Beneficial Autoimmunity to Proinflammatory Mediators Restrains the Consequences of Self-Destructive Immunity

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Summary

Therapies that neutralize the function of TNF-α suppress rheumatoid arthritis (RA) but not osteoarthritis (OA). We show that patients suffering from RA but not OA have significant levels of autoantibodies directed to TNF-α. Thus, the immune system can selectively generate autoimmunity to proinflammatory mediators when such a response is beneficial for the host. A well-defined model of RA was used to elaborate the contribution of beneficial autoimmunity to the regulation of disease. We show that during the disease autoantibody production is elicited against few inflammatory, but not regulatory, mediators. Selective amplification of these beneficial antibodies by targeted DNA vaccines provided protective immunity. Epitope mapping revealed that anti-TNF-α immunity is highly restricted and excretes no crossreactivity to other known gene products. Its selective exclusion substantially exacerbated the disease. Administration of anti-TNF-α antibodies could then override this aggravation. This substantiates the significance of beneficial autoimmunity in restraining self-destructive immunity.

Introduction

T cell-mediated autoimmune diseases result from a breakdown of tolerance to self-components such as myelin proteins in multiple sclerosis, β-islets components in type I diabetes, or cartilage in rheumatoid arthritis (RA). In all of these diseases, proinflammatory cytokines and chemokines are believed to play a pivotal role in the attraction of leukocytes to the site of autoimmunity and in the initiation and progression of the inflammatory process. Thus, proinflammatory cytokines and chemokines and their receptors constitute an important target for therapeutic intervention (Feldmann et al., 1996; Huang et al., 2000; Izikson et al., 2000; Wildbaum et al., 1998). Karpus and colleagues first demonstrated the critical role of chemokines in autoimmunity, using antibodies to the C-C chemokine, MIP-1α, to suppress experimental autoimmune encephalomyelitis (EAE) (Karpus et al., 1995). Subsequently, Gong et al. suppressed experimentally induced arthritis using an antagonist to another C-C chemokine, MCP-1 (Gong et al., 1997).

We have explored the use of naked DNA vaccination in order to induce a beneficial autoimmune response against endogenous proinflammatory mediators, in the context of two different autoimmune diseases: EAE, a model for multiple sclerosis (MS), and adjuvant-induced arthritis (AA), a model for RA (Wekerle et al., 1994). Our approach entails immunization with a mammalian expression vector, encoding a selected proinflammatory cytokine or chemokine, under the control of a strong viral promoter (CMV). The same vector also drives the expression of bacterial CpG as a recurring immunostimulatory sequence (ISS) (Raz et al., 1996; Sato et al., 1996), which signals via TOLL receptor 9 (Finotto et al., 2002; Wen-Ming et al., 2000), triggering a breakdown of tolerance and establishing an immune response against gene products encoded by the vaccines. We demonstrated that “vaccination” via administration of plasmid DNA constructs encoding various C-C chemokines followed by later induction of EAE elicited antibody production against the chemokines, which were encoded by these genes used for “vaccination,” and at the same time suppressed the disease (Youssef et al., 1998). Chemokine-specific purified antibodies isolated from these protected animals could then be used to transfer the beneficial effects of each vaccine (Youssef et al., 1998). Later, we showed that this strategy could be used to rapidly treat ongoing autoimmunity and that neutralizing antibodies generated in response to the gene product of each vaccine can adaptively transfer the high state of resistance (Salomon et al., 2002; Wildbaum and Karin, 1999; Wildbaum et al., 2002a, 2000b; Youssef et al., 2000, 1998).

Throughout these studies, we have repeatedly observed some unexpected phenomena. The elicitation of beneficial autoantibody production was dependent on induction of an autoimmune disease and was regulated by the immune system in accordance with disease progression. Thus, autoantibody production against the vaccine-encoded product regressed to baseline levels shortly after remission in acute EAE, whereas in chronic AA these antibodies were continuously produced, at a very high titer (Salomon et al., 2002; Wildbaum and Karin, 1999; Wildbaum et al., 2002a, 2000b; Youssef et al., 2000, 1998). Moreover, therapy of ongoing diseases led to a very rapid (within less than 48 hr) production of highly specific beneficial autoantibodies (IgG2a) that could effectively transfer disease resistance (Salomon et al., 2002; Wildbaum and Karin, 1999; Wildbaum et al., 2002a, 2000b; Youssef et al., 2000, 1998). The most reasonable explanation for these unexpected findings is that targeted DNA vaccines amplify a preexisting anti-self-regulatory response, which, by itself, is capable of limiting, albeit not preventing, the emerging autoimmune condition. The current study uses a variety of complementary strategies to explore this hypothesis.
Figure 1. Beneficial Autoimmunity to Proinflammatory but Not Regulatory Mediators Participates in the Regulation of AA

(A) Lewis rats were immunized with CFA to induce active AA and separated into 14 groups of three rats each. On days 17 and 19, they were injected with plasmid DNA encoding MIP-1α, MIF, IP-10, MCP-1, TNF-α, IL-15, IL-17, IFN-γ, IL-4, IL-10, IL-18BP, or FasL. Sera were collected on day 22, and the development of antibody titer to the relevant gene product of each vaccine was determined, as described in Experimental Procedures, in vaccinated (black bars) or control AA rats (gray bars). These titers were also recorded in sera of rats immunized with CFA to hind footpads for the induction of a local inflammatory response rather than polyarthritis (striped bars) and in sera from naive rats (white bars). Results are shown as the mean of three samples ± SE.

(B) A group of Lewis rats was subjected to active induction of AA. On day 17, the rats were divided into seven groups of nine equally sick rats and subjected to the administration of naked DNA vaccines (days 17 and 19, 300 μg/rat) using DNA constructs encoding MIF (open circles), IP-10 (filled diamonds), TNF-α (filled circles), IL-15 (filled triangles), or IL-17 (open triangles), as described (Wildbaum et al., 2000b). Control groups were injected with a plasmid DNA construct encoding soluble intracellular β-actin (filled squares) (Wildbaum et al., 2000b) or were not treated (open squares). The clinical manifestations of disease were monitored by an observer blind to the experimental protocol as described before (Wildbaum et al., 2000b). Clinical scores were also histologically verified (not shown). Results are shown as the mean score of nine rats ± SE.

(C) AA rats were subjected to plasmid DNA therapy as in Figure 1B (days 17 and 19, 300 μg/rat). Each group included 30 rats. On day 22, all rats were sacrificed and blood sera were purified in two steps: first on an IgG purification column and then on CNBr columns loaded with the gene product of each vaccine. Each purified antibody was adoptively transferred to AA rats (100 μg/rat every other day beginning at the onset of disease) as follows: anti-MIF (open circles), anti-IP-10 (filled diamonds), anti-TNF-α (filled circles), anti-IL-15 (filled triangles), or anti-IL-17 (open triangles). Control rats were injected with IgG purified from β-actin plasmid-vaccinated rats (filled squares), or from untreated rats (open squares). An observer blind to the experimental protocol monitored the clinical manifestations of the disease. Results are shown as the mean score of six rats ± SE.

Results

Beneficial Autoimmunity to Proinflammatory but Not Regulatory Mediators Participates in the Regulation of AA

We followed the development of autoantibody production to various proinflammatory and regulatory mediators during AA and the ability of targeted DNA vaccines to amplify each potential response (Figure 1). We show that during the course of adjuvant arthritis, there are autoantibody responses only to proinflammatory mediators including: MIP-1α, MIF, IP-10, MCP-1, TNF-α, IL-15, and IL-17 (Figure 1A, log2Ab titer of 14.3 ± 0.26, 16 ± 0.45, 14.3 ± 0.26, 13 ± 0.45, 16 ± 0.45, 14.3 ± 0.26, and 14.3 ± 0.26, respectively, compared to control sera from naive donors: log2Ab titer of 6.6 ± 0.26, 6.6 ± 0.26, 6.6 ± 0.26, 6.6 ± 0.26, 6.6 ± 0.26, and 6 ± 0, p < 0.01 for each reciprocal comparison). The baseline titer of naive rats to each of the detected gene products remained at baseline levels (log2Ab titer of 5–6). Plasmid DNA vaccines encoding proinflammatory mediators could not elicit anti-self responses under these conditions (log2Ab titer of 5–6 sera to all cytokines/chemokines). These data imply that DNA vaccines encoding self-proinflammatory mediators can rapidly amplify but not generate autoantibody responses.

Following base of the tail immunization of heat-killed Mycobacterium tuberculosis emulsified in oil, Lewis rats develop a long-term form of polyarthritis in all limbs, whereas the administration of the same adjuvant to footpads leads to a local DTH response (Pearson, 1956). To determine whether elicitation of a high antibody titer to proinflammatory mediators could be associated with the development of the autoimmune condition, we have compared titers that developed in AA rats to those appearing following immunization of CFA in hind footpads. The respective Ab titers in AA rats were significantly higher than the corresponding titer in rats immunized
with CFA in hind footpads (p < 0.05). AA rats never mounted a notable response (over background of log2Ab titer = 6) against regulatory cytokines such as IL-10, IL18 binding protein (IL-18BP), and IL-4 (Figure 1A). Even the response to IFN-γ that plays a pleiotropic role in the regulation of autoimmunity (Krakowski and Owens, 1996) was at background levels. Moreover, the immune response was different toward two members of the same gene family: TNF-α, a key proinflammatory mediator, and Fas-L, that plays a dual role in the regulation of autoimmunity (Suvannavej et al., 2000; Wildbaum et al., 2000a). An autoantibody response against TNF-α but not Fas-L was observed (Figure 1A).

**DNA Vaccination to Proinflammatory Cytokines Suppressed Ongoing Arthritis**

Administration of plasmid DNA vaccines encoding each proinflammatory mediator, during an ongoing disease, could very rapidly accelerate autoantibody production to each mediator (Figure 1A, p < 0.01 for each comparison). At the same time, these DNA vaccines suppressed ongoing disease (Figure 1B, Mean Max score of 13.2 ± 1 and 14 ± 0.8 in control groups versus 4 ± 0.5, 4.8 ± 0.6, 6.5 ± 0.9, 6 ± 0.9, and 4.5 ± 0.8 in rats treated with plasmid DNA encoding TNF-α, MIF, IL-15, IL-17, or IP-10, p < 0.01, respectively). Vaccinating AA rats with an empty vector did not lead to a significant amplification of Ab titer against each proinflammatory gene product beyond the one caused by the disease itself (data not shown). These results are consistent with our previous observations (Salomon et al., 2002; Wildbaum and Karin, 1999; Wildbaum et al., 2002a, 2000b; Youssef et al., 2000, 1998).

**Immunization with DNA Vaccine Is Highly Specific**

We checked whether the administration of a DNA vaccine encoding one inflammatory mediator might induce a nonspecific amplification of autoantibody response to other inflammatory mediators. Serum obtained from AA rats treated with TNF-α encoding DNA vaccine (titer of log2 24) was tested for antibody titer to MIP-1α, MCP-1, IP-10, and IL-15. All titers varied between log2 14 and log2 16, which is similar to titers obtained in sera from control AA rats (see Figure 1). This implies that despite the CpG sequence included in these plasmids, the vaccines amplify responses in a specific manner.

**Adoptive Transfer of Autoantibodies Elicited by DNA Vaccines Suppresses Arthritis**

The beneficial effect of each vaccine could be adoptively transferred by autoantibodies that were produced in DNA vaccinated rats (Figure 1C, p < 0.01 for the comparison of each treated group with each of the two control groups). These results, together with our previous observations (Wildbaum et al., 2002a, 2000b; Youssef et al., 2000), strongly suggest that DNA vaccines amplify a regulatory response aimed to restrain the pathological consequences of autoimmunity.

**Beneficial Autoimmunity Is Restricted to Highly Selective Determinants**

To further explore the above hypothesis, we decided to focus on one of these mediators, TNF-α. This proinflammatory cytokine has been preferentially selected because of its key function not only in experimental models of autoimmunity, but also in human RA (Arend et al., 1994; Choy et al., 2002; Feldmann et al., 1997). At first, we mapped determinant that autoantibodies from AA rats, which were or were not subjected to a TNF-α encoding DNA vaccine, bind on TNF-α (Figure 2A). Autoantibodies produced in AA rats bound only 3 out of 24 different fragments that were constructed from the whole cytokine (Figure 2A): Fragment 6 (p145–160, QGCPDYVLLTHTVSR), Fragment 8 (p207–218, GDDLSSLNLPK), and Fragment 11 (p161–178, FAISYQEKVSSLLSAIR). Targeted DNA vaccine encoding TNF-α amplified only the response to these 3 fragments (Figure 2A). The sequence of each peptide fragment was analyzed in the Protein Data Bank (ENTREZ), and we could not find any matching sequence to each of these fragments in any known protein (except for TNF-α). This demonstrates the selective breakdown of tolerance against self and also implies that DNA vaccines indeed amplify a response initiated by the disease itself. It should be noted, however, that using this procedure one could record only binding to linear fragments and that potential conformational epitopes are being excluded. Western blot analysis was then conducted to further corroborate the specificity of DNA vaccine-based anti-TNF-α antibodies. Figure 2B indicates that these antibodies are indeed highly specific for TNF-α and do not bind to other members of the TNF family including TRANCE and Fas L. They also do not bind other proinflammatory mediators such as IL-18 or MIP-1α.

**Selective Inclusion of Anti-TNF-α Immunity Aggravates the Course of AA**

The contribution of anti-TNF-α Ab produced during natural regulation of disease was then evaluated by complementary experiments. At first, the IgG fraction of blood sera obtained from AA rats (peak of disease) was purified on a CNBr-TNF-α purification column and tested for anti-TNF-α neutralizing competence in an in vitro system. Figure 3A shows that these anti-TNF-α Ab could effectively abolish the cytotoxic activity of TNF-α on U937 human T cells (p < 0.001). They were then tested for their competence to suppress AA in adoptive transfer experiments. Figure 3B shows that TNF-α-specific antibodies produced in AA rats (that were not subjected to plasmid DNA therapy) can effectively suppress ongoing AA (mean maximal score of 4.66 ± 1 versus 10.3 ± 1.5 and 10 ± 1 in control groups, p < 0.01). This suggests that these antibodies can potentially be involved in the regulation of disease. To further explore this possibility, we have looked for a way to abolish their in vivo elicitation in a specific manner. Neonatal administration of a self-antigen may result in an inability to mount a significant immune response against it in adult life (i.e., neonatal tolerance) (Gammon et al., 1986). This approach has recently been extended to plasmid DNA vaccines (Mor et al., 1996). We used our TNF-α encoding DNA plasmid to induce neonatal tolerance to its gene product and studied the consequences of this abolition on the development and progression of AA (Figure 4). At first, we looked for a way to obtain a moderate form of disease, which potentially could be aggravated. We found that
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Figure 2. Beneficial Autoimmunity Is Restricted to Highly Selective Determinants

Rat TNF-α (a 237 amino acid protein) was subjected to trypsin proteolysis followed by HPLC purification, resulting in 24 different fragments that comprise the whole protein. Each fragment was sequence-verified, coated on an ELISA plate, and used to map the epitopes recognized by the sera of TNF-α DNA-vaccinated rats. Groups of 3 rats were subjected to active AA induction. On day 18, just before the peak of disease, these rats were (black columns) or were not (striped columns) treated with a TNF-α encoding DNA plasmid. Three days later (day 21), blood serum from each group was tested for its ability to bind each of the different TNF-α fragments described above. Results are shown as mean of triplicates ± SE.

(B) Equal amounts of IL-18 (R&D systems), TNF-α (PeproTech), TRANCE, (R&D systems), MIP-1α (PeproTech), or Fas L (R&D systems) were separated on an SDS-PAGE gel and stained by Coomassie (left) or detected by Western blotting with the sera from AA rats treated with a TNF-α-encoding DNA vaccine (right). Only TNF-α could be detected by the antisera, demonstrating the specificity of the vaccine-induced autoimmune response.

under our working conditions administration of 0.2–0.4 mg of MT in 100 μl oil (rather than 1 mg) leads to the development of a milder form of AA (Figure 4A). Rats that were subjected to a neonatal administration of TNF-α encoding DNA plasmid failed to elicit anti-TNF-α Ab titer during AA (Figure 4B, log2 Ab of 14.2 ± 0.23 in AA rats that were not subjected to neonatal tolerance versus 6.2 ± 0.6 in those neonatally administered with TNF-α encoding DNA vaccine, b versus d, p < 0.01). These rats with impaired anti-TNF-α responses developed an extremely severe form of disease (Figure 4A, mean maximal score of 13.3 ± 0.9 versus 5.3 ± 0.6 in control AA rats). This group mounted similar levels of Ab titers to MCP-1 or IP-10 to those measured in AA rats that were not subjected to neonatal administration of TNF-α encoding DNA (log2 Ab titer of 8 ± 0 each). Thus, abrogation of the competence to elicit antibodies to TNF-α was specific. These experiments imply that autoantibodies to TNF-α contribute to the natural regulation of disease. Replacement therapy with TNF-α-specific antibodies reversed the effect of neonatal tolerance (Figure 4C). Clinical scores were verified histologically. Rats that were subjected to a neonatal administration of TNF-α encoding DNA vaccines exhibited an extremely enhanced in-
Paramount Role of TNF-α in Autoimmune Arthritis

Finally, we have used the system described above to determine whether TNF-α plays a dominant role in the pathogenesis of AA. Lewis rats were subjected to neonatal administration of TNF-α encoding DNA plasmid and were thus tolerized to TNF-α. As a result, these tolerized rats could not mount antituberculosis response to neutralize endogenous TNF-α. Under these conditions, replacement therapy with neutralizing antibodies to MCP-1 could not suppress severe AA induced by suboptimal dose of adjuvant (Figure 8). These antibodies could significantly reduce the severity of AA induced by an optimal dose of adjuvant in control AA rats (Figure 1C). This further substantiates the paramount role of TNF-α in the regulation of autoimmune arthritis.
Figure 4. Selective Exclusion of Anti-TNF-α Immunity Aggravates the Course of AA

During the first 24 hr after birth, groups of six Lewis rats were subjected to a single administration of 10 ng of plasmid DNA encoding TNF-α (filled squares), an empty plasmid (open squares), or PBS (filled circles). Another group was administered with the same TNF-α encoding DNA plasmid at 4 weeks of age (open triangles). At 8 weeks of age, all rats were subjected to the induction of a moderated form of AA and monitored for the development of disease.

(A) The mean clinical score of the rats, observed in one of three experiments done under the same conditions with similar results. Results are shown as mean clinical score ± SD.

(B) At the peak of disease in control animals (day 22), sera were obtained from all rats and the TNF-α-specific Ab titer was determined as follows: a, naive rats, no disease; b, AA rats; c, naive rats previously subjected to neonatal administration of TNF-α encoding DNA plasmid; d, AA rats previously subjected to neonatal administration of TNF-α encoding DNA plasmid; e, AA rats subjected to “adult life” administration of TNF-α encoding DNA plasmid. Results are shown as log₂Ab titer ± SE of triplicates.
Beneficial Autoimmunity to Inflammatory Mediators

Figure 6. Replacement Therapy with Anti-MCP-1 Antibodies Cannot Override Exclusion of Anti-TNF-α Immunity

During the first 24 hr after birth, four groups of six Lewis rats were subjected to a single administration of 10 ng of plasmid DNA encoding TNF-α. At 8 weeks of age, these rats were subjected to the induction of moderated AA by a suboptimal dose of CFA. Beginning at the onset of disease, these rats were treated (100 μg/rat every other day) with neutralizing antibodies to TNF-α (open squares), or to MCP-1 (open triangles), or with IgG purified from rats that were treated with an empty plasmid (filled squares), or were not treated (open circles). Naive rats were also subjected to the administration of a suboptimal form of disease (filled circles). The results of one out of two experiments done under the same conditions with similar results are presented as mean clinical score ± SD.

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 even though mRNA encoding proinflammatory mediators is transcribed at the site of injection, for as long as 4 weeks, the actual amount of gene products produced there is extremely low and is probably rapidly neutralized by autoantibodies, like those shown to exist against TNF-α (Wildbaum et al., 2002a). According to our current study, an 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. This may explain, in part, why administration of DNA vaccines encoding a self-autoimmune antigen plus IL-4 leads to the generation of antigen-specific Th2 cells (Garren et al., 2001).

Figure 5. RA Patients but Not Those Suffering from OA Mount Anti-TNF-α Immunity

Blood sera were obtained from 22 patients suffering from RA, 10 patients suffering from OA, and 12 healthy individuals. Synovial fluid was also taken from all RA patients.

(A) The log₂Ab titer of anti-TNF-α of each individual is shown. A one-way ANOVA test (with the Bonferroni correction) showed a significant elevation (p < 0.05) in antibody titer determined in sera of RA patients compared to healthy individuals and those suffering from OA. No significant difference could be observed between the last two groups.

(B) The correlation between the Ab titer to TNF-α developed in blood sera and synovial fluid of the above RA patients is shown.

Discussion

The current study shows that during an autoimmune condition, the immune system mounts a beneficial autoantibody response to proinflammatory mediators. This response counteracts, to a certain extent, the autoimmune pathology. This natural counteraction is illustrated in animal models of autoimmunity, and evidence is provided that it occurs in the human disease rheumatoid arthritis.

(C) Using the same procedure, rats were subjected to TNF-α encoding DNA plasmid neonatally, and to active induction of AA at 8 weeks of age. These rats were then separated into different groups that were or were not administered (every other day beginning at the onset of disease) with anti-TNF-α Ab (IgG fraction of blood sera obtained from control AA rats, purified on a CNBr-TNF-α purification column). The mean maximal clinical score of six rats per group ± SE is shown.

(D) Histological evaluation of the experiment described in (C). Joint samples from naive (a), control AA rats (b), AA rats previously subjected to neonatal administration of TNF-α encoding DNA plasmid (c), and AA rats previously subjected to neonatal administration of TNF-α encoding DNA plasmid and during disease to replacement therapy with anti-TNF-α Ab (d) were subjected to histological analysis (joints from two rats were removed 30 days after the induction of disease; 12 sections per each group were analyzed). Representative samples from each group are presented (×10). The arrowheads point to the synovial lining (b, bones; S, synovial membrane).
We have previously shown that during experimentally induced autoimmune diseases, the immune system mounts a beneficial autoantibody response to proinflammatory mediators (Salomon et al., 2002; Wildbaum and Karin, 1999; Wildbaum et al., 2002a, 2000b; Youssef et al., 2000, 1998). It is an open question, however, whether these findings that were obtained in animal models are relevant to human autoimmune diseases, like RA. We show here (Figure 5) that patients suffering from RA but not osteoarthritis develop beneficial autoimmune responses against such gene products could then be amplified to provide protective immunity.

Perhaps the most surprising finding here is the wide range of anti-cytokine antibodies that are elicited during the disease (Figure 1A). This implies that each of these molecules might be important to development of the full-blown syndrome of adjuvant arthritis. The hierarchical importance of these cytokines and chemokines to which the human immune system mounts “beneficial autoimmunity” as a potential therapy for different autoimmune diseases. It may also provide a tool for identifying important inflammatory mediators in other autoimmune diseases in human. The responses against such gene products could then be amplified to provide protective immunity.

Experimental Procedures

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

Immunizations and Active Disease Induction
Lewis rats were immunized subcutaneously at the base of the tail with 0.1 ml of CFA (incomplete Freund’s adjuvant supplemented with 10 mg/ml heat-killed Mycobacterium tuberculosis H37Ra in oil) (Difco Laboratories Inc., Detroit, MI) and were monitored daily for clinical signs by an observer, blind to the treatment protocol. Severity of clinical signs 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 the entire limb, 4 = severe disease evident in the entire limb. The arthritic clinical score for each animal is the sum of the scores for all four limbs (0–16). In addition, the severity of disease was assessed histologically as described below.

Histopathology
Joints were removed, fixed with 10% buffered formalin, decalcified in 5% ethylenediaminetetraacetic acid in buffered formalin, embedded in paraffin, and sectioned along the midline through the metatar-

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sal region (Bacha et al., 1992). Sections were stained with hematoxylin and eosin and analyzed by a histopathologist who was a blind observer to the experimental procedure. Evaluation was based upon the presence of an inflammatory mononuclear cell infiltrate in the synovial membrane, the thickness of the synovial lining, joint space narrowing, and peristeal new bone formation. The histological score was determined as follows: 0 = no evidence of disease, 1 = mild lymphocytic infiltrate, 2 = widespread mononuclear infiltrate and thickening of the synovial lining, and 3 = severe bone destruction, new bone formation, and destruction of the synovial lining (Bacha et al., 1992).

DNA Vaccination
Plasmid DNA vaccines encoding MIP-1α, MCP-1, TNF-α, and FasL have been previously described (Salomon et al., 2002; Wildbaum et al., 2000a, 2000b; Yousset et al., 2000, 1998). cDNA encoding rat MIF, IL-15, IL-17, IFN-γ, IL-10, IL-4, and IL-18BP was amplified, using primers based on the published sequence of each gene, as follows: MIF sense, 5′-CATGCTTCTGCTTCAAGAAGCGA-3′; antisense, 5′-TCAAGGCAAGTGTGAACCCGC-3′; IL-15 sense, 5′-CATGAAAATTTGAAAACCCAT-3′; antisense, 5′-TCAAGACGTTGGTATGAC-3′; IL-17 sense, 5′-AGCAGCAGCTGATCAAG-3′; antisense, 5′-GGGTTCTCTAGGTCGTA-3′; IL-10 sense, 5′-CATGCTGGCTCAAGGACGA-3′; antisense, 5′-CGTCCCTTCTACTGCTGAT-3′; IL-4 sense, 5′-CATGCGGCTACCCCCACCT-3′; antisense, 5′-TTAGAGACCAGGAGGGACGAGG-3′; IFN-γ sense, 5′-CATGAGGCCTACACCGCGCGTGTGCGT-3′; antisense, 5′-TCAAGACGATTCACTTCTTTCCG-3′; IL-18BP sense, 5′-CATGAGACACTGTGGCTGTGAACACC-3′; antisense, 5′-TCATGCTTGGCTCAGCAC-3′. Each PCR product was sequence-verified and cloned into a pcDNA3 vector (Invitrogen, San Diego, CA). Large-scale preparation of plasmid DNA was performed using Mega prep (Qiagen Inc., Chatsworth, CA). DNA vaccination was performed as we previously described (Yousset et al., 1998). Each DNA vaccine was tested by injection into naive Lewis rats. Four to five days later, RT-PCR was applied on tibialis anterior muscle samples to verify that the relevant insert of each gene is transcribed in the injected muscle.

Production and Purification of Recombinant Gene Products
Each of the PCR-amplified cDNAs described above was recloned previously with the relevant recombinant gene product. Bound antibodies were detected using goat anti-rabbit antibodies from sera (IgG fraction) were loaded on the column, eluted by an acidic elution buffer (glycine pH 2.5).

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

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Evaluation of Ab Titer in Sera Samples
The titer of cytokine- or chemokine-specific antibodies in the sera of AA rats was determined using a direct ELISA assay. ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 ng/well commercially available recombinant rat MIP-1α, rat TNF-α, rat IL-4, rat IL-10, rat MCP-1, rat IFN-γ, murine IP-10, murine IL-15 (PeproTech, Rocky Hill, NJ), murine IL-17, murine IL-18BP, murine FasL, or human MIF (R&D systems, Minneapolis, MN). All results obtained using murine or human gene products were verified using recombinant rat gene products, generated from each cloned product using a POE expression vector as described above. Following blocking of the wells with 1% BSA/PBS, sera samples were added in serial dilutions from 2^−6 to 2^−2 to wells that were or were not coated previously with the relevant recombinant gene product. Bound antibodies were detected using goat anti-rat IgG conjugated to alkaline phosphatase (Sigma) and the soluble substrate, p-nitrophenyl phosphate (p-NPP) (Sigma). Titer was determined by comparing the OD measured in wells coated with the appropriate cytokine or chemokine to those not coated with this recombinant gene product. Results of triplicates were calculated as log₂, antibody titer = SE. As sera were added in serial dilutions from 2^−6 to 2^−2, the baseline of the sensitivity of the test is log₂Ab titer − 6.

CNBr Purification of Cytokine- or Chemokine-Specific Antibody
Recombinant chemokines or cytokines (5 mg) were bound to a CNBr-activated sepharose column according to the manufacturer’s instructions (Pharmacia Biotech, catalog number 17-0820-01). Specific antibodies from sera (IgG fraction) were loaded on the column, eluted by an acidic elution buffer (glycine pH 2.5).


