Targeting the Function of IFN-γ-Inducible Protein 10 Suppresses Ongoing Adjuvant Arthritis[1]

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IFN-γ-inducible protein 10 (IP-10) is a CXC chemokine that is thought to manifest a proinflammatory role because it stimulates the directional migration of activated T cells, particularly Th1 cells. It is an open question whether this chemokine is also directly involved in T cell polarization. We show here that during the course of adjuvant-induced arthritis the immune system mounts a notable Ab titer against self-IP-10. Upon the administration of naked DNA encoding IP-10, this titer rapidly accelerates to provide protective immunity. Self-specific Ab to IP-10 developed in protected animals, as well as neutralizing Ab to IP-10 that we have generated in rabbits, could inhibit leukocyte migration, alter the in vivo and in vitro Th1/Th2 balance toward low IFN-γ, low TNF-α, high IL-4-producing T cells, and adoptively transfer disease suppression. This not only demonstrates the pivotal role of this chemokine in T cell polarization during experimentally induced arthritis but also suggests a practical way to interfere in the regulation of disease to provide protective immunity. From the basic science perspective, this study challenges the paradigm of in vivo redundancy. After all, we did not neutralize the activity of other chemokines that bind CXCR3 (i.e., macrophage-induced gene and IFN-inducible T cell α chemoattractant) and yet significantly blocked not only adjuvant-induced arthritis but also the in vivo competence to mount delayed-type hypersensitivity. ***The Journal of Immunology*****, 2002, 169: 2685–2693.**

Rheumatoid arthritis (RA)[3] is an inflammatory disorder characterized by infiltration of leukocytes into synovial tissue and synovial fluid of joints (1). Depending on the mode of immunization, a single administration of CFA may result in the development of a local inflammatory process or chronic poly adjuvant-induced arthritis (AIA, also termed AA), which histologically and clinically resembles human RA (2). In both diseases, proinflammatory cytokines and chemokines are believed to play a pivotal role in the attraction of leukocytes to the site of inflammation and in the initiation and progression of the inflammatory process. The role of key cytokines in the regulation of disease has been well characterized in experimental models that have been expanded in clinical trails (3–9). Chemokines play a major role in the massive recruitment of leukocytes at the site of inflammation during inflammatory conditions (for a recent review, see Ref. 10). This also includes autoimmune disorders. About 6 years ago, Karpus et al. blocked experimental autoimmune encephalomyelitis (11) in mice by immunizing them with rabbit anti-mouse polyclonal Ab against macrophage-inflammatory protein 1α (MIP-1α). Thereafter, Gong et al. (12) used an antagonist of monocyte chemoattractant protein 1 (MCP-1) to inhibit arthritis in the MRL/*lpr* mouse model. Later, Barnes et al. (13) used antihuman RANTES to ameliorate AA in the Lewis rat. Last, in a very recent study Fife et al. (14) used anti-IFN-γ-inducible protein 10 (IP-10) Ab to inhibit experimental autoimmune encephalomyelitis.

We have used an alternative approach to generate antichemokine protective immunity. The basic idea was to clone various chemokine/cytokine, 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 (15–18) and then to use them as vaccines against the product of each inserted gene (19–24). In these studies, we also demonstrated that this response includes T-dependent Ab production and generation of immunological memory that is turned on during an autoimmune condition to provide protective immunity (19–24). 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 (19–24).

We have found much interest in exploring the role of the CXC chemokine IP-10 in the regulation of T cell-mediated autoimmunity. Our interest in this particular chemokine flows from recent studies demonstrating its ability to stimulates the directional migration of activated T cells, particularly Th1 cells (25–27), including those of human T cells in SCID mice (28), and from other studies suggesting a possible role for IP-10 in T cell-mediated autoimmunity (14, 29–32). 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 experimentally induced arthritis.

Materials and Methods

Rats

Female Lewis rats, ~6 wk old, were purchased from Harlan (Jerusalem, Israel) and maintained under clean conditions in our animal facility.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; IP-10, IFN-γ-inducible protein 10; AA, adjuvant-induced arthritis; MIP-1α, macrophage-inflammatory protein 1α; MCP-1, monocyte chemoattractant protein 1; CNBr, cyanogen bromide; MIG, macrophage-induced gene; PPD, purified protein derivative; ITAC, IFN-inducible T cell α chemoattractant; DTH, delayed-type hypersensitivity.

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Immunizations and active disease induction

Rats were immunized s.c. in the base tail with 0.1 ml of CFA (supplemented with 10 mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra in oil; Difco, Detroit, MI). Rats were then monitored for clinical signs daily by an observer blind to the treatment protocol. Severity of the disease was quantified subjectively by scoring each limb on a scale of 0–4 to indicate the severity of peripheral joint swelling and erythema: 0, no signs of disease; 1, disease evident in a small number of distal joints of the limb; 2, disease evident in all of distal joints of the limb; 3, disease evident in all of the limb; and 4, severe disease evident in all of the limb. The arthritic clinical score was determined as the sum of the scores of all four limbs from each animal (0–16 limbs). In addition to clinical evaluation in all experiments, severity of disease was also assessed histologically as described below.

Cloning of rat IP-10

IP-10-specific oligonucleotide primers were designed based on its published sequence (National Center for Biotechnology Information accession number U22520) as follows: rat IP-10 sense, 5′-CATGAACCCAAGT GCTGCTGTCGT-3′; and rat IP-10 antisense, 5′-TTACGGAGCTCTTT TAGACCTTCT-3′. RT-PCR was then applied on mRNA form the inflamed AA joint. PCR products were 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; Upstate Biotechnology, Cleveland, OH) 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 (22). 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 Mega prep (Qiagen, Chatsworth, CA). Cardiotoxin (Sigma-Aldrich, St. Louis, MO) was repeatedly injected into the tibia's anterior muscle of 4- to 6-wk-old female Lewis rats (10 μM/leg). One week following injection, rats were injected with 100 μg DNA in PBS. Four to 5 days after the first immunization, one rat from the group previously subjected to IP-10 DNA vaccination was sacrificed and transcription of IP-10 was verified using RT-PCR on tibia anterior muscle samples. For treatment of ongoing disease, AA rats were subjected to the administration of 500 μg DNA in PBS into the tibia's anterior muscle (no cardiotoxin was used).

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-supper 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 for IP-10-specific Ab in DNA-vaccinated rats

Our recombinant rat IP-10, produced as described above, and commercially available recombinant mouse IP-10 (PeproTech, Rocky Hill, NJ) 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) was used as primary Ab. Goat anti-rat biotin-conjugated Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a second step, followed by streptavidin HRP (Jackson ImmunoResearch Laboratories), Western blotting Luminol Reagent kit (Santa Cruz Biotechnology, Santa Cruz, CA) was 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 antisera, 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.

Cyanogen bromide (CNBr) purification of IP-10-specific Ab

Recombinant rat IP-10 (5 mg) was bound to a 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).

In vitro chemotaxis assay

The in vitro Boyden chamber chemotaxis assay was conducted as we have previously described (22). A total of 1.2×10^6 purified protein derivative (PPD)-specific T cells, selected as described elsewhere (24), was added to the upper well. Commercially available (Chemicon International, Temecula, CA) IP-10 (200 ng/ml) was used as a chemoattractant. fMLF (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.

Delayed-type hypersensitivity (DTH)

Rats were immunized intradermally into the dorsal surface of the ear with 10 μg PPD (Pasteur Merieux Connaught, Toronto, Ontario, Canada) in 25 μl of PBS (or with PBS alone). Ear thickness was measured using a caliper (Lange Skinfold Caliper; Cambridge Scientific Industries, Cambridge, MA). The ear thickness at time 0 was subtracted from the 24-h measurement to give the amount of Δ ear swelling in response to PPD (34).

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

T cells were positively selected using a MACS magnetic cell sorting 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 the 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 was added to each well and incubated for 72 h in full medium enriched with Con A supernatant as a source of IL-2 (35).

Cytokine determination in cultured primary spleen cells

The protein level of various cytokines was determined 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, Grand Island, NY); 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 (24050D OX-81; BD PharMingen) as a capture Ab and rabbit anti-rat IL-4 biotin-conjugated polyclonal Ab (2411-2D; BD PharMingen) as second Ab. Recombinant rat IL-4, purchased from R&D Systems (504-RL; R&D Systems, Minneapolis, MN), was used as a standard; and 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 used before (20). Before being subjected to intracellular staining, cells were suspended with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (0.2 mM; Sigma-Aldrich), and monensin (0.2 mM; Sigma-Aldrich) for 5 h. For intracellular staining, PE-labeled mouse anti-rat IFN-γ mAb (BioSource International, Nivelles, Belgium), PE-labeled mouse anti-rat TNF-α mAb (BioSource International), and FITC-labeled mouse anti-rat IL-4 mAb (BioSource International) were used. 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

Joints were removed, fixed with 10% buffered Formalin, decalcified in 5% EDTA in buffered Formalin, embedded in paraffin, and sectioned along the midline through the metatarsal region (36). Sections were stained with H&E and analyzed by a histopathologist who was a blind observer to the experimental procedure. Evaluation was made based upon inflammatory

mononuclear cell infiltrate in the synovial membrane, thickness of the synovial lining, joint space narrowing, and periosteal new bone formation. The histological score was determined as follows: 0, no evidence of disease; 1, mild lymphocytic infiltrate; 2, widespread mononuclear inflammation and thickening of the synovial lining; and 3, severe bone destruction, new bone formation, and destruction of the synovial lining (36).

Statistical analysis

Significance of differences was examined using Student's *t* test. A value of $p < 0.05$ was considered to be significant. Mann-Whitney *U* 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 to be significant.

Results

IP-10-encoding DNA vaccine prevents AA

Under our working conditions, AA manifests a long-lasting form of disease that includes an acute phase, peaking around day 20, early chronic phase that persists until days 50–60, and late chronic phase that persists >90 days after disease induction. At this time, AA rats display low clinical score, yet their histological examination shows a vigorous progression of disease (21, 24). To explore the effect of IP-10-encoding DNA vaccines of disease manifestation, rats were subjected to four weekly injections of IP-10-encoding DNA vaccines. Following the last vaccination, the development of self-specific Ab titer to IP-10 was carefully monitored. Within 4 wk, this titer regressed to background levels (data not shown). At that time, rats previously administered IP-10-encoding construct and control rats that were repeatedly injected either with the pcDNA3 vector alone, pcDNA3-β-actin construct, or with PBS were subjected to the induction of active AA. Fig. 1 represents one of three experiments with very a similar pattern of results. All control groups (six per group) developed a severe form of disease (mean maximal clinical score of 12.33 ± 1.2, 12.6 ± 1.1, and 13 ± 0.66, respectively, Fig. 1). In contrast, rats subjected to the administration of the IP-10 construct developed a significantly reduced form of disease (mean maximal score of 7.1 ± 0.6, $p <$ 0.001compared with each of the control groups). A significantly reduced form of disease was also observed in these animals during the early chronic phase of disease (day 42, 2.33 ± 0.6 vs 6.33 ± 0.54, 5.66 ± 0.23, and 6.83 ± 1, $p < 0.001$). Clinical scoring has also been verified by measuring the changes in the degree of paw swelling, once again by an observer blind to the experimental procedure. At all times during the acute and early chronic phases of disease, IP-10 DNA-vaccinated rats exhibited a marked reduction

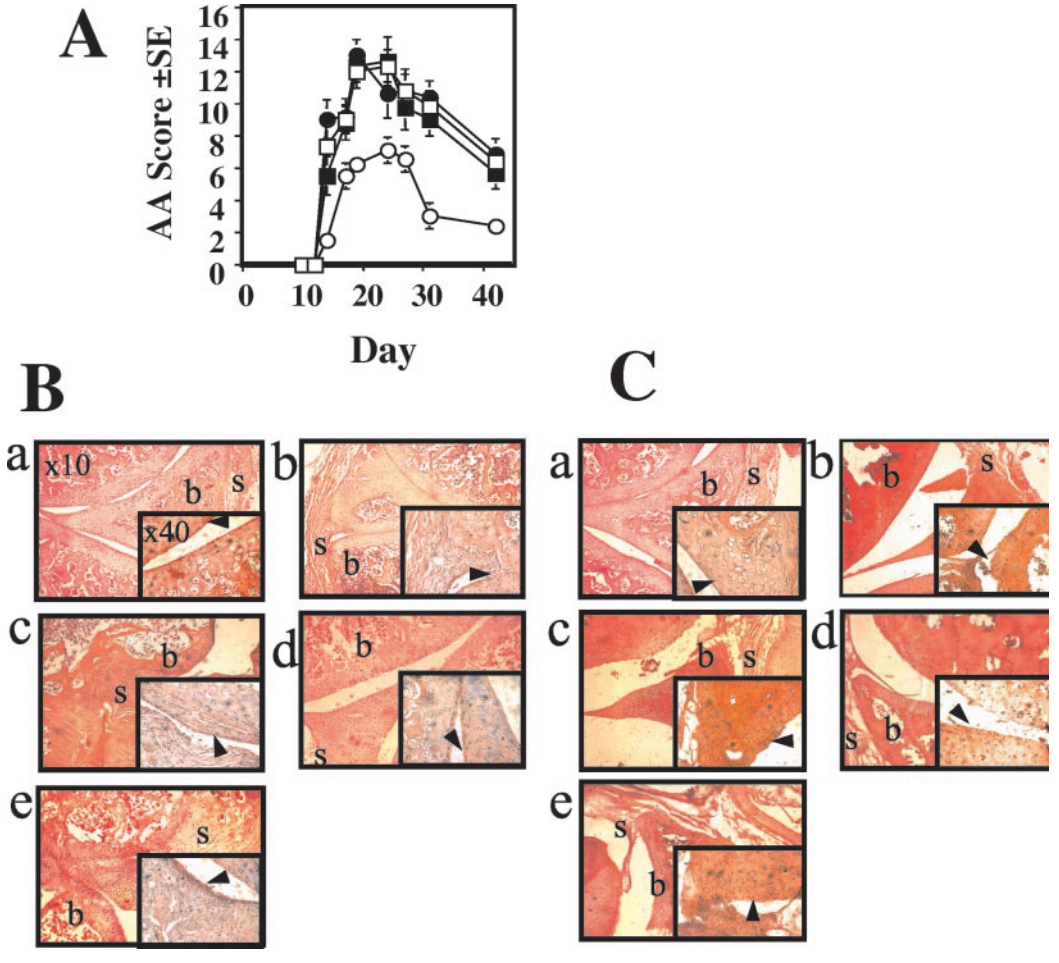

FIGURE 1. Prevention of AA by IP-10-encoding DNA vaccine. Four groups of Lewis rats were subjected to 4 weekly injections of IP-10-encoding DNA vaccines (○). Control rats that were repeatedly injected either with the pcDNA3 vector alone (■), pcDNA3-β-actin construct (●), or with PBS (□). One month later, all rats were subjected to the induction of active AA. Data are from one of three independent experiments with very a similar pattern of results. Six rats per group were monitored for clinical manifestation of disease by observers blind to the experimental protocol. Other rats were sacrificed at different time points for histological evaluation. *A*, The mean clinical score ± SE of six rats per group ($b < a$, $p < 0.001$). *B*, histological evaluation conducted 30 days after disease induction. *C*, Histological evaluation conducted 90 days after disease induction. At each time, joint samples from a naive rat (*a*) or from two AA rats treated with PBS (*b*), pcDNA3 alone (*c*), IP-10-encoding construct (*d*), or β-actin-encoding construct (*e*) were subjected to histological analysis (12 sections each group). The arrowheads point to the synovial lining. b, Bones; s, synovial membrane.

in Δpaw swelling compared with each of the control groups ($p < 0.001$, data not shown). Additionally, representative joint sections from all experimental groups (four animals per group) were obtained on days 30 (Fig. 1*B*) and 90 (Fig. 1*C*) and were screened for histological inflammatory mononuclear cell infiltrate in the synovial membrane, thickness of the synovial lining, joint space narrowing, and periosteal new bone formation. Sections obtained from IP-10 DNA-vaccinated rats displayed a marked reduction in each of the above parameters as compared with control and pcDNA3-treated AA rats. Thus, IP-10-encoding DNA vaccine can serve as a powerful tool to prevent not only the inflammatory process during the acute and early chronic phases of AA (Fig. 1, *A* and *B*), but also the long-lasting damage to the joint during the late chronic phase of disease (Fig. 1*C*).

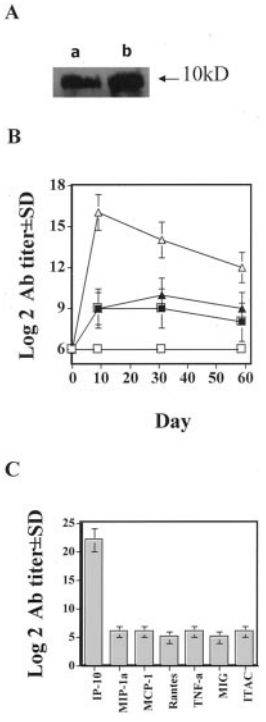

FIGURE 2. IP-10-encoding DNA construct augments production of neutralizing Ab to its gene product. At different time points during the experiment described in Fig. 1, blood serum samples were detected for the development of Ab titer to IP-10. *A,* A Western blot analysis in which serum obtained from IP-10-encoding DNA-vaccinated rats was determined for its ability to bind our recombinant rat IP-10 (10 kDa) and also the commercially available mouse IP-10 (PeproTech). *Lane a* shows binding to our recombinant gene product, while *lane b* shows binding to commercial rIP-10. These Abs also bound natural rat IP-10 from supernatant of activated myelin basic protein p68–86-specific cultured T cells (data not shown). *B,* The kinetics of Ab titer to self-IP-10 developed in naive rats (□), AA rats (■), AA rats that were subjected to the administration of β-actin-encoding construct (▲), and AA rats administrated the IP-10-encoding construct (△). Results are given as mean Ab titer obtained from three rats per group ± SD. *C,* After CNBr-IP-10 purification, our anti-IP-10 Abs were tested for specificity of binding to IP-10 compared with other proinflammatory mediators including MIP-1α, MCP-1, RANTES, TNF-α, MIG, and ITAC by ELISA. In this assay, we have used rMIP-1α, MCP-1, RANTES, and TNF-α that we have generated before (19–22, 24), rIP-10 generated as described above, and rMIG and ITAC generated using their published sequence and the same protocol. These Abs were then used for adoptive transfer experiments that are summarized in Fig. 3.

Table I. *Self-specific Ab to IP-10 inhibits IP-10-induced migration of T cells*[a]

		No. of Migrating Cells		
	Chemoattractant	No Ab	Control rat IgG (10 μg/ml)	Anti-IP-10 Ab (10 μg/ml)
a	Medium	76 ± 2	80 ± 4	72 ± 4
b	fMLF (10^{-7} M)	282 ± 14	268 ± 17	260 ± 12
c	IP-10 (100 ng/ml)	141 ± 20[a]	136 ± 18[b]	80.1 ± 5[c]*

[a] A Boyden chamber chemotaxis assay was conducted to determine the competence of the IP-10-specific Ab to inhibit IP-10- or fMLF-induced migration of PPD-specific T cells. Results are shown as mean triplicates ± SE.

*, $p < 0.001$ for c compared with either b or a.

IP-10-encoding DNA vaccine augments production of neutralizing Ab to its gene product

We determined 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 IP-10 DNA construct as described in Fig. 1. One month after the last immunization, when IP-10-specific Ab titer maintained a baseline level, the rats were injected with CFA to induce active AA. At different times serum 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 binds not only our recombinant rat IP-10, but also the commercially available mouse IP-10 fragment (Fig. 2*A*). These Abs did not bind C-C chemokines including MCP-1, MIP-1α, or RANTES (data not shown). Then the kinetics of Ab production along the course of disease was measured (Fig. 2*B*). Rats with developing AA displayed a significant Ab titer to IP-10 (at the onset of disease $\log_2$ Ab titer of 9 ± 0.4 in AA rats vs 6 ± 0 in control rats, $p < 0.01$) even without DNA vaccination. This titer was dramatically accelerated just before the onset of disease in DNA-vaccinated rats (Fig. 2*B*, $\log_2$ Ab titer of 16.5 ± 0.66 in DNA-vaccinated rats vs 9 ± 0.4 in control AA rats, $p < 0.001$), possibly, to provide protective immunity. To further characterize the function of these Abs, we have tested their ability to affect the migration properties of spleen T cells from AA rats (Table I), on the ability of IP-10 to induce T cell polarization (Table II), and, most importantly, their competence to adoptively transfer to AA resistance (Fig. 3).

Table II. *Self-specific Ab to IP-10 redirects IP-10-induced polarization of anti-CD3-activated T cells*[a]

●●●		IL-4 (pg/ml)	IFN-γ (ng/ml)
a	+Anti-CD3	120 ± 8[a]	1.2 ± 0.3[a]
b	+Anti-CD3$^+$ recombinant β-actin	112 ± 11[a]	1 ± 0.2[a]
c	+Anti-CD3 + IP-10	15 ± 2[b]	2.8 ± 0.4[b]
d	+Anti-CD3 + IP-10 + anti-IP-10	85 ± 9[a]	1.4 ± 0.3[a]

[a] CD4$^+$ T cells were separated from naive spleens by magnetic beads and subjected to anti-CD3 activation. Different wells were or were not supplemented with recombinant mouse IP-10 (PeproTech) or recombinant β-actin (50 ng/ml each), with or without the addition of Ab to IP-10 (10 μg/ml). Levels of IL-4 and IFN-γ were recorded by ELISA after 72 h. Results are shown as mean triplicates ± SE. Without anti-CD3 stimuli levels of IFN-γ in primary cultures of naive T cells were very low and no differences could be observed between cells that were cultured in the presence of control Ab or anti-IP-10 Ab. IL-4 levels were below sensitivity of commercially available ELISA kits.

*, $p < 0.001$ in each column for b compared with a.

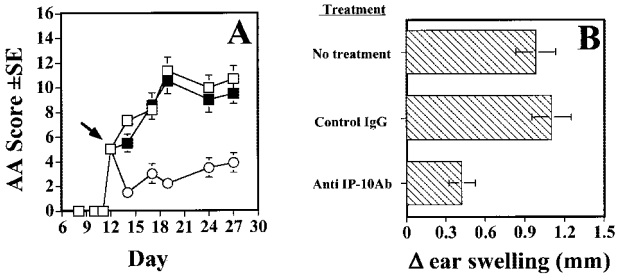

FIGURE 3. IP-10-specific Ab produced in DNA-vaccinated rats transfer protective immunity. Lewis rats were injected with CFA to induce AA. One day after the onset of disease, three groups of equally sick rats (six rats each) were selected and administered, every other day, either purified anti IP-10 Ab (○), IgG from naive rats (■), or PBS (□). An observer blind to the experimental protocol determined clinical scores. *A.* The mean clinical score ± SE of six rats per group of one of two independent experiments done with the same procedure with very similar results. *B,* DTH response to PPD of these animals as was recorded at the peak of disease in control groups. Results are shown as mean Δ ear swelling ± SE.

IP-10-specific Abs produced in DNA-vaccinated rats alter IP-10-induced polarization of T cells and inhibit their migratory properties

After CNBr-IP-10 purification, anti-IP-10 Abs from DNA-vaccinated AA rats were determined for their specificity (Fig. 2*C*) and for their competence to inhibit the migratory properties of activated T cells (Table I). From various proinflammatory mediators (MIP-1α, MCP-1, RANTES, TNF-α, macrophage-induced gene (MIG), IFN-inducible T cell α chemoattractant (ITAC)), these purified Abs specifically bound IP-10 (Fig. 2*C*, $\log_2$ Ab titer of 22 ± 0.9 vs 6 ± 0.4, 6 ± 0.4, 5 ± 0.4, 6 ± 0.4, 5 ± 0.4, and 6 ± 0.4, $p < 0.001$). To evaluate their competence to inhibit the migratory properties of activated T cells, spleen T cells from AA rats were activated (in vitro) with PPD, and subjected to an in vitro migration assay in a Boyden chamber. Recombinant mouse IP-10 (Pepro Tech) significantly enhanced the migratory properties of these cells (Table I, 141 ± 20 vs 76 ± 2, $p < 0.001$), so did fMLF (Table I, $p < 0.001$). Anti-IP-10 Ab could entirely reverse the chemoattraction properties of IP-10 (80.1 ± 5 in the presence of anti-IP-10 Ab and 76 ± 2 in their absence), but had no effect on fMLF-induced attraction (Table I). Thus, the inhibitory effect of the Ab is chemokine specific.

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, naive T cells undergoing activation produced a Th2 cytokine profile with high levels of IL-4 (Table II, 112 ± 11 pg/ml) and low levels of IFN-γ (1.2 ± 0.3 ng/ml). The addition of IP-10 redirected T cell polarization into Th1 (Table II, IFN-γ, 2.8 ± 0.4 ng/ml; IL-4 15 ± 2 pg/ml, $p < 0.001$ for each compression). Addition of anti-IP-10 Ab from DNA-vaccinated AA rats reversed this effect (Table II). Thus, IP-10 polarizes T cells into Th1. A function that can be effectively reversed by neutralizing Ab produced in DNA-vaccinated protected animals.

IP-10-specific Ab produced in DNA-vaccinated rats transfer protective immunity and alter the in vivo polarization of T cells

The in vitro characteristics of anti-IP-10 Ab purified from protected rats (Tables I and II) suggests that these Abs may participate in the regulation of disease in DNA-vaccinated rats. To determine this possibility, we have detected their ability to adoptively transfer AA resistance (Fig. 3*A*). Beginning 2 days after the onset of disease, AA rats were administered, every other day, either purified anti-IP-10 Ab, IgG from naive rats, or PBS. Only the subsequent administration of anti-IP-10 Ab led to a marked reduction in disease severity (day 19: 6 rats per group, mean maximal score ± SE of 11.33 ± 1.1 and 10.5 ± 1 in control and IgG-treated rats, respectively, vs 2.17 ± 0.6 in anti-IP-10-treated rats, $p < 0.001$ for each comparison). At the peak of disease, the ability of these rats to mount a DTH response to PPD was determined (Fig. 3*B*). It appears that anti-IP-10 Ab could effectively ($p < 0.0001$) inhibit the ability of the immune system to mount a proinflammatory DTH response. Inhibition of DTH could be associated with altered migration of proinflammatory cells (Table I), altered polarization of T cells (Table II), or both. To determine whether neutralizing IP-10 affects T cell polarization in vivo, popliteal and inguinal lymph node CD^+ T cells were subjected to intracellular staining of IL-4, IFN-γ, and TNF-α (Fig. 4). Apparently the subsequent administration of anti-IP-10 Ab redirected in vivo polarization of $CD4^+$ T cells into low IFN-γ, high IL-4 (Fig. 4, *B* vs *A*) low TNF-α (Fig. 4, *D* vs *C*)-producing cells. Very similar results were obtained using rabbit anti-rat IP-10 polyclonal Ab which we have also generated against our rIP-10. That is administration of these Abs using the same protocol described above significantly suppressed AA and blocked DTH (data not shown).

DNA vaccine encoding IP-10 treats ongoing AA

From the practical perspective, it is important to evaluate the competence of the IP-10 encoding naked DNA to interfere with the progression of an ongoing disease. Lewis rats were immunized with CFA to induce active AA and divided into four random

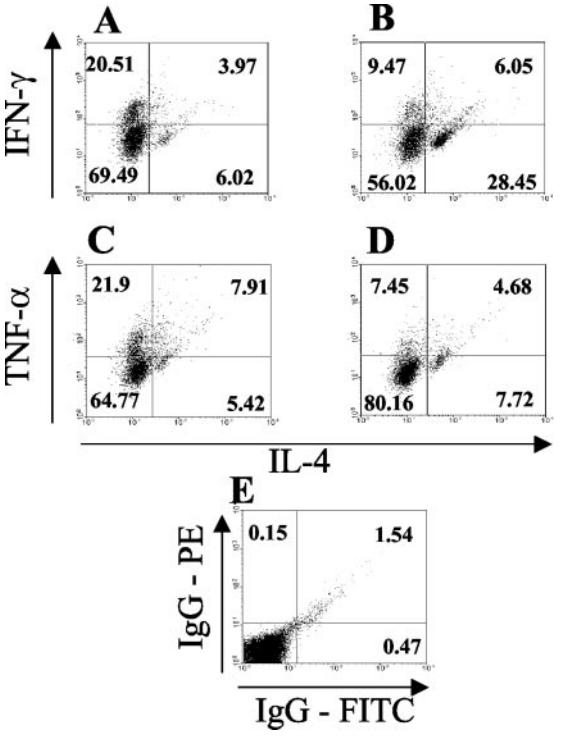

FIGURE 4. Neutralizing IP-10 affects the in vivo polarization of T cells. Rats were immunized with CFA to induce active AA. On days 5 and 7, these rats were treated with anti-IP-10 Ab (*B* and *D*), PBS (*A* and *C*), or normal rat IgG (data not shown). On day 9, popliteal and inguinal lymph node $CD4^+$ T cells were subjected to intracellular staining of IL-4, IFN-γ, or TNF-α (*A–D*). Staining with isotype-matched labeled control Ab is also shown (*E*). Repeated injections of IgG from normal rat serum had no effect on T cell polarization as compared with PBS treatment (data not shown).

groups. Two days after the onset of disease (day 13) and on days 15 and 18, each group was subjected to the administration of PBS, pcDNA3 alone, β-actin construct, or the IP-10-encoding construct (500 μg/rat). Although all control and pcDNA3-treated rats continued to develop severe AA, those exposed to the IP-10 DNA vaccine exhibited a marked reduction of disease severity (Fig. 5 *A*, day 24, mean maximal score of 11.25 ± 1, 11.5 ± 0.9, and 12 ± 0.95, respectively, in rats treated with either PBS, β-actin construct, or pcDNA3 alone vs 3.8 ± 0.25, $p < 0.001$ for the comparison of IP-10 DNA-vaccinated rats to each control group). The rapid production of anti-IP-10 Ab in rats treated with IP-10-encoding DNA vaccines (Fig. 5*B*) suggests an amplification of a pre-existing response can explain, in part, the rapid beneficial effect of the therapy. The notable reduction maximal clinical score in rats treated with empty plasmid or β-actin-encoding plasmid (Fig. 5*A*) could not be attributed to elicitation of anti-IP-10 Ab titer (Fig. 5*B*) and is probably associated with the nature of the plasmid itself. Clinical scores were confirmed histologically (Fig. 5*C*), including the chronic phase (day 60) at which the massive cartilage loss, bone erosion, and periosteal new bone formation characterized control and pcDNA3-treated rats were entirely absent in joint sections of rats treated with IP-10 naked DNA vaccine.

Discussion

Administration of the cytokine/hormone-encoding gene in a plasmid (i.m.) might be used as a way of eliciting the production of its

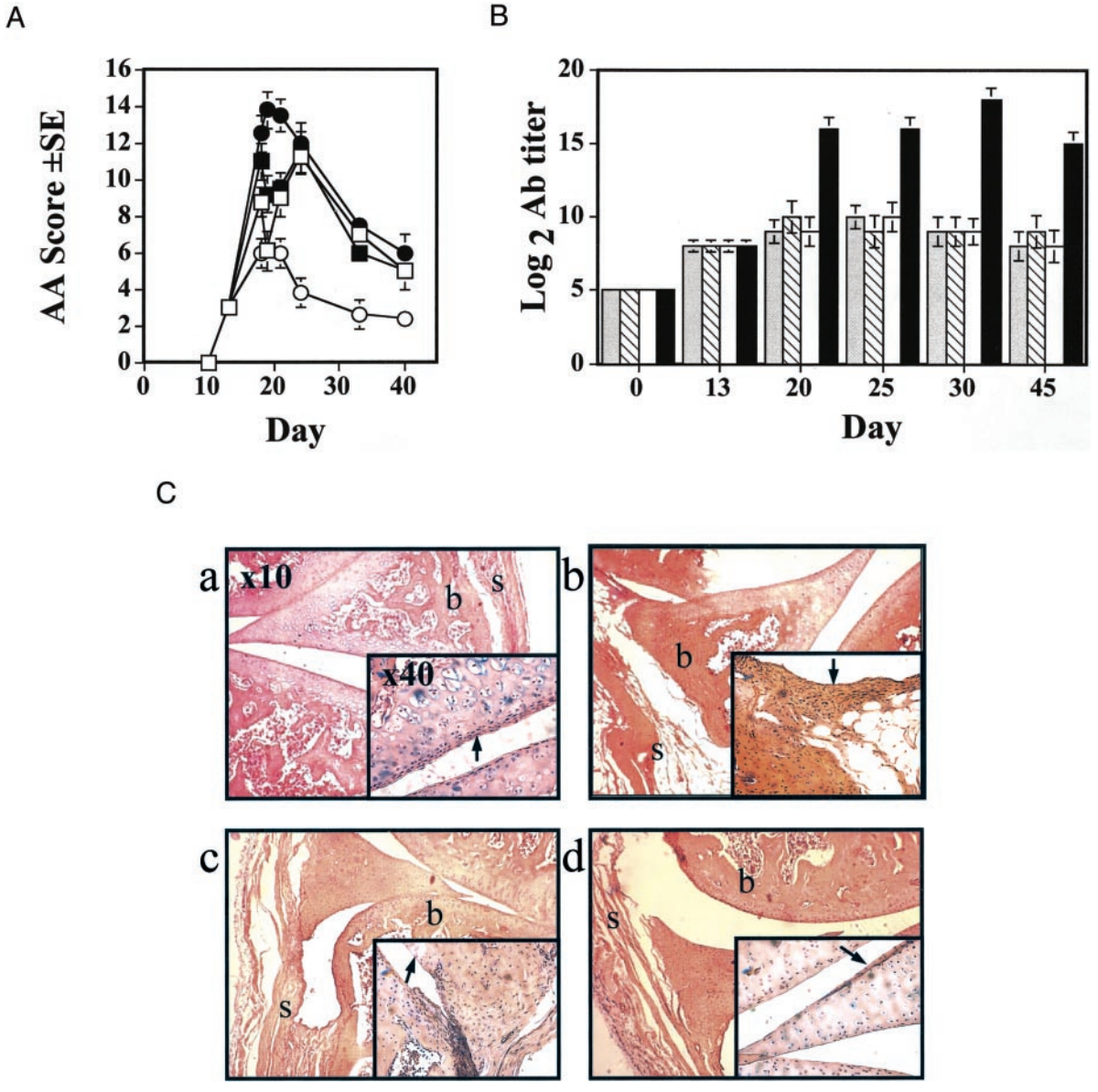

FIGURE 5. DNA vaccine encoding IP-10 treats ongoing AA. *A*, Lewis rats were immunized with CFA to induce active AA and divided into four random groups. Two days after the onset of disease (day 13) and on days 15 and 18, each group was subjected to administration of PBS (□), pcDNA3 alone (■), β-actin construct (●), or the IP-10-encoding construct (○; 500 μg/rat). Six rats per group were monitored for clinical manifestation of disease by an observer blind to the experimental protocol. Other rats were sacrificed at different time points for histological evaluation. Results of one of two independent experiments done under the same experimental procedure, with very similar data. Results are shown as mean clinical score ± SE of six rats per group at different time points. *B*, IP-10-specific Ab titer developed in control AA (▨) rats and rats treated with either empty vector (▧), β-actin-encoding plasmid (□), or IP-10-encoding DNA vaccine (■). Results are shown as mean triplicates ± SE. *C*, Histological evaluation conducted 60 days after disease induction. Joint samples form a naive rat (*a*) or from two AA rats treated with PBS (*b*), pcDNA3 alone (*c*), or the IP-10-encoding construct (*d*) were subjected to histological analysis (12 sections each group). The arrowheads point to the synovial lining. b, Bones; s, synovial membrane.

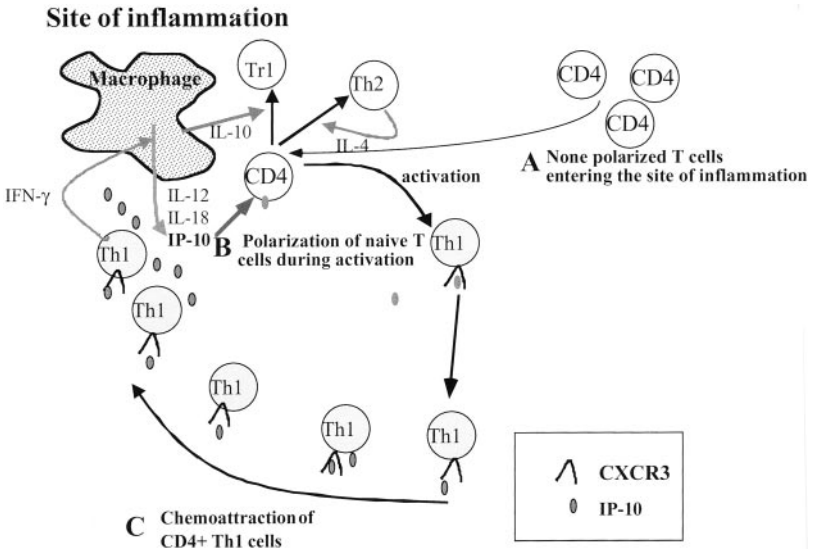

FIGURE 6. The role of IP-10 in the development of inflammation. IP-10 is involved in polarizing naive T cells entering the site of inflammation into Th1. These cells, along with Th1 cells directly attracted by IP-10, then produce high levels of IFN-γ that further induce IP-10 production by APC of the innate immune system.

gene product, which can be used for targeted delivery of gene products (37–39), or as a way to elicit and immune response against the gene product encoded by this construct (19, 22, 40, 41). The mechanistic basis leading to these opposing effects is not fully understood yet and may be dependent, in part, by the nature of the selected plasmid. The existence or absence of repeated immunostimulatory sequence (CpG) may provide a partial explanation for the ability of certain plasmids to serve as adjuvants (42).

We have previously demonstrated that naked DNA vaccines encoding self-proinflammatory mediators, particularly soluble cytokines and chemokines, may be effectively used to breakdown immunological tolerance to their gene products, resulting in the generation of anti-self-immunity (19–24). The current study defines a significant anti-self-response to IP-10 in rats immunized with CFA to induce active AA (Fig. 2*B*). We believe that this response is part of a regulatory mechanism in which the immune response mounts protective immunity against self proinflammatory mediators to restrain other activities against self (i.e., autoimmune diseases). Administration of the actin-encoding construct had no significant effect on the development of this titer (Fig. 2*B*) that was not sufficient to provide protective immunity (Fig. 1). This titer rapidly accelerated in IP-10-encoding DNA-vaccinated rats (Fig. 1), even when vaccines were administered after the onset of disease (Fig. 5), leading to AA suppression. 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 (21). 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. Because these Abs include IgG isotypes, it likely that the response is a T-dependent one. To further elaborate this interesting issue, anti-IP-10 Ab generated in AA rats that were not subjected to IP-10-encoding DNA vaccines were purified from blood sera (day 14) and evaluated for their neutralizing properties. These Abs, at a concentration of 10 μg/ml, could significantly inhibit T cell migration induced by 100 ng/ml rIP-10 (155 ± 12 without addition of Ab, 148 ± 17 in the presence of total IgG from naive rats, and 84 ± 8 in the presence of Ab from AA rats, $p < 0.001$). This suggests that during the course of AA the immune system mounts an immune response against IP-10 as a part of its attempt to restrain the autoimmune condition. This may also explain why naked DNA vaccines encoding IP-10 are so effective. After all, it probably augments a pre-existing response that, to begin with, is not sufficient to entirely suppress the development of an autoimmune condition. Interestingly, targeting the function of a single proinflammatory mediator suppresses a disease regulated by various cytokines and chemokines (13, 43–50). These results are very surprising since different chemokines bind and activate common chemokine receptors. This is also true for CXC chemokines (i.e., ITAC, MIG, and IP-10 bind the same receptor). Two possible explanations should be considered: 1) Despite competition on common receptors, the absence of one chemokine could be overcome by others in an in vivo system. 2) The clinical readout of disease resides from a balance between the activity of various proinflammatory cytokines/chemokines such as TNF-α, IL-1, RANTES, MCP-1, and regulatory cytokines like IL-10 and IL-4. Thus, blockade of the function of one proinflammatory mediator would significantly alter this balance. Our results showing that blockade of IP-10 not only alters the clinical balance of disease, but also inhibits DTH (Fig. 3*B*) challenges the paradigm of chemokine redundancy.

Recent studies demonstrated the ability of IP-10 to stimulate the directional migration of activated Th1 cells (25–27). Neutralizing the in vivo activity of IP-10 could alter the in vivo Th1/Th2 balance (Fig. 4) 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 the ability of IP-10 to directly drive naive T cells into Th1 (Table II). It is unclear whether alteration of the Th1/Th2 balance toward Th2 may provide protection from AA since IL-4-producing Th2 cells are regulatory cells, or because alteration of this balance preferentially polarizes Ag-specific T cells that produce a very low level of TNF-α in vivo (Fig. 4, *D* vs *C*). Hence, it is clear that targeted DNA vaccines encoding IP-10 can be used to effectively circumvent potential pro-Th1 polarization of T cells resulting from CpG-mediated activation of cells of the innate immune system (18), probably via Toll receptor 9 (17). This is an alternative way to the one recently suggested by Garren et al. (51) who used IL-4-encoding DNA vaccines to redirect T cell polarization.

A major disadvantage in treating chronic diseases with xenogenic neutralizing Abs lies in their immunogenicity. This has motivated investigators to develop chimeric humanized Abs and mAbs engineered with human Ig H and L chain yeast artificial chromosome (52). However, following repeated immunization, these engineered Abs can trigger allotypic responses. The therapeutic strategy suggested here has an advantage over the above methods since it resulted in the generation of immunity to autologous Ags that accelerate during the course of an autoimmune condition in accordance with disease progression. Even though this makes this type of therapy a very promising means of treatment for RA and possibly other T cell-mediated autoimmune diseases (19, 22), one should be also aware of its limitations. Patients developing a chronic form of disease will have to spend the rest of their lives with Abs to their own IP-10. We therefore believe that such vaccines would be used in humans only after Ab/soluble receptor therapy has been explored.

We have recently demonstrated that RA patients, but not patients suffering from osteoarthritis, mount a significant Ab titer against TNF-α (G. Wildbaum, A. M. Nahir, S. Youssef, Y. Geron, A. Admon, and N. Karin, manuscript in preparation). Possible self-specific titer to various chemokines, including IP-10, will also be determined. Patients with RA are usually administered immunosuppressive drugs. In light of our observations, it could be that these drugs suppress a natural mechanism aimed to restrain the severity of disease. On the other hand, while planning intervention in an ongoing disease by IP-10-or TNF-α-encoding DNA vaccines, one should take into consideration that such drugs might affect the efficiency of the vaccines. Finally, the data presented in the current study, together with our very recently published manuscript (53), may suggest a novel role for IP-10 in the polarization of naive cells entering an autoimmune site of inflammation (Fig. 6). This further emphasizes the key role of this chemokine in T cell-mediated autoimmunity.

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