Plasmid DNA Encoding IFN-γ-Inducible Protein 10 Redirects Antigen-Specific T Cell Polarization and Suppresses Experimental Autoimmune Encephalomyelitis[1]

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IFN-γ-inducible protein 10 (IP-10) is a CXC chemokine that stimulates the directional migration of activated T cells, particularly Th1 cells. We demonstrate in this work that during activation this chemokine drives naive CD4$^+$ T cells into Th1 polarization. Administration of plasmid DNA encoding self IP-10 was found capable of breaking down immunological tolerance to IP-10, resulting in the generation of self-specific immunity to the gene product of the vaccine. Despite the CpG motif that drives T cells into Th1, the vaccine redirected the polarization of myelin basic protein-specific T cells into Th2 and conferred the vaccinated recipients a high state of resistance against experimental autoimmune encephalomyelitis, a T cell-mediated autoimmune disease of the CNS. The vaccine also suppressed full-blown ongoing disease in a mouse model of multiple sclerosis. Self-specific Ab to IP-10 developed in protected animals could inhibit leukocyte migration, alter the in vitro Th1/Th2 balance of autoimmune T cells, and adoptively transfer disease suppression. This demonstrates not only the pivotal role of a chemokine in T cell polarization and function but also its potential implications for plasmid DNA gene therapy. *The Journal of Immunology*, 2002, 168: 5885–5892.

Experimental autoimmune encephalomyelitis (EAE)[3] is an autoimmune disease of the CNS that serves as a model for the human disease multiple sclerosis. In both diseases circulating leukocytes penetrate the blood brain barrier and damage myelin, resulting in impaired nerve conduction and paralysis (1, 2). Chemokines are chemoattractants that mediate leukocyte attraction and recruitment. As such, they are likely to be key mediators in the massive recruitment of leukocytes at the site of inflammation during the course of T cell-mediated inflammatory conditions, including autoimmune disorders. Based on the positions of the first two cysteines, the chemokines can be divided into four highly conserved but distinct supergene families: C-C, C-X-C, C, and C-X3-C (3–7). The C-C family is primarily involved in the activation of endothelium and chemoattraction of T cells and monocytes to the site of inflammation. This has motivated several investigators, including us, to explore anti-C-C chemokine immunity as a potential way of suppressing EAE and other T cell-mediated autoimmune diseases. About 5 years ago Karpus et al. (8) blocked EAE in mice by immunizing them with rabbit anti-mouse polyclonal Ab against macrophage-inflammatory protein-1α (MIP-1α). Thereafter, Gong et al. (9) used an antagonist of monocyte chemoattractant protein 1 (MCP-1) to inhibit arthritis in the MRL-*lpr* mouse model. Later Barnes et al. (10) used anti-human RANTES to ameliorate adjuvant-induced arthritis in the Lewis rat. We have used an alternative approach to generate anti-chemokine protective immunity. The basic idea was to clone various chemokines/cytokines, encoding genes into plasmid vectors with a strong CMV promoter and a repeated immunostimulatory sequence (i.e., CpG motif) that serves as a DNA adjuvant (11–14), and then to use them as vaccines against the product of each inserted gene (15–20). In these studies we also demonstrated that this response includes T-dependent Ab production and generation of immunological memory, which is turned on during an autoimmune condition to provide protective immunity (15–20). This can potentially provide a patient with an ongoing autoimmune condition, a powerful tool with which the immune system would restrain its own harmful activities (15–20). Hence, a point of concern in our approach is that as an adjuvant the CpG may select Th1 cells that might potentially aggravate autoimmunity. Thus, recently we have found much interest in exploring the role of the CXC chemokine IFN-γ-inducible protein 10 (IP-10) in the regulation of EAE. Our interest in this particular chemokine flows from recent studies demonstrating its ability to stimulate the directional migration of activated T cells, particularly Th1 cells (21–23), including those of human T cells in SCID mice (24), and from other studies suggesting a possible role for IP-10 in T cell-mediated autoimmunity (25–29). Very recently Fife et al. (29) have demonstrated the competence of anti-IP-10 Ab to inhibit EAE in the SJL mice. The current study explores IP-10-encoding DNA vaccination as a potential way to interfere in T cell polarization and provide long-lasting protective immunity against autoimmune disorders.

Materials and Methods

Animals

Female Lewis rats, SJL mice, and C57BL/6 mice (~6 wk old) were purchased from Harlan (Jerusalem, Israel) and maintained under specific pathogen-free conditions in our animal facility.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; IP-10, IFN-γ-inducible protein 10; SCF, spinal cord fluid; MIP-1α, macrophage-inflammatory protein-1α; MCP-1, monocyte chemoattractant protein 1; MBP, myelin basic protein; CNBr, cyanogen bromide; MOG, myelin oligodendrocyte glycoprotein.

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Peptide Ags

Myelin basic protein (MBP)p68–86, myelin oligodendrocyte glycoprotein (MOG) p33–55, and proteolipid protein p139–151 were all synthesized on a MilliGen 9050 peptide synthesizer (MilliGen, Burlington, MA) by standard 9-fluorenylmethoxycarbonyl chemistry and purified by HPLC. Sequence was confirmed by amino acid analysis and the correct mass was checked by mass spectroscopy. Only peptides that were >95% pure were used in our study.

Immunizations and active disease induction

Active induction of EAE in each experimental model was done as described elsewhere (30–32). Animals were then monitored for clinical signs daily by an observer blind to the treatment protocol. 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 limb and front limb paralysis.

RT-PCR analysis

RT-PCR analysis was used on brain samples according to the protocol we described elsewhere (18). IP-10-specific oligonucleotide primers were designed based on its published sequence (National Center for Biotechnology Information accession no. U22520) as follows: rat IP-10 sense, 5′-CAT GAACCCAAGTGCTGCTGTCGT-3′; rat IP-10 antisense, 5′-TTACG GAGCTCTTTTAGACCTTCT-3′. The PCR product was then cloned and its sequence was verified as described below.

Cloning and sequencing of PCR products

The PCR product described above was cloned into a pUC57/T vector (T-cloning kit K1212; MBI Fermentas, Vilnius, Lithuania) and transformed to *Escherichia coli* according to the manufacturer's protocol. Each clone was then sequenced (Sequenase version 2; USB, Cleveland, Ohio) according to the manufacturer's protocol. PCR products were selected to be used as constructs for naked DNA vaccination only after cloning and sequence verification.

DNA vaccination

DNA vaccination was performed as we previously described (18). Sequenced PCR products of rat IP-10 were transferred into a pcDNA3 vector (Invitrogen, San Diego, CA). Large-scale preparation of plasmid DNA was conducted using MegaPrep (Qiagen, Chatsworth, CA). Cardiotoxin (Sigma-Aldrich, St. Louis, MO) was injected into the tibia's anterior muscle of 4- to 6-wk-old female Lewis rats (10 μM per leg). One week following injection rats was injected with 100 μg DNA in PBS. To verify the transcription of IP-10-encoding mRNA at the injected tibia's anterior muscle and to follow the kinetics of this transcription, rats were sacrificed at different time points (after 4 days, 7 days, 2 wk, and 4 wk) and transcription of IP-10 was determined using RT-PCR on tibia's anterior muscle samples. Under our working conditions IP-10 transcription peaked after 7 days and gradually declined by wk 4 (data not shown).

Production and purification of rIP-10

PCR product was recloned into a PQE expression vector, expressed in *E. coli* (Qiagen), and then purified by an Ni-NTA super-flow affinity purification of 6xHis proteins (Qiagen). After purification, the purity of rIP-10 was verified by gel electrophoresis followed by sequencing (N terminus) by our sequencing services unit.

Western blot analysis

Our recombinant rat IP-10, produced as described above, and commercially available recombinant mouse IP-10 (Cytolab, Rehovot, Israel) were each subjected to Western blot analysis according to the protocol described in details elsewhere (33), with the minor modification of using a 12% (rather than 8%) running gel. IgG from IP-10 DNA-vaccinated rats or IgG from normal rat serum (final concentration of 1/500 each) were used as primary Ab. Goat anti-rat alkaline phosphate-conjugated Ab (Sigma-Aldrich) was used a second step. 5-Bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml; Sigma-Aldrich) and nitroblue tetrazolium (0.3 mg/ml; Sigma-Aldrich) were then used as a substrate.

Evaluation of anti-IP-10 Ab titer in sera of DNA-vaccinated rats

A direct ELISA has been used to determine the anti-IP-10 Ab titer in DNA-vaccinated rats. The rIP-10, which we have produced, was coated onto 96-well ELISA plates (Nunc, Roskilde, Denmark) at concentrations of 50 ng/well. Rat anti-sera, in serial dilutions from 2^8 to 2^{30}, were added to ELISA plates. Goat anti-rat IgG alkaline phosphatase-conjugated Ab (Sigma-Aldrich) was used as a labeled Ab. *p*-Nitrophenyl phosphate (Sigma-Aldrich) was used as a soluble alkaline phosphatase substrate. Results are shown as $\log_2$ Ab titer $\pm$ SE.

CNBr purification of IP-10-specific Ab

Recombinant rat IP-10 (5 mg) was bound to a cyanogen bromide (CNBr)-activated Sepharose column according to the manufacturer's instructions (catalog no. 17-0820-01; Pharmacia Biotech, Uppsala, Sweden). IP-10-specific Ab from sera (IgG fraction) of DNA-vaccinated rats were loaded on the column and then eluted by an acidic elution buffer (glycine, pH 2.5). Isotype determination of the purified Ab (ELISA) revealed that purified Ab is mostly of the IgG2a isotype (data not shown).

T cell separation and activation with anti-CD3$^+$ Ab

T cells were positively selected using MACS kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. FITC-anti-CD4 (OX-35, catalog no. 22024D; BD PharMingen, San Diego, CA) was used as a first Ab, and anti-FITC magnetic beads (catalog no. 130-048-701; Miltenyi Biotec) were used as a second step. Purity of separation was confirmed by FACS analysis using an Ab specific for CD4$^+$ T cells (W3/25; Serotec, Oxford, U.K.). Purified anti-rat CD3 (catalog no. 220100; BD PharMingen) at a concentration of 10 μg/ml was coated to 96-well plates for 1.5 h and washed twice. A total of 2×10^5 CD4$^+$ T cells were added to each well and incubated for 72 h in full medium enriched with Con A supernatant as a source of IL-2 (34).

Selection of MBP-specific T cell lines

MBP-specific T cell lines were selected according to the method developed by Ben-Nun et al. (34), with our minor modifications (35).

In vitro chemotaxis assay

In vitro Boyden chemotaxis chamber chemotaxis assay was conducted as we have previously described (18). A total of 1.2×10^6 MBP-specific T cells, selected as described elsewhere (18), were added to the upper well. Commercially available IP-10 (200 ng/ml; PeproTech, Rocky Hill, NJ) was used as a chemoattractant. fMLP (10^{-7} M; Sigma-Aldrich) was used as a positive control for chemoattraction. Purified Abs (IgG purification) were added at a concentration of 10 μg/ml. Result are shown as mean of triplicates $\pm$ SE.

Cytokine determination in cultured primary spleen cells

Spleen cells from EAE donors were stimulated in vitro (10^7 cells/ml) in 24-well plates (Nunc) with 100 μM p68–86. After 72 h of stimulation, supernatants were assayed for the protein level of various cytokines using semi-ELISA kits: 1) IFN-γ, rabbit anti-rat IFN-γ polyclonal Ab (CY-048; Innogenetics, Gent, Belgium) as a capture Ab, biotinylated mouse anti-rat mAb (CY-106, clone BD-1; Innogenetics) as a detection Ab, and alkaline phosphatase-streptavidin (catalog no. 43-4322; Zymed Laboratories, San Francisco, CA) with rat rIFN-γ as a standard (catalog no. 3281SA; Life Technologies, Rockville, MD); 2) TNF-α, commercial semi-ELISA kit for the detection of rat TNF-α (catalog no. 80-3807-00; Genzyme, Cambridge, MA); 3) IL-4, mouse anti-rat IL-4 mAb (OX-81, catalog no. 24050D; BD PharMingen) as a capture Ab, and rabbit anti-rat IL-4 biotin-conjugated polyclonal Ab (catalog no. 2411-2D; BD PharMingen) as second Ab. Recombinant rat IL-4 (504-RL), purchased from R&D Systems (Minneapolis, MN), was used as a standard; 4) IL-10, commercial semi-ELISA kits for the detection of rat IL-10 (BD PharMingen).

FACS analysis

FACS analysis was conducted according to the basic protocol we described in details elsewhere (16). Intracellular staining of IFN-γ and IL-4 was done using a commercially available kit (LEUCOPERM, catalog no. BUF9; Serotec) according to the manufacturer's protocol. FITC-labeled mouse anti-rat IFN-γ mAb (BioSource, Nivelles, Belgium) and PE-labeled mouse anti-rat IL-4 mAb (BioSource) were used for direct staining. Cells were analyzed using a FACSCalibur (BD Biosciences, Mountain View, CA). Data were collected for 10,000 events and analyzed using a CellQuest program (BD Biosciences).

Histopathology

Histological examination of H&E-stained sections of formalin-fixed, paraffin-embedded sections of the lower thoracic and lumbar regions of the spinal cord was performed. Each section was evaluated without knowledge of the treatment status of the animal. The following scale was used: 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 ± SE was calculated for each treatment group.

Statistical analysis

Significance of differences was examined using Student's *t* test. A value of $p < 0.05$ was considered significant. Mann-Whitney sum of ranks test was used to evaluate significance of differences in mean of maximal clinical score. A value of $p < 0.05$ was considered significant.

Results

IP-10-encoding DNA vaccine alters cytokine production and prevents EAE

Cloned PCR products of rat IP-10, obtained as described in *Materials and Methods*, were ligated into a pcDNA3 eukaryotic expression vector and used as constructs for naked DNA vaccination. Rats were subjected to four weekly injections of the above construct. Control rats were injected with the pcDNA3 vector alone, pcDNA3-β-actin construct, or PBS. Two months after the last immunization all rats were immunized with p68–86/CFA to induce active EAE. Fig. 1 shows representative data of one of three independent experiments that were conducted under the same protocol. All control groups developed active disease that persisted for 5–6 days (Fig. 1*A*, six of six in each group with a maximum clinical score of 2.83 ± 0.18, 2.5 ± 0.23, and 2.33 ± 0.23 in PBS-, pcDNA3-β-actin-, and pcDNA3-immunized rats, respectively). In contrast, in rats injected with the IP-10-encoding DNA vaccine a disease of a markedly reduced degree was developed (Fig. 1, incidence of six of six with a maximum clinical score of 1.16 ± 0.18; $p < 0.016$ vs PBS-treated rats and $p < 0.02$ vs each of the other control groups). Disease suppression was accompanied by a marked reduction, but not complete disappearance, of perivascular mononuclear cell infiltration (Fig. 1, *Bc* vs *Bb*). We determined whether the IP-10-encoding DNA vaccine altered Ag-specific T cell polarization. Spleen T cells from these rats were cultured with MBPp68–86 and the level of Th1 vs Th2 cytokines was determined (Fig. 1*C*). Rats administered with IP-10-encoding DNA vaccine showed a remarkable elevation in the ex vivo production of IL-4 (790 ± 85 vs 240 ± 30 and 190 ± 20 pg/ml in empty vector- or PBS-treated rats, $p < 0.0001$ for each comparison). This was accompanied with a marked reduction in TNF-α production (220 ± 35 vs 880 ± 60 and 1130 ± 90 pg/ml in empty vector- or PBS-treated rats, $p < 0.0001$ for each comparison) and also a significant decrease in the production of IFN-γ (42 ± 4 vs 68 ± 5 and 62 ± 4 ng/ml in empty vector- or PBS-treated rats, $p < 0.01$ for each comparison). Based on these findings we decided to further investigate the possibility that targeted DNA vaccine encoding IP-10 leads to in vivo neutralization of IP-10 and thus alters T cell polarization into IL-4^{high}IFN-γ^{low}TNF-α^{low}-producing T cells.

IP-10-encoding DNA vaccine induces breakdown of tolerance to its gene product and generates immunity to native IP-10

To determine whether the subsequent administration of IP-10 DNA vaccine may elicit or amplify breakdown of tolerance to self IP-10, Lewis rats were subjected to four weekly injections of the

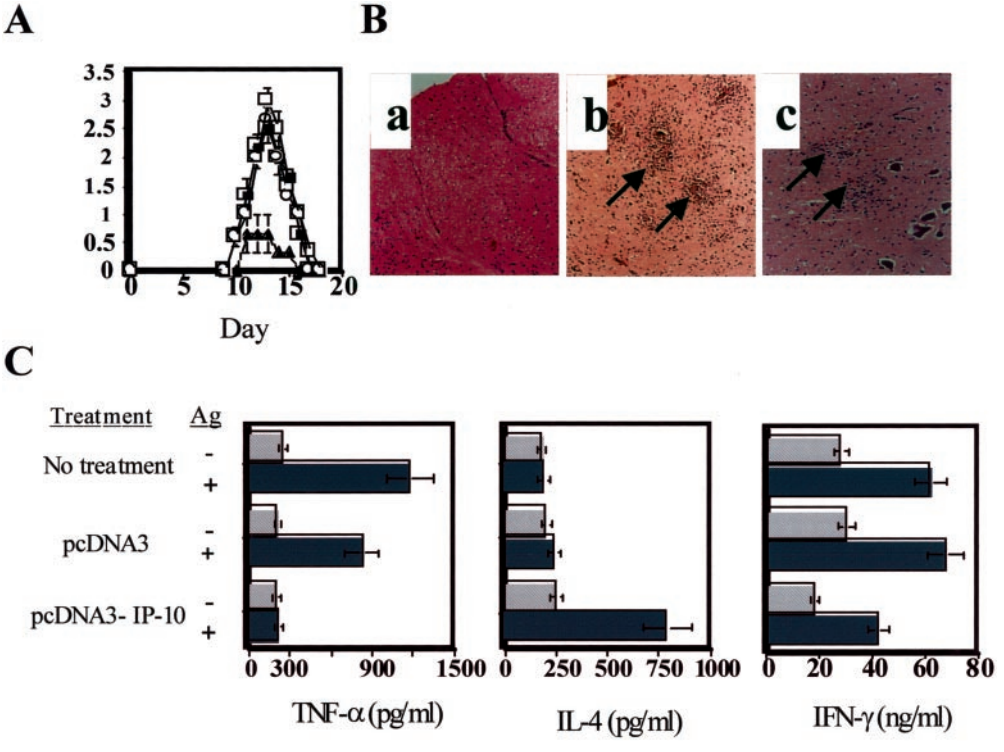

FIGURE 1. IP-10-encoding DNA vaccine alters cytokine production and prevents EAE. A group of 12 Lewis rats was subjected to four weekly injections of naked DNA encoding IP-10 (▲). Control rats (12 per group) were injected with an empty vector (○), pcDNA3 vector encoding soluble β-actin (■), or PBS (□). Two months after the last immunization all rats were immunized with p68–86/CFA to induce active EAE. Just before the onset of disease (day 9) three rats per groups were sacrificed and spleen T cells were cultured together with MBPp68–86 for cytokine determination. Three other rats per group were sacrificed at the peak of disease for histological evaluation. The remaining six rats were monitored for the development of active EAE by an observer blind to the experimental protocol. *A*, Mean maximal score of six rats ± SE of these groups. The data presented summarize representative data of one of three independent experiments that were conducted under the same conditions. *B*, Representative histological sections of lumbar spinal cord section of naive (*a*), positive control (*b*), and IP-10-encoding DNA-vaccinated (*c*) rats. *C*, The levels of TNF-α, IL-4, and IFN-γ that were determined (mean triplicates ± SE) in supernatants of spleen cells cultured in the presence or absence of the target Ag (MBPp68–86).

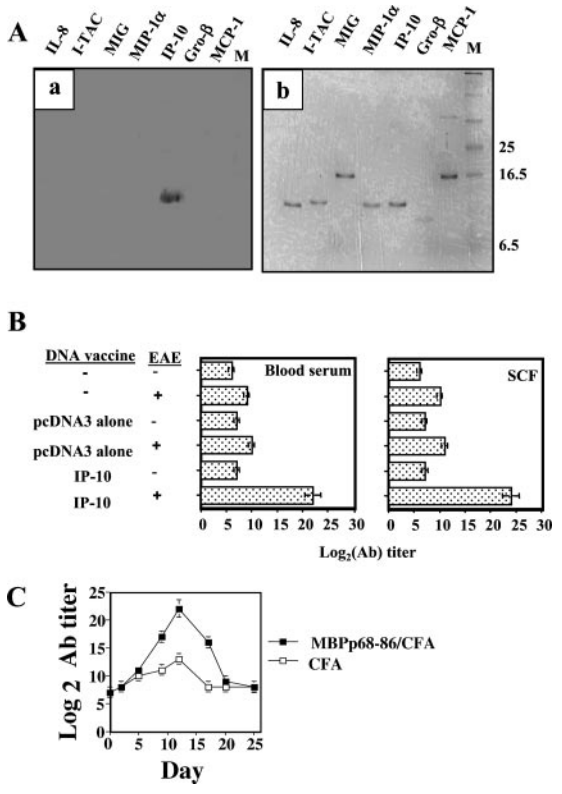

FIGURE 2. IP-10-encoding DNA vaccine induces breakdown of tolerance to its gene product and generates immunity to native IP-10. Groups of Lewis rats were subjected to a repeated administration of IP-10-encoding DNA vaccines and then to active induction of EAE as described in Fig. 1. An additional group was subjected to IP-10-encoding DNA vaccine and later on immunized with CFA alone to induce a local inflammatory response. At different times blood serum was obtained and evaluated for IP-10-specific Ab titer. On day 12 (peak of disease in control rats) SCF was also obtained and subjected to the same evaluation. *Aa*, Western blot shows that our self-specific anti-IP-10 Ab binds mouse IP-10, but not IL-8, IFN-inducible T cell α chemoattractant, monokine induced by IFN-γ, MIP-1α, IP-10, growth-related oncogene β, or MCP-1 (PeproTech). Coomassie staining verified the appearance of each chemokine on the loaded gel (*Ab*). These Ab also bound natural rat IP-10 from supernatant of activated MBPp68–86-specific cultured T cells (data not shown). *B*, The IP-10-specific Ab titer developed in blood serum and SCF of representative rats from each of the above groups (mean Ab titer obtained from three rats per group ± SE). *C*, The kinetics of Ab titer to self IP-10 developed in pcDNA3-IP-10-vaccinated rats that were subjected to either active induction of EAE or administration of CFA alone (mean Ab titer obtained from three rats per group ± SE).

IP-10 DNA construct as described in Fig. 1. Two months after the last immunization, when IP-10-specific Ab titer maintained a baseline level (Fig. 2*B*), the rats were injected with p68–86/CFA to induce active EAE, or with CFA alone to induce a local inflammatory reaction. At different time points serum and spinal cord fluid (SCF) from representative rats were analyzed for the presence of Ab to IP-10. At first, SDS-PAGE (under reducing conditions) followed by Western blot analysis confirmed that the Ab produced in IP-10-encoding DNA-vaccinated rats bind not only our recombinant rat IP-10 (a 10-kDa fragment) but also the 8.7-kDa commercially available mouse IP-10 fragment (data not shown). Then, using Western blot analysis, we determined the specificity of these Ab (Fig. 2*Aa*). The Abs bind IP-10, but not several other ELR and non-ELR CXC chemokine ligands, including IFN-inducible T cell α chemoattractant, monokine induced by IFN-γ, IL-8, and growth-related oncogene β. They also do not bind MIP-1α and MCP-1 (Fig. 2*A*). Thus, autoantibodies produced by DNA vaccination are highly specific. The kinetics of Ab production along the course of disease was then measured (Fig. 2, *B* and *C*). An elicited Ab titer to the above gene product was observed in both serum and SCF of IP-10 DNA-vaccinated rats. This titer reached its maximal level on day 13 (Fig. 2*B*, $p < 0.001$ compared with all other groups). Immunization with CFA alone also significantly elicited the production of self-specific Ab to IP-10 in DNA-vaccinated rats, though to a much lesser extent than in the case of active disease (Fig. 2*C*, $p < 0.001$). Interestingly, each titer regressed almost to background levels following recovery (Fig. 2*C*). Taken together our results suggest that IP-10 naked DNA vaccination leads to a breakdown of immunological tolerance, resulting in the generation of an immunological memory to its gene product, and that this memory is turned on upon EAE induction to provide protective immunity. To determine this possibility, the in vitro and in vivo characteristics of these Abs, including the competence to adoptively transfer EAE resistance, were determined.

IP-10-specific Ab generated in DNA-vaccinated EAE rats inhibit migratory properties and alter cytokine production in primary cultures

Fig. 3*A* shows the in vitro chemoattractive properties of IP-10 on $CD4^+$ T cells. IP-10-specific Ab produced in DNA-vaccinated rats could significantly inhibit the migration induced by our recombinant rat IP-10, as well as by the commercially available 8.7-kDa fragment of mouse IP-10 ($p < 0.001$). These Abs had no effect on MCP-1-induced migration of these T cells (data not shown). Thus, the inhibitory effect of the Ab is chemokine specific. Once added to MBP-specific (p68–86) cultured spleen cells our IP-10-specific Ab altered their cytokine production, leading to a significant decrease in levels of TNF-α and IFN-γ (Fig. 3*B*, 1240 ± 60 vs 460 ± 20 pg/ml, $p < 0.001$, and 29.8 ± 1.9 vs 18.1 ± 1.2 ng/ml, $p < 0.01$, respectively). This reduction was accompanied by a marked increase in IL-4 production (Fig. 3*B*, 48 ± 7 vs 294 ± 12 pg/ml, $p < 0.0001$). These results motivated us to further explore the possibility that IP-10 directs T cell polarization.

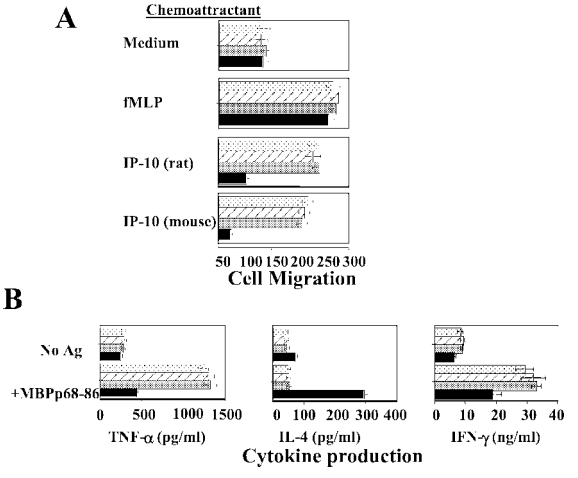

FIGURE 3. IP-10-specific Ab generated in DNA-vaccinated EAE rats inhibit migratory properties and alter cytokine production in primary cultures. Our IP-10-specific Ab (filled bars; IgG, CNBr purified), control medium without IgG (light gray bars), IgG from normal rat serum (dark gray bars) and IgG from EAE rats previously administered with an empty plasmid (hatched bars) were tested for their ability to inhibit the migration of our encephalitogenic line (L68-86) in a Boyden chemotaxis chamber assay (*A*) and for their ability to alter the cytokine profile of primary cultured T cells (*B*).

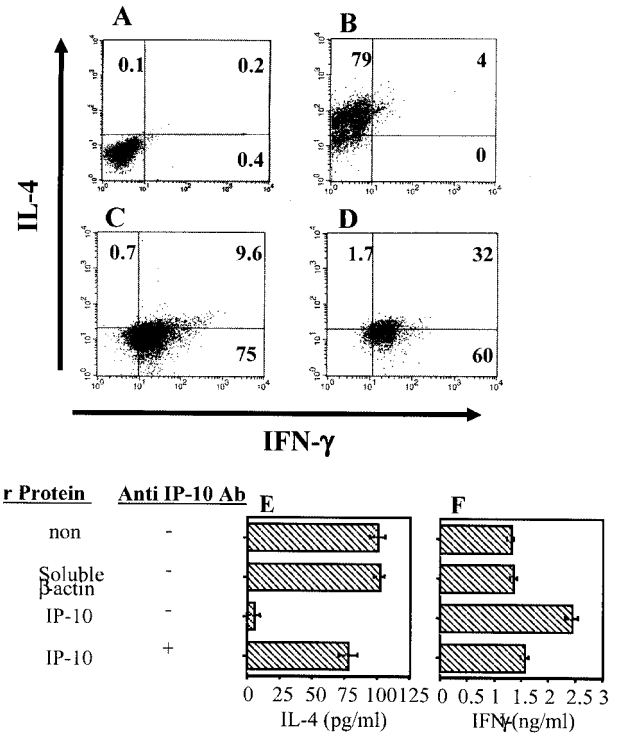

FIGURE 4. IP-10 directs T cell polarization into Th1. $CD4^+$ T cells were separated from naive spleens by magnetic beads and subjected to anti-CD3 activation as described in *Materials and Methods*. Different wells were or were not supplemented with an 8.7-kDa fraction of mouse IP-10 (Cytolab) or recombinant β-actin (50 ng/ml each), with or without the addition of Ab to IP-10 (10 μg/ml) or to IFN-γ (5 μg/ml; Cytolab). Representative wells were subjected to intracellular FACS analysis of IL-4 and IFN-γ after 72 h. *A*, Naive $CD4^+$ T cells that were not subjected to anti-CD3 activation. *B*, Anti-CD3 activation with no addition of IP-10. *C*, Anti-CD3 activation with the addition of IP-10. *D*, Anti-CD3 activation with the addition of IP-10 and anti-IFN-γ. Another group was subjected to anti-CD3 activation with or without the addition of anti-IP-10 Ab alone. Addition of these Abs alone did not affect T cell polarization (data not shown). After 72 h supernatants from all cultures (except for those supplemented with anti-IFN-γ Ab) were also analyzed for IL-4 (*E*) and IFN-γ (*F*) levels. An additional group was subjected to the addition of mouse IP-10 (Cytolab) and our anti-IP-10 neutralizing Ab (10 μg/ml). The results summarize one of two independent experiments done under the same experimental conditions with very similar data. They are shown as mean triplicates $\pm$ SE.

IP-10 directs T cell polarization

To determine whether IP-10 directs T cell polarization $CD4^+$ T cells were purified from naive Lewis rats and then subjected to anti-CD3-induced activation in IL-2-enriched medium. Under these conditions the vast majority of $CD4^+$ cells were driven into Th2 (Fig. 4*B*, 79% IL-4^{high}IFN-γ^{low}, 0% IFN-γ^{high}IL-4^{low}). The addition of IP-10 redirected T cell polarization into IFN-γ^{high}IL-4^{low}-producing T cells (Fig. 4, *C* vs *B*, 75 vs 0%). The addition of neutralizing Ab to IFN-γ could not reverse IP-10-induced Th1 polarization (Fig. 4*D*, 60% IFN-γ^{high}IL-4^{low}-producing T cells), suggesting that the pro-Th1 effect of IP-10 on naive T cells could be IFN-γ independent. Interestingly, in the presence of IP-10 and neutralizing Ab to IFN-γ a significant portion of naive $CD4^+$ T cells were polarized into a IFN-γ^{high}IL-4^{high}-producing subtype (32 vs 9.6 and 4% accordingly). Cytokine level in supernatant was also recorded by ELISA (Fig. 4, *E* and *F*), and these confirmed the intracellular FACS analysis. Additionally, the competence of our IP-10-specific neutralizing Ab to reverse the effect of IP-10 on T cell polarization was confirmed by ELISA (Fig. 4, *E* and *F*).

Administration of self-specific Ab to IP-10 redirects Ag-specific T cell polarization toward Th2 and suppresses EAE

The in vitro properties of our self-specific anti-IP-10 Ab suggest that they might affect the in vivo function of autoimmune T cells and thus the regulation of EAE. Their subsequent administration, starting 5 days before the onset of disease, led to a marked reduction in the clinical (Fig. 5*A*) and histological scores of disease (see below). That is, while all control groups developed severe EAE (Fig. 5*A*, mean maximal score $\pm$ SE of 3.83 $\pm$ 0.25, 3.3 $\pm$ 0.8, and 3 $\pm$ 0.3), those treated with purified anti-IP-10 Ab exhibited a mild form of disease (mean maximal score of 1.16 $\pm$ 0.23, $p < 0.001$ compared with each control group). Disease inhibition was accompanied by a significant, though incomplete, reduction in parenchymal mononuclear cell infiltration (mean histological score of 0.83 $\pm$ 0.16 vs 2.6 $\pm$ 0.2, 2.3 $\pm$ 0.3, and 2.16 $\pm$ 0.3 in control groups, respectively). To determine the possibility that neutralizing Ab to IP-10 affected the in vivo polarization of T cells, spleen cells were obtained from these rats and subjected to intracellular staining of IFN-γ and IL-4 (Fig. 5*B*). Results were also confirmed by an ELISA (Fig. 5*C*). About 60% of control $CD4^+$ T cells (from rats treated with normal IgG) were found to be IFN-γ^{high}-producing T cells, including IFN-γ^{high}IL-4^{low} (32%)- and IFN-γ^{high}IL-4^{high} (27%)-producing T cells and only 18% of IL-4^{high}IFN-γ^{low}-producing Th2 cells (Fig. 5*B*). In contrast, primary T cells ($CD4^+$) isolated from rats treated with anti-IP-10 Ab exhibited a significant shift toward Th2 (Fig. 5*B*, 44% IL-4^{high}IFN-γ^{low}-producing T cells and only 6% IFN-γ^{high}IL-4^{low}-producing cells). Analysis of the supernatant levels of TNF-α, IL-4, and IFN-γ also demonstrated a significant decrease in TNF-α (430 $\pm$ 33 vs 810 $\pm$ 68 and 890 $\pm$ 70 pg/ml in control IgG- or PBS-treated rats, $p < 0.001$ for each comparison) and IFN-γ (72 $\pm$ 6 vs 109 $\pm$ 9 and 113 $\pm$ 8 ng/ml in control IgG- or PBS-treated rats, $p < 0.01$ for each comparison) production. This reduction was accompanied by a substantial ($p < 0.0001$) increase in the levels of IL-4 in supernatant of these rats (Fig. 5*C*).

Altered polarization of Ag-specific T cells toward Th2 could provide protective immunity against EAE, either because these cells are regulatory T cells capable of suppressing the function of autoimmune cells or because their polarization competes with polarization of Th1 cells. To further investigate this important question we have selected MBP-specific T cell lines from control and IP-10 DNA-vaccinated rats and determined their in vivo capabilities. The control line produced high levels of IFN-γ and TNF-α (55 $\pm$ 4 ng/ml and 80 $\pm$ 6 pg/ml, respectively) and low levels of IL-4 (<20 pg) and was highly encephalitogenic (i.e., 2×10^6 cells or more could effectively transfer EAE (six of six sick rats); mean maximal score 2 $\pm$ 0). The line selected from IP-10 DNA-vaccinated EAE-resistant rats produced low levels of IFN-γ and TNF-α (26 ng and 24 pg, respectively) and high levels of IL-4 (240 pg) and was not encephalitogenic. Even 20×10^6 cells from this Th2 line could never transfer EAE (0 of 12 sick rats). In contrast, transferring these cells to sick rats ($2–10 \times 10^6$ cells per rat) did not have any beneficial effect. Very similar results were also obtained using T cell lines selected from EAE rats treated with anti-IP-10 Ab (data not shown). This suggests that alteration of T cell polarization toward Th2 does not necessarily select regulatory T cells, but rather drives potential autoreactive T cells to become nonencephalitogenic.

Interference of IP-10-encoding DNA vaccines in established EAE suggests a preexisting network

We determined whether IP-10-encoding DNA vaccines interfere in the regulation of established EAE. Lewis rats were immunized

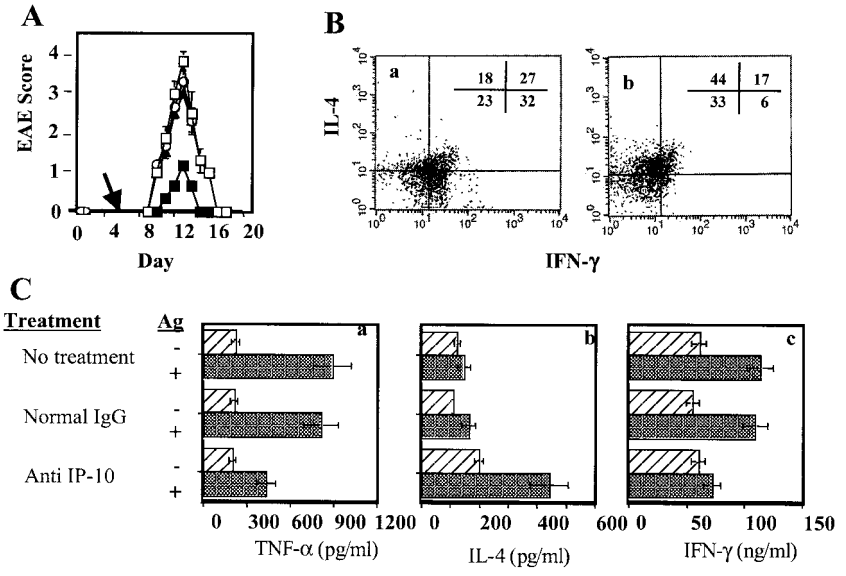

FIGURE 5. Administration of self-specific Ab to IP-10 redirects Ag-specific T cell polarization toward Th2 and suppresses EAE. Four groups of nine rats were subjected to active induction of EAE. Beginning 5 days after disease induction these rats were repeatedly (every other day) subjected to injections of 100 μg per rat of anti-IP-10 Ab (■), IgG from pcDNA3-vaccinated EAE rats (▲), or IgG from normal rat serum (○). Another control group was administered with PBS (□). *A*, Mean maximal score ± SE of these groups. The data shown are representative of one of three independent experiments done under the same conditions. Ten days after disease induction three rats per group were sacrificed and their spleen T cells were cultured together with MBPp68–86. At this time FACS analysis (gated on $CD4^+$ T cells) for IFN-γ vs IL-4 was recorded (*B*). *Ba* shows intracellular staining of CD4 T cells from control untreated rats, whereas *Bb* shows intracellular staining of CD4 T cells from anti-IP-10-treated rats. Intracellular staining of $CD4^+$ T cells of control rats treated with normal IgG was very similar to the one obtained from the control untreated group showed in Fig. 1*Ba* (data not shown). Twenty-four hours later levels of TNF-α, IL-4, and IFN-γ were determined by ELISA. *C*, The levels of these cytokines as mean triplicates ± SE.

with MBPp68–86/CFA to induce active EAE. Five, 6, and 7 days later they were injected with either IP-10 or soluble β-actin-encoding DNA vaccines and monitored for the development and progression of disease by an observer blind to the experimental procedure (Fig. 6*A*). While control and β-actin DNA-vaccinated rats developed severe disease (mean maximal score of 3.5 ± 0.23 and 3.3 ± 0.23, respectively), those treated with IP-10-encoding DNA developed a significantly lower form of disease (mean maximal score of 1.5 ± 0.66, $p < 0.01$ compared with each control group) and quickly went into remission. On day 16 sera from these rats were analyzed for IP-10- or β-actin-specific Ab titer (IgG). In accordance with the results summarized in Fig. 2 control rats with developing EAE displayed a significant ($p < 0.05$) Ab titer to IP-10 during the course of disease that was amplified ($p < 0.001$) following DNA vaccination (Fig. 6*B*). EAE rats did not mount an increased Ab titer to self β-actin. Also, exposure to β-actin-encoding DNA did not rapidly elicit Ab production against the gene product of this construct (Fig. 6*B*). This further suggests that naked DNA vaccination encoding proinflammatory mediators augments a preexisting response, which possibly plays a regulatory function in T cell-mediated autoimmunity.

Finally, in the Lewis rat the disease manifests a short acute form that persists for only 5–6 days, making this model impractical for exploring the ability of IP-10-encoding DNA vaccines to treat a full-blown disease. Under our working conditions C57BL/6 mice immunized with the encephalitogenic residue of MOGp33–55/CFA develop a long-lasting form of disease that persists for ~20 days or more (Fig. 7). This makes this model more practical for determining the effect of IP-10-encoding DNA vaccines on the dynamics of ongoing disease. C57BL/6 mice were subjected to

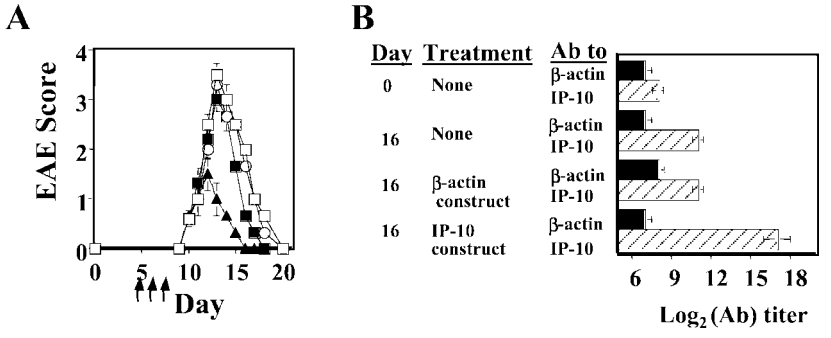

FIGURE 6. IP-10-encoding DNA vaccines interfere in the regulation of established EAE. Lewis rats (six per group) were immunized with MBPp68–86/CFA to induce active EAE, and 5, 6, and 7 days later they were injected with IP-10 (▲)- or soluble β-actin (■)-encoding DNA vaccines, an empty vector (○; 300 μg per rat per injection), or PBS (□) and monitored for the development and progression of disease by an observer blind to the experimental procedure. Representative data of one of two independent experiments conducted under the same experimental protocol are summarized in *A*. The results are shown as mean maximal score ± SE. *B*, Sixteen days after disease induction blood sera were obtained from three representative rats per group and determined for IP-10- and β-actin-specific Ab titer. Results are shown as mean $\log_2$ Ab titer of three samples ± SE.

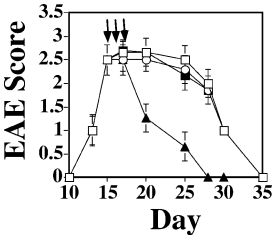

FIGURE 7. IP-10-encoding DNA vaccine suppress ongoing EAE. C57BL/6 mice were subjected to active induction of EAE. On day 15 these mice were separated into four groups of equally sick mice (12 mice per group) and subjected to a repeated administration (three times, days 15, 16, and 17) of IP-10 (▲)- or soluble β-actin (■)-encoding DNA vaccines, an empty vector (○; 100 μg per rat per injection), or PBS (□) and monitored for clinical signs by an observer blind to the experimental protocol. Results are shown as mean maximal score ± SE. On day 25 sera from three mice per group were analyzed for the development of anti-IP-10 and anti-β-actin Ab as described in Fig. 6. Data analysis is summarized in *Results*.

active induction of EAE. On day 15 these mice were separated into four groups of equally sick mice (12 mice per group) and subjected to a repeated administration (three times, days 15, 16, and 17) of IP-10, soluble β-actin-encoding DNA vaccine, an empty vector (100 μg each), or PBS. Only those administered with the IP-10-encoding DNA vaccine went into a rapid remission (Fig. 7, day 25, 0.66 ± 0.26 vs 2.2 ± 0.3, 2.3 ± 0.23, and 2.5 ± 0.3, respectively, $p < 0.01$). At this time sera from three mice per group were analyzed for the development of anti-IP-10 and anti-β-actin Ab as described in Fig. 6. Mice subjected to IP-10-encoding DNA vaccine developed a significantly elevated Ab titer to IP-10 ($\log_2$ Ab titer of 22 ± 1 vs 13 ± 0.5 in control EAE ($p < 0.001$), and 8 ± 0 in naive mice). Thus, IP-10-encoding DNA vaccines can serve as a powerful way to prevent and treat ongoing EAE in various models of the disease.

Discussion

An ideal way to treat a T cell-mediated autoimmune condition is by redirecting the immune system to use the tools with which it generates autoimmunity to generate protective immunity. Our approach in accomplishing this task has been to use a naked DNA vaccination strategy. We have previously demonstrated that naked DNA vaccines encoding self proinflammatory mediators, particularly soluble cytokines and chemokines, may be used to break down immunological tolerance to their gene products, resulting in the generation of anti-self immunity (15–20). Interestingly, the immunological memory generated following vaccination could be effectively turned on to rapidly elicit the production of anti-self Ab during an autoimmune condition, but not following the induction of a local inflammatory condition (Fig. 2). The current study also defines a significant anti-self response to IP-10 in rats immunized with MBPp68–86/CFA to induce active EAE, but not in those immunized with CFA alone to induce a local inflammatory response (Fig. 2*B*). Furthermore, this response accelerates in EAE rats following DNA vaccination (Fig. 2*C*) to provide protective immunity (Fig. 1). Again, this type of intervention was turned on rapidly and could thus effectively suppress an established disease (Fig. 6), even when administered after the onset of disease (Fig. 7). In a very recent study we have demonstrated that administration of TNF-α-encoding DNA vaccine at the peak of a full-blown experimentally induced arthritis can lead to a rapid production of neutralizing Ab to self TNF-α and thus reverse this long-lasting disease (17). In both studies we could not observe development of anti-self Ab response to regulatory gene products such as IL-10-, IL-4-, or IL-18-binding protein (data not shown). It is possible that TNF-α and IP-10 are among few proinflammatory mediators against which the immune system mounts a natural autoimmune response as a part of its attempt to restrain autoimmunity (Fig. 2) (17, 18). As these Ab include IgG isotypes, it likely that the response is a T-dependent one. The possible existence of a preexisting regulatory network may also explain why naked DNA vaccines encoding IP-10 or TNF-α are so effective. After all, they probably augment a preexisting response that, to begin with, is not sufficient to entirely suppress the development of an autoimmune condition.

Recent studies demonstrated the ability of IP-10 to stimulate the directional migration of activated Th1 cells (21–23). Neutralizing the in vivo activity of IP-10 could alter the in vivo Th1/Th2 balance (Fig. 5), either due to an increase in selective accumulation of effector T cells at the autoimmune site and/or following a direct effect of IP-10 on T cell polarization. It is indeed an open question whether IP-10 can directly drive T cell polarization into Th1. The current study demonstrates, for the first time, the ability of IP-10 to directly drive naive T cells into Th1 (Fig. 4). We have previously shown that neutralizing Ab to IL-18 not only direct the in vivo polarization of autoimmune T cells but also suppress EAE (36). It is an open question whether factors like IL-18 or IP-10 direct T cell polarization via their positive effect on IFN-γ production. Our study shows that IP-10 can drive T cells into Th1, even when IFN-γ is being neutralized (Fig. 4*D*).

The mechanistic basis of the adjuvant effect of naked DNA vaccines with CpG motif has been solved very recently (13, 14). Either by activating Toll-like receptor 9 (13) and/or via the catalytic subunit of DNA-PKcs (14), CpG initiates a signal transduction cascade in APCs (i.e., dendritic cells), resulting in their activation. An abundant use of naked DNA vaccines with plasmids barring this motif is for the increase of the proinflammatory (Th1) immune response against infectious agents such as tuberculosis, HIV, and allergens such as mite proteins (11, 12, 37–41). Interestingly, repeated administrations of DNA vaccines that include the CpG motif did not ameliorate T cell-mediated autoimmunity in experiments done by us (15, 17–20) or independently by others (42, 43). Our recent observations suggest that CpG selects in vivo a TNF-α^{low}-producing subtype of $CD4^+$ Th1 cells (S. Youssef, G. Wildbaum, and N. Karin, manuscript in preparation). This can explain, in part, why pro-Th1 DNA vaccines do not aggravate autoimmunity. Hence, the current study demonstrates how a CpG DNA vaccine could be constructed to preferentially select IL-4^{high}IFN-γ^{low}TNF-α^{low}-producing T cells. Such a construct could be used alone or together with a construct encoding a self-autoimmune target Ag (such as MBP, proteolipid protein, or MOG for multiple sclerosis) to effectively direct regulatory T cells to an autoimmune site. An alternative, complementary, way by which a target autoimmune Ag could be coinserted to a plasmid DNA constructed to codeliver IL-4, and thus alter T cell polarization, has very recently published by others (44).

Finally, it is unclear whether alteration of the Th1/Th2 balance toward Th2 may protect from EAE because IL-4-producing Th2 cells are regulatory cells or because alteration of this balance preferentially polarizes Ag-specific T cells that are neither pathogenic nor protective, and thus a smaller amount of potential autoaggressive Th1 cells are being polarized at a given time point. On one hand our current study demonstrates that Th2 cells selected (in vitro) in the absence of IP-10 are not encephalitogenic, but cannot transfer EAE resistance; on the other hand, alteration of the in vivo Th1/Th2 balance, by neutralizing IP-10, could provide protective immunity, very fast, even at late stages of disease (Fig. 7). One possible explanation is that some of the cells selected in vivo in the

absence of IP-10 are regulatory cells that are not being selected in vitro under these conditions. It could also be that the dynamics of an autoimmune condition require a continuing entry of effector autoimmune T cells to replace those undergoing apoptosis at this site (16, 45, 46), and thus altering the balance toward Ag-specific T cells that are not encephalitogenic would reduce the in vivo level of Th1 effector cells and excrete a rapid beneficial effect even without activating regulatory T cells.

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