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A Fusion Protein Encoding the Second Extracellular (E2) Domain of CCR5 Arrests Chemokine-Induced Cosignaling and Effectively Suppresses Ongoing Experimental Autoimmune Encephalomyelitis

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CCR5 is a key CCR that is highly expressed on CD4+ T cells. It binds three different ligands: CCL3 (MIP-α), CCL4 (MIP-β), and CCL5 (RANTES). Recent studies suggested that the interaction between CCR5 and its ligands is essential not only for attracting these CCR5+ T cells but also substantial for transducing cosignals for their activation. The current study explores, for the first time, the in vivo consequences of CCR5 as a costimulatory molecule. First, we show redundancy between CCR5 ligands not only in chemoattractive properties but also in their ability to induced cosignals via CCR5. This has motivated us to generate a soluble receptor-based fusion protein that would selectively bind and neutralize all three CCR5 ligands. We show in this study that a 30-aa–based CCR5-Ig fusion protein encoding the second extracellular domain of receptor selectively binds and neutralizes all three CCR5 ligands and, when administered during ongoing experimental autoimmune encephalomyelitis, rapidly suppressed the disease while arresting Ag-specific effector T cell functions. Finally, our results clearly show that although CCR5 ligands induced cosignaling for IL-2 production is directed by CCR5, other proinflammatory properties of these ligands, such as TNF-α, IL-17, and IFN-γ production, are CCR5 independent and therefore likely to be mediated by the other receptors for these ligands. These findings imply that implementing a CCR5-Ig-based therapy would be advantageous over blockade of this receptor or of the use of mAbs for targeting a single CCR5 ligand. The Journal of Immunology, 2010, 185: 000–000.

Chemokines are small (~8–14 kDa), structurally related proteins that regulate cell trafficking via interactions with a subset of seven-transmembrane, G protein-coupled receptors (1–4). Most of the attention has been drawn to their key role in the inflammatory process (5) and cancer diseases (6, 7). As key mediators of inflammatory processes, some of them were found to be attractive targets for therapy of a variety of autoimmune diseases including multiple sclerosis, rheumatoid arthritis (RA), type I diabetes (TIDM), atherosclerosis, myocarditis, and others (5, 8–14).

Aside from their chemotactic properties, chemokines display other biological features, some of which are fundamental for the development and regulation of the inflammatory process. For example, the CXCR3 ligand CXCL10 polarizes CD4+ T cells into effector Th1 (15, 16), whereas CXCL12 skews T cell polarization into IL-10–producing Ag-specific regulatory T cells (17). Another example for the variety of biological properties of chemokines is their role in the activation of adhesion receptors, an essential step allowing rapid influx of leukocytes to target organs (18, 19).

CCR5 is a key CCR that binds three different ligands: CCL3 (MIP-α), CCL4 (MIP-β), and CCL5 (RANTES) (2). In a recent study, Molon et al. (20) showed that signals transduced by CCL5, via CCR5, are required for effective T cell activation. Subsequently, Contento et al. (21) showed that during T cell activation by APCs, the chemokine receptors CCR5 and CXCR4 are recruited into the immunological synapse, where they deliver costimulatory signals mediated by their hetero-oligomerization. Very recently, Camargo et al. (22) showed that the interaction between CCR5 and its ligands induces signals via NFAT translocation, leading to IL-2 production by CD4+ T cells, and that IL-2 production is highly related to CCR5 expression. The in vivo implications of these findings have never been explored. In the current study, we initially show that each of the three CCR5 ligands, CCL3, CCL4, and CCL5, can effectively compensate for lack of their counterpart ligand-transduced costimulatory signals; thus, only a combined targeted neutralization of all three ligands led to arrest in effector T cell activation. This motivated us to generate a soluble CCR5-Ig fusion protein that selectively neutralized all three ligands and was also used to explore its biological properties.

Materials and Methods

Mice

Six- to 7-wk-old female C57BL/6 mice were purchased from Harlan (Jerusalem, Israel) and maintained under specific pathogen-free conditions in our animal facility. All animal studies were conducted according the National Institutes of Health guideline and were approved by the Technion ethics committee for experiments in animals.
Thyl.1 mice and CCR5<sup>−/−</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

**Peptides**

Myelin oligodendrocyte glycoprotein (MOG)<sub>p35-55</sub> was constructed by the Beckman Center at Stanford University (Stanford, CA). After purification by HPLC, the sequence was confirmed by amino acid analysis, and the correct mass was checked by mass spectroscopy. Purification of the peptide that was used in the current study was >95%.

**Induction and treatment of active disease**

Experimental autoimmune encephalomyelitis (EAE) was induced by immunizing mice with MOG<sub>p35-55</sub>/CFA, as described by Tompkins et al. (23). Briefly, 6- to 7-week-old female C57BL/6 mice were immunized s.c. with 200 μl of an emulsion containing 800 μg Mycobacterium tuberculosis H37Ra and 200 μg MOG<sub>p35-55</sub>. Animals were then monitored for clinical signs by an observer blind to the treatment protocol. In ongoing disease treatment, 1 d after onset of disease, mice were divided to equal score groups and were treated i.p. with repeated administration (every other day) of 250 μg/mice with either a CCR5(E2)-IgG, isotype IgG control, or with PBS. An observer blind to the experimental procedure scored EAE daily.

**Prophylactic treatment in active-induced EAE**

Twice a week, beginning 4 d after the induction of the disease, mice were injected i.p. with 250 μg/mice with a CCR5(E2)-Ig, isotype IgG control, anti-CLL3 pDNA-based Abs, control rat sera, or PBS. An observer blind to the experimental procedure scored EAE daily.

**EAE scores**

EAE scores are as follows: 0, normal; 1, flaccid tail; 2, hind limb paralysis; 3, total hind limb paralysis, accompanied by an apparent front limb paralysis; 4, total hind limb and front limb paralysis; and 5, death.

**Induction of adoptive transfer disease**

Adoptive disease was induced as described elsewhere (24). Briefly, active EAE was induced, and the mice were treated in prophylactic manner as described previously. On days 10 postdisease induction, draining lymph nodes (DLNs) and spleens were harvested, and cells were cultured for 72 h in stimulation medium containing DMEM, 5% FBS, 2 mM L-2, medium pyruvate, MEM nonessential amino acids, and penicillin-streptomycin and supplemented with 50 μg/ml MOG<sub>p35-55</sub>. After 72 h, cells were washed, counted, and resuspended in PBS and were administered to naive mice i.v. An observer blind to the experimental procedure scored EAE daily.

**Induction of adoptive transfer disease in Thy1.1 mice**

Active EAE was induced in Thy1.1 mice. On day 9 postdisease induction, DLNs and spleens were harvested, and cells were cultured for 72 h in stimulation medium supplemented with 50 μg/ml MOG<sub>p35-55</sub>. After 72 h, cells were washed, counted, and resuspended in PBS and were administered to naive C57BL/6 mice (i.v. 20 × 10<sup>6</sup> cells/mouse). Mice were then treated every other day with CCR5(E2)-Ig or control isotype-matched IgG. The infiltration of Thy1.1 cells into the spinal cord was evaluated using immunostaining.

**Cytokine measurement by ELISA**

IL-2, IL-4, IL-12, IL-17, TNF-α, and IFN-γ ELISA kits (BioLegend, San Diego, CA), IL-10 (R&D Systems, Minneapolis, MN), and TGFB (BD Biosciences, Franklin Lakes, NJ) were used according to the manufacturer’s protocols. Human cytokine level was evaluated using human (h)TNF-α, and hIFN-γ ELISA kits (BioLegend).

**Cells isolation and stimulation**

Spleens and lymph nodes (LNs) were harvested, and single-cell suspension was created. For costimulatory experiments, CD4<sup>+</sup> T cells were isolated by MACS beads (Miltenyi Biotec, Bergisch-Gladbach, Germany), and successful purification (>95%) was verified by flow cytometry and then suspended in culture plates bound anti-CD3 (0.2 μg/ml) (BioLegend). CCL3, CCL4, CCL5, CCL2, or CXCL16 (R&D Systems) was added at a final concentration of 10 ng/ml of for 5 d. During the last 18 h of activation, [3H]dThd was added, and the proliferation index was determined by quantifying [3H]dThd uptake assay.

In the in vitro MOG<sub>p35-55</sub> restimulation experiments, active EAE was induced. On days 9–10 postdisease induction, DLNs and spleens were harvested, and cells were cultured for 72 h in stimulation medium with 50 μg/ml MOG<sub>p35-55</sub> and the addition of supplements as mentioned in the different experiments. The proliferation rate was evaluated as described before using [3H]dThd uptake assay.

For isolation of spinal cords cells, the spinal cords excluded and were digested for 2 h with 300 U/ml type IV collagenase (Worthington, Lakewood, NJ) in DMEM. Cells were then separated by Percoll-Ficoll, gradient, washed, and stained.

**Flow cytometry**

Flow cytometry analysis was conducted according to the protocol described in detail elsewhere (25). For intracellular staining, the Cytotox/Cytoperm kit (BD Biosciences, Piscataway, NJ) was used, and anti-mouse TNF-α, IL-17, IFN-γ, IL-4, and IL-10 Abs were all purchased from BD Biosciences. For extracellular staining, anti-CD4 mAb, anti-mouse CCR5, and anti-human CCR5 were purchased from BD Biosciences, and anti-mouse CD 11b, anti-mouse CCR1, and anti-mouse CCR3 were purchased from BioLegend.

**Construction of soluble receptors**

Human and mouse cDNA encoding the C region (Fc, Hinge-CH2-CH3) IgG were constructed as follows: human Fc was generated by RT-PCR on RNA extracted from hPBMCs that was cultured for 4 d with LPS and IL-4. The primers used for this reaction were as follows: ctcgagccacaattccttga-CAAAAC (sense) and ggcgccctTTCCCGGCACGGGAGA (AP275383) (antisense). The mouse Fc was extracted from Con A-stimulated spleen cells, and the primers were cggccgctagtgacgaggtg (sense) and gaa-catcgggctggacctgaggg (antisense).

The PCR products were digested with XhoI and Apal and ligated into mammalian expression vector pcScTag2/Hygro B (Invitrogen Life Technologies, San Diego, CA). A different set of primers, 5′ (sense) and 3′ (antisense), were used to generate a DNA encoding the different human and mouse domains of CCR5 as previously identified by Boring et al. (26) and Raport et al. (27). Primers were designed as follows: hCCR5-N terminus, ccaagcttagatttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); hCCR5-E1, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); hCCR5-E2, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); hCCR5-E3, ccaagctttcgccgctagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); mouse CCR5-N terminus, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); mouse CCR5-E1, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); mouse CCR5-E2, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense); mouse CCR5-E3, ccaagcttcatgtagttcatagctggactagc (sense) and cccgctagggagacgcgagcgtg (antisense). Each PCR product was digested with HindIII and XhoI and subcloned into the vector containing the human/mouse IgG1 fragment. Each fused fragments was sequenced by dyeoxyxucleotide sequencing at our facility (Sequenase version 2; Upstate Biotechnology, Cleveland, OH).

**Expression and purification of fusion proteins**

Expression and purification of the various fusion proteins was done using CHO dfr<sup>−/−</sup> (DG44) cells (provided by L. Chasin from Columbia University, New York, NY) according to the method described in detail in Ref. 28. The fusion protein was expressed as a disulfide-linked homodimer similar to IgG1, and it was purified from the culture medium by the High-Tarp protein A (GE Healthcare, Piscataway, NJ) affinity column (BD Biosciences, Piscataway, NJ).

**Binding properties of the soluble receptors**

The binding capacity of the soluble receptors was detected by an ELISA as follows: each well was coated with 10 ng of the detected proteins CCL3, CCL4, and CCL5 (R&D Systems) and incubated at 4°C overnight. Wells were incubated with 200 μl 1% BSA/PBS blocking buffer for 1 h at room temperature. Soluble chemokine receptors were added (1 μg/ml) in 1% BSA/PBS buffer (100 μl/well) and incubated overnight at 4°C and washed four times with PBS/Tween 20 (0.05%). Then 100 μl goat anti-hIgG-HRP or goat anti-mIgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) was added at 1/10,000 in 1% BSA/PBS for 1 h and washed four times with PBS/Tween 20 (0.05%). The substrate solution (tetramethylbenzidine) was then added at 100 μl/well. When a blue color appeared, the reaction was terminated by adding 100 μl H<sub>2</sub>SO<sub>4</sub> (1 M). OD was determined at 450 nm with the reference filter set to 620 nm. Binding specificity was evaluated in similar experiment in which the wells were coated with murine CCL1, CCL2, CCL3, CCL4, CCL5, CXCL9.
CXCL11, and CXCL16 (R&D Systems) or the human chemokines CCL2, CCL3, CCL4, CCL5, CXCL11, and CCL22 (R&D Systems) in hCCR5 (E2)-Ig binding assays.

**In vitro chemotaxis assays**
Chemotaxis assays were performed in a Transwell system (Corning Costar, Cambridge, MA). Transwells of 5-μm pore size were used for THP-1 cells and 8-μm for RAW 264 cells. Briefly, 10^6 cells were loaded into the upper chamber of the two systems. The lower chambers were loaded with the chemokines, according to the supplier recommended dosage (10 ng/ml CCL3, 15 ng/ml CCL4, or 50 ng/ml CCL5 [R&D Systems]) and different concentrations of neutralizing Abs or CCR5(E2)-Ig, as described in the legend of each figure. Cells migrate for 3 h under a humidified 7.5% CO2 atmosphere at 37°C. The content of the lower chambers was collected and counted using the FACSCalibur System (BD Biosciences, Piscataway, NJ). The chemotaxis index was then calculated by dividing the number of migrating cells in the presence of chemotractant by the number of cells migrated in its absence. The ND50 calculation was performed by Origin8 software (OriginLab, Northampton, MA).

**In vivo BrdU uptake**
Mice were administered with MOGp35–55/CFA to induce active EAE and then injected i.p. with 1 mg BrdU (Sigma-Aldrich) daily for 7 d. On day 10 post-EAE induction, spleen cells and inguinal LNs were stained with anti-CD4 and anti–CD44-PE. Stained cells were fixed using BD Cytofix/ Cytoperkit. The cells were treated with DNase and incubated with 0.25 μg anti-BrdU-FITC (B44; BD Biosciences). BrdU incorporation was analyzed on gated CD4+CD44+T cells.

**hPBMC isolation and stimulation**
PBMCs were isolated by Ficoll gradient from collected blood. The intermediate phase containing the PBMCs was separated; cells were washed twice, counted, and plated for activation with Con A (α) for 72 h (1 μg/ml).

**Peritoneal macrophages isolation and stimulation**
C57BL/6 mice were injected 3 ml thioglycollate 25% (Difco, Lawrence, KS) reducing agent into the peritoneal cavity. After 7 d, cells were collected by PBS washing steps of the peritoneum and plated at a concentration of 1 × 10^6 cells/ml. Macrophages were further purified with a panning step, which included two PBS wash cycles to remove nonadherent cells. Macrophages were stimulated with 1 μg/ml LPS for 48 h.

**Histopathology and immunostaining**
The lumbar spinal cord was dissected, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Next, 5-μm-thick sections were stained with H&E. Immunostaining of T cells and macrophages were conducted using anti-CD3 (Serotec, Oxford, UK.), anti-F4/80 (Serotec) mAbs, or anti-CD90.1 (eBioscience, San Diego, CA).

**Statistical analysis**
Statistical analysis was conducted according to Refs. 29–32. A two-tailed Student t test was applied for statistical comparison of two groups, or where appropriate, the two-way ANOVA, followed by Bonferroni’s post-hoc test for multiple comparisons and a Mann-Whitney U test for non-parametric data (EAE scoring). A p value of ≤0.05 was considered significant. In all figures, significance appears as follows: *p < 0.05; **p < 0.01; and ***p < 0.001 versus control.

**Results**

**Redundancy in CCR5 ligand induced costimulatory function**
At first, naive CD4+-isolated cells were stimulated by anti-CD3 mAb in cultures that were supplemented with different CCR5 ligands, including CCL3, CCL4, and CCL5. We show in this study that each of these ligands, at a final concentration of 10 ng/ml (we first verified by flow cytometry that the addition of 10 ng/ml of each chemokine did not lead to receptor desensitization [Supplemental Fig. 1]), significantly enhanced the proliferative response of these cells (p < 0.05) (Fig. 1A). Addition of CCL2, or CXCL16 at the same concentrations, did not affect T cell proliferation response (Fig. 1A). We then proceeded to selectively neutralize the activity of each of the CCR5 ligands in primary cultures of spleen cells from EAE mice undergoing MOGp35–55-specific activation (Fig. 1B) and recorded the effects on Ag-specific T proliferation. Although the neutralization of each of the CCR5 ligands (neutralizing mAbs, 10 μg/ml each [R&D Systems]) had no significant effect on the proliferative response of these cells, combined neutralization of all three ligands led to a 2.5-fold decrease in the proliferative response of these cells compared with controls (p < 0.01) (Fig. 1B). Cytokine analyses of samples from these cultured cells show that a reduced proliferative response was associated with a marked reduction in IFN-γ (7.7 versus 1.9 ng/ml; p < 0.001), TNF-α (4.2 versus 2.2 ng/ml; p < 0.05), IL-2 (1117 versus 650 pg/ml; p < 0.01), and IL-17 (2.9 versus 2.2 ng/ml; p < 0.05) but not in IL-12 concentration that is majorly produced by APCs (Fig. 1C). There was no significant change in the supernatant levels of several other cytokines including IL-4, IL-10, TGF-β, and IL-6 (data not shown). Taken together, these results imply for redundancy in cosignaling induced by the CCR5 ligands. This has motivated us to generate a soluble CCR5-based receptor that would co-neutralize all three CCR5 ligands.

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom)
A fusion protein encoding 30 aa of the second extracellular domain of CCR5 selectively binds all CCR5 ligands and neutralizes their biological function.

As G protein-coupled receptors, chemokine receptors span the plasma membrane seven times, generating three extra cellular domains, aside from the N-terminal domain (i.e., first, second, and third extracellular domains [E1, E2, and E3]). We have recently mapped the binding sites of CCR2 to CCL2 and generated a functionally binding CCR2-Ig soluble receptor (33). In this study, we used this strategy once again; first, we have cloned the extracellular loops of murine CCR5 (Fig. 2Aa). Each of the four extracellular domains of CCR5 (N-terminal domain, E1, E2, and E3) was expressed in eukaryotic expression system as a fusion protein stabilized by murine Ig (Fc) (Fig. 2Ab) (i.e., E1-Ig, E2-Ig, E3-Ig, and Nter-Ig). First we analyzed the binding specificity of each domain to all three CCR5 ligands. Fig. 2B shows that all four fusion proteins only CCR5(E2)-Ig binds all three CCR5 ligands (10 ng/ml) (OD at 450 nm of 0.20 ± 0.012, 1.7 ± 0.014 of the binding of mCCR5[E2]-Ig to mCCL3, mCCL4, and mCCL5, respectively, compared with OD < 0.03 for the binding of all other domains to each ligand; p < 0.001). We have then determined the ability of this fusion protein to inhibit the chemokine-induced migration of RAW 264 cells (murine macrophage cell line), induced by each of the different CCR5 ligands, in comparison with each of the other three fusion proteins. Fig. 2C shows that only CCR5 (E2)-Ig (20 µg/ml) could inhibit ≥80% of the mCCL3 (10 ng/ml), mCCL4 (15 ng/ml)-, and mCCL5 (50 ng/ml)-induced migration of these cells (p < 0.001). The other fusion proteins (20 µg/ml) showed no inhibitory effect compared with PBS (Fig. 2C) or isotype-matched IgG (data not shown). To further examine the binding specificities of CCR5(E2)-Ig, we examined its ability to bind various other CC and CXC chemokines, including CXCL16, CXCL11, CXCL9, CCL2, and CCL1. None of these chemokines (10 ng/ml) bound CCR5(E2)-Ig. Fig. 2D shows that the binding of CCR5(E2)-Ig is selective to CCR5 ligands and not to any of the other chemokines. Finally, we compared the ability of CCR5 (E2)-Ig to inhibit the migration of RAW 264 cells, in response to each CCR5 ligand, as compared with commercial anti-mCCL3, anti-mCCL4, and anti-mCCL5 neutralizing Abs (R&D Systems). In addition, the neutralizing dose of CCR5(E2)-Ig required to inhibit 50% of the chemokine-induced migration (ND50) was calculated. Fig. 2E shows that CCR5(E2)-Ig neutralizes in a dose-dependent manner chemokine induces migration of CCR5**-expressing cells. The following ND50 values of CCR5 (E2)-Ig were recorded: 20 nM for CCL3 (10 ng/ml), 15 nM for CCL4 (15 ng/ml), and 29 nM for CCL5 (50 ng/ml). Biacore analysis of binding kinetics between CCR5(E2)-Ig to each of the above ligands, showed varying results between three independent experiments, with an average affinity (Kd) of ~2 × 10−7 M.

![Image](http://www.jimmunol.org/)
M ± 30% for each detected chemokine, which is comparable to the one of CCL2 to CCR2 (E3-Ig) (33), and is ~200-fold lower than the binding affinity of each reciprocal mAb (data not shown). This may explain, in part, the functional differences in the ability to block chemokine induced attraction between mAb and CCR5(E2)-Ig (Fig. 2E, for CCL3 ND50 of 1.7 nM compared with 20 nM, for CCL4 ND50 of 1.3 nM compared with 15 nM, and for CCL5 ND50 of 1.1 nM compared with 29 nM; p < 0.01).

Combined neutralization of CCR5 ligands by mCCR5(E2)-Ig reduces inflammatory cytokine secretion and proliferation of Ag-specific T cells in primary cultures

We have first determined whether the addition of CCR5(E2)-Ig, compared with CCR5(E3)-Ig, which does not bind CCR5 ligands, to Ag-specific primary T cells undergoing MOGp35–55 stimulation would affect cytokine production by these cells, as detected by ELISA (Fig. 3A) and intracellular FACS analysis (Fig. 3B). In these experiments, the proliferating rate of these cells was also

**FIGURE 3.** Combined neutralization of CCR5 ligands by mCCR5(E2)-Ig reduces inflammatory cytokine secretion and proliferation of primary cell culture. Splenocytes were acquired from mice on day 9 postactive EAE induction and subjected to an in vitro MOG (p35–55)-specific stimulation (A–C). A, ELISA-based quantification of in vitro cytokine secretion levels from isolated primary cells in the presence of increasing doses of soluble receptor compared with control IgG. Results are shown as mean of duplicates ± SE of one of five independent experiments with similar results. B, Complementary intracellular FACS analysis of proinflammatory cytokines producing immune cell subtypes in the same primary culture: a–c gated on CD4+ cells; and d gated on CD11b+ cells. The results show one of three independent experiments with a similar pattern of results. C, In vitro proliferation assay of primary cells in response to addition of mCCR5(E2)-Ig. The proliferation index was determined by quantifying [3H]TdR incorporation. The results represent mean of six replicates ± SE with similar results shown in another experiment. D, Cytokine response of LPS-activated macrophages treated with CCR5(E2)-Ig, PBS, or control IgG. Results are shown as mean of duplicates ± SE. Identical results were shown in another independent experiment. E, ELISA-based quantification of in vitro cytokine secretion levels of cultured cells obtained from C57BL/6 or CCR5−/− mice, undergoing MOGp35–55-specific stimulation in the presence of mCCR5(E2)-Ig. Results are shown as mean of duplicates ± SE of one of three independent experiments with similar results.
CD4+ T cells from CCR5

We have also recorded the proliferative response of these CD4 + IL-2, TNF-

Intracellular FACS analysis (Fig. 3B) clearly showed a marked reduction in the relative number of Th1 (CD4^+IFN-γ^highIL-17^low) (9.88 compared with 18 and 18.8%, respectively) and Th17 (CD4^+ IL-17^highIFN-γ^low) (2.6 compare with 5.5 and 5.9%, respectively) cells in these primary cultures. We could not record significant changes in the relative number of IL-10-producing CD4^+ T cells, or the IL-4–producing cells (Fig. 3B), or in the IL-10 levels as recorded by ELISA (Fig. 3A). Within the CD4^+ T cell subsets Th1 and Th17 are the major producers of TNF-α (35). This may explain the reduced production of TNF-α as documented by ELISA (Fig. 3A) and verified by intracellular analysis (Fig. 3B, d). We have also recorded the proliferative response of these CD4^+ T cells (thymidine uptake) showing that only the addition of CCR5(E2)-Ig led to a reduction in the proliferative response of these cells (reduction of 70%; Fig. 3C), which could be explained by the reduced level of IL-2 as recorded by ELISA (Fig. 3A).

Finally, intracellular analysis of TNF-α production in CD11b^+ cells in the above primary cultures showed a reduced number of TNF-α high-producing cells (15.3 compared with 23.45 and 23.9% in the controls; Fig. 3B). One possibility is that blockade of the autocrine effect of CCR5 ligands on their macrophage receptor is essential for executing the proinflammatory activities of these cells. Alternatively it could be that the interplay between CD4^+ T cells activated in the presence of CCR5 ligands and macrophages enhances the proinflammatory activities of these cells. To distinguish between these possibilities, purified peritoneal macrophages were activated in vitro by LPS in cultures supplemented with of CCR5(E2)-Ig (20 μg/ml), control IgG (20 μg/ml), or PBS (Fig. 3D). Our results clearly show that targeted neutralization of CCR5 ligands (by CCR5[E2]-Ig) had no effect on TNF-α, IL-6, IL-10, or IL-12 production by these cells, suggesting the suppression of TNF-α production by CD11b^+ cells in primary cultures results from a possibly T cell-dependent paracrine effect.

Reduced production of IFN-γ, IL-17, and TNF-α in the presence of CCR5(E2)-Ig could result from inhibiting the interaction of one or more of these ligands with CCR5 or because of their interactions with other chemokine receptors (i.e., CCR1 and CCR3). To elaborate on this question, we have subjected CCR5^-/- mice and wild-type (WT) C57BL/6 to active induction of EAE. On day 9, primary CD4^+ T cells from each group were subjected to MOG_p35-55 induces immunization. Cultured cells were then supplemented with or without soluble CCR5(E2)-Ig (20 μg/ml). After 72 h, levels of IL-2, TNF-α, IFN-γ, and IL-17 were determined. Fig. 3E shows that CD4^+ T cells from CCR5^-/- mice displayed significantly lower levels of IL-2 (0.29 ± 0.05 compared with 1.37 ± 0.3; p < 0.001), but not of each of the other cytokines (TNF-α, IFN-γ, and IL-17), and that the addition of CCR5(E2)-Ig to these cultures that suppressed the production of IL-2 in WT to baseline levels of CCR5^-/-, also suppressed the production of TNF-α, IFN-γ, and IL-17 in both CCR5^-/- and WT mice (p < 0.001 for each reciprocal comparison; Fig. 3E). Taken together, these results suggest that although IL-2 production is directed mostly via CCR5, the production of other inflammatory cytokine by CD4^+ T cells is directed by the interaction of CCR5 ligands with other chemokine receptors.

It is not clear why blockade of CCR5 ligands inhibits TNF-α production in CD4^+ T cells but not in the APCs. One possibility is that they differentially express each of the three chemokine receptors for these ligands. We therefore compared CCR1, CCR3, and CCR5 expression on peritoneal macrophages undergoing LPS-activated activation and primary CD4^+ T cells stimulated by MOG_p35-55. Supplemetnal Fig. 2 shows a significant elevation of CCR5 expression in CD4^+ T cells (33 compared with 13.6% on peritoneal macrophages) and moderate differences in CCR3 (12.8 compared with 8.3% on peritoneal macrophages) and CCR1 expression in these cell types (4 compared with 6.78% on peritoneal macrophages). We therefore cannot conclude, at this juncture, that the differential levels of expression of CCR1 or CCR3 led to distinctive effect of CCR5 ligands on TNF-α production by each cell subtype. One possibility is that different signal transduction pathways are triggered by these ligands in each cell type.

Repeated administration of CCR5(E2)-Ig during ongoing EAE suppresses the disease and its pathological consequences

In the in vivo consequences of combined neutralization of CCR5 ligands have not been studied so far. We have conducted three independent experiments, in which CCR5(E2)-Ig was administered during ongoing EAE (score 1 ± 0). Fig. 4A shows the results of a representative experiment with CCR5(E2)-Ig, in which three groups of equally sick C57BL/6 mice (day 13, mean score 1 ± 0, six per group) were injected (every other day) with 250 μg/mice of a CCR5(E2)-Ig, isotype IgG control, or PBS. Although mice administered with either PBS or control IgG continued to develop a progressive disease that persisted for more than 30 d, those administered with mCCR5(E2)-Ig went into fast remission (day 20, mean score of 0.33 ± 0.1 compared with 1.83 ± 0.23 and 1.66 ± 0.23; p < 0.01). At the peak of the disease (day 20), cells were purified from the lumbar spinal cords of representative mice. Before being subjected to intracellular cytokine analysis (Fig. 4C), total number of cells recovered from the spinal cord of the different groups were counted. Fig. 4B shows that the total cell number recovered from the spinal cord of control EAE mice, and those treated with control IgG (1.97 × 10^6 ± 0.37 and 2.0110^6 ± 0.43, respectively) were significantly higher than those recovered from mice treated with CCR5(E2)-Ig (0.5 × 10^6 ± 0.2; p < 0.05). These results are not surprising because the observed reduction in the severity of EAE developed in CCR5(E2)-Ig–treated mice was likely to have been associated with reduced inflammatory cell accumulation within the spinal cord.

Intracellular flow cytometry analyses showed that CCR5(E2)-Ig administration led to a significantly reduced production of the key inflammatory cytokine TNF-α by both CD11b^+ macrophages/DCs (from 11 to 4.5%) and by CD4^+ T cells (from 6.0 to 3.6%), with a concomitant reduction in the relative number of Th1 cells (IFN-γ^highIL-17^low) (from 20.2 to 7.91% of CD4^-gated cells) and Th17 cells (IFN-γ^lowIL-17^high) (from 8.4 to 4.9% of CD4^-gated cells), both of which are major TNF-α producing CD4^+ T cells. Histological analysis of lumbar spinal cord of representative mice showed a marked reduction in mononuclear cell accumulation around high endothelial venules (Fig. 4D) compared with b and c). Immunostaining for T cells (anti-CD3) and macrophages (F4/80) showed a reciprocal reduction in the accumulation of these cells (Fig. 4E, 4F). This further confirms the reduction in the clinical manifestation of diseases following CCR5(E2)-Ig therapy (Fig. 4A).

It is possible that T cells or monocytes that entered the CNS in CCR5(E2)-Ig–treated mice display soluble CCR5(E2)-Ig bound to
In an identical experiment to the one performed in section a, at the peak of the disease cells were purified from the lumbar spinal cords, counted (B), and subsequently subjected to intracellular FACS staining (C). Co and Ch, Gated on viable cells; Cc, gated on CD4+ cells. Similar results were seen in another experiment. D–F, Histological evaluation of spinal cord sections. At the peak of the disease, lumbar spinal cord sections of naive (a, e), control PBS (b, f), control IgG (c, g), or CCR5(E2)-Ig (d, h)–treated mice were analyzed for mononuclear infiltrating cells using H&E staining (D) (original magnification ×10 [a–d] and ×40 [e–h]) or immunostaining for anti-CD3 (E) (original magnification ×40) or anti-F4/80 (F) (original magnification ×40). Each panel shows a representative of six sections from three different mice per group.

Combined targeted neutralization of all CCR5 ligands arrests the selection of Ag-specific T cells that adoptively transferring EAE

Several studies, including ours, previously showed that targeted neutralization of single CCR5 ligands, particularly CCL3 (10, 13) or CCL5 (36) suppresses EAE without affecting effector cell polarization or function (13). In this study, we conducted a comparative experiment in which either CCR5(E2)-Ig or anti–CCL3-neutralizing Abs (rat anti-rat/mouse, generated by the DNA vaccination technology [10]) were repeatedly administered during the course of EAE (twice a week, beginning 4 d after the induction of the disease). To exclude xenogenic effects anti-CCL3 Ab therapy was limited to day 20. This group was controlled by a group injected with Abs from rats immunized with an empty vector. We show in Fig. 5Aa that although both CCR5(E2)-Ig and anti–CCL3 Abs effectively suppressed EAE (mean maximal score of 0.65 and 1.3, respectively, compared with 2.54 [PBS], 2.46 [IgG], and 2.75 [rat IgG]; p < 0.01 and p < 0.05, accordingly), the beneficial outcome of CCR5(E2)-Ig on disease manifestation was significantly more effective than of Ab-based therapy (p < 0.05). Clinical scoring was confirmed by histopathological evaluation (Fig. 5Ab). Moreover, in three different experiments, only Ag-specific T cells selected from mice treated with CCR5(E2)-Ig were incapable of transferring EAE (Fig. 5B), as verified by clinical (Fig. 5Ba) and histopathological examination (Fig. 5Bb).

In vitro cytokine quantification (Fig. 5C) showed that these cells differ from those isolated from control mice or from anti–CCL3-treated mice by the level of proinflammatory cytokines including IL-17 (reduction of ∼60% compared with the controls), TNF-α (reduction of ∼70% compare with the controls), and IFN-γ (reduction of ∼37% compared with the controls). Moreover, cells from CCR5(E2)-Ig–treated mice also had a marked reduction in their rate of proliferation as determined by direct measurement of IL-2 (reduction of ∼80% compared with the controls) or thymidine uptake (Fig. 5D), reduction of ∼40% in splenocyte proliferation compared with the controls and ∼50% in DLN cells proliferation compared with the controls).

In attempting to determine the affect of CCR5(E2)-Ig–based therapy on the in vivo proliferative response of CD4+CD44+ T cells during EAE, the same protocol described in the legend to Fig. 5A was repeated. Hence, mice treated with CCR5(E2)-Ig were also injected with BrdU and monitored for BrdU incorporation by FACS analysis gated on CD4+CD44+ cells. Fig. 5E shows a significant reduction in the percentage of CD4+CD44+ T cells incorporating BrdU as determined in the spleen (18.4 ± 1.7 in control mice compared with 10.3 ± 0.9 in CCR5(E2)-Ig–treated
mice; \( p < 0.05 \) and DLNs (21.7 ± 2.6 in control mice compared with 12.1 ± 1.2 in CCR5[E2]-Ig–treated mice; \( p < 0.05 \)). This further substantiates the role of CCR5 ligands as costimulatory molecules in vivo that promote CD4+ T cell proliferation.

Suppression of EAE in mice treated with CCR5(E2)-Ig could result from reduced production of inflammatory mediators by CCR5+ T cells migrating to the CNS and/or from the direct effect of these ligands on cell migration to the inflammatory site. To further elucidate the contribution of these possible ligands, involvement in the pathogenesis of EAE MOG(p35–55)-specific CD4+ T cells from C57BL/6 mice with a Thy1.1 background (isolated 9 d after active disease induction) were transferred into WT mice. Recipient mice were either subjected or not to CCR5[E2]-Ig–based therapy as described in the legend for Fig. 5A. Another control group was administered with isotype-matched control IgG (six per group) developed severe EAE (day 12 mean maximal score of 2.5 ± 0.23 and 2.65 ± 0.4, respectively), those administered with CCR5[E2]-Ig displayed a significantly lower form of EAE (day 12 mean maximal score of 0.66 ± 0.35; \( p < 0.001 \)). At this time, lumbar spinal cord sections were subjected to immunostaining for relative number of Thy1.1-infiltrating cells. Fig. 5Fa shows the results of a representative slide (1 of 18) from each group. Analyses of the average number of Thy1.1 T cells in the CNS of CCR5E2- and control-treated mice is presented in Fig. 5Fb, showing a marked reduction in the relative number of infiltrating Thy1.1 cells in the CNS of CCR5E2- and control-treated mice.

**FIGURE 5.** MOG(p35–55)-specific T cells selected in CCR5(E2)-Ig–treated mice are not encephalitogenic. Aa, Prophylactic treatment of active EAE: mice received prophylactic treatment of CCR5(E2)-Ig, anti-CCL3 DNA vaccination-based Abs, isotype-matched IgG control, control rat sera or PBS, and followed for the development of disease. Results are shown as mean clinical score ± SE of six mice per group. Ab, Histological evaluation of spinal cord sections from Aa. Each panel shows a representative of six sections from different mice per groups (original magnification ×40). B–D, CCR5(E2)-Ig reduces the activation state of MOG(p35–55)-specific cells: in a subsequent set of experiments conducted under the same conditions, on day 10, splenocytes and DLNs from mice treated with CCR5(E2)-Ig, PBS, isotype-matched IgG, or anti-CCL3 Abs were harvested and restimulated with MOG(p35–55). Ba, The MOG(p35–55) specific cells were determined for their ability to transfer EAE (four mice per group mean maximal clinical scores ± SE). Bb shows histological evaluation of representative spinal cord sections of each group (original magnification ×40). Cytokine production by MOG(p35–55)-specific cells as determined by ELISA (mean of triplicates ± SE) (C) or for their proliferative response by quantifying [3H]TdR uptake (mean of four replicates ± SE) (D). Similar results were obtained in another experiment performed under the same conditions. E, In an additional experiment conducted as described above (A), the in vivo proliferation of CD4+CD44+ T cells from spleens and DLNs of EAE mice was measured by applying a BrdU incorporation assay. Results are shown as percentage of CD4+CD44+ incorporating BrdU cells and represent mean of six mice per group ± SE. F, Adoptive transfer of MOG(p35–55)-specific cells from Thy1.1 to naïve C57BL/6 mice and evaluation of the migratory changes mediated by CCR5(E2)-Ig using immunostaining. Each panel shows a representative of three sections from six different mice per group; similar results were obtained in another independent experiment (original magnification ×40). The average number of Thy1.1 T cells in the CNS of CCR5E2- and control-treated mice is presented in Fb.
1.1 cells only in CCR5(E2)-Ig–treated mice. This may suggest that in addition to their proinflammatory activities on T cells CCR5 ligands are essential for targeted migration of CD4⁺ T cells to the CNS.

hCCR5(E2)-Ig selectively binds CCR5 ligands, neutralizes their biological activities, and suppresses inflammatory cytokine production by hPBMCs

To determine the relevance of these findings to human, we have constructed the reciprocal hCCR5 fusion proteins. Fig. 6A shows that, similarly to mouse, of the four fusion proteins only hCCR5 (E2)-Ig binds all three CCR5 ligands (OD 450nm of 0.174, 0.167, and 0.146 to CCL3, CCL4, and CCL5, respectively), compared with OD < 0.04 for the binding of all other domains to each ligand (p < 0.05). Before using THP1 cells in our migration assays, we confirmed (flow cytometry) that these cells express CCR5 (Fig. 6B). We then determined the ability of this fusion protein to inhibit the chemokine-induced migration of THP1 cells mediated by each of the different CCR5 ligands, in comparison with a fusion protein that does not bind hCCR5 ligands (hCCR5[E1]-Ig) or PBS. Fig. 6C shows that only hCCR5(E2)-Ig could inhibit ≥50% of the CCL3-, CCL4-, and CCL5-induced migration of these cells (p < 0.01). Other fusion proteins showed no inhibitory effect compared with PBS (Fig. 6C) or isotype-matched IgG (data not shown).

Finally, either CCR5(E2)-Ig or CCR5(E1)-Ig was added, at different concentrations, to hPBMCs undergoing Con A-induced activation. Levels of IFN-γ (Fig. 6Da) and TNF-α (Fig. 6Db) were then recorded. Our results clearly show that the addition of CCR5 (E2)-Ig led to a significant reduction in the supernatant level of each cytokine, in a dose dependent manner compared with control CCR5(E1)-Ig (at a level of 10 ng/ml; IFN-γ, 14.7 ± 1.4 compared with 34.3 ± 3.5 ng/ml [p < 0.001]; TNF-α, 0.63 ± 0.1 compared with 3.35 ± 0.4 ng/ml [p < 0.001]).

Discussion

Chemokines have been thought as major targets for therapy of inflammatory autoimmune diseases including multiple sclerosis, RA, TIDM, atherosclerosis, myocarditis, and others (5, 8–14). Intriguingly, although many of 50 known chemokines overlap in their basic feature of attracting T cells, monocytyc cells, and
neutrophils to the autoimmune site, only the neutralization of a few of them—mostly CCL2, CCR5 ligands, and one of the three CXCR3 ligands, CXCL10—could effectively suppress inflammatory autoimmunity (15, 16). One possible explanation for this paradigm is that beyond their function as chemoattractants, which could be overcome in the absence of a single chemokine, chemokines also execute other biological activities that direct the biological function of their target cells, and these features are fundamental in the dynamics of the inflammatory process. For example, the CCL2–CCR2 interaction is essential for monocyte emigration from bone marrow during inflammatory processes (37), which may explain why CCR2−/− or CCL2−/− mice develop an attenuated form of EAE (8, 38), and why targeted neutralization of CCL2 suppresses the disease so effectively (33). Chemokines are also involved in T cell and monocytic cell polarization. On this subject, we have previously shown that of the different CXC ligands CXCL10, but not CXCL11, polarizes CD4+ T cells to effector Th1 and, by doing so, promotes the development and progression of different inflammatory autoimmune diseases and makes this chemokine an attractive target for therapy of these diseases (15, 16). More recently, we have shown that the chemokine CXCL12 (SDF-1α) functions as an anti-inflammatory chemokine that polarizes macrophages to become IL-10–producing anti-inflammatory macrophages (17), also known as M2 macrophages (39), and Ag-specific T cells, to become IL-10–producing regulatory T cells (17), also known as Tr1 (40).

As for CCR5 and its ligands, it has been shown that CCL5, via CCR5, is required for effective activation of CCR5, thus serving as co-stimulatory signals in T cell activation (20), and that this interaction signals via NFAT translocation leading to IL-2 production by CD4+ T cells (22). Our results showing that all three CCR5 ligands may intercompensate one another to effectively induce this signal, and thus, only concurrent targeted neutralization of all of them would inhibit CD4+ T cell proliferation (Fig. 1B), and effector cytokine production (Fig. 1C) may suggest the use of CCR5–Ig that target all of them concurrently as a favorable way of therapy. Tentatively, two of the CCR5 ligands, CCL3 and CCL5, bind more than one chemokine receptor. That is, CCL3 binds CCR1 and CCR5, whereas CCL5 binds CCR1, CCR3, and CCR5 (4). Thus, it could be that more than a single chemokine receptor is involved in CCR5 ligand-induced cosignaling. The observation that T cells from CCR5−/− mice and from human with a functional mutation in CCR5 (CCR5 Δ32Δ32 homozygotes) display a significantly reduced proliferative response and IL-2 production (22) further support the hypothesis that CCR5 ligands induce their cosignaling activation via CCR5. Intriguingly, Tran et al. (41) reported that CCR5−/− and CCL3−/− mice develop full-blown EAE with no significant difference from WT. To further elaborate on this subject, we have generated a soluble receptor-based fusion protein that would bind and neutralize all three CCR5 ligands. From the functional perspective, the efficiency of CCR5(E2)-Ig in inhibiting CCR5 ligands induced migration of CCR5+ cells is ~10-fold lower than mAb-based neutralization (Fig. 2E).

An open-ended question that remains unsolved is why targeted neutralization of either CCL3 or CCL5 but not CCL4 is effective in suppressing T cell-mediated autoimmune disease, such as EAE or experimentally induced arthritis (10, 42, 43). One possibility is preferential expression at the target autoimmune site. For example, it has been previously shown that CCL3 is preferentially expressed at the autoimmune site of NOD mice developing TIDM and that, subsequently, anti-CCL3 Abs suppress the development of disease (14). Very recently, we have show complementary evidence supporting the relevance of these data in human TIDM (44). Alternatively, it could be that other biological characteristics that are specific to individual CCR5 ligands also take place in the regulation of these diseases. For example, it has been shown that of the various CCR5 ligands, CCL5 (and the CCR2 ligand CCL2) mediates leukocyte adhesion to the inflamed endothelium in EAE (45).

From a clinically oriented perspective, the current paper further supports the idea that combined neutralization of CCR5 ligands, or their ability to bind CCR5, could be an effective strategy for treating inflammatory autoimmunity. This could be obtained by using mAbs that effectively block the ability of all CCR5 ligands to induce cosignaling, small molecules with similar features, and CCR5-based soluble receptors, as the one presented in this paper.

How would treating autoimmunity with CCR5(E2)-IG be advantageous over direct blockade of CCR5 using CCR5 antagonists, such as TAK-779 (46)? This subject could be viewed from two different perspectives: 1) our study distinguishes between cosignaling induced via CCR5, which are involved in cosignaling for IL-2 production, and CD4+-mediated proinflammatory properties directed by CCR5 ligands in a CCR5-independent manner. This includes TNF-α, IL-17, and INF-γ production by CD4+ T cells. Thus, CD4+ T cells from CCR5−/− mice produce these cytokines in levels that are comparable to WT but could be significantly reduce by CCR5(E2)-Ig (Fig. 3E). This may suggest that CCR5-Ig–based therapies may be significantly advantageous over direct blockade of CCR5 using CCR5 antagonists (46). 2) We show in this paper that blockade of these ligands inhibited CD4+ T cells migration to the CNS (Fig. 5F). Blockade of CCR5 alone would still preserve the ability of CCL3, CCL4, and CCL5 to induced migration of CD4+ T cells and monocytic cells via CCR1 and CCR3, compensating for the absence of accessible CCR5.

Tran et al. (41) previously showed that CCR5−/− mice are EAE susceptible; others, including us, showed that targeted neutralization of CCR5 ligands, particularly CCL3, suppresses EAE (10). Our current results, using primary T cells from CCR5−/− mice and CCR5–Ig, distinguished between the role of these ligands in providing co-stimulatory signals via CCR5 and other proinflammatory functions directed by other receptors for these ligands (i.e., CCR1 and CCR3) (Fig. 3E). It is thus plausible that in the absence of CCR5 these ligands direct inflammatory properties in these cells via CCR1 and/or CCR3. This may explain, in part, why CCR1−/− mice display an attenuated form of disease, compared with WT (47). Taken together, these data may explain why CCR5–Ig–based therapy could be of advantageous over direct blockade of CCR5 using CCR5 antagonists, such as TAK-779 (46), or over inhibition of a single CCR5 ligand, using mAb-based therapy.

Finally, previous experiences with other diseases showed that some individuals better respond to one drug and others to different drugs, all of which operate with a similar mechanism of action. For example, one of the major targets for therapy of RA is the inflammatory cytokine TNF-α. This includes Ab-based therapy (48) and soluble TNF-α receptor (49). Recent studies show that some patients are better responders to Ab-based therapy, whereas others to the administration of the soluble receptor (50). We assume that having a variety of means for targeting the interaction of CCR5 and its ligands may be beneficial to a larger variety of patients of treatment and suffering from inflammatory autoimmune diseases, some of which might be refractory to the current modalities of treatment.

Disclosures
N.K., G.W., and Y.S. hold a pending patent on therapy of inflammatory autoimmunity and cancer using CCR5–Ig.

References
Supplemental Figure 1: CCR5 expression following anti CD-3 activation
CD4 positive cells were isolated from naïve C57BL/6 mice (MACS beads) and stimulated with anti CD3 mAb in cultures supplemented with either CCL3, CCL4, CCL5, CCL2 (10-500 ng/ml). The expression of the receptor was analyzed by flow cytometry. Results represent one of three independent experiments with a similar pattern of results.
Supplemental Figure 2: CCR1, CCR3, CCR5 expression on T cells and macrophages

(A) Splenocytes were acquired from mice on day 9-post active EAE induction and subjected to 72 hours of an in vitro MOG_{p33-55} specific stimulation. Live cells were separated by ficoll gradient and expression of CCR1, CCR3 and CCR5 was analyzed by flow cytometry from CD4⁺ positive cells.

(B) Peritoneal macrophages were harvested and activated in vitro by LPS for 48 hours. Expression of CCR1, CCR3 and CCR5 was analyzed by flow cytometry from CD11b⁺ positive cells. Similar results were obtained in another independent experiment.