMI-1640 containing 0.5% HSA, 10 mM HEPES and then chemokine was added to the lower well. The wells were washed and resuspended at 4 × 10^6 cells/ml for Th1/Th2 lymphocytes and at 10^6 cells/ml for resting CD4 lymphocytes in RPMI 1640 0.5% HSA and 10 mM HEPES. An aliquot of 200 μl of cell suspension (input of 8 × 10^6 cells and 2 × 10^6 cells, respectively) was added to each insert. After 2 to 4 h, the inserts were removed and the number of cells that had migrated through the ECV304 monolayer to the lower well counted for 60 s on a Becton Dickinson FACScan with the gates set to acquire the cells of interest. Using this technique, 100% migration would be 25,000 cells for Th1/Th2 cells and 75,000 cells for resting CD4 lymphocytes, in which this number represents the cells in the lower well counted on the FACScan over 1 min. To study the phenotype of migrating cells, identical experiments with CD4 lymphocytes were performed with six-well plates using 24-mm diameter inserts. Chemotaxis assays were identical to ECV304 migration assays but fibronectin-coated inserts (10 μg/ml) were used. In all cases, the data points were the result of duplicate wells, with the mean value shown and the error bars representing the standard deviation.

Ca mobilization assay

A total of 10^7 cells/ml in DPBS were labeled for 30 min with Fura 2 dye (Molecular Probes, Eugene, OR) at 2 mM, washed three times in DPBS and resuspended at 10^6 cells/ml in DPBS containing 1 mM CaCl_2, 0.5 mM MgCl_2, 10 mM HEPES, and 5.5 mM glucose. The cells were then analyzed on a fluorometer using 10 nM Nonidet P-40 and 10 mM EDTA to establish the maximum and minimum Ca^2+ mobilizations.

Relative quantification of mRNA levels by quantitative TaqMan RT-PCR

RNA was isolated from resting Th1 and Th2 lymphocytes using a Qiagen RNasey Midi Kit (Chatsworth, CA). Relative mRNA levels were determined by real time fluorometric measurement of cDNA amplification as described in (22) and the TaqMan PCR Reagent Kit (PE Applied Biosystems, Foster City, CA) using an Applied Biosystems Prism 7700 Sequence Detection System. Messenger RNA for 18S rRNA served as the internal standard. Values determined for mRNA of chemokine receptors were normalized by dividing by mRNA for 18S rRNA. Forward, reverse, and Taq-Man probe primers were used as follows: CCR1 (CTG CAT CTC CCT CCC CAT A, GGC GCC AAT GTA GGC AAA, TCC TCG GAC ACC ACA CCC TGC TG), CCR3 (GGA AGT ACT TGC ACT TCT, GGA AGG AAT GGG ATG TAT CCT GC, CCA CAG GCA CTT GCT CAT GCA CCT), CCR4 (CAA TAC TGT GGG CTC CCT CAA, TCA ATG GTG GAC TGC GTG TA, TTA TCT TGC TGA CAC CCC CAG CTC ATC), CXCR3 (GTG GCC AAG TCG GTC ACC T, TTG ACC CCT AAG GCA TAG AG, ACA TGC ACT GTC GCC TCA ACC CG), and 18S GCG CCG TAG AGG TAG AAT TCT, CAT TCT TGG CAA ATG CCT TCC, AGC GGC AGA CGG ACC AGA). In all cases samples were run in triplicates and the mean and SD are shown.

Results

STCP-1 acts on a subset of memory CD4 lymphocytes that produce a distinctive set of cytokines on TCR stimulation

In contrast to MCP-1 and MCP-3, STCP-1 did not act as a chemotactic agent for freshly isolated CD4 lymphocytes (Fig. 1A). However, after 24 h in culture, a small subset of CD4 lymphocytes, representing approximately 1% of the CD4 lymphocytes, migrated to STCP-1 (Fig. 1A). In the six experiments performed with 24-h-old CD4 lymphocytes, the migration observed varied from 0.2 to 2.9%, depending on the individual and also the particular time at which blood was drawn for an individual. We also observed increased migration of CD4 lymphocytes to RANTES, IL-8, MCP-1, MCP-3, MIP-1α, and MIP-1β. To show that the chemokines were acting on subsets of CD4 lymphocytes, we combined various chemokines in order to increase the migration, based on the assumption that the various chemokines would act on different subsets in many cases. By this approach we were able to increase migration at least 100-fold. Also, a polyclonal to STCP-1 was able to inhibit the migration of CD4 lymphocytes to STCP-1 (Fig. 1B) and therefore the migration was mediated by STCP-1. The CD4 lymphocytes, which migrated to STCP-1, were CD45RO memory CD4 lymphocytes as well as CD58°, CD29high, and CD11a high.
(Fig. 1C). We also found that STCP-1-responsive CD4 lymphocytes produced almost no IFN-γ and less TNF-α upon stimulation with anti-CD3/CD28 mAbs when compared with the total CD4 lymphocyte population (Fig. 2, A and C). However, IL-10 production by STCP-1 responsive CD4 lymphocytes was identical to that of total CD4 lymphocytes (Fig. 2B). Following one activation using plate-bound anti-CD3 mAb/anti-CD28 mAb, the migrated and starting populations were examined for their production of IL-4, IL-5, and IL-13 upon cross-linking of TCR and CD28 (Fig. 2, D, E, and F). Production of Th2 cytokines IL-4, IL-5, and IL-13 was restricted to STCP-1-responsive CD4 lymphocytes.

**STCP-1 acts specifically on chronically activated Th2 lymphocytes**

As STCP-1-responsive CD4 lymphocytes display a Th2 cytokine profile, we examined the response of Th1 and Th2 lymphocytes (third-cycle activation) to STCP-1. By ELISA, Th1 lymphocytes produced no IL-4, but high amounts of IFN-γ on stimulation, while Th2 lymphocytes produced IL-4, but little IFN-γ (Fig. 3, C and D). Both Th1 and Th2 lymphocytes chemotaxed to MCP-1, but only Th2 lymphocytes chemotaxed to STCP-1 (Fig. 3, A and B). In a general screen using a panel of chemokines, only STCP-1, TARC, eotaxin and eotaxin-2 were Th2 specific (Fig. 4A). The CXC chemokine IP-10 displayed some selectivity for Th1 lymphocytes, but was not completely specific for these cells (Fig. 4A). TARC and IP-10 were retested at several concentrations and identical results were obtained (Fig. 4, B and C), confirming that TARC is Th2 specific and that IP-10 has some selectivity for Th1 lymphocytes. Furthermore, Ca flux experiments confirmed our chemotaxis experiments showing that STCP-1 and TARC act specifically on Th2 lymphocytes, while MCP-1 was active on both Th1 and Th2 lymphocytes (Fig. 5).

**STCP-1 acts specifically on primary mouse Th2 lines generated to OVA**

As STCP-1 is active on mouse cells, we also examined in vitro-derived mouse Th1 and Th2 cells for their response to STCP-1. As in the human system, STCP-1 was found to act specifically on in vitro-derived mouse Th2 lymphocytes (Fig. 6, A and B). Also, when primary Th1 and Th2 Ag-specific T cell lines to OVA were generated in C57/BL mice and BALB/c mice, STCP-1 showed better activity on the Th2 line than on the Th1 line, MCP-1 and MIP-1α were equally effective on both Th1 and Th2 lines (Fig. 6C). Therefore, Ag-specific Th2 lymphocytes generated in vivo are responsive to STCP-1, while Th1 lymphocytes are not.
TARC binds to the STCP-1 receptor

Due to the similarity in the expression of TARC and STCP-1 reported in Northern blots of human tissues, and the similar target cells of these two chemokines, we tested TARC and STCP-1 against each other in desensitization Ca\(^{2+}\) flux experiments. TARC was found to desensitize Th2 lymphocytes to a second stimulation with TARC (Fig. 7B), while prestimulation of Th2 cells with STCP-1 prevented Ca\(^{2+}\) flux on a second stimulation with STCP-1 (Fig. 7A). Prestimulation of Th2 cells with MCP-1 did not prevent TARC (Fig. 7E) or STCP-1 (Fig. 7F) from stimulating Ca\(^{2+}\) flux in these cells; however, prestimulation of Th2 cells with STCP-1 prevented these cells from responding to TARC (Fig. 7C). Surprisingly, prestimulation of Th2 cells with TARC did not prevent Ca\(^{2+}\) mobilization on a second stimulation with STCP-1 (Fig. 7D). Also, further experiments in which Th2 lymphocytes were either stimulated with higher doses of TARC or were prestimulated twice with TARC did not completely desensitize the cells to STCP-1 (data not shown).

FIGURE 2. The STCP-1-responsive CD4 lymphocytes display a distinctive cytokine profile on T lymphocyte activation. A starting population of CD4 lymphocytes (approximately 4 \times 10^8 CD4 lymphocytes and the CD4 lymphocytes that migrated across ECV304 monolayers to STCP-1 (from 1 to 4 \times 10^6 CD4 lymphocytes) were compared for the levels of IFN-\(\gamma\) (A), IL-10 (B), and TNF-\(\alpha\) (C) that they produced upon stimulation. In D, E, and F, we used migrated and starting CD4 lymphocytes, which had been placed on petri dishes coated with anti-CD3/CD28 for 4 days followed by culture in IL-2 for 4 days. These cells were then compared for the levels of IL-4 (D), IL-5 (E), and IL-13 (F) that they produced upon stimulation. Various concentrations of anti-CD3 mAb OKT3 in the presence of 10 \(\mu\)g/ml anti-CD28 were used to stimulate the cells. These experiments were performed twice with similar results.
CCR3, CCR4, and CXCR3 are differentially expressed on Th1/Th2 lymphocytes

In the absence of mAbs to CCR3 and CCR4, we examined the expression of these chemokine receptors on Th1 and Th2 lymphocyte lines using quantitative Taqman RT-PCR. CCR1 mRNA was expressed at similar levels on Th1/Th2 lymphocytes, but CCR3 and CCR4 mRNA were expressed at higher levels on Th2 lymphocytes (Fig. 8). CXCR3 mRNA expression was twofold higher in Th1 lymphocytes (Fig. 8). While mRNA for CCR3 was detected specifically in Th2 lymphocytes, the levels of mRNA were much lower than those observed for CCR4. This may reflect decreased expression of CCR3 on Th2 lymphocytes, or expression of CCR3 on a subset of Th2 lymphocytes in comparison with CCR4.

Expression of STCP-1 is restricted to immune cells and is under the control of cytokines IL-4, IL-10, IL-13, and GM-CSF

We examined several cell types for their ability to produce STCP-1 upon stimulation with a panel of cytokines. We also measured IL-8, TNF-α, or RANTES production by these different cell types to ensure that the various cell populations were activated by the treatments used. While HUVEC, astrocytes, synoviocytes, Th1, Th2 lymphocytes, and neutrophils produced IL-8 or RANTES on appropriate stimulation, they did not produce STCP-1 (Fig. 9). Lung fibroblasts and smooth muscle cells were also unable to produce STCP-1 upon stimulation with this panel of cytokines (data not shown); however, cross-linking of CD3 on freshly isolated CD4 lymphocytes resulted in STCP-1 production (Fig. 10E), while APCs such as dendritic cells (Fig. 10A), B cells (Fig. 10D), and macrophages (data not shown) constitutively secreted STCP-1. Freshly isolated NK cells (Fig. 10C) and monocytes (Fig. 10B) also produced STCP-1 upon appropriate stimulation.

Having established the cell types that produce STCP-1 upon stimulation, we examined in greater detail the cytokines that stimulate STCP-1 production by monocytes. IL-4 and IL-13 were potent stimulators of STCP-1 secretion, while GM-CSF proved a weak activator of STCP-1 production (Fig. 11A). We also observed that preincubation of monocytes with IL-10 blocked the production of STCP-1 on stimulation with IL-4 30 min later (Fig. 11B). IL-10 also inhibits the constitutive production of STCP-1 by monocytes, dendritic cells, NK cells, and B cells (Fig. 10).

Discussion

We report here that the CC chemokines STCP-1 and TARC act specifically on Th2 lymphocytes. Primary stimulated Th2 lymphocytes showed a preference for STCP-1 and TARC, and by the second round of stimulation, only Th2 lymphocytes responded to STCP-1 and TARC (data not shown). This Th2 specificity remained for as long as we maintained Th1 and Th2 lymphocytes
FIGURE 4. Screen of a panel of chemokines on Th1/Th2 lymphocytes. A, Th1 and Th2 lymphocytes (third stimulation) were tested for their ability to cross monolayers of ECV304 endothelial cells in response to various chemokines at 100 ng/ml. TARC (B) and IP-10 (C), along with STCP-1, were then tested at several concentrations for their ability to act as chemoattractants for Th1 and Th2 lymphocytes. As a positive control, MCP-1 at 100 ng/ml was used and chemoattracted 8236 ± 170 Th1 lymphocytes, and 6657 ± 827 Th2 lymphocytes. The two experiments were repeated twice with similar results.

FIGURE 5. Chemokines STCP-1 and TARC differentiate between Th1/Th2 lymphocytes in Ca²⁺ flux experiments. Th1 (A, C, E) and Th2 (B, D, F) lymphocytes were loaded with Fura-2 dye for 30 min and then washed and examined on a fluorometer for their ability to flux to chemokines STCP-1 (A, B) and TARC (C, D), both at 100 ng/ml. MCP-1 at 100 ng/ml served as a positive control (E, F), inducing Ca²⁺ flux in both Th1 and Th2 lymphocytes. These experiments were repeated four times with identical results.
cells were then tested for their response to various concentrations of STCP-1 and MCP-1 in the presence of various cytokines to direct to Th1 or Th2. These Th1/Th2 cells were activated with plate-bound anti-CD28/CD3 antibody (BA) and BALB/c mice (C) were activated with plate-bound anti-CD3. These experiments were repeated twice with similar results.

FIGURE 6. STCP-1 is a chemoattractant for Th2, but not Th1, mouse T cell lines. CD62L<sup>−</sup> T cells isolated from the spleens of and C57/BL mice (A) and BALB/c mice (B) were activated with plate-bound anti-CD28/CD3 in the presence of various cytokines to direct to Th1 or Th2. These Th1/Th2 cells were then tested for their response to various concentrations of STCP-1 and MCP-1. C. C57/BL and BALB/c mice were immunized with OVA and the harvested lymph node T lymphocytes restimulated in vitro to generate Th1/Th2 lines, which were then tested for their ability to chemotax to 100 ng/ml of STCP-1, MCP-1, and MIP-1α. These experiments were repeated twice with similar results.

up to five rounds of stimulation). Eotaxin was recently reported to act specifically on Th2 lymphocytes (22) and also proved Th2 specific in our experiments, as did eotaxin-2, which also binds to CCR3 (23). Unlike TARC and STCP-1, several rounds of stimulation of Th2 lymphocytes was necessary to obtain optimal chemotactic responses to eotaxin and eotaxin-2 (data not shown). Our functional data is in part validated by the selective expression by Th2 lymphocytes of CCR3 and CCR4. We have also been able to extend our studies to blood CD4 lymphocytes in which the small subset of CD4 lymphocytes that respond to STCP-1 were found to be of memory phenotype and to display a Th2 cytokine profile.

Our experiments have focused on the ability of chemokines to act as chemoattractants for Th1 and Th2 lymphocytes. However, it is also possible that these chemokines act in other ways on Th1 and Th2 lymphocytes. RANTES and MIP-1α modulate T lymphocyte activation, inducing T lymphocyte proliferation and cytokine secretion (12, 13). While cytokines IL-12 and IL-4 direct development of Th1 and Th2 lymphocyte populations, chemokines also modulate the secretion of cytokines by Th1/Th2 lymphocytes (24), as well as IgE secretion by B lymphocytes (25). Recent studies in chemokine receptor-deficient mice have shown that, in the absence of CCR-1, Th1 responses are enhanced while CCR-2 deficiency results in defective Th1 responses (26, 27). Therefore, chemokines may also play a important role in the development of Th1/Th2 lymphocytes. STCP-1 and TARC, as well as acting as chemoattractants for Th2 lymphocytes, may also modulate cytokine production of these cells.

In our experiments, TARC also acted as a Th2-specific chemokine. The expression pattern for TARC and STCP-1 are identical. Both are expressed highly in thymus, lymph nodes, and appendix, but not in spleen. The Ca<sup>2+</sup> flux induced by TARC on chronic Th2 lymphocytes was blocked by STCP-1, but surprisingly the response of these cells to STCP-1 was not blocked by TARC. Our data agree with recent reports showing TARC (28) and STCP-1/MDC (29) bind to CCR4; however, the fact that TARC does not block STCP-1-mediated Ca<sup>2+</sup> flux of Th2 lymphocytes indicates that STCP-1 may also bind to another chemokine receptor in addition to CCR4 on Th2 lymphocytes.

Our ELISA data show that STCP-1 is selectively produced by immune cells. As previously reported (17), we found HUVEC did not produce STCP-1 on stimulation with TNF-α. We also report here that neutrophils, Th1/Th2 lymphocytes, astrocytes, and synovocytes do not produce STCP-1 upon stimulation, as well as smooth muscle cells and fibroblasts (data not shown). The constitutive expression of STCP-1 by macrophages and dendritic cells, as also reported in Northern blot studies (17), as well as by B cells, may reflect a role for STCP-1 in either Ag presentation to naive T lymphocytes or development of these T lymphocytes into effector cells. While a previous study showed no expression of STCP-1 by IL-2-cultured NK cells (17), we detected STCP-1 expression upon activation of freshly isolated NK cells. This difference may result from the use of IL-2-cultured NK cells as opposed to fresh NK cells in these two reports, as well as the fact that we activated our NK cells. Alternatively, the difference may result from the presence of CD8<sup>−</sup>CD56<sup>−</sup> cells in our NK cell preparations, as we used anti-CD56 beads to isolate our NK cells. The ability of freshly isolated CD4 lymphocytes, but not chronically activated Th1/Th2 lymphocytes, to produce STCP-1 on stimulation was a surprising result. It is possible that just as surface molecules CD31, CD62L, and CD45RA are down-regulated on chronic T lymphocyte stimulation (16), production of certain chemokines such as STCP-1 are also down-regulated on chronic stimulation. While, as previously reported, monocytes made very little STCP-1 (17), IL-4 and IL-13 were potent stimulators of STCP-1 production by monocytes. Therefore, while our chemotaxis data demonstrate that STCP-1 acts specifically on chronically activated Th2 lymphocytes, our expression studies indicate that Th2 cytokines also stimulate the production of STCP-1. STCP-1, to our knowledge, is the first chemokine whose production by monocytes is selectively stimulated by IL-4 and IL-13. The production of RANTES, MIP-1α, and IL-8 by monocytes is also stimulated by LPS and various
proinflammatory cytokines. By contrast, STCP-1 expression is under tight control, as very few cytokines positively modulate STCP-1 production, while IL-10 inhibits its secretion.

In our experiments, we identified chemokines that act specifically on Th2 lymphocytes. However, chemokines specific for Th1 lymphocytes proved harder to define. The CXC chemokine IP-10, which binds to the chemokine receptor CXCR-3, did show some specificity for Th1 lymphocytes, but was by no means Th1 specific. Using quantitative TaqMan RT-PCR, the mRNA for the receptor for IP-10, CXCR3, was also found to be expressed at higher levels on Th1 lymphocytes. Our observation of an association between IP-10 and Th1 lymphocytes is all the more relevant, given the fact that IFN-γ is a cytokine associated with a Th1 environment and is a potent inducer of IP-10 production (30). Therefore, IP-10 would be predicted to be expressed at sites of inflammation associated with IFN-γ production, which may favor the recruitment of Th1 lymphocytes to these sites. In fact, IP-10 is expressed at sites of cutaneous delayed-type hypersensitivity reactions, which are associated with Th1 responses (31). In our studies MIP-1α and MIP-1β acted on both Th1 and Th2 lymphocytes, while other reports have shown that CCR5 is expressed selectively on Th1 lymphocytes. It is possible that the interaction of MIP-1αβ with other chemokine receptors such as CCR1 and CCR8 (32) on Th2 lymphocytes may account for the migration we observed.

FIGURE 7. STCP-1 desensitizes Th2 cells to TARC stimulation. Th2 lymphocytes were loaded with Fura-2 for 30 min and then washed and examined on a fluorometer. Cells were stimulated twice at time points 50 and 150 s with chemokines at 100 ng/ml in the following order: STCP-1/STCP-1 (A), TARC/TARC (B), STCP-1/TARC (C), TARC/STCP-1 (D), STCP-1/MCP-1 (E), and MCP-1/TARC (F). The experiment was performed four times with similar results.

FIGURE 8. CCR3, CCR4, and CXCR1 are differentially expressed on Th1 and Th2 lymphocytes. RNA was isolated from Th1 and Th2 lymphocytes at the third cycle of activation at day 4 in IL-2. We then used quantitative TaqMan RT-PCR to compare the levels of mRNA of CCR1, CCR4, and CXCR3 (A), and CCR3 (B), in Th1/Th2 lymphocytes using mRNA levels for 18S rRNA to normalize the levels. Underlined values represent the ratio of Th2 to Th1. All points were performed in triplicate with means and SDs shown. The experiment was repeated twice with similar results.
Also, studies in CCR5-deficient mice have shown that activated T lymphocytes still respond to MIP-1α (33).

Chemokine receptors are expressed on several subsets of T lymphocytes. For example, CXCR-4, the receptor for SDF-1β, is specifically expressed by naive T lymphocytes (34). A new chemokine, DC-CK-1, produced by dendritic cells, acts specifically on naive T lymphocytes. Presumably, the receptor for this chemokine is expressed selectively on naive T lymphocytes (35). Conversely,
CCR5 and CCR2 chemokine receptors are expressed on subsets of memory CD4 lymphocytes (36). Just as differential expression of adhesion molecules on subsets of T lymphocytes control the trafficking of these cells to different sites, differential expression of chemokine receptors on T lymphocyte subsets may also be involved in selective trafficking of these subsets. Our data indicate that the receptor for STCP-1 is expressed on only 0.2 to 2% of circulating CD4 lymphocytes, and that this subpopulation represents a small subset of memory CD4 lymphocytes. Given the specific action of STCP-1 on chronically activated Th2 lymphocytes, and the low expression of IFN-γ by this subset, it is probable that this population is composed of Th2 lymphocytes.

The selective expression of chemokine receptors on Th2 lymphocytes may represent a point of therapeutic intervention in Th2-mediated chronic inflammatory diseases such as asthma and allergic rhinitis. Chemokine receptors selectively expressed on Th2 lymphocytes may play an important role in the trafficking of these lymphocytes to the lung or, alternatively, in development/effector action of Th2 lymphocytes. In an asthmatic attack, Th2 lymphocytes responsive to allergen disappear from the blood. The presence of Th2 lymphocytes in bronchoalveolar lavage would indicate that these cells traffic to the lung after exposure to allergen (37, 38). Our experiments indicate that Th2 lymphocytes represent only a small proportion of total CD4 cells (0.2–2%) in the circulation. Just as
mAbs to CD52 (39) and CD4 (40) have previously been used to deplete specific lymphocytes in inflammatory diseases, complement-fixing mAbs to chemokine receptors such as CCR4 or chemokine-ricin conjugates theoretically could be used to remove pathogenic Th2 lymphocytes, while leaving the rest of the immune system intact. Depletion of T lymphocytes in asthma models results in reversal of asthma (41). Depletion of chronically activated Th2 lymphocytes may have a similar effect.

In summary, we show that STCP-1 acts specifically on Th2 lymphocytes. Interestingly, STCP-1 expression is restricted to the lymphoid system cells and Th2 cytokines IL-4 and IL-13 are potent stimulators of STCP-1 production by monocytes, while IL-10 antagonizes secretion of STCP-1. Our desensitization Ca\textsuperscript{2+} flux experiments indicate that, as recently reported (29), STCP-1 binds to CCR4.

Note. While this manuscript was in preparation, other studies reported that demonstrated CCR4 is expressed preferentially by Th2 lymphocytes (42, 43), confirming several of the experiments reported here.

Acknowledgment

We thank Dr. Werner Risau for providing the bEND3 mouse endothelial cell line.

References