

CXCL12 (SDF-1 α) suppresses ongoing experimental autoimmune encephalomyelitis by selecting antigen-specific regulatory T cells

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Experimental autoimmune encephalomyelitis (EAE) is a T cell-mediated autoimmune disease of the central nervous system induced by antigen-specific effector Th17 and Th1 cells. We show that a key chemokine, CXCL12 (stromal cell-derived factor 1 α), redirects the polarization of effector Th1 cells into CD4⁺CD25⁻Foxp3⁻interleukin (IL) 10^{high} antigen-specific regulatory T cells in a CXCR4-dependent manner, and by doing so acts as a regulatory mediator restraining the autoimmune inflammatory process. In an attempt to explore the therapeutic implication of these findings, we have generated a CXCL12-immunoglobulin (Ig) fusion protein that, when administered during ongoing EAE, rapidly suppresses the disease in wild-type but not IL-10-deficient mice. Anti-IL-10 neutralizing antibodies could reverse this suppression. The beneficial effect included selection of antigen-specific T cells that were CD4⁺CD25⁻Foxp3⁻IL-10^{high}, which could adoptively transfer disease resistance, and suppression of Th17 selection. However, in vitro functional analysis of these cells suggested that, even though CXCL12-Ig-induced tolerance is IL-10 dependent, IL-10-independent mechanisms may also contribute to their regulatory function. Collectively, our results not only demonstrate, for the first time, that a chemokine functions as a regulatory mediator, but also suggest a novel way for treating multiple sclerosis and possibly other inflammatory autoimmune diseases.

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Abbreviations used: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PI, propidium iodide; SDF-1 α , stromal cell-derived factor 1 α .

Chemokines are small (~8–14 kD), structurally cytokine-like, secreted proteins that regulate cell trafficking through interactions with a subset of seven-transmembrane, G protein-coupled receptors (1). Members of this molecular superfamily share structural similarities, including four conserved cysteine residues that form disulphide bonds, which are crucial to the tertiary structures. Chemokines can be divided into four subclasses: the C, C-C, C-X-C, and C-X3-C chemokines, depending on the location of the first two cysteine residues in their protein sequence. The interaction of these soluble proteins with their specific receptors mediates their biological effects. Most of the attention has been devoted to elucidating the key role of these mediators in inflammatory processes (2), with special interest in inflammatory autoimmune diseases, mainly multiple sclerosis (MS) and its experimental models (2–4). Except for their

role in inflammatory diseases, chemokines are also involved in allergic responses and cancer (5).

The CXC chemokine CXCL12 (stromal cell-derived factor 1 α [SDF-1 α]) was originally identified as a growth factor for mouse pre-B cells (6, 7). CXCL12 is constitutively expressed by various cells and tissues, and exhibits chemoattractive activity for monocytes, bone marrow neutrophils, and early stage B cell precursors. It is also a highly efficient and potent chemoattractant for T cells, as well as a co-stimulator of their activation (8). Furthermore, CXCL12 induces the adhesion of T cells to intercellular adhesion molecule 1 (CD54) (9) by up-regulating the binding activity of lymphocyte function-associated antigen 1 (CD11a/CD18), and also modulates the α 4- β 7 integrin-mediated

M. Meiron and Y. Zohar contributed equally to this paper.

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lymphocyte adhesion to mucosal addressin cell adhesion molecule 1 and fibronectin (10). As a result of these activities, it is thought to play an important role in the attraction of T cells into specific sites. In addition to these observations, CXCL12 has been associated with the regulation of rheumatoid arthritis and nephritis in a mouse lupus model (11, 12). Interestingly, this chemokine is preferentially expressed in the healthy central nervous system (CNS), where it also serves as a survival and migratory factor for neuronal and oligodendrocyte precursors that express CXCR4 (13).

Experimental autoimmune encephalomyelitis (EAE) is an experimentally induced autoimmune disease of the CNS that serves as an animal model for MS (14). The expression of CXCL12 within the CNS was found to be up-regulated in the MS brain, particularly by astrocytes, which are likely to attract dendritic cells, macrophages, and T cells to the perivascular areas of the CNS (15–17). This motivated us to explore the role of this chemokine in the regulation of EAE and its clinical implications.

RESULTS

Neutralization of CXCL12 during ongoing EAE aggravates its manifestation

We first explored the possibility that the endogenously produced CXCL12 participates in the natural regulation of disease. To test this hypothesis, EAE mice were administered with a mAb to CXCL12, or an isotype-matched control IgG, after the onset of disease. Our results (Fig. 1) indicate that these mice, but not those administered with the control antibodies, developed an exacerbated, long-term form of disease (mean maximal score of 3 ± 0.28 vs. 2.166 ± 0.18 in both control groups; $P < 0.03$). The data shown represent one out of three experiments with very similar observations. These results suggest that CXCL12 may function as an antiinflammatory chemokine in regulating an ongoing disease.

CXCL12 directs the functional polarization of macrophages and T cells into high IL-10, low inflammatory mediator-producing cells

How does CXCL12 suppress inflammation? We explored the possibility that this chemokine functions as a potential regulatory and antiinflammatory mediator. At first, rCXCL12 was added, in different concentrations, to primary spleen cells isolated from EAE mice that were cultured with their target antigen (myelin oligodendrocyte glycoprotein [MOG]_{p35-55}). Fig. 2 A shows that supplementing these cultured cells with this chemokine induced an increase in IL-10 production ($P < 0.01$ in cultures supplemented with 100 ng/ml CXCL12), and at the same time a significant decrease in IL-12 and TNF- α production ($P < 0.01$ in cultures supplemented with 100 ng/ml CXCL12), all in a dose-dependent manner. Because the CXCR4 receptor is expressed on both CD4⁺ T cells and macrophages (1), we determined the direct effect of this chemokine on the production of various cytokines in each cell type separately. Initially, rCXCL12 was added in different concentrations to freshly isolated, LPS-activated peritoneal mac-

rophages, and the effect on the production of various key cytokines was determined 48 h later. A dose-dependent increase in IL-10 production and, at the same time, a decrease in TNF- α and IL-12 were recorded ($P < 0.01$ in cultures supplemented with 100 ng/ml CXCL12 for all comparisons; Fig. 2 B). To determine whether CXCL12 directly affects IL-10 production by CD4⁺ T cells, we isolated and purified CD4⁺ T cells from spleens of naive C57BL/6 mice (MACS beads) and subjected them to anti-CD3-induced activation in the presence of CXCL12. Fig. 2 C shows a significant, dose-dependent elevation in IL-10 production ($P < 0.01$ in cultures supplemented with 100 ng/ml CXCL12), accompanied by a significant reduction in TNF- α ($P < 0.01$ in cultures supplemented with 100 ng/ml CXCL12), and a dose-dependent increase in IL-2 production (Fig. 2 C). The increased production of IL-2 may suggest that the increased production of IL-10 results, in part, because of the differential proliferation of IL-10-producing cells, but it cannot explain the reduced levels of TNF- α produced by the cultured cells. These results may thus suggest a possible role for this chemokine as a regulatory mediator, and they motivated us to determine its potential use for the treatment of ongoing EAE.

CXCL12-Ig fusion protein suppresses ongoing EAE

Chemokines possess a very short half-life time in vivo, and therefore, their potential use as drugs is limited. To overcome this, we adopted the strategy of generating Ig-based fusion proteins and constructed a chimeric protein composed of CXCL12 fused to IgG1 (Fc). The fusion protein was expressed as a

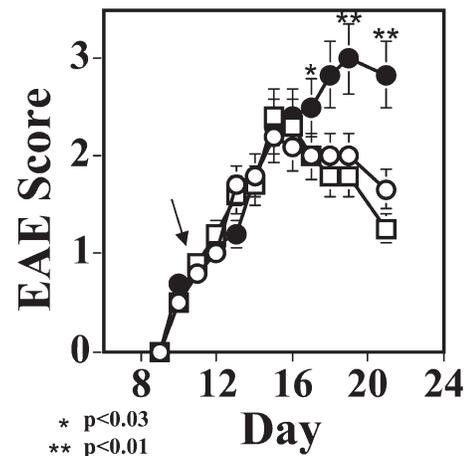


Figure 1. Neutralization of CXCL12 during ongoing EAE aggravates ongoing EAE. C57BL/6 female mice ($n = 6$ per group) were subjected to active induction of MOG_{p35-55}-induced EAE, and at the onset of disease (day 10) were separated into three equally sick groups ($n = 6$ mice per group). On days 11, 13, 15, and 17 after the induction of disease, mice were injected i.v. either with PBS (open circles), 50 μ g/mouse of anti-CXCL12 mAb (closed circles), or control antibody (open squares). An observer blind to the experimental protocol monitored the development and progression of disease. The results of one out of three independent experiments ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE. The arrow indicates the first day of anti-CXCL12 antibody administration.

disulphide-linked homodimer, similar to IgG1, and it had a molecular mass of ~ 72 kD, consisting of two identical 36-kD subunits (Fig. 3 A). Next, we determined whether our CXCL12-Ig maintains the functional properties of the chemokine, including its ability to attract human THP-1 monocytic cell line cells ($P < 0.001$; Fig. 3 B), as well as Jurkat cells (not depicted), in a Transwell system. CXCL12-Ig was also tested for its ability to elicit IL-10 production in LPS-activated peritoneal macrophages (Fig. 3 C), and in primary T cells undergoing antigen-specific *in vitro* activation (Fig. 3 D). Both the commercially available rCXCL12 and our fusion protein could significantly ($P < 0.01$) induce IL-10 production in these cells.

In an attempt to identify which of the two receptors that bind CXCL12—CXCR4 or CXCR7—dominates CXCL12-induced IL-10 production, we have used the commercially available CXCR4 antagonist AMD3100 (Sigma-Aldrich), which specifically blocks CXCR4 (18). As CXCR4 exclusively mediates CXCL12-induced migration (19), we first determined the optimal concentration of AMD3100 that completely blocked CXCL12-induced migration of anti-CD3-activated naive T cells. Fig. 3 E shows that at AMD3100 concentrations >50 nM, CXCL12-induced migration of anti-CD3-activated T cells was entirely blocked. Similar results were obtained using mouse and human monocytic cell lines (unpublished data). We then determined whether the addition of 100 nM AMD3100 could block the increase in IL-10 production induced by CXCL12 in these cells. We clearly show that blocking CXCR4 completely reversed IL-10 production induced by CXCL12 (879 ± 34 vs. 413 ± 28 pg/ml; Fig. 3 F) but not IL-2 production ($3,153 \pm 158$ vs. $3,353 \pm 226$ pg/ml; Fig. 3 G), indicating the pivotal role of CXCR4 in IL-10 production by these cells.

We then explored the ability of this fusion protein to suppress ongoing EAE. Fig. 4 A shows that repeated administration of this fusion protein, but not of a control fusion protein comprised from soluble β -actin-Ig, could effectively and rapidly suppress the disease. Although all control mice continued to develop a semichronic form of disease that persisted >4 wk, all CXCL12-Ig-treated mice went into remission within 7–8 d (day 20 control group mean maximal score of 2.1 ± 0.166 and 2.3 ± 0.26 vs. 0.166 ± 0.16 ; $P < 0.001$). Histological analysis conducted on lumbar spinal cord sections on day 20 verified the clinical results (mean histological score of 0.4 ± 0.3 vs. 2.3 ± 0.3 and 2.1 ± 0.3 in control groups). Representative sections were also subjected to immunohistochemical analysis of IL-10, showing the existence of IL-10-producing cells within the few perivascular infiltrates in sections from CXCL12-Ig-treated mice but not control groups (Fig. 4 B). In a subsequent set of experiments, spleen cells from these groups were cultured in the presence of their target antigen (MOG_{p35-55}), and the levels of IL-10, IL-4, IL-12, TGF- β , IL-17, IL-23, and TNF- α were recorded (Fig. 4 C). The significantly higher levels of IL-10 detected in cultures from CXCL12-Ig-treated mice ($1,450 \pm 170$ compared with 750 ± 65 and 790 ± 70 pg/ml; $P < 0.01$) was accompanied by a reduced production of macrophage proin-

flammatory mediators, including IL-12 (330 ± 27 vs. 920 ± 80 and $1,230 \pm 140$ pg/ml; $P < 0.01$), IL-23 (13 ± 1.2 vs. 30 ± 4.3 and 32 ± 3.1 pg/ml; $P < 0.001$), and TNF- α (780 ± 55 vs. $1,540 \pm 130$ and $1,420 \pm 60$ pg/ml; $P < 0.01$), which is also largely produced by Th1 cells (20). No significant changes in TGF- β or IL-4 levels were noted. Thus, therapy with CXCL12-Ig promotes antiinflammatory cytokine production, particularly IL-10, whereas blocking the production of proinflammatory cytokines, including those directing the polarization of Th1 and Th17 cells, particularly the cytokine IL-17 (160 ± 22 vs. 820 ± 115 and 780 ± 95 pg/ml; $P < 0.001$).

In an attempt to elucidate the long-term effect of therapy on the manifestation of disease, we have established a chronic form of disease by subsequent administrations of the encephalitogenic peptide (21) and determined the long-term effect of CXCL12-Ig on the manifestation of disease. Fig. 4 D summarizes the results of one out of three independent experiments with similar data, showing a marked, long-lasting suppression of disease in treated mice (day 40 mean EAE score of 0.33 ± 0.16 in treated mice compared with 2.66 ± 0.3 and 2.83 ± 0.5 in control groups; $P < 0.001$).

CXCL12 was found capable of inducing CD4⁺ T cell apoptosis via up-regulation of the Fas (CD95)–Fas ligand (CD95L) pathway (22). To elucidate whether under our working conditions the administration of CXCL12-Ig induced apoptosis of antigen-specific CD4⁺ T cells, primary T cells from the cervical lymph node of treated and control EAE mice (Fig. 4 D, a) were obtained just before the peak of disease (day 24) and subjected to *in vitro* activation in the presence of their target antigen. Fig. 4 D (b) shows no significant

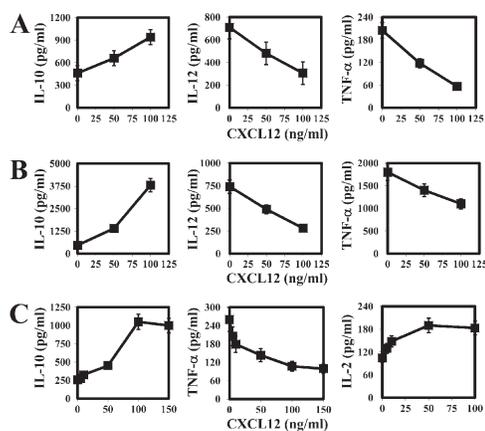


Figure 2. CXCL12 directs the functional polarization of macrophages and T cells into high IL-10, low inflammatory mediator-producing cells. (A–C) CXCL12 was added at different concentrations to primary whole spleen culture taken from EAE mice and stimulated with their target MOG_{p35-55} antigen for 72 h (A), freshly isolated peritoneal macrophages stimulated with 0.5 μ g/ml LPS for 48 h (B), or purified naive CD4⁺ T cells activated with anti-CD3/anti-CD28 for 48 h (C). Cytokine concentrations were measured in triplicates using a standard ELISA method. Results shown in this figure represent three independent experiments with similar results and are presented as means \pm SE.

differences in IL-2 production or in the proliferative response of T cells isolated from CXCL12-Ig-treated mice compared with those injected with β -actin-Ig or PBS, or in the number of apoptotic cells, as determined by Annexin V/propidium iodide (PI) staining of CD4⁺ T cells (Fig. 4 D, c). Thus, CXCL12-Ig therapy suppresses EAE without inducing a significant alteration in T cell proliferation or apoptosis rates. It should be noted that under controlled in vitro conditions, the addition of CXCL12 to anti-CD3-activated naive T cells led to a significant increase in IL-2 production (Fig. 2 C).

CXCL12 therapy selects antigen-specific T cells that suppress EAE in adoptive transfer experiments

The suppression of EAE after CXCL12-Ig therapy could result from the reduced production of proinflammatory mediators by macrophages, including those selecting Th17 and Th1 effector T cells (IL-23 and IL-12; Fig. 4 C), and/or from possible selection of antigen-specific regulatory T cells, potentially capable of suppressing an ongoing disease in adoptive transfer experiments. To explore this possibility, mice were subjected to active induction of EAE and then to the

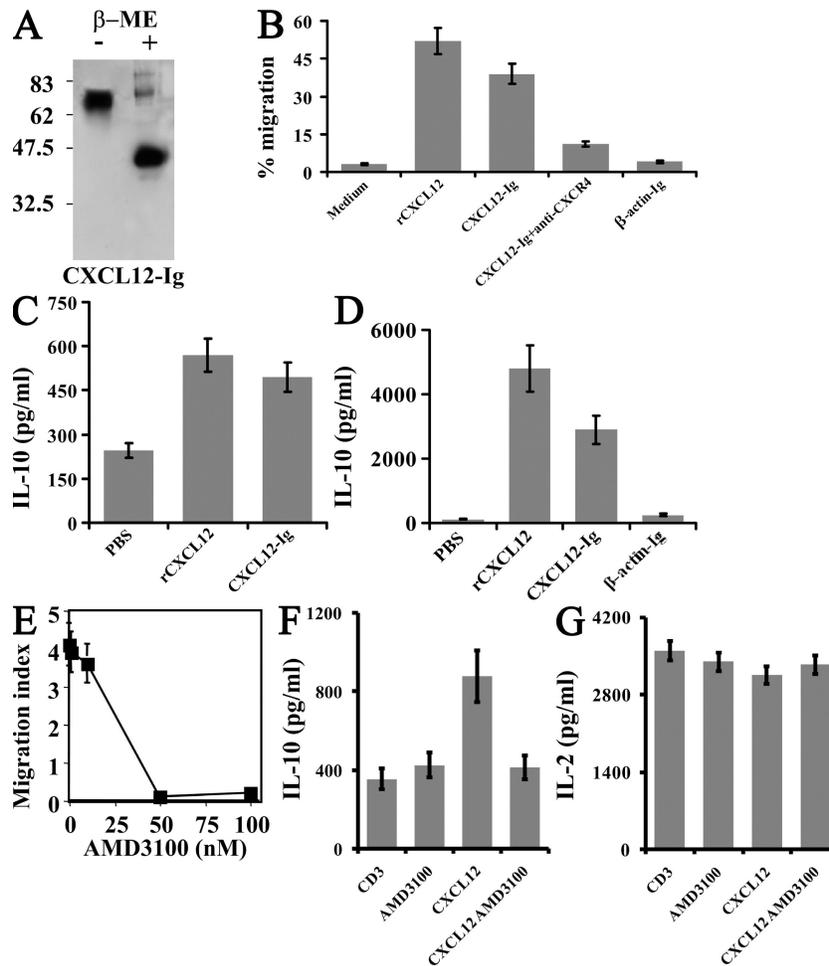


Figure 3. CXCL12-Ig preserves the biological activities of native CXCL12. (A) Purified CXCL12-Ig was separated on 12% SDS-PAGE and subjected to Western blot analysis under reducing and nonreducing conditions (with or without β -mercaptoethanol) using anti-CXCL12 mAb (clone 79014) as a primary antibody (molecular masses are shown). (B) THP-1 cells (human monocytic cell line) were subjected to a migration assay using a Transwell system. Lower chambers were supplemented with culture medium, rCXCL12, CXCL12-Ig, CXCL12-Ig plus anti-CXCR4 mAb, or β -actin-Ig. The number of cells migrating to the lower chamber was counted by FACS 3 h later. Results shown represent three independent experiments and are the mean of the migration percentage (number of cells that migrated to the lower chamber divided by the number of cells originally plated in the upper chamber) \pm SE. (C) Freshly isolated peritoneal macrophages were supplemented with PBS, rCXCL12, or CXCL12-Ig. Supernatants were collected 48 h later, and the IL-10 concentration was determined by ELISA. The results shown represent three experiments done in triplicates, and are the mean IL-10 concentration \pm SE. (D) Primary spleen cell cultures responding to their target MOG_{p35-55} antigen were supplemented with PBS, rCXCL12, CXCL12-Ig, or β -actin-Ig. Supernatants were collected 48 h later, and IL-10 levels were determined by standard ELISA. The results represent three experiments done in triplicates, and are the mean IL-10 concentration \pm SE. (E) Dose-dependent inhibition of CXCL12-induced migration of anti-CD3/anti-CD28-activated spleen T cells from naive C57Bl/6 mice. Results are shown as the mean \pm SE of three independent experiments with similar results. (F and G) IL-10 and IL-2 production of anti-CD3/anti-CD28-activated spleen T cells in the presence of 100 ng/ml CXCL12, 100 nM AMD3100, or CXCL12 plus 100 nM AMD3100. The results represent three experiments done in triplicates and are shown as the mean cytokine concentration \pm SE.

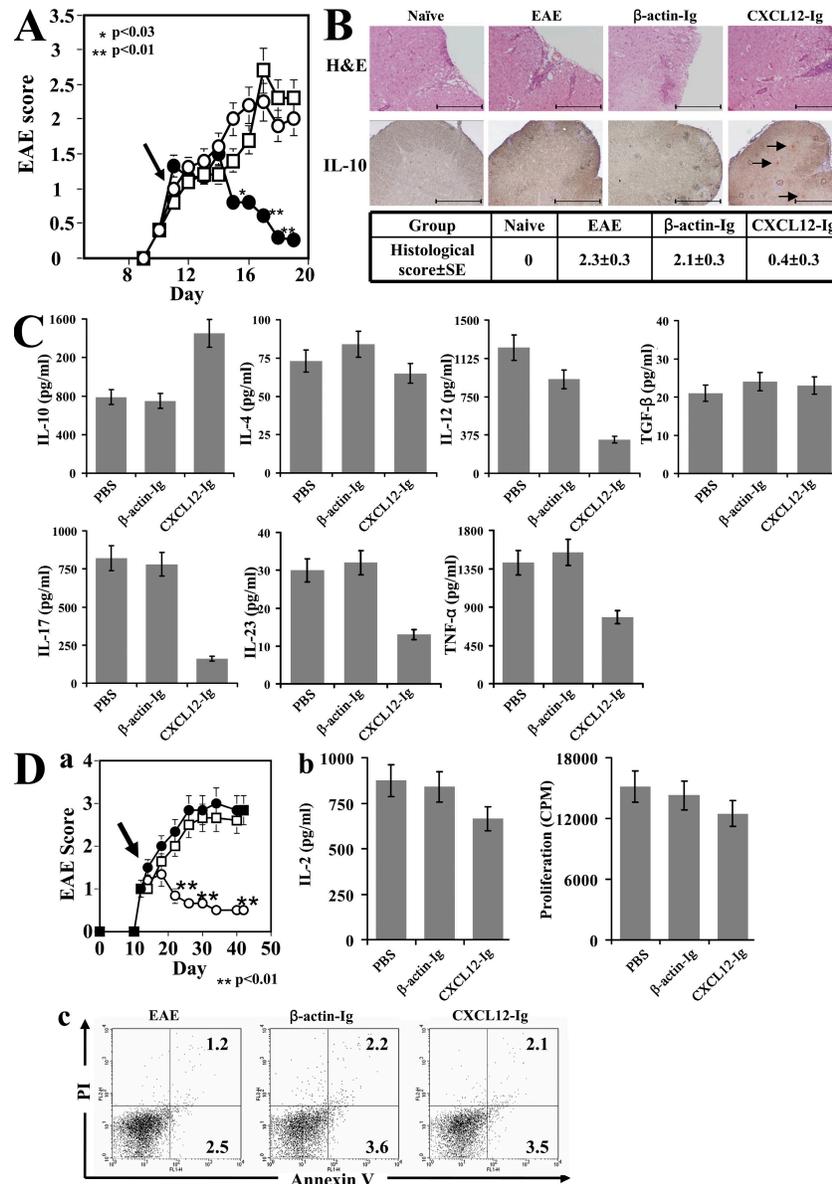


Figure 4. CXCL12-Ig suppresses ongoing EAE. (A) C57BL/6 female mice were subjected to active induction of EAE (MOG_{p35-55}/CFA), and just after the onset of disease (day 11), they were separated into equally sick groups ($n = 6$ mice each). On days 11, 13, 15, and 17, these groups were injected i.v. with either PBS (open circles), CXCL12-Ig (closed circles), or β -actin-Ig (open squares) and were monitored for the progression of disease by an observer blind to the experimental protocol. Results of one out of three independent experiments ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE. The arrow indicates the first day of CXCL12-Ig administration. (B) On day 20, three representative mice from each group were subjected to histological analysis of the lumbar spinal cord (eight sections per sample). A scale ranging from 0 to 3, based on the number of perivascular lesions per section, was used to quantify the histological score of disease, as described in Materials and methods. The table presents the quantification analysis of these sections, and a representative section from each group is also shown. Representative sections were also subjected to immunohistochemistry for IL-10. Arrows indicate cells stained positive for IL-10. Bars, 200 μ m. (C) In a subsequent experiment, conducted under the same experimental protocol, mice were killed on day 15, and spleen cells from each group were cultured in the presence of their target antigen (MOG_{p35-55}). After 24 h, levels of IL-10, IL-4, TGF- β , IL-12, IL-17, IL-23, and TNF- α were recorded by ELISA. Results are shown as the mean of triplicates \pm SE. (D, a) C57BL/6 female mice were subjected to active induction of a long-term form of disease (reference 21), and just after the onset of disease they were separated into equally sick groups ($n = 6$ mice each). Twice a week, these groups were injected i.v. with PBS (closed circles), CXCL12-Ig (open circles), or β -actin-Ig (open squares) and monitored for the development and progression of disease by an observer blind to the experimental protocol. Results of one out of three independent experiments ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE. The arrow indicates the first day of CXCL12-Ig administration. (b) Just before the peak of disease (day 24), primary T cells from the cervical lymph nodes of PBS-, β -actin-Ig-, and CXCL12-Ig-treated mice were subjected to MOG_{p35-55}-induced activation. The proliferative response and levels of IL-2 were recorded. (c) Apoptosis in CD4⁺ T cells in these cultures was determined by flow cytometry using Annexin V/PI staining (percentages are shown).

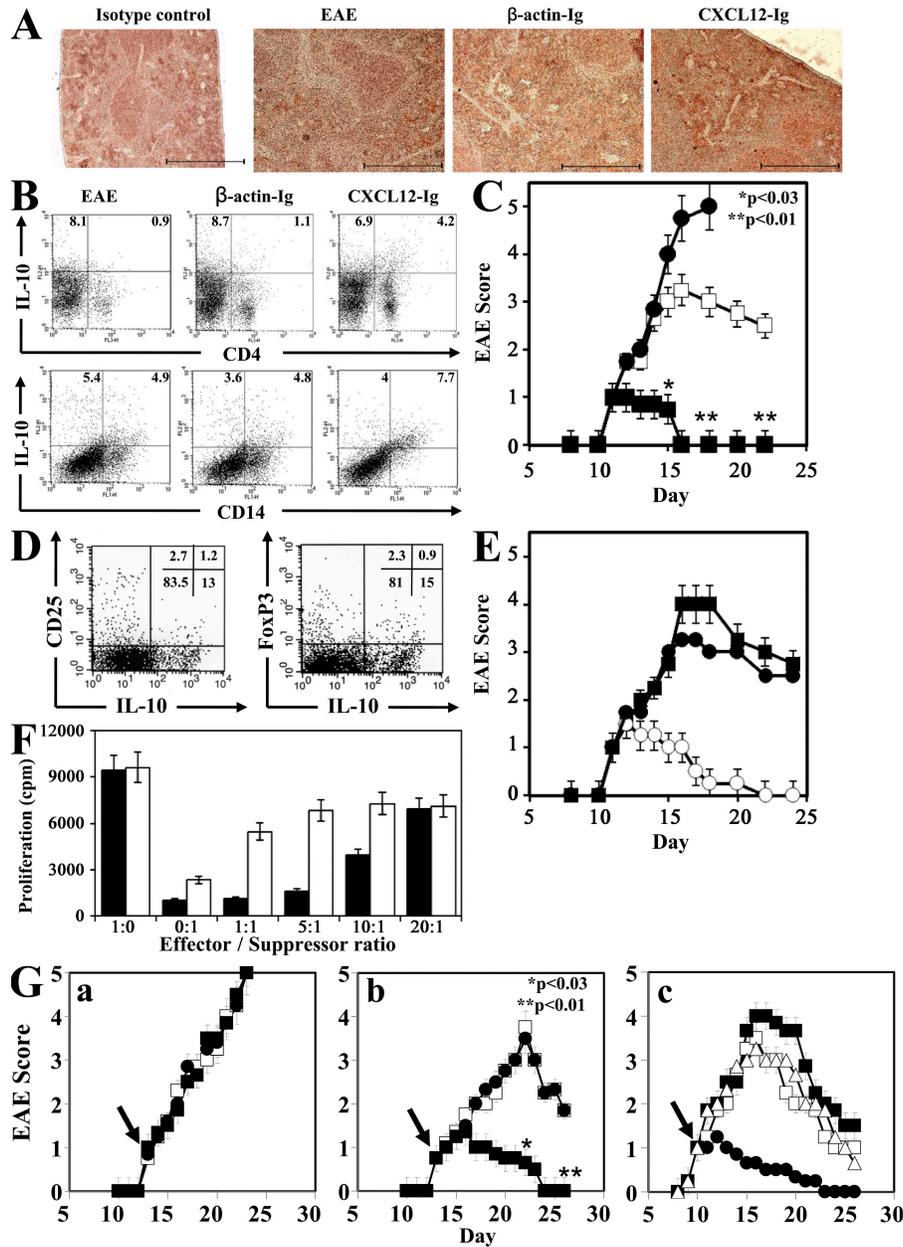


Figure 5. Antigen-specific T cells selected in the presence of CXCL12 suppress EAE. C57BL/6 female mice were subjected to active induction of EAE (MOG_{p35-55}/CFA), and just after the onset of disease (day 11), they were separated into equally sick groups ($n = 6$ mice each). On days 11 and 13, these groups were injected i.v. either with PBS, CXCL12-Ig, or β -actin-Ig. On day 15, the spleens were removed. (A) Spleen sections were subjected to immunohistochemical analysis for IL-10 expression. Bars, 200 μ m. (B) Spleen cells from the different groups were cultured with the target antigen for 72 h and were then subjected to flow cytometry analysis for intracellular staining of IL-10 in macrophages/dendritic cells (CD14⁺) and in CD4⁺ T cells (percentages are shown). (C) Spleen cells isolated from treated mice (in B) were subjected to antigen-specific activation and were injected (20×10^6 cells per mouse) into recipient EAE mice at the onset of disease ($n = 6$ mice per group) either with cells isolated from CXCL12-Ig mice (closed squares) or from β -actin-Ig-treated EAE mice (closed circles). A third group of recipients was administered with PBS (open squares). All groups were monitored for the development and progression of disease by an observer blind to the experimental protocol. Results of one out of three independent experiments ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE. (D) Before being administered to EAE mice (in C), IL-10^{high} T cells selected in CXCL12-Ig-treated mice were tested for the expression of CD25 and FOXP3 (percentages are shown). (E) Spleen cells from EAE mice that were treated with CXCL12-Ig, as described in C, were subjected to antigen-specific in vitro activation and separated into CD4⁺ and CD14⁺ (MACS beads). 10×10^6 cells per mouse were injected into recipient EAE mice at the onset of disease ($n = 6$ mice per group) as follows: CD4⁺ cells isolated from CXCL12-Ig mice (open circles) and CD14⁺ cells isolated from CXCL12-Ig mice (closed circles). Control EAE mice were administered with PBS (closed squares). All groups were monitored for the development and progression of disease by an observer blind to the experimental protocol. Results of one out of three independent experiments ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE. (F) IL-10^{high} T cells selected in CXCL12-Ig treated mice were tested for their ability to suppress the proliferative response of antigen-specific effector T cells from control EAE mice

administration of CXCL12-Ig, β -actin-Ig, or PBS, as described in the legend to Fig. 4 A. On day 15, when the therapeutic effect of CXCL12-Ig was highly significant (Fig. 4 A), spleens were removed. Immunohistochemical analysis of representative sections revealed high IL-10 expression in spleen sections from CXCL12-Ig-treated mice (Fig. 5 A). Intracellular flow cytometry analysis, conducted on samples of cultured cells from these groups, clearly showed a significant increase in IL-10^{high} CD4⁺ T cells (4.2 vs. 1.1 and 0.9%, respectively; Fig. 5 B), as well as in IL-10^{high} CD14⁺ macrophages/dendritic cells (7.7 vs. 4.8 and 4.9%, respectively) in the CXCL12-Ig-treated mice. T cells from donors treated with protective CXCL12-Ig or β -actin-Ig were then administered to mice suffering from active EAE. After antigen-specific activation, these cells were administered to EAE recipients (just after the onset of disease). Fig. 5 C shows that although the administration of spleen cells from EAE donors, treated with β -actin-Ig, aggravated the severity of disease (day 18 mean score of 5 ± 0 vs. 3 ± 0.26 ; $P < 0.01$), the administration of spleen cells from CXCL12-Ig-treated mice led to a rapid recovery (day 18 mean score of 0 ± 0 ; $P < 0.001$). Further analysis of the transferred cells showed that the vast majority of IL-10-producing T cells from protected donors were Foxp3⁻ (96%), CD25⁻ (86%; Fig. 5 D). In an attempt to elucidate the possibility that these cells direct disease suppression, we have repeated the adoptive transfer experiment described in Fig. 5 C. Hence, spleen cells from EAE mice treated with CXCL12-Ig were separated (MACS beads, negative selection) to either CD4⁺ or CD14⁺ cells, and only then were they injected into recipient EAE mice (10×10^6 cells per mouse). Our results clearly show that under these conditions only CD4⁺ T cells could effectively ($P < 0.01$) suppress the disease (Fig. 5 E). Thus, CXCL12-Ig selects antigen-specific regulatory CD4⁺ T cells that are IL-10^{high}CD25⁻Foxp3⁻, which are capable of suppressing EAE in adoptive transfer experiments.

To further investigate the mechanistic basis of action of these cells, we conducted mixed culture experiments in which isolated CD4⁺ T cells from protected donors were mixed at different ratios with effector CD4⁺ T cells (effector/regulatory ratio ranging from 1:0 to 20:1). Fig. 5 F shows that these cells suppressed the proliferative response of control primary cells responding to their MOG_{p35-55} target antigen in a dose-dependent manner. Careful analysis of the ability of anti-IL-10 mAb to reverse this effect showed that under saturating conditions, anti-IL-10 antibodies could reverse up to 70% of suppression

(Fig. 5 F). This implies that although IL-10 is a dominant mediator of the regulatory function of these IL-10^{high}CD25⁻Foxp3⁻CD4⁺ T cells, other mechanisms, yet to be identified, may also contribute to their suppressive function.

Finally, to determine whether the effect of CXCL12-Ig-based therapy is IL-10 dependent, we have tested the ability of our fusion protein to suppress EAE in IL-10^{-/-} mice (Fig. 5 G, a) compared with wild-type C57BL/6 EAE mice (Fig. 5 G, b). We show that although CXCL12-Ig rapidly suppresses the disease in control mice (day 23 mean EAE score of 3.83 ± 0.18 compared with 0.5 ± 0.13 ; $P < 0.001$), it had no effect on IL-10^{-/-} mice. To further investigate whether this suppression was IL-10 dependent, CXCL12-Ig-treated EAE mice, as well as control EAE mice, were repeatedly injected with anti-IL-10 mAb. Fig. 5 G (c) shows that the suppressive effect of CXCL12-Ig could be also reversed in vivo by IL-10 blockade.

CXCL12-Ig redirects the polarization of antigen-specific effector (Th1) cells into IL-10-producing regulatory T cells that suppress EAE

Primary T cells from EAE donors were subjected to two subsequent stimulation cycles in the presence of recombinant mouse IL-12 and anti-IL-4 neutralizing antibodies, and then to a subsequent stimulation in cultures that were or were not supplemented with 50 μ g/ml CXCL12-Ig. Intracellular flow cytometry analysis showed that in the absence of CXCL12-Ig, the vast majority of the polarized CD4⁺ T cells were IFN- γ ^{high}IL-4^{low} Th1 cells, whereas the addition of CXCL12-Ig to the culture medium only during the third stimulation cycle redirected the polarization of a significant portion of these cells into IL-10^{high}IL-4^{low} cells (from 1.6 to 23%; Fig. 6 A), resulting in a >10-fold increase in the level of secreted IL-10, as determined by ELISA (from 40 ± 5 to 580 ± 25 pg/ml; $P < 0.0001$; Fig. 6 B). Notably, the relative number of IL-4^{high}IL-10^{low} CD4⁺ T cells was also significantly increased (from 0.1 to 9%), and so did the level of IL-4, as determined by ELISA (from 64 ± 6 to 215 ± 20 pg/ml; $P < 0.001$), together with a significant reduction in the production of IFN- γ (from $5,850 \pm 430$ to $1,930 \pm 210$ pg/ml; $P < 0.001$) and TNF- α (from 440 ± 55 to 180 ± 24 pg/ml; $P < 0.001$). No changes were observed in the level of TGF- β (Fig. 6 B). Intracellular analysis of IL-10 and IFN- γ in these cells clearly showed a highly significant increase in IL-10^{high}IFN- γ ^{low} CD4⁺ T cells (from 1.8 to 22.5%), accompanied by a reciprocal decrease in the number of IL-10^{low}IFN- γ ^{high} (from 37 to 7.8%) CD4⁺ T cells after

when added at different effector/regulatory ratios (shaded bars) and in the presence of 50 μ g/ml of neutralizing anti-IL-10 antibody (open bars). (G) IL-10^{-/-} mice (a) and C57BL/6 mice (b) were subjected to the treatment protocol described in Fig. 4 A. On day 13, mice were injected with either PBS (open squares), β -actin-Ig (closed circles), or CXCL12-Ig (closed squares). (c) C57BL/6 female mice were subjected to active induction of EAE, and just after the onset of disease (day 9), they were separated into equally sick groups ($n = 6$ mice each). On days 10, 12, and 14, mice were injected i.v. with PBS (open squares), CXCL12-Ig (closed circles), anti-IL-10 mAb (closed squares), or CXCL12-Ig followed by anti-IL-10 mAb injected 6 h later (open triangles). Mice were monitored daily for the progression of the disease by an observer blind to the treatment protocol. The arrow indicates the first day of CXCL12-Ig administration. Results of one out of three independent experiments with similar results ($n = 6$ mice per each group) are shown as the mean maximal score \pm SE.

reselection in the presence of CXCL12-Ig (Fig. 6 A). These data further demonstrate the apparent shift from Th1- to IL-10–producing regulatory T cells in the presence of CXCL12.

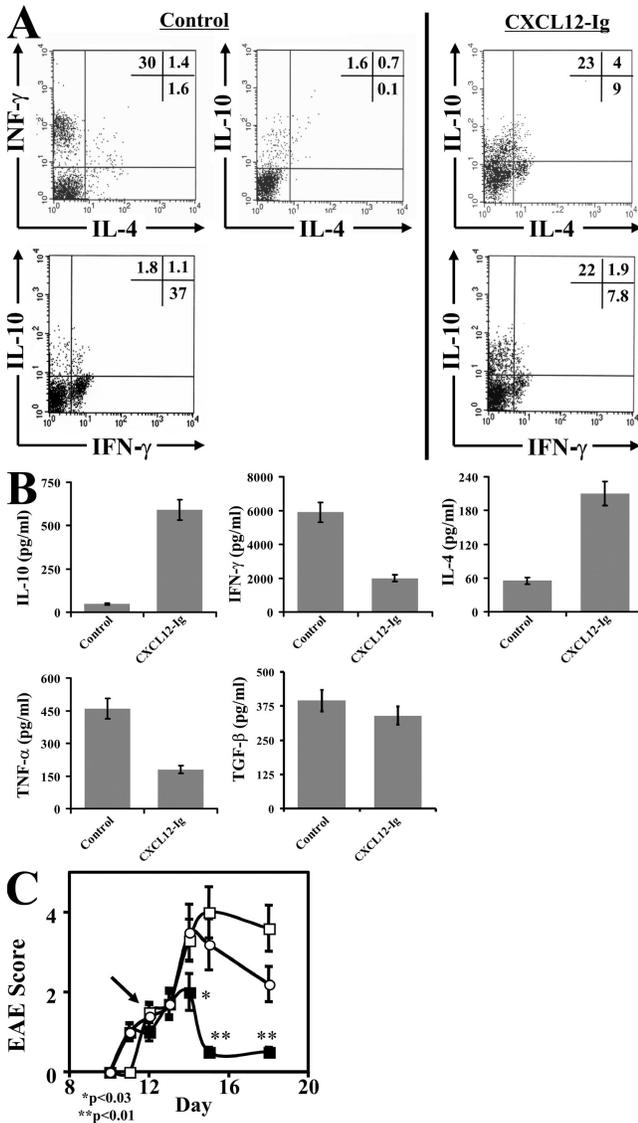


Figure 6. CXCL12-Ig redirects the polarization of antigen-specific effector (Th1) cells into IL-10–producing regulatory T cells that suppress EAE. (A) The MOG_{p35-55} CD4⁺ T cell line was selected during two subsequent stimulation cycles in the presence of the target antigen and the combination of recombinant mouse IL-12 and anti-IL-4–neutralizing antibodies, and were activated in the third cycle in the presence or absence of 50 μ g/ml CXCL12-Ig. Cells were subjected to intracellular cytokine staining (percentages are shown). (B) Cytokine levels in the culture media were also recorded using a standard ELISA method. (C) 3×10^6 T cells per mouse from the CXCL12-Ig–supplemented MOG_{p35-55} line (closed squares), the MOG_{p35-55} line (open squares), or PBS (open circles, no cells) were administered to EAE mice on day 12. Mice were monitored daily for the progression of the disease. Results of one out of two independent experiments with similar data ($n = 6$ mice per each group) are shown as the mean EAE score \pm SE. The arrow indicates the day of cell therapy.

This has motivated us to explore the therapeutic competence of these cells in adoptive transfer experiments. Fig. 6 C shows the results of one out of three experiments with very similar results. EAE mice that were treated just after the onset of disease with 3×10^6 line cells that were previously selected in the presence of CXCL12-Ig went into fast remission within 4–5 d, whereas those administered with control line cells continued to develop a progressive form of disease (day 15 mean maximal score of 0.66 ± 0.3 compared with 3.3 ± 0.6 ; $P < 0.01$). The administration of cells from the same line that were not co-cultured with CXCL12-Ig aggravated the disease (day 15 mean maximal score of 4 ± 0.3 compared with 3.3 ± 0.6 ; $P < 0.05$).

DISCUSSION

CXCL12 is constitutively expressed, at low levels, in the healthy CNS, and its expression is up-regulated in the MS brain (15–17), where it is expressed by various cell types, including astrocytes, that are likely to attract dendritic cells, macrophages, and T cells to areas of the inflamed CNS (15–17). It is likely that in healthy individuals endogenous CXCL12, produced by various resident cells within the CNS, is involved in modulating the migration of leukocytes that are essential for the regular “policing” of this partially immune-privileged area. The development of progressive multifocal leucoencephalopathy after anti-very late antigen 4 therapy (23) may serve as an example for the importance of such policing of the CNS by T cells and macrophages. Our earlier working hypothesis was therefore that CXCL12 functions as a proinflammatory chemokine and, as such, neutralization of its activity during ongoing EAE would be beneficial for the host. Our data show that the administration of anti-CXCL12 antibodies aggravated EAE (Fig. 1), which motivated us to revise our working hypothesis. We show in this paper that a chemokine may function as an antiinflammatory mediator, which not only attracts T cells to the site of inflammation but also polarizes them, including inflammatory Th1 cells, into antiinflammatory regulatory T cells.

Depending on their cytokine profile, the CD4⁺ T cells can be categorized into different subsets, including (a) Th1 cells that produce large amounts of IFN- γ and TNF- α and low levels of IL-4; (b) Th2 cells that mostly produce IL-4, IL-5, and IL-13, and to a much lesser extent, IFN- γ and TNF- α (24); (c) Th3 cells producing high levels of TGF- β and to a much lesser extent other cytokines (25); and (d) Tr1 cells that produce high levels of IL-10 (26), CD4⁺CD25⁺ regulatory T cells (27, 28), and the recently defined Th17 cells, selected in the presence of IL-6 and TGF- β (29), and that in response to IL-23 produce high levels of the inflammatory cytokine IL-17 (30). The inflammatory process during EAE is driven by at least two types of effector cells: the newly discovered IL-17–producing Th17 cells, and the IFN- γ – and TNF- α –producing Th1 cells. Of these effector cell subtypes, the Th17 cells are likely to be more potent initiators of the inflammatory process in the CNS than Th1 cells (31). Their activity is later suppressed, in part by IFN- γ , which is

largely produced by Th1 cells (32, 33). Although IL-12 is involved in selecting Th1 cells, IL-23 has been reported to be dominant in selecting Th17 cells (34, 35). We show a significant reduction in the levels of both IL-23, which drives Th17 polarization, and IL-12, which promotes Th1 polarization (Fig. 4) after CXCL12-Ig therapy. This may well explain the significant reduction in IL-17 and TNF- α in primary cultures (Fig. 4), and the function of CXCL12 as an antiinflammatory chemokine. Several studies have previously shown that by altering their Th1/Th2 balance, C-C chemokines may affect the polarization of antigen-specific T cells (36–38). In addition to these studies, we have shown that of the three CXCR3 ligands—CXCL9, CXCL10, and CXCL11—only CXCL10 directs the polarization of antigen-specific T cells into Th1 cells, and therefore its neutralization effectively suppresses EAE (39) and adjuvant-induced arthritis (40). Our recent observations suggest that CXCL11, which effectively competes with CXCL10 in binding their common CXCR3 receptor, antagonizes this function, and thus acts as an antiinflammatory chemokine (unpublished data). The possibility that CD4⁺CD25⁻Foxp3⁻ Th1 cells could potentially be converted into IL-10-producing regulatory T cells has been very recently raised by Anderson et al. (41), showing that Th1 cells redirect their polarization during chronic cutaneous leishmaniasis (for review see O'Garra and Vieira [42]). We show in this paper, for the first time, that a chemokine may alter the polarization of antigen-specific effector Th1 cells into IL-10-producing T cells. This may explain the rapid effect of CXCL12-Ig therapy on ongoing EAE (Fig. 4, A and D), and it could also suggest a novel way of targeted cell therapy in which effector T cells from patients are being activated *in vitro* in the presence of CXCL12-Ig and returned back to the patient.

The current study shows, for the first time, that a well-defined chemokine functions as a regulatory mediator during the course of diseases in two complementary ways: (a) down-regulation of inflammatory cytokine production and up-regulation of IL-10 production in macrophages, and (b) selection of IL-10-producing regulatory T cells capable of transferring the beneficial effect of therapy (Figs. 5 and 6). Both mechanisms are related because IL-10 directly suppresses proinflammatory mediator production by activated macrophages (43). To determine whether disease suppression is mostly IL-10 dependent, we have conducted three sets of experiments. The first shows that primary T cells from protected mice suppress the MOG_{p35-55}-specific proliferative response of primary T cells from EAE donors in an IL-10-dependent manner (Fig. 5 F). The second shows that IL-10-deficient mice, which develop a more severe form of disease (44), are resistant to CXCL12-Ig-induced therapy (Fig. 5 G, a and b), whereas the third experiment shows that disease suppression, induced by CXCL12-Ig, could be reversed *in vivo* by anti-IL-10 antibodies (Fig. 5 G, c). These experiments are complementary and emphasize the pivotal role of IL-10 and IL-10-producing regulatory T cells in disease suppression. Nevertheless, as IL-10 neutralization could not fully recover the ability of antigen-specific regulatory T cells, selected in

CXCL12-Ig-treated mice, to inhibit effector T cell proliferation (Fig. 5 F), it is plausible that other mechanisms that are IL-10 independent also contribute to their function. This issue is further discussed in the last paragraph of this section.

An alternative interpretation for our data could be that by creating a strong chemokine gradient, which could potentially direct activated T cells away from the CNS, the trafficking of inflammatory cells into the CNS may be altered. This effect might also exist in addition to the selection of IL-10-producing regulatory T cells and IL-10-producing macrophages. However, the results showing that CXCL12-Ig exerts no therapeutic effect on IL-10^{-/-} mice (Fig. 5 G) emphasize the IL-10-dependent function of this chemokine as its major, but not necessarily exclusive, mechanism of action.

The function of CXCL12 as a regulatory mediator that suppresses immune activity extends beyond the regulation of inflammation and inflammatory autoimmune diseases. More specifically, this chemokine is preferentially produced by various cancer cells and, therefore, serves as a potential target for treating these diseases (45, 46). It has been previously proposed that this chemokine assists in suppressing immunity against ovarian cancer by attracting myeloid dendritic cells that produce IL-10 at the tumor site (47). Our results show that CXCL12 is not only involved in attracting antigen-presenting cells and T cells but also in directing their antiinflammatory properties, and may also explain how tumors that largely produce CXCL12 escape antitumor immunity.

At least two different receptors bind CXCL12 on macrophages and T cells, CXCR4 and CXCR7 (19). Of these receptors, only CXCR4 mediates CXCL12-induced migration (19). We show in this paper that the blockade of CXCR4 completely blocked CXCL12-induced production of IL-10, and thereby the selection of antigen-specific IL-10-producing regulatory T cells. Recently, McCandless et al. (48) showed that *in vivo* administration of the CXCR4 antagonist AMD3100 affects the mobilization of hematopoietic precursor cells to the CNS, thus aggravating EAE. Our data clearly show that a major role of CXCR4 in the regulation of EAE is to direct CXCL12-induced IL-10 production by CD4⁺ T cells and macrophages, and by so doing to select antigen-specific regulatory T cells that transfer disease resistance.

Notably, *in vivo* administration of CXCL12 significantly increased IL-10 but not IL-4 production in primary T cells (Fig. 4). Yet *in vitro* selection in the presence of CXCL12-Ig also led to the selection of IL-4-producing Th2 cells (Fig. 6 A) and an elevated level of IL-4 (Fig. 6 B). It is thus plausible that CXCL12, as an antiinflammatory chemokine, is also involved in directing the Th1/Th2 balance in favor of Th2. However under our *in vivo* conditions this type of selection is not dominant, as it is under *in vitro* selective conditions.

We have previously shown that IL-10-producing Tr1 cells participate in the regulation of EAE, including limiting the consequences of disease during determinant spread, and that administering the soluble form of the target determinant leading to the spread amplifies this beneficial function (49). Based on this study and the present one, we suggest that a

combined therapy that would include soluble peptide therapy together with CXCL12-Ig could be considered. Such a therapeutic approach may be useful for rapidly selecting antigen-specific regulatory T cells that would provide selective tolerance. One should not ignore the possibility that because of its role in attracting T cells and monocytes for the regular “policing” of the CNS, a targeted expression of CXCL12 in the CNS at preclinical stages of MS might increase the recruitment of inflammatory cells into the CNS, and thus put in risk patients subjected to CXCL12-Ig-based therapy. However, it is likely that, at that particular stage of the disease, the blood–brain barrier is not activated enough to allow the entry of molecules as large as CXCL12-Ig into the CNS.

Finally, if the suppressive effect of CD4⁺ T cells from protected donors is solely mediated by IL-10, why then does simply adding rIL-10 to effector T cell cultures not mediate the same suppression as shown in Fig. 5 F? (50) Moreover, if this suppression was solely directed by IL-10, then antibodies to IL-10 could completely reverse the suppressive effect of regulatory T cells in mixed culture experiments. It is therefore likely that even though IL-10 is a dominant mediator of the regulatory function of CD25⁺Foxp3⁺IL-10^{high} CD4⁺ T cells, and that anti-IL-10 antibodies reverse the *in vivo* therapeutic effect of CXCL12-Ig, other yet to be identified mechanisms also contribute to their suppressive function.

Our data, showing that on one hand IL-10^{-/-} mice are resistant to CXCL12-Ig based therapy (Fig. 5 G, a and b) and anti-IL-10 antibodies reverse tolerance in CXCL12-Ig treated mice (Fig. 5 G, c), whereas IL-10, even though a key player in CXCL12-induced tolerance, is not the solitary mean by which IL-10-producing regulatory T cells, selected by CXCL12-Ig, suppress effector T cell activity (Fig. 5 F), are conflicting. These observations strongly suggest that IL-10, even though being a major component in IL-10-producing regulatory T cell-induced tolerance, is not solely responsible for the protection induced by these cells, and that other mechanisms, yet to be identified, contribute to their function. It is likely that the dynamics of EAE result from the balance between proinflammatory activities and antiinflammatory activities, some of which are directed by CXCL12 that selects IL-10-producing regulatory T cells to suppress inflammation by various means. Thus, the blockade of the IL-10-directed suppression does not eradicate all IL-10-producing regulatory T cell activities, but it is sufficient to shift the antiinflammatory/inflammatory balance and reverse CXCL12-induced tolerance.

MATERIALS AND METHODS

Mice. 6-wk-old female C57BL/6 mice were purchased from Harlan and were maintained under specific pathogen-free conditions in our animal facility.

Breeders of IL-10^{-/-} C57BL/6 mice were purchased from the Jackson Laboratory, from which our colony was set under pathogen-free conditions. All animal handling was approved by the Technion ethics committee for experiments in animals.

Peptides. MOG_{p35-55} was constructed by the Protein and Nucleic Acid Facility of the Beckman Center of Stanford University. 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%.

Antibodies and recombinant proteins. Anti-mCXCL12 mAb, anti-mIL-4 mAb, anti-mIL-10 mAb, anti-hCXCR4 mAb, recombinant mCXCL12, and recombinant mouse IL-12 were all purchased from R&D Systems. Anti-CD3ε mAb and anti-CD28 used for T cell activation were purchased from BD Biosciences.

Induction of active and adoptively transferred disease. EAE was induced by immunizing mice with MOG_{p35-55}/CFA, as described by Tompkins et al. (51). Animals were then monitored daily for clinical signs by an observer blind to the treatment protocol. Adoptive disease was induced as described previously (52). EAE was scored as follows: 0, clinically normal; 1, flaccid tail; 2, hind limb paralysis; 3, total hind limb paralysis, accompanied by an apparent front limb paralysis; 4, total hind and front limb paralysis; and 5, death.

Construction of CXCL12-Ig. cDNA encoding the constant region of Fc (Hinge-CH2-CH3) of mouse IgG1 was constructed from RNA extracted from mouse splenocytes that were cultured for 96 h in the presence of LPS and mIL-4. The primers used for this reaction were 5'-CTCGAGGTGCC-CAGGGATTGTGGTTG-3' (sense) and 5'-GGGCCCTTTACCAGGAG-AGTGGGAGA-3' (anti-sense). The PCR product was then digested with XhoI and ApaI, and ligated into the mammalian expression/secretion vector pSecTag2/Hygro B (Invitrogen). A different set of primers, 5'-GCTAG-CATGGACGCCAAGGTCGTCGC-3' (sense) and 5'-CTCGAGCT-TGTTTAAGGCTTTGTCC-3' (anti-sense), was used to amplify cDNA encoding mCXCL12. Because alterations in the amino acid sequence at the N-terminus of chemokines might change their properties, by using NheI for the second cloning procedure, the original mouse κ chain leader sequence found in the pSecTag2/Hygro B vector was replaced by a mCXCL12 leader sequence. Hence, the second PCR product was digested with NheI and XhoI and subcloned into the vector containing the mouse IgG1 fragment. The fused fragments were sequenced by dideoxynucleotide sequencing in our facility (Sequenase version 2; Millipore).

Expression and purification of CXCL12-Ig fusion protein. Expression and purification of CXCL12-Ig fusion protein was done using Chinese hamster ovary dhfr^{-/-} (DG44) cells (provided by L. Chasin, Columbia University, New York, NY) according a previously described method (53). The fusion protein was purified from the culture medium by a High-Trap protein A affinity column (GE Healthcare).

Western blot analysis. Purified protein were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to Western blot analysis, using anti-CXCL12 (R&D Systems) as a primary antibody. Donkey anti-mouse horseradish peroxidase-conjugated antibody was used as a secondary antibody (Jackson ImmunoResearch Laboratories). Signals were detected with an electrochemiluminescence detection kit (Biological Industries).

Cell cultures. Primary spleen cells were collected from mice 15 d after induction of EAE. Cells were cultured for 72 h in stimulation medium supplemented with DMEM, 5% FBS, 2 mM β-mercaptoethanol, Na pyruvate, MEM nonessential amino acids, and Pen-Strep (Biological Industries) in a humidified 7.5% CO₂ atmosphere at 37°C and stimulated with 50 μg/ml MOG_{p35-55} peptide.

Human monocytic (THP-1) cells were differentiated into macrophage-like cells by culturing 10⁶ cells in 24-well plates in RPMI 1640 (Biological Industries) supplemented with 10% FCS (Biological Industries) and 30 nM PMA for 96 h in a humidified 7.5% CO₂ atmosphere at 37°C. Cells were washed three times with PBS and were cultured in RPMI 1640 supplemented with 5% FCS for an additional 24 h. Human macrophage-like cells were stimulated with 0.5 μg/ml LPS.

Cell separation. CD4⁺ T and CD14⁺ cells were separated from whole spleen culture using MACS magnetic beads (Miltenyi Biotec), according to the manufacturer's instructions.

Anti-CD3/anti-CD28 activation. Anti-CD3 ϵ mAb (BD Biosciences) was immobilized to a 96-well plate (2 μ g/ml, 50 μ l/well) for 1 h at 37°C. Wells were washed three times with PBS, and isolated CD4⁺ T cells were plated at 3×10^5 cells per well in culture medium supplemented with 1 μ g/ml anti-CD28 mAb (BD Biosciences). Cells were cultured for 48 h in a humidified 7.5% CO₂ atmosphere at 37°C.

Isolation of peritoneal macrophages. C57BL/6 mice were injected intraperitoneally with 3 ml of 2.5% thioglycolate broth (BD Biosciences). 5–7 d later, peritoneal cells were obtained by peritoneal lavage with PBS. Cells were washed three times and plated at 10^6 cells per well in a 24-well plate in stimulation medium. After 24 h, nonadherent cells were removed by washing the plates twice with PBS. The remaining adherent cells (macrophages) were stimulated with 0.5 μ g/ml LPS.

Cytokine ELISA. IL-2, IL-4, IL-10, IL-12, IL-17, TNF- α , and IFN- γ ELISA kits were purchased from BioLegend. A TGF- β ELISA kit was purchased from BD Biosciences. An IL-23 ELISA kit was purchased from R&D Systems.

Selection of MOG_{p35-55}-specific T cell lines. Selection of MOG_{p35-55}-specific T cell lines was done by repeated antigen-specific selections according to the basic protocol previously developed for the selection of myelin basic protein-specific T cell lines in another system (54). To rapidly select antigen-specific Th1 cells, cultured media were supplemented with 10 ng/ml of recombinant mouse IL-12 (R&D Systems) and 10 μ g/ml of purified anti-IL-4 neutralizing antibodies (BD Biosciences).

Migration assay. 10^6 THP-1 cells were placed in the upper chamber of a 6.5-mm diameter, 5- μ m pore polycarbonate Transwell culture insert (Costar). The lower chamber contained 10 ng/ml rCXCL12 (R&D Systems) or 100 ng/ml CXCL12-Ig fusion protein. Cells were allowed to migrate for 3 h at 37°C in 7.5% CO₂, and cells that migrated were collected and counted using a FACSCalibur (BD Biosciences). The percentage of cell migration was calculated as the number of cells that migrated to the lower chamber divided by the number of cells originally placed in the upper chamber.

[³H]Thymidine incorporation proliferation assay. 3×10^5 cells were plated in 96-well in triplicates with 50 μ g/ml of their MOG_{p35-55} target antigen and cultured for 72 h at 37°C in 7.5% CO₂. Each well was pulsed with 1 μ Ci [³H]thymidine for the last 16 h of stimulation. Cells were harvested on fiberglass filter paper and counted in β counter. The proliferative response is expressed as cpm \pm SD.

Flow cytometry. Flow cytometry analysis was conducted according to a previously described protocol (55). An Annexin V/PI kit was purchased from Bender Medsystems. For intracellular staining, a Cytotfix/Cytoperm kit and anti-mouse IL-4, IL-10, and IFN- γ antibodies were purchased from BD Biosciences. A FOXP3/CD4/CD25 flow cytometry kit was purchased from BioLegend.

Histopathology. The lumbar spinal cord was dissected, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Next, 5- μ m-thick sections were stained with hematoxylin and eosin. Each section was evaluated for tissue damage and mononuclear infiltration using the following scale: 0, no mononuclear cell infiltration; 1, 1–5 perivascular lesions per section, with minimal parenchymal infiltration; 2, 5–10 perivascular lesions per section, with parenchymal infiltration; and 3, >10 perivascular lesions per section, with extensive parenchymal infiltration. The mean histological score \pm SE was calculated for each treatment group.

Immunohistochemistry. Spleens and lumbar spinal cords were dissected, fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Next, 5- μ m-thick sections were mounted on Superfrost slides, deparaffinized, and blocked using normal donkey serum (Jackson ImmunoResearch Labora-

tories). Slides were subjected to immunohistochemistry analysis using goat anti-IL-10 antibody (R&D Systems). Donkey anti-goat biotinylated antibody (Jackson ImmunoResearch Laboratories) was used as a secondary antibody, and streptavidin-conjugated peroxidase (Invitrogen). Aminoethyl carbazole (Invitrogen) was used as a substrate.

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