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Suppression of Ongoing Adjuvant-Induced Arthritis by Neutralizing the Function of the p28 Subunit of IL-27

Ruth Goldberg,*[‡] Gizi Wildbaum,* Yaniv Zohar,* Gila Maor,[‡] and Nathan Karin^{1,*[‡]}

IL-27 is a recently defined family member of the long-chain four-helix bundle cytokines, which consists of EBI3, an IL-12p40-related protein, and p28, an IL-12p35-related polypeptide. The role of IL-27 in the regulation of inflammatory autoimmune diseases has never been studied. The current study uses the DNA vaccination technology, and highly specific Abs to the p28 subunit of IL-27 that were generated by this technology, to delineate its role in the regulation of adjuvant-induced arthritis in Lewis rats. Neutralizing the in vivo function of IL-27 by targeted DNA vaccines and by Abs against IL-27 p28 that were produced in protected donors could rapidly suppress an ongoing disease. Disease suppression was associated with a reduced ex vivo production of inflammatory cytokines. We then used these Abs to investigate the mechanistic basis of disease suppression, showing that IL-27 is not only involved in directing the polarization of naive T cells, but also affects the proliferative response and cytokine production of Ag-specific effector/memory Th1 cells. This may explain, in part, its important role in the regulation of inflammatory autoimmune diseases, and also suggest novel ways of therapy. *The Journal of Immunology*, 2004, 173: 1171–1178.

Rheumatoid arthritis (RA)² is an inflammatory disorder characterized by infiltration of leukocytes into synovial tissue and synovial fluid of joints. The prominent T cell infiltrate in RA suggests that these cells are involved in the pathogenesis of this disease. The role of these cells in the regulation of RA has been largely explored in different experimental models such as collagen-induced arthritis in mice and adjuvant arthritis in rats (1).

Depending on their cytokine profile, CD4⁺ T cells fall into different subsets including Th1 cells that produce large amounts of IFN- γ and TNF- α and low levels of IL-4, Th2 cells that mostly produce IL-4, IL-5, and IL-13, and, to a much lesser extent, IFN- γ and TNF- α (2, 3). Th3 cells that produce high levels of TGF- β and, to a much lesser extent, other cytokines (4, 5), and Tr1 cells that produce high levels of IL-10 (6) and CD4⁺CD25⁺ suppressor T cells (7, 8). The pivotal role of Th1 cells in the initiation and progression of the inflammatory process in several autoimmune diseases, including RA, has been well documented. Thus, neutralization of IL-12, IL-18, or IFN- γ -inducible protein 10 (CXCL10) suppresses experimentally induced arthritis while shifting Ag-specific T cell polarization from Th1 to Th2 (9–12).

IL-27 is a newly defined family member of the long-chain four-helix bundle cytokines (13). It is a heterodimeric cytokine that consists of EBI3, an IL-12p40-related protein, and p28, an IL-12p35-related polypeptide (13). This cytokine is an early product of activated APCs and drives rapid clonal expansion of naive CD4⁺ T cells (13). IL-27 acts synergistically with IL-12 to trigger

IFN- γ production of naive CD4⁺ T cells and mediates its biologic effects through the orphan cytokine receptor WSX-1/TCCR (13–17). WSX-1 signaling activates T-bet through activation of STAT1 during initial Th1 commitment (18–20). It is therefore possible, although has yet to be proven, that neutralization of IL-27 may affect the regulation of Th1-mediated inflammatory diseases. Very recently its expression was identified in tissue samples from patients suffering from granulomatous diseases (21). However, the role of IL-27 in the regulation of inflammatory autoimmune diseases has never been elucidated. The current study explores, for the first time, the role of this cytokine in the regulation of experimentally induced RA.

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 (AA) that resembles both histologically and clinically human RA (22). We have previously shown that during AA, but not in a local inflammatory response, the immune system selectively breaks down tolerance to key proinflammatory mediators (mostly chemokines and cytokines) that are associated with the regulation of disease. This breakdown of tolerance includes production of “beneficial autoantibodies” to these gene products (23). We also showed that targeted DNA vaccines encoding these mediators could selectively amplify these responses to suppress ongoing AA (11, 23–25). In a very recent study, we showed that this natural response directly participates in the regulation of the disease since a selective exclusion of “protective autoimmunity” to a single key mediator (TNF- α) radically aggravated its severity (23). In this particular study, we have also shown the relevance of these observations to human RA. It appears those RA patients, but not those suffering from osteoarthritis, display a significant autoantibody response of neutralizing Abs to TNF- α . Anti-TNF- α Abs suppressed RA but displayed no beneficial effect on osteoarthritis. Thus, breakdown of tolerance selectively occurs when it is beneficial to the host (23).

The current study shows that during AA rats manifest a significant autoantibody titer to the p28 subunit of IL-27 (IL-27 p28) and that targeted DNA vaccines encoding IL-27 amplify this Ab production to suppress the disease. It also shows that disease suppression is associated with a marked reduction in the inflammatory activity of Ag-specific T cells. Finally, we used these Abs and a

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² Abbreviations used in this paper: RA, rheumatoid arthritis; AA, adjuvant-induced arthritis; CNBr, cyanogen bromide; MT, *Mycobacterium tuberculosis*.

long-term autoimmune Th1 cell line to delineate the mechanistic basis of disease suppression.

It should be noted that in the current study we did not determine the role of the other subunit of IL-27 (EB13) in the functional regulation of IL-27 and its relevance for autoimmunity. It is not yet clear whether EB13 is solely required for the secretion of p28 or displays a functional role by itself. This should be addressed by future studies aiming at determining the role of EB13 in the regulation of autoimmunity.

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.

Immunizations and active disease induction

Rats were immunized (s.c.) in the base tail with 0.1 ml of CFA (IFA supplemented with 10 mg/ml heat-killed *Mycobacterium tuberculosis* (MT) H37Ra; Difco, Detroit, MI). Rats were then monitored for clinical signs daily by an observer blind to the treatment protocol, as we described elsewhere (23, 25). 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 distal joints of the limb; 3, disease evident in all of the limbs; and 4, severe disease evident in all of the limbs. The arthritic clinical score was determined as the sum of the scores of all four limbs from each animal (sum, 0–16). An observer blind to the experimental protocol quantified the degree of arthritis, indicated by swelling, by measuring front and hind limb circumference using a caliper (Lange Skinfold Caliper; Cambridge Scientific Industries, Cambridge, MA).

Cloning of mouse IL-27 p28

IL-27 p28-specific oligonucleotide primers were designed based on its published sequence (National Center for Biotechnology Information accession number U22520) as follows: mouse IL-27 p28 sense, 5'-ATGGGCCAG GTGACAGGAGACCTTGGCT-3' and mouse IL-27 p28 antisense, 5'-TTAGGAATCCAGGCTGAGCCTGGGGCGC-3'. RT-PCR was then applied on mRNA from the inflamed AA joint. PCR products were cloned into a pUC57/T vector (T-cloning kit K1212; MBI Fermentas, Vilnius, Lithuania) and transformed into *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

The sequenced PCR product of mouse IL-27 p28 was transferred into a pcDNA3 vector (Invitrogen, San Diego, CA). Large-scale preparation of plasmid DNA was conducted using Mega Prep (Qiagen, Chatsworth, CA). In a preliminary study, we verified that IL-27 p28 is transcribed at the site where the construct was administered. Four to 5 days after administration of plasmid DNA encoding IL-27, rats were sacrificed and RT-PCR was applied on tibialis anterior muscle samples. Indeed, muscle samples exhibited an elevated transcription of IL-27 p28. This construct and a control vector encoding soluble β -actin (11) were later used to treat ongoing AA.

Production and purification of rIL-27 p28

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-tagged proteins (Qiagen). After purification, the purity of rIL-27p28 was verified by gel electrophoresis followed by sequencing (N terminus) by our sequencing services unit.

Western blot analysis

Our recombinant mouse IL-27 p28, produced as described above and commercially available recombinant mouse IL-18, IL-12, and IFN- γ (Pepro-Tech, Rocky Hill, NJ) were each subjected to Western blot analysis according to the protocol described in detail elsewhere (23, 25), with the minor modification of using a 12% (rather than 8%) running gel. IgG from IL-27 p28 DNA-vaccinated rats or IgG from normal rat serum (final dilution of 1/500 each) was used as primary Abs. Goat anti-rat biotin-conju-

gated Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary Ab, followed by streptavidin-HRP (Jackson ImmunoResearch Laboratories). A Western blotting Luminol Reagent kit (Santa Cruz Biotechnology, Santa Cruz, CA) was then used as a substrate.

Evaluation of anti-IL-27 p28 Ab titer in sera of DNA-vaccinated rats

A direct ELISA has been used to determine the anti-IL-27 p28 Ab titer in DNA-vaccinated rats. The rIL-27 p28, 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 25 to 230 ng/ml, were added to ELISA plates. Goat anti-rat IgG alkaline phosphatase-conjugated Ab (Sigma-Aldrich, St. Louis, MO) 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.

Purification of anti-IL-27 Ab from sera

A High-Trap Protein G column (Pharmacia, Piscataway, NJ) was used according to the manufacturer's protocol to purify the IgG fraction. Then IL-27 p28-specific Abs were purified using a cyanogen bromide (CNBr)-activated Sepharose column as follows: recombinant mouse IL-27 p28 (5 mg) was bound to a CNBr-activated Sepharose column according to the manufacturer's instructions (catalogue no. 17-0820-01; Pharmacia Biotech, Uppsala, Sweden). IL-27 p28-specific Abs from sera (IgG fraction) of DNA-vaccinated rats were loaded onto the column and then eluted by an acidic elution buffer (glycine, pH 2.5). Isotype determination of the purified Abs (ELISA) revealed that purified Abs are mostly of the IgG2a isotype (data not shown).

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 (catalogue no. 43-4322; Zymed Laboratories, San Francisco, CA) with rat rIFN- γ as a standard (catalogue no. 3281SA; Life Technologies, Grand Island, NY); and 2) TNF- α , a commercial semi-ELISA kit for the detection of rat TNF- α (catalogue no. 80-3807-00; Genzyme, Cambridge, MA).

Histopathology

Joints were removed, fixed with 10% buffered Formalin, decalcified in 20% EDTA in buffered Formalin, embedded in paraffin, and sectioned along the midline through the metatarsal region. Sections were stained with Alcian blue and H&E. Evaluation was made based upon inflammatory mononuclear cell infiltrate in the synovial membrane, thickness of the synovial lining, cartilage destruction, 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 (23).

Immunohistochemistry

Immunohistochemistry was performed as described in detail elsewhere (26). Deparaffinized paraffin sections were incubated for 2 h at room temperature with the specific Ab, mouse anti-collagen type II (catalogue no. MAB 8887 Chemicon International, Temecula, CA), following incubation with an appropriate biotinylated secondary Ab and with streptavidin-peroxidase conjugate and *S*-(2-aminoethyl)-L-cysteine as a substrate (Histostain-SP kit; Zymed Laboratories); counterstaining was done with hematoxylin.

Results

During the development and progression of AA the immune system mounts autoantibody response to the p28 subunit of IL-27

Depending on the mode of immunization, a single administration of CFA may result in the development of a local inflammatory process or chronic poly-AA that histologically and clinically resembles human RA (22). We show here that at the onset of disease (Fig. 1A) and during the course of disease (Fig. 1C), the immune system mounts a significant ($p < 0.01$) Ab titer to the p28 subunit of IL-27 (Fig. 1A, \log_2 Ab titer of 12 ± 0.8 compared with 6 ± 0 in naive rats), but not to the regulatory cytokine IL-4 (\log_2 Ab titer

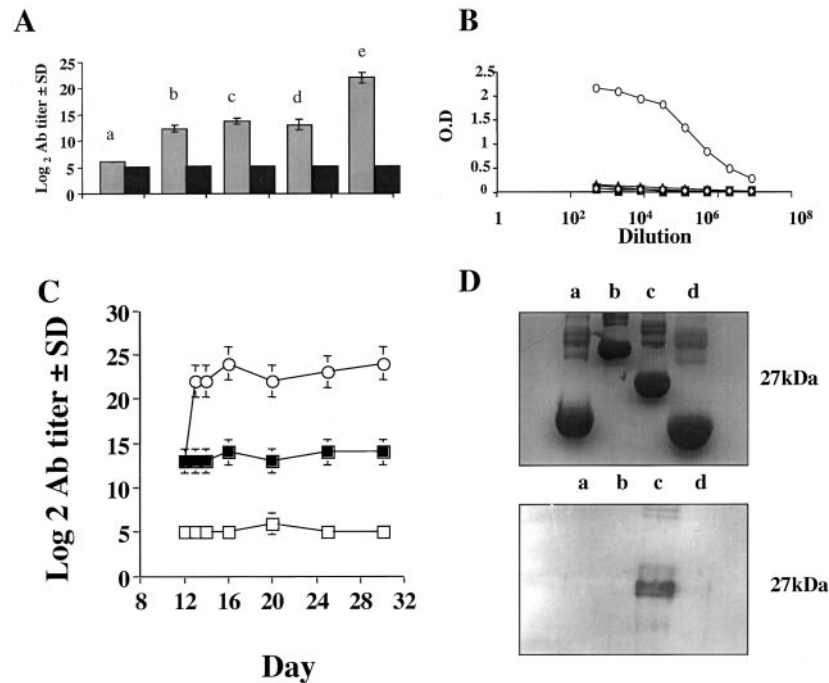


FIGURE 1. IL-27 p28-encoding DNA construct augments production of neutralizing Ab to its gene product. Lewis rats were immunized with CFA to induce active AA and divided into four random groups of 12 rats each. At the onset of disease (day 10) and on days 12, 14, and 16, each group was subjected to administration of PBS, empty construct, β -actin-encoding construct, or an IL-27 p28-encoding plasmid (300 μ g/rat). On days 12, 13, 15, 20, 25, and 30, serum samples were detected for the development of Abs to IL-27 p28. **A**, The Ab titer against IL-27 p28 developed in naive rats (*a*), AA rats (*b*), AA rats that were subjected to the administration of an empty vector (*c*), β -actin-encoding construct (*d*), or the IL-27 p28-encoding plasmid (*e*) as determined in samples obtained 2 days after the administration of the plasmid DNA construct. Gray columns display titers determined in sera of rats administered IL-27-encoding DNA, whereas black columns show titers of sera from rats administered an empty vector. Results are shown as mean \log_2 Ab titer of three rats \pm SE. **B**, Examines the affinity of each titer in representative rats by measuring the OD at different serum dilutions: naive rats (\blacklozenge), AA rats (\square), AA rats that were subjected to the administration of an empty vector (\blacksquare), β -actin-encoding construct (\blacktriangle), or the IL-27 p28-encoding construct (\circ). **C**, Follows the dynamics of this titer at different time points in naive rats (\square), AA rats (\blacksquare), and the IL-27 p28-encoding construct (\circ). Results are shown as mean \log_2 Ab titer of three rats \pm SE. **D**, The results of Western blot analysis in which serum obtained from IL-27 p28-encoding DNA-vaccinated rats was determined for its ability to bind our recombinant mouse IL-27 p28 (lane *c*, 27 kDa) in comparison to the commercially recombinant mouse IL-18, IL-12, and IFN- γ (lanes *a*, *b*, and *d*, respectively).

of 5 ± 0 either in AA or naive rats). Immunization of CFA to the hind footpad that resulted in a local inflammatory response and increased Ab titer did not induce an increased autoantibody response to IL-27 p28 (data not shown), implying that the immune system selectively distinguishes an autoimmune condition from a local inflammatory process. These results are consistent with our recently published observations (23). In a preliminary set of experiments, we have detected the ability of our plasmid DNA vaccine encoding IL-27 p28 to prevent AA in a prophylactic manner. Lewis rats (six per group) were subjected to three intervals of IL-27-encoding plasmid DNA vaccine, empty vector, or PBS. Five weeks after the last administration, these rats were subjected to active induction of AA and monitored for clinical manifestation of disease and also for the development of Ab titer to IL-27. These experiments clearly showed that prophylactic exposure to the targeted DNA plasmid encoding IL-27 led to the development of a significant Ab titer to IL-27 and marked suppression of disease (data not shown). These experiments motivated us to explore the ability of targeted DNA vaccines encoding IL-27 p28 to amplify the Ab titer that is produced against this gene product during an ongoing disease (Fig. 1).

Administration of a targeted DNA plasmid encoding IL-27 p28 rapidly amplified autoantibody production against its gene product and at the same time suppressed an ongoing disease

We have previously shown that targeted DNA vaccines encoding self-chemokines/cytokines could be used to selectively amplify au-

toantibody responses that are being mounted in inflammatory autoimmune diseases, probably as a natural part of their regulation (23).

To determine whether the administration of a targeted DNA vaccine encoding IL-27 p28 in ongoing AA would also rapidly elicit the production of Abs to this gene product, the p28 subunit of IL-27 was cloned, ligated onto the pcDNA3 plasmid, and injected into AA rats immediately following the onset of disease. These rats were then monitored for the levels of anti-IL-27 p28 Abs (Fig. 1, A–C), for their specificity (Fig. 1D), and for the effect of this vaccine on the development and progression of disease (Fig. 2). The administration of IL-27-encoding DNA plasmid (p28) rapidly amplified autoantibody production to its gene product (Fig. 1Ae compared with Ab, \log_2 Ab titer of 23 ± 1.1 compared with 12 ± 0.8 , $p < 0.001$). This treatment did not elicit immunity to IL-4 (Fig. 1A), showing that amplification is specific to the gene product encoded by the vaccine. These Abs continued to be produced in AA rats during the acute and the chronic phase of disease (Fig. 1C). Immunization with an empty plasmid or a plasmid encoding a control gene (β -actin) did not enhance immunity to IL-27 (Fig. 1A). Subsequently, administration of the targeted DNA plasmid encoding IL-27 p28 did not elicit an accelerated response to its gene product in rats injected with CFA to induce a local inflammatory process (data not shown). Thus, administration of a targeted DNA plasmid encoding IL-27 p28 selectively amplifies a rapid autoantibody production against its gene product during an autoimmune inflammatory process. It should be noted that this

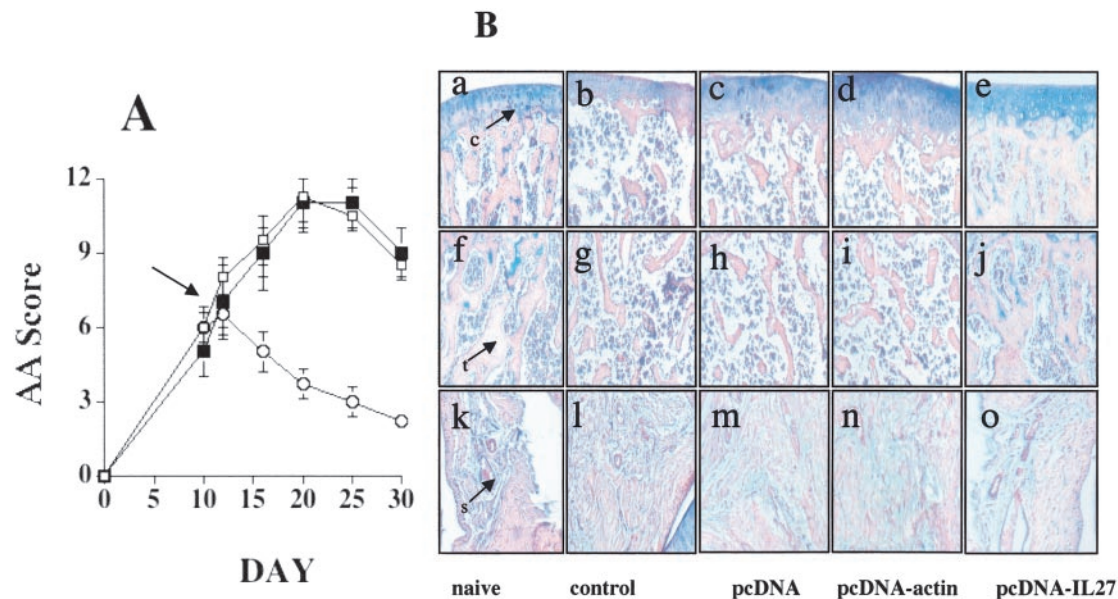


FIGURE 2. A DNA vaccine encoding IL-27 p28 suppresses ongoing AA. Lewis rats were subjected to immunization with CFA to induce active AA and at the onset of disease were separated into four groups of equally sick rats (10/group), 6 of which were monitored for clinical manifestation of disease (A) and the others were sacrificed for histological evaluation as shown in B. These rats were then treated with an empty plasmid (■), β -actin-encoding construct (▲), the IL-27 p28-encoding construct (○), or PBS (□) according to the protocol described in the legend to Fig. 1. All rats were monitored for clinical manifestation of disease by observers blind to the experimental protocol. Results are shown as mean clinical score \pm SE of six rats per group. Rats treated with IL-27 p28-encoding DNA vaccine developed a significantly less severe disease than each of the other groups ($p < 0.001$). Histological evaluation (B) was conducted 30 days after disease induction. At this time, joint samples from a naive rat (a, f, and k) or from AA rats treated with PBS (b, g, and l), pcDNA3 alone (c, h, and m), β -actin-encoding construct (d, i, and n), or IL-27p28-encoding construct (e, j, and o) were subjected to histological analysis (12 sections for each group). Arrows indicate cartilage (c), trabeculae (t), and synovial membrane (s).

amplification occurred very rapidly, within the first 24 h after administering the plasmid, as if it amplifies a pre-existing response (Fig. 1C). The specificity of our DNA vaccination-based anti-IL-27 Abs was verified by Western Blot analysis showing that they bind IL-27 but not IL-12, IL-18, or IFN- γ (Fig. 1D). We then verified by ELISA that our Abs do not bind other mouse type I cytokines including IL-2, IL-3, IL-4, IL-5, IL-6, IL-15, GM-CSF, and G-CSF (BD PharMingen, San Diego, CA). It should be noted that all gene products that were used in this experiment and throughout the article are commercially available murine cytokines. Therefore, we do not exclude the possibility that Abs that can potentially bind rat, and not mouse cytokines, are being produced.

The DNA-vaccinated AA rats were monitored for the development and progression of disease by an observer blind to the experimental procedure. Fig. 2 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 ± 1.2 , 12 ± 1 , and 11.25 ± 1 , respectively, Fig. 2A). By contrast, rats subjected to the administration of the IL-27 p28-encoding construct developed a significantly reduced form of disease (mean maximal score of 6.5 ± 0.5 , $p < 0.001$ compared 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 30, 2.25 ± 0.5 compared with 7.5 ± 0.6 , 8.5 ± 0.6 , and 8 ± 1 , and $p < 0.001$). Clinical scoring was also verified by measuring the changes in the degree of paw swelling, once again by an observer blind to the experimental procedure. The IL-27 p28-DNA-vaccinated rats exhibited a marked reduction in paw 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 day 30 (Fig. 2B) and were screened for histological in-

flammatory mononuclear cell infiltrate in the synovial membrane, thickness of the synovial lining, cartilage destruction, and periosteal new bone formation. Sections obtained from IL-27 p28 DNA-vaccinated rats displayed a marked reduction in each of the above parameters as compared with control and pcDNA3-treated AA rats. Thus, IL-27 p28-encoding DNA vaccine can serve as a powerful tool to prevent the inflammatory process during the acute and chronic phases of AA (Fig. 2).

Finally, a comparative analysis of all 18 rats that were subjected to IL-27-encoding DNA vaccine (three experiments) revealed that the therapy significantly increased the Ab titer against IL-27 in 16 rats (88%). The remaining rats (12%) that did not exhibit a significant increase in this Ab titer continued to develop severe AA. This may suggest that anti-IL-27-neutralizing Abs produced in DNA-vaccinated rats manifest a significant role in the regulation of the disease.

Anti-IL-27 p28 Abs developed in DNA-vaccinated rats transfer the beneficial effect of the vaccine and suppress ongoing severe AA

Purified Abs (IgG fraction, protein G purification followed by CNBr purification) were obtained from protected donors (Fig. 2A) and evaluated for their competence to suppress ongoing AA (Fig. 3). It should be noted that Ab transfer was conducted after the neutralizing competence of these Abs has been verified as described in the legend to Fig. 4. Beginning at the onset of AA (day 10), rats were challenged (i.v.) with 300 μ g of each of our DNA vaccination-based anti-IL-27 p28-specific Abs. Control rats were injected with either PBS, IgG from naive rats, or IgG from AA rats administered with an empty vector (data not shown). Repeated administration of IL-27 p28-specific Abs from protected rats led to a marked reduction in disease severity as compared with all control

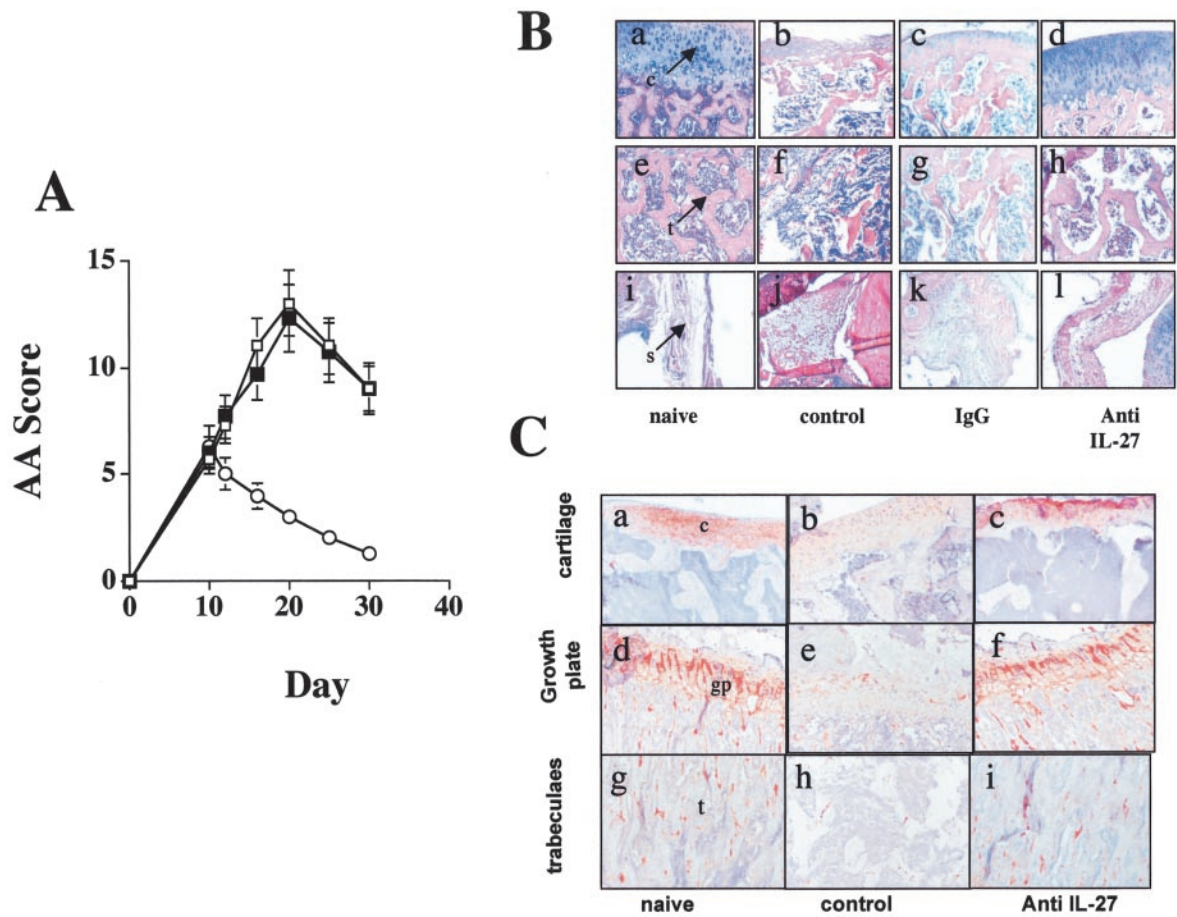


FIGURE 3. Anti-IL-27 p28 Abs produced by DNA vaccination provide protection from severe AA. Lewis rats were subjected to immunization with CFA to induce active AA and at the onset of disease were separated into four groups of equally sick rats (10/group), 6 of which were monitored for clinical manifestation of disease (A) and the others were sacrificed for histological evaluation as shown in B and C. These rats were challenged (i.v.) every other day either with 300 μ g of purified anti-IL-27 p28 Ab (\circ), IgG from naive rats (\square), PBS (\blacksquare), or IgG from AA rats treated with an empty vector (data not shown). An observer blind to the experimental procedure scored AA daily. Results are shown as mean clinical score of six rats in each group \pm SE. Mean maximum AA score of rats treated with anti-IL-27 p28 Ab was significantly lower than that of all of the other groups ($p < 0.001$) that showed no difference between each other. Histological evaluation (B) was conducted 30 days after disease induction. At each time, joint samples from naive rats (a, e, and i), AA rats treated with PBS (b, f, and j), IgG from naive rats (c, g, and k), or IL-27 p28 Abs from DNA vaccination (d, h, and l) were subjected to histological analysis (12 sections each group). Arrows indicate cartilage (c), trabeculae (t), and synovial membrane (s). C, Immunohistochemistry of collagen type II (red) in the extracellular matrix of cartilage and bone zones (c, articular cartilage; gp, growth plate; and t, trabeculae) in AA rats treated with PBS (b, e, and h) in comparison to those treated with anti-IL-27 p28 (c, f, and i). Expression of collagen type II in naive rats is also presented (a, d, and g).

groups, which did not differ from each other (day 20, mean maximal score of 2.25 ± 0.7 compared with 9.5 ± 1.6 , 10 ± 1.4 , 10 ± 0.7 , and 10.5 ± 1.4 and 10 ± 1.4 in AA rats treated with either PBS, or IgG from naive rats, or IgG from AA rats treated with an empty vector, $p < 0.001$ for the comparison with each of the control groups; Fig. 3A). Clinical scoring was also verified histologically (Fig. 3B). Type II collagen is a major component of the cartilaginous matrix. Its secretion by chondrocytes indicates a normal process of cartilage differentiation. To further characterize the beneficial effect of anti-IL-27 p28 Abs on the cartilage of treated rats, the thickness and morphology of type II collagen were examined in sections obtained from treated and control rats. Collagen type II levels were localized immunohistochemically using mouse anti-collagen type II. Fig. 3C shows that in control (b) and IgG-treated AA rats (data not shown) the expression of collagen type II is decreased in comparison to that of naive rats (a). The administration of anti-IL-27 p28 Abs reversed this effect. In fact, a very similar expression of collagen type II was demonstrated by immunohistochemistry (red color) in the extracellular matrix of cartilage and bone zones (c, articular cartilage; gp, growth plate; and t, trabeculae) of naive and treated rats (Fig. 3C).

Protective administration of anti-IL-27 Abs suppresses proinflammatory cytokine production and Ag T cell reactivity

We determined the possibility that the adoptive transfer of anti-IL-27 p28 Abs suppresses AA by altering the cytokine production by Ag-specific T cells. Rats immunized with CFA to induce active AA were treated on days 6, 8, and 10 with our anti-IL-27 p28 Abs, PBS, or normal rat IgG. On day 11, spleen cells were stimulated in with 100 μ M heat-killed MT. After 72 h of stimulation, supernatants were assayed for the protein level of different cytokines including IFN- γ , TNF- α , and IL-4. These primary spleen cells were also evaluated for their ability to mount an Ag-specific proliferative response. Our results clearly show that Ag-specific T cells from rats treated with protective anti-IL-27p28 Abs mounted a significantly lower proliferative response to MT ($6,150 \pm 470$ compared with $11,300 \pm 650$ with backgrounds of $\sim 2,000$, $p < 0.001$) and also produced a significantly lower level of IFN- γ (120 ± 5 pg/ml compared with 230 ± 10 pg/ml, $p < 0.001$) and TNF- α (60 ± 4 pg/ml compared with 125 ± 12 pg/ml, $p < 0.001$). One possibility is that anti-IL-27 Abs block the in vivo activation of naive T cells in response to MT, and thus reduce the relative

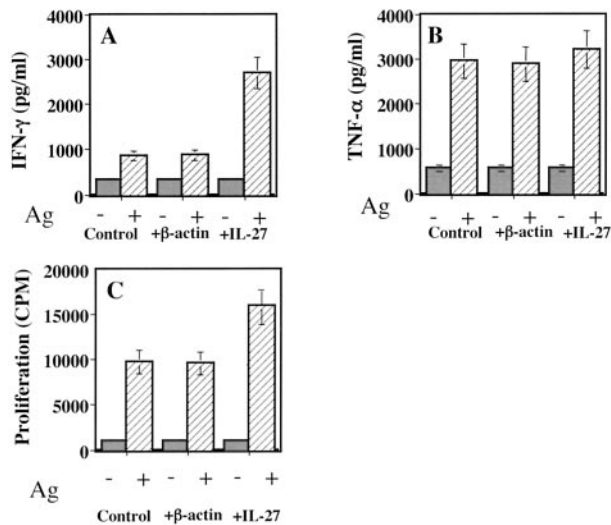


FIGURE 4. IL-27 p28 elicits the proliferative response and IFN- γ production by primary T cells responding to their target Ag. Lewis rats were injected with CFA to induce active AA. Ten days later, draining inguinal lymph nodes were isolated and cultured with MT. Cultures were supplemented with IL-27 p28 (10 ng/ml), soluble β -actin, or medium and 72 h later levels of IFN- γ , TNF- α (pg/ml), and proliferative response (cpm) were determined. Results are shown as mean triplicates \pm SE.

number of primed Ag-specific T cells and the proinflammatory cytokines they produce. Alternatively, IL-27 may also act on activated/memory T cells to increase their Ag-specific proliferative response as well as IFN- γ production. The paragraph below explores this possibility.

IL-27 acts on activated/memory CD4⁺ Th1 cells to increase their IFN- γ , but not TNF- α production

Neutralization of IL-27 p28 that suppressed ongoing AA led to a reduction in TNF- α production (Fig. 5B). Two different potential mechanisms could contribute to this significant reduction. One possibility is that neutralizing IL-27 directly affects TNF- α production by Ag-specific T cells. Another possibility is that by blocking the inflammatory process, neutralizing IL-27 suppresses TNF- α production. After all, during the course of disease, the production of this cytokine by various types of cells, particularly macrophages, is dependent on the progression of the inflammatory process (27, 28). To detect whether neutralizing IL-27 directly affects TNF- α production by Ag-specific T cells, Lewis rats were injected with CFA to induce active AA. Ten days later, draining inguinal lymph nodes were isolated and cultured with MT. Cultures were supplemented with IL-27 p28, soluble β -actin, or medium, and, 72 h later, levels of IFN- γ , TNF- α , and proliferative response were determined. Figure 4 clearly shows that IL-27 p28 elicits a substantial increase in IFN- γ production ($2,690 \pm 230$ compared with 860 ± 120 , $p < 0.001$), but not TNF- α production,

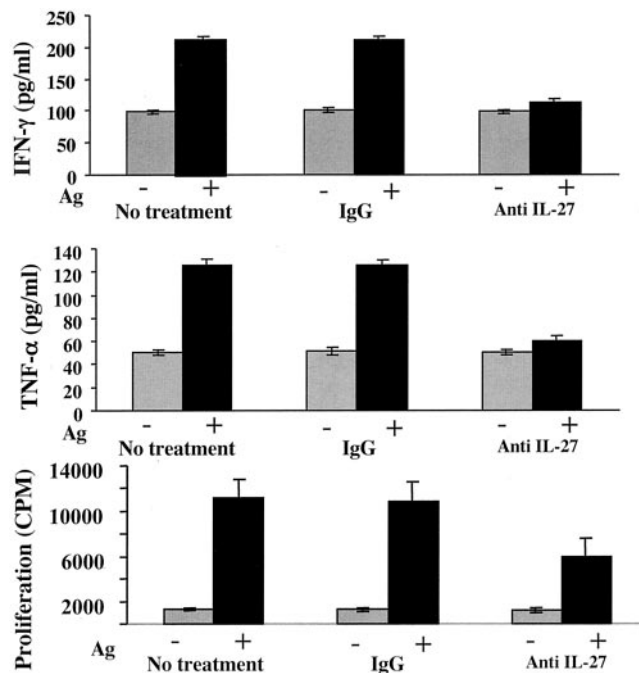


FIGURE 5. Neutralizing IL-27 p28 affects the in vivo cytokine production of T cells. Groups of three rats were immunized with CFA to induce active AA. On days 6, 8, and 10, these rats were treated with anti-IL-27 p28 Ab, PBS, or normal rat IgG. On day 11, popliteal and inguinal lymph node cells were cocultured with MT. Levels of IFN- γ , TNF- α , and IL-4 (data not shown) were then determined by ELISA. Levels of IL-4 were very low (< 10 pg/ml) in all cultures (data not shown). Results are shown as mean of triplicates \pm SE. At the same time, the proliferative response to MT was also recorded. Results are shown as mean cpm of triplicates \pm SE.

and that this increase is accompanied by a significant increase in the proliferative response of these cells ($15,960 \pm 270$ compared with 940 ± 110 , $p < 0.01$). To detect the possibility that IL-27 also acts on long-term activated/memory T cells, we determined the effect of IL-27 on the proliferative response and cytokine production of our long-term encephalitogenic memory/effector (Th1) CD4⁺ T cell line (29). This line was cultured in the presence of our rIL-27 p28, or recombinant β -actin, that was constructed under the same conditions. The addition of rIL-27 p28 could very effectively increase IFN- γ production by these cells ($1,130 \pm 75$ pg/ml compared with 520 ± 40 , $p < 0.001$). Our anti-IL-27-neutralizing Abs could successfully reverse this effect (Table I). Control IgG had no effect on this response (data not shown). Thus, IL-27 can act as a potent proinflammatory mediator not only on naive cells undergoing pro-Th1 polarization (13), but also on T cells that have previously been polarized into Th1. The addition of IL-27 also significantly increased their proliferative response (Table I, $\sim 60\%$ increase, $p < 0.005$), but had no effect on TNF- α production by these cells (Table I). As was expected, IL-4 was produced at very

Table I. IL-27 elicits proliferation and IFN- γ production by myelin basic protein-specific CD4⁺ T cell line

	+Ag				
	No Ag	+ β -Actin (50 ng/ml)	+IL-27 (50 ng/ml)	+IL-27 + Anti-IL-27 Ab	
IL-4 (pg/ml)	12.7 \pm 3.5	25 \pm 7.8	23 \pm 6	30 \pm 5	27 \pm 6
IFN- γ (pg/ml)	452 \pm 25	520 \pm 40	490 \pm 50	1,130 \pm 75	480 \pm 60
IL-10 (pg/ml)	12 \pm 5	12 \pm 4	11 \pm 3	12 \pm 3	10 \pm 4
TNF- α	295 \pm 35	460 \pm 80	450 \pm 70	485 \pm 50	480 \pm 40
Proliferation (cpm)	880 \pm 240	8,800 \pm 930	8,950 \pm 740	14,580 \pm 720	8,300 \pm 890

low levels by these Th1 cells. The addition of IL-27 did not increase the production of this cytokine by these cells. Taken together these results show, for the first time, that the function of IL-27 is not limited to naive T cells. This can explain, in part, why neutralizing IL-27 rapidly and effectively suppresses an ongoing inflammatory autoimmune disease (Figs. 2 and 3).

Discussion

The current study shows that during the course of experimentally induced RA, the immune system mounts a significant Ab titer against IL-27 p28 and that a targeted DNA plasmid encoding this gene product amplified this autoantibody titer and suppressed ongoing disease. Adoptive transfer experiments showed that, indeed, anti-IL-27-based Ab therapy suppresses ongoing disease and that the mechanism for this suppression could be the reduction in the proliferative response of Th1 cells accompanied by a marked reduction in IFN- γ and TNF- α production. We also show that IL-27 affects not only naive T cells undergoing Ag-specific activation, but also effector/memory Th1 cells.

Our laboratory had previously shown, in numerous articles, that in experimentally induced autoimmune disease, the immune system selectively elicits a beneficial autoantibody response to key inflammatory mediators that are involved in the regulation of these diseases and that targeted DNA vaccines can amplify these responses (11, 23–25, 30–33). Others have recently adopted this strategy to define the role of IL-18 in the regulation of systemic lupus erythematosus (34). In a very recent study, we showed that this type of beneficial autoimmune response also participates in the regulation of RA. Thus, patients suffering from RA, but not osteoarthritis, display a significant autoantibody response to TNF- α . Anti-TNF- α Abs suppress RA but display no beneficial effect on osteoarthritis (23). Moreover, the exclusion of this response in AA rats led to the manifestation of a very severe form of disease that could be attenuated by a replacement therapy of anti-TNF- α neutralizing Abs (23).

DNA vaccines encoding proinflammatory mediators have the potential capacity to augment autoimmunity. After all, if the entire gene product of each vaccine is produced at the site of injection, it could function, at the protein level, to promote the inflammatory process. We have recently shown that during AA the immune system mounts a beneficial autoimmune response against several, but not all, proinflammatory cytokines/chemokines and that administration of DNA plasmids encoding these gene products rapidly elicits autoantibody response that not only suppress the function of the functional protein produced by the plasmid itself, but also the natural mediator that participates in the initiation and progression of disease (23). In contrast, administration of a DNA vaccine encoding a regulatory cytokine, such as IL-4, during inflammation would not elicit anti-IL-4 immunity. Under these conditions, the low amount of IL-4 produced at the site of injection would be functional (35). The rapid production of autoantibodies against IL-27 p28 in DNA-vaccinated AA rats (within 24 h) may explain why the administration of this particular plasmid results in the neutralization of IL-27 rather than generation of a functional gene product.

Amplification of beneficial autoimmunity holds a highly significant therapeutic potential. Hence, due to regulatory considerations, its implantation in human diseases has to be carefully considered. We believe that this therapeutic approach would be applicable for chronic autoimmune diseases after the long-term consequences of Ab therapy against the relevant target will already be explored in clinical trials. It should be noted that amplification of beneficial immunity to IL-27 could also be potentially achieved

by immunizing patients against modified versions of IL-27 at the protein level.

Two lines of evidence question the relevance of IL-27 as a target for suppressing ongoing inflammation: 1) The interaction of IL-27 with its receptor, WSX-1, was shown to promote Th1 differentiation in naive, but not activated, CD4⁺ T cells (13). Yet, in our study we injected anti-IL-27-neutralizing Abs after the onset of disease, in which Ag-specific T cells have already been primed (in vivo). 2) In a very recent study conducted in mice lacking the IL-27 p28 receptor (WSX-1^{-/-} mice), it was found that the absence of this particular receptor did not reduce the competence of these mice to provoke an inflammatory response against *Toxoplasma gondii* (36). Another recent study also disagrees with the role of IL-27 in the differentiation of nonpolarized T cells into Th1 (37). In contrast, Nagai et al. showed that IL-27 does promote Th1 polarization in humans (38). Our results clearly show that neutralizing the p28 subunit of IL-27 exerts an anti-inflammatory effect (Fig. 5) that leads to a rapid suppression of an inflammatory autoimmune disease of the joints. We clearly show that IL-27 also acts on primed T cells (Fig. 4), including long-term memory/effector Th1 cells, to promote Ag-specific proliferation and IFN- γ production (Table I). This could explain, in part, its key role in the regulation of autoimmune diseases and may also suggest a novel way for treating RA and possibly other T cell-mediated autoimmune diseases.

We show in the current article that IL-27 affects the proliferative response and IFN- γ , but not TNF- α production by Th1 cells (Fig. 4 and Table I). Yet its in vivo neutralization markedly reduced the ex vivo production of this cytokine (Fig. 5), and by doing so rapidly suppressed the disease (Fig. 3). At the autoimmune site, TNF- α is produced by Th1 and by macrophages. It is believed that Ag-specific Th1 cells interacting with their target autoantigen attract monocytes to the autoimmune site. Upon activation, these cells become the major TNF- α producing inflammatory cells there (39, 40). Their elimination could therefore rapidly suppress these diseases (40). It is possible that anti-IL-27 therapy that does not directly affect TNF- α production by Th1 cells alters their ability to attract/activate inflammatory macrophages, and thereby reducing TNF- α production (Fig. 5) and suppressing AA (Fig. 3).

Finally, even though alteration of the Th1/Th2 balance to Th2 suppresses various experimentally induced Th1-mediated autoimmune diseases, including arthritis (9–12), it is not clear what contributes more to this beneficial effect of this shift. Is it the increased relative number of Ag-specific Th2 cells that produce IL-4 and IL-13? Or is the reduced relative number of Ag-specific inflammatory Th1 cells by itself sufficient to suppress inflammation? The current study shows that anti-IL-27 therapy does not significantly select Th2 cells, but rather reduces the relative number of Ag-specific inflammatory T cells, further suggesting that suppression of Th1 function would be sufficient for suppressing AA and possibly RA.

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