Interleukin-27 and Interferon-γ Are Involved in Regulation of Autoimmune Arthritis

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Inflammation underlying immune pathology and tissue damage involves an intricate interplay between multiple immunological and biochemical mediators. Cytokines represent the key immune mediators that trigger a cascade of reactions that drive processes such as angiogenesis and proteolytic damage to tissues. IL-17 has now been shown to be a pivotal cytokine in many autoimmune diseases, supplanting the traditional Th1-Th2 paradigm. Also, the dual role of proinflammatory IFN-γ has unraveled new complexities in the cytokine biology of such disorders. A major hurdle in fully understanding the effector pathways in these disorders is the lack of information regarding the temporal kinetics of the cytokines during the course of the disease, as well as the interplay among the key cytokines. Using an experimental model of arthritic inflammation, we demonstrate that the temporal expression of cytokines during the incubation phase is a critical determinant of disease susceptibility. The susceptible rats raised a vigorous IL-17 response early, followed by IFN-γ and IL-27 response in that sequence, whereas the resistant rats displayed an early and concurrent response to these three cytokines. Accordingly, treatment with exogenous IFN-γ/IL-27 successfully controlled arthritic inflammation and inhibited the defined mediators of inflammation, angiogenesis, cell survival, apoptosis, and tissue damage. Furthermore, IFN-γ enhanced IL-27 secretion, revealing a cooperative interplay between the two cytokines. Our results offer a novel immunobiochemical perspective on the pathogenesis of autoimmune arthritis and its therapeutic control.

Inflammation is a characteristic feature of a variety of autoimmune diseases (e.g. rheumatoid arthritis and multiple sclerosis) resulting from dysregulation of the immune responses. Both genetic and environmental factors play a role in the initiation and propagation of autoimmune inflammation (1–9). Experimental models of inflammatory autoimmune diseases (10–13) are an invaluable resource for examining critical aspects of disease pathogenesis that are difficult to test in patients, e.g. the temporal cytokine profiles at different phases of the disease and the in vivo regulation among the immunological and biochemical mediators involved. To gain insights into how the newer cytokines as well as established cytokines with a dual action (14–17) modulate the mediators of inflammation and tissue damage in autoimmune arthritis, we investigated the antigen-specific cytokine profiles at different phases of adjuvant-induced arthritis (AA),2 an animal model of human rheumatoid arthritis. Also examined was the interplay among IL-17, IFN-γ, and IL-27 in vivo as well as in vitro. AA serves as an experimental model for rheumatoid arthritis, and it can be induced in Lewis rats (RT.1b) by subcutaneous injection of heat-killed Mycobacterium tuberculosis (Mtbb) H37Ra. Lewis rats are highly susceptible to AA (18), whereas the major histocompatibility complex-compatible Wistar Kyoto (WKY) rats are resistant to disease induction following an arthritogenic challenge. However, WKY rats are as capable as Lewis rats in raising T cell proliferative response to the disease-related antigens (19), raising the possibility that qualitative differences such as cytokine responses contribute to the differential outcome of the pathogenic events in these two rat strains.

IL-17, IL-23, and IL-27 represent relatively newer cytokines in infection and autoimmunity (20–24). IL-17 is considered to be a pathogenic cytokine that plays a critical role in certain autoimmune diseases (20, 25), and IL-23 is important for the expansion of Th17 cells (26, 27). The transcription factors retinoic acid receptor-related orphan receptor (ROR)-γt and STAT3 are vital for Th17 differentiation and IL-17 secretion (23, 28, 29). Unlike IL-23, IL-27 is a negative regulator of Th17 cells (30, 31). IL-27 belongs to the IL-12/IL-23 family and is a heterodimeric cytokine composed of p28, an IL-12p40-related subunit, and EBI3 (Epstein-Barr virus-induced gene 3), an IL-12p40-related subunit (32). IL-27 is produced by activated antigen-presenting cells, macrophages, and fibroblasts. IL-27 plays a very important role in infection and some autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE). However, the precise role of IL-27 in promoting or suppressing autoimmune inflammation has yet to be resolved (33–37). Both proinflammatory and anti-

2 The abbreviations used are: AA, adjuvant-induced arthritis; Mtbb, M. tuberculosis; WKY, Wistar Kyoto; ROR-γt, retinoic acid receptor-related orphan receptor-γt; Inc, incubation; Ons, onset; HBSS, Hanks' balanced salt solution; LNC, lymph node cell(s); SAC, spleen-adherent cell(s); SIC, synovium-infiltrating cell(s); FLS, fibroblast-like synoviocyte(s); MMP, matrix metalloproteinase.

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inflammatory roles of IFN-γ in autoimmune models have been reported (14, 16, 38–40), and the precise interaction between IFN-γ and IL-27 is not defined.

Our results demonstrate a regulatory anti-inflammatory role of IL-27 and IFN-γ in AA, as well as a novel cooperative interaction between IFN-γ and IL-27 such that IFN-γ up-regulates IL-27. These cytokine-driven events in turn influence the key biochemical mediators of inflammatory arthritis. These results are of significance in advancing the mechanistic understanding of the pathogenesis of autoimmune arthritis.

EXPERIMENTAL PROCEDURES

Animals

Lewis (LEW/Hsd, RT.11) and Wistar Kyoto (WKY/NHsd, RT.11) rats were purchased from Harlan Sprague-Dawley and housed in the vivarium of the University of Maryland School of Medicine (Baltimore, MD). Male rats (4–6 weeks old) were used in this study, and these rats were treated according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Induction and Evaluation of AA

Lewis rats were immunized subcutaneously at the base of the tail with 1 mg/rat heat-killed Mtb H37Ra (Difco, Detroit, MI) suspended in oil (Sigma). Thereafter, these rats were observed regularly and graded for the severity of arthritis on the basis of erythema and swelling of the paws on a scale of 0–4 as described previously (19, 41). Different phases of AA were labeled as follows: incubation (Inc), onset (Ons), peak, and recovery phase. In most of the experiments, these phases corresponded to the day after Mtb immunization as follows: early incubation, day 3; late incubation, day 5; Ons, day 9; peak, day 18; and recovery, day 24.

Testing the Effect of Cytokine Treatment in Vivo on AA in Lewis Rats

Before Onset of AA—Lewis rats were immunized with Mtb, and 3 days later, subgroups of these rats received two to three injections (intraperitoneal) on alternate days of a particular cytokine, namely IL-17 (3 μg/rat; Shenandoah Biotechnology Inc.), IFN-γ (3 μg/rat; PeproTech), or IL-27 (2 μg/rat; R&D Systems). Control rats received equal amount of PBS instead of the cytokine.

After Onset of AA—Mtb-immunized rats were injected with IFN-γ (3 μg/rat) or IL-27 (2 μg/rat) after the onset of arthritis. Control rats were injected with PBS. Thereafter, all rats were observed regularly for signs of arthritis, and the disease was graded as described above.

Preparation of Different Types of Cells and Their in Vitro Restimulation with Antigen and/or Cytokines

Lymph Node Cells (LNC)—Mtb-immunized Lewis rats were killed at different phases of AA, and their draining lymph nodes (para-aortic, inguinal, and popliteal) were harvested. For comparison, LNC of WKY rats immunized with Mtb were harvested at the time points corresponding to different phases of AA in Lewis rats.

Thereafter, a single-cell suspension of LNC was prepared, and the cells were washed three times with Hanks’ balanced salt solution (HBSS; Invitrogen). These LNC were cultured (2.5 × 10^6 cells/well) in a flat-bottomed 96-well plate in HL-1 serum-free medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mm l-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate in an atmosphere of 95% air and 5% CO2. The cells were restimulated with antigen for different time points (6–72 h) (supplemental Fig. S1) in the presence or absence of a cytokine. In some experiments, culture supernatants of LNC were collected for analysis by Multiplex assay (University of Maryland Baltimore Cytokine Core Facility).

Spleen-adherent Cells (SAC)—Spleens were harvested from arthritic Lewis rats at different phases of AA. For comparison, spleens of WKY rats immunized with Mtb were harvested at the corresponding time points. Thereafter, a single-cell suspension of spleen cells was prepared, and the cells were washed three times with HBSS. These spleen cells were cultured in 60 × 15-mm culture dishes (Corning, Inc., Corning, NY) at 37°C in RPMI 1640 medium supplemented with 5% FBS, 2 mm L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. After 90 min, the nonadherent cells were removed by washing with HBSS, yielding the SAC (41). These SAC (~1.5–2.0 × 10^6 cells/dish) were restimulated with sonicated Mtb (10 μg/ml) for different time points (3–12 h) (supplemental Fig. S2) in the presence or absence of a cytokine.

Synovium-infiltrating Cells (SIC)—Hind paws of Mtb-immunized Lewis rats were removed at the peak phase of the disease, and SIC were collected by opening the joint using a sterile surgical blade. These SIC (total SIC) were washed three times with HBSS and then distributed equally into 60 × 15-mm culture dishes. This was followed by restimulation of SIC with antigen in serum-free HL-1 medium for 24 h. For adherent cells, the total SIC were cultured for 90 min in a 60 × 15-mm dish in RPMI 1640 medium containing 5% FBS. The nonadherent cells were removed by washing the culture dish with HBSS. The remaining cells (adherent SIC) were restimulated for 6 h with Mtb (10 μg/ml) in RPMI 1640 medium containing 5% FBS.

Fibroblast-like Synoviocytes (FLS)—SIC were collected from arthritic Lewis rats as described above and cultured in DMEM supplemented with 10% FBS, 2 mm L-glutamine, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. After overnight incubation, the culture medium was replaced with fresh medium to remove nonadherent cells. Thereafter, the remaining adherent cells were split after they reached confluence, and cells in passage 2 were stimulated for 24 h with different concentrations of IFN-γ, IL-17, and IL-27 in serum-free medium. The supernatant was collected and analyzed for matrix metalloproteinase (MMP) activity using a gelatin zymogram and for VEGF using an ELISA kit (University of Maryland, Baltimore Cytokine Core Facility).

Preparation of RNA and cDNA from Cells

RNA was isolated from LNC, SAC, and SIC using TRIzol reagent (Invitrogen), and then cDNA was prepared from RNA
using an iScript cDNA synthesis kit (Bio-Rad). The resultant cDNA was used for analysis of mRNA expression levels of different cytokines as described below.

**Quantitative RT-PCR (qRT-PCR) for Quantification of Cytokine mRNA**

Appropriate primers (synthesized at the University of Maryland, Baltimore Biopolymer Core Facility) were designed using the Primer Express 2.0 program (Applied Biosystems, Foster City, CA) for the detection of mRNAs for IFN-γ, IL-6, IL-10, IL-17, IL-23p19, IL-27p28, IL-12p35, IL-12p40, TGF-β1, MMP9, and hypoxanthine-guanine phosphoribosyltransferase.

The cDNA prepared from the RNA that was obtained from cells was amplified in an ABI Prism 7900HT cycler (Applied Biosystems) by qRT-PCR using SYBR Green PCR Master Mix (Applied Biosystems). The mRNA levels of the genes of interest were normalized to the hypoxanthine-guanine phosphoribosyltransferase gene, and the relative gene expression levels were determined. “-Fold increase” over mRNA levels of untreated cells was then determined. In our preliminary experiments using this method of cytokine testing, we had observed a clear correlation between mRNA and protein for IL-17, and the ratios were normalized to the hypoxanthine-guanine phosphoribosyltransferase gene, and the relative gene expression levels were determined. “-Fold increase” over mRNA levels of untreated cells was then determined. In our preliminary experiments using this method of cytokine testing, we had observed a clear correlation between mRNA and protein for IL-17, IFN-γ, and IL-10 (supplemental Fig. S3, A–F). Henceforth, for brevity, we will refer to cytokine mRNA expression as cytokine.

**Testing the Effect of Cytokines on Transcription Factors**

Cytokine Treatment of LNC in Vitro—LNC obtained from Mtb-immunized Lewis rats were restimulated (in a 12-well plate) for 24 h with antigen in the presence or absence of a cytokine. These cells were lysed using cell lysis buffer (Cell Signaling Technology, Danvers, MA). The supernatants were cleared by centrifugation at 16,000 × g for 10 min, and their protein concentration was determined by BCA protein assay kit (Pierce). These supernatants (25 μg of protein) were used in Western blotting with rabbit anti-phospho-STAT3 and anti-ROR-γt antibodies (Cell Signaling Technology).

**LNC of Cytokine-treated Rats—LNC of IFN-γ- or IL-27-treated animals were restimulated with antigen for 24 h.** Thereafter, RNA was collected for IL-17 mRNA expression studies. Cell lysates were used to study ROR-γt and phospho-STAT3 levels using Western blotting.

**Testing the Effect of Cytokines on Signaling Molecules Using Western Blotting**

FLS at passage 2 (in a 30-mm dish) were first starved for 6 h in serum-free medium and then stimulated for 30 min with different concentrations of IFN-γ, IL-17, or IL-27. Thereafter, these cells were sonicated briefly in a cell lysis buffer and protein content of the lysate was estimated. The proteins (25 μg) in the lysate were separated by 4–20% SDS-PAGE and transferred onto a PVDF membrane. The membrane was first blocked with 5% milk in Tris-buffered saline containing 0.05% Tween and then probed with rabbit antibodies (anti-phospho-ERK and anti-phospho-Akt; Cell Signaling Technology) (Akt is “thymoma viral proto-oncogene”; synonymous with protein kinase B). Secondary anti-rabbit antibody (Pierce) was used at 1:1000 dilution. The membrane was probed with antibody against total ERK (Santa Cruz Biotechnology, Santa Cruz, CA) or total Akt. GAPDH was used as a protein loading control. Protein bands were visualized with SuperSignal West Dura chemiluminescent detection reagents (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer’s directions.

**Detection of MMP Activity Using a Gelatin Zymogram**

A zymogram assay was carried out as described previously (42). Briefly, supernatant from FLS treated with IL-17, IFN-γ, or IL-27 was loaded onto a gelatin-coated, precasted polyacrylamide gel (Bio-Rad). Standard MMP9 and MMP2 (Sigma) were used as positive controls. Electrophoresis was carried out under SDS nonreducing conditions at constant voltage. The gel was incubated with 2.5% Triton X-100 at room temperature for 1–2 h to remove SDS. The gel was then washed three to four times with water to remove Triton X-100 and incubated overnight at 37 °C in developing buffer (Tris-HCl, pH 7.4) containing 5 mM CaCl2, 0.2 mM NaN3, and 0.02% Brij 35. Thereafter, the gel was stained with Coomassie Brilliant Blue R-250. The metalloprotease activity was visualized and scanned using a scanner after destaining.

**Assay for Caspase-3/7 Activity**

FLS (25,000/well) were cultured in a black 96-well plate with a clear bottom in DMEM supplemented with 10% FBS. The next day, the culture medium was replaced with DMEM without FBS, and the cells were stimulated for 12 h with IFN-γ, IL-17, or IL-27 (20 ng/ml). Thereafter, caspase-3/7 activity was assayed using the SensoLyte homogeneous AMC caspase-3/7 assay kit (AnaSpec, La Jolla, CA). Cells and assay reagents were mixed according to the manufacturer’s recommendation and then incubated for an additional 24 h on a plate shaker at room temperature. The fluorescence of 7-amino-4-methylcoumarin released as a result of caspase action on N-acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin substrate was measured using a plate reader at excitation/emission of 360/460 nm, and results are presented as relative fluorescence units.

**Statistics**

Cytokine mRNA levels of Lewis and WKY rats at the corresponding time points (see Fig. 1) were analyzed by Student’s t test. MMP9 mRNA expression, VEGF data, and cytokine mRNA (Fig. 2) were analyzed by repeated measures of analysis of variance with Dunnett’s multiple comparison tests. IL-17 expression in Fig. 3 and caspase-3/7 activity were analyzed by Student’s t test, whereas two-way analysis of variance with the Bonferroni post-test was carried out for in vivo experiments. A p value of <0.05 was considered to be significant. Data are presented as mean ± S.E. from at least three independent experiments.

**RESULTS**

Lewis and WKY Rats Reveal Differential Temporal Cytokine Profiles following Immunization with Mtb—We examined the temporal cytokine mRNA expression profiles of IL-17, IFN-γ, and IL-10 using Bhsp65 (mycobacterial heat-shock protein
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**FIGURE 1. Cytokine mRNA expression profiles in Lewis and WKY rats following an arthritogenic challenge.** Mtb-immunized Lewis and WKY rats were killed at different time points corresponding to the phases of AA in the Lewis rat. The draining LNC harvested from these rats were restimulated with Bhs65 for 24 h (A, B, and D), whereas the SAC were restimulated with sonicated Mtb (10 μg/ml) for 6 h (C). The cytokine mRNA expression (n = 4 per group at each phase) was quantified using real-time qRT-PCR. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA served as an internal control. *, p < 0.05 when cytokine mRNA levels at the corresponding time point were compared. Pk, peak phase; Rec, recovery phase.

65)-restimulated LNC (Fig. 1, A, B, and D) from arthritis-susceptible Lewis rats and arthritis-resistant WKY rats at different time points after Mtb challenge. In view of the importance of the draining lymph nodes in the initiation of immune responses leading to disease induction following an arthritogenic challenge (14), we tested the whole LNC population to gain an insight into the *in vivo* cytokine milieu in that lymphoid tissue. In parallel, we probed the profiles of dendritic cell/macrophage cytokines (IL-27 (p28 subunit), IL-6, IL-12p35, IL-12p40, and IL-23 (p19 subunit)) in SAC stimulated with sonicated Mtb (Fig. 1C and supplemental Fig. S4).

In Lewis rats, IL-17 was highest at the Inc phase, followed by a rapid decline at the onset of AA and thereafter (Fig. 1A). In contrast, IFN-γ (Fig. 1B) followed the disease profile, and its highest level was observed at the peak phase of the disease. IL-27 showed a small but gradual increase, peaking at the recovery phase (Fig. 1C). However, IL-10 (Fig. 1D) had a profile similar to that of IL-17 except for much less decline after the Inc phase. In WKY rats, the profiles of IL-17, IFN-γ, and IL-27 were almost identical, and each of these cytokines reached its peak level at the Inc phase. However, IL-10 was unchanged throughout the disease course. For the remaining SAC cytokines, in Lewis rats, IL-6 showed a gradual increase, and in WKY rats, IL-12p40 followed the pattern of IFN-γ response. However, IL-23 did not display a clear relation either with the disease course (Lewis) or with the time after Mtb injection (WKY) (supplemental Fig. S4).

**Exogenously Added IFN-γ and IL-27 Inhibit the Production of Antigen-induced IL-17 in Vitro**—To examine the interplay among IL-17 and IFN-γ/IL-27, Mtb-primed LNC from Lewis rats were harvested at the Ons phase of AA and restimulated with Bhs65 in the presence or absence of IFN-γ, anti-IFN-γ/ 

isotype control antibody, IL-27, or IL-17. Significantly reduced IL-17 mRNA (Fig. 2A and C), as well as IL-17 protein (Fig. 2, D and E), was evident following the addition of IFN-γ or IL-27, whereas enhanced IL-17 mRNA was observed in the presence of anti-IFN-γ antibody (Fig. 2B). The suppressive action of IFN-γ and IL-27 on IL-17 expression was further validated by testing the levels of ROR-γt and phosphorylated STAT3, which are required for Th17 differentiation and IL-17 production. Interestingly, both IFN-γ and IL-27 reduced the level of ROR-γt (Fig. 2F) but had a differential effect on the phosphorylated STAT3 level, with IL-27 decreasing but IFN-γ increasing it at higher cytokine concentration (Fig. 2F). However, no significant additive or synergistic effect of IFN-γ and IL-27 on IL-17 mRNA suppression was observed (supplemental Fig. S5A). Moreover, the addition of IL-27 to LNC increased neither IFN-γ (supplemental Fig. S5B) nor IL-10 (supplemental Fig. S5C). Similarly, IL-17 failed to suppress IFN-γ expression (supplemental Fig. S5D). Thus, the cytokine cross-regulatory effect was unidirectional, with IFN-γ/IL-27 suppressing IL-17.

We also examined the effect of IFN-γ and IL-17 on cytokine expression in Mtb-primed SAC representing antigen-presenting cells. Interestingly, IFN-γ up-regulated IL-27 significantly in the presence as well as the absence of Mtb (Fig. 2G), but it down-regulated TGF-β1 (Fig. 2H). However, neither IL-17 nor IFN-γ altered IL-23 significantly (Fig. 2I). IL-23 and TGF-β1 (with IL-6) are known to facilitate induction of the Th17/IL-17 response, whereas IL-27 suppresses IL-17. These results (Fig. 2G) brought out a novel positive interplay between IFN-γ and IL-27, which might be of physiological significance considering that both these cytokines can inhibit IL-17 response (see Fig. 6). This positive effect of IFN-γ on IL-27 also helps to explain the ordered temporal appearance of IFN-γ before IL-27 in Lewis rats (Fig. 1, B and C). As for other cytokines, the addition of IFN-γ or IL-17 did not have a significant effect on their expression (supplemental Fig. S6, A–D).

**Treatment with IFN-γ/IL-27 Suppresses AA**—To further examine the *in vivo* effects of the three major cytokines, we performed direct injection of IFN-γ, IL-27, or IL-17 into Mtb-immunized Lewis rats. IFN-γ injection at the Inc phase (Fig. 3B) and IL-27 injection at both the Inc and Ons phases (Fig. 3, E and H) significantly down-modulated AA. However, IFN-γ injection at the Ons phase did not affect the course of AA (Fig. 3D). As anticipated from the previous report of attenuation of AA by the *in vivo* neutralization of IL-17 during the incubation phase of AA (43), supplementation with exogenous IL-17 caused an increase in disease severity (Fig. 3A). The effect of these cytokines on clinical AA was further validated by histopathological examination of the joint sections (Fig. 3I). The joint space was clear with minimal mononuclear cell infiltration of the synovial tissue in IFN-γ- or IL-27-treated rats compared with PBS- or IL-17-treated rats. This arthritis-suppressing effect of IFN-γ and IL-27 correlated with the *in vivo* (Fig. 3, C and F) as well as *in vitro* (Fig. 2, A and C) suppression of IL-17 by these two cytokines. The expression of ROR-γt and phosphorylated STAT3 was also reduced in LNC of IL-27-injected Lewis rats (Fig. 3G).
Pattern of Cytokine Expression in the Arthritic Joint Diffsers from That in the Draining Lymph Node Cells of Lewis Rats—

We also examined the cytokine expression in the target organ (the joint) of arthritic Lewis rats. Because of the technical limitation of obtaining an adequate number of SIC at other phases of AA, the cytokine levels could be tested only at the peak phase of AA. The total SIC were restimulated with Bshps65 for T cell cytokines (Fig. 4A), whereas adherent SIC were restimulated with sonicated Mtb for cytokines derived from macrophages, fibroblasts, and related myeloid cells (Fig. 4B). The level of IL-17-related cytokines (IL-17, IL-23, and IL-6) was significantly higher than that of the IL-12-related cytokines (IFN-γ, IL-12, and IL-27). These results further highlight the aforementioned inverse correlation between IL-17 and IFN-γ/IL-27. Furthermore, a comparison of the cytokine levels in the target organ (SIC) and the peripheral lymphoid tissue (LNC/SAC) revealed that IL-17 (supplemental Fig. S7A) and its related cytokines (IL-6 and IL-23) were increased, whereas its counter-regulatory cytokines were either reduced (IL-27) (supplemental Fig. S7B) or unchanged (IFN-γ) (supplemental Fig. S7C).

IL-17 and IFN-γ/IL-27 Differentially Regulate MMP9, VEGF, Akt, and Caspase-3/7—To further examine the role of IL-17, IFN-γ, and IL-27 in the target organ, we studied the mediators of inflammation and tissue damage in synovial fibroblast-like cells obtained from the joints of Lewis rats. Also studied were the signaling molecules and caspase-3/7 activity. The latter represents the marker for apoptosis. VEGF is an angiogenic factor that is involved in neovascularization (44), whereas MMPs are extracellular matrix-degrading enzymes that damage bone and cartilage (45, 46). The addition of IL-17
to FLS in vitro enhanced MMP9 activity (Fig. 5A) as well as MMP9 mRNA expression (Fig. 5B) but not VEGF secretion (Fig. 5C), whereas the addition of IFN-γ and IL-27 reduced not only the activity of MMP9 and its mRNA expression but also VEGF secretion. However, none of the cytokines tested modulated MMP2 activity (Fig. 5A). All three cytokines tested (IL-17, IFN-γ, and IL-27) increased ERK phosphorylation considerably. On the other hand, phosphorylated Akt, which is an anti-apoptotic (prosurvival) (47) kinase, was increased by IL-17 but decreased by IFN-γ and IL-27 (Fig. 5D). Furthermore, IFN-γ and IL-27 increased caspase-3/7 activity, whereas IL-17 had no significant effect (Fig. 5E). These results reveal an overall anti-apoptotic effect of IL-17 compared with IFN-γ and IL-27. In view of the quantitative dominance of IL-17 over IFN-γ and IL-27 in the joints, the net effect of this cytokine balance might be the enhanced survival of pathogenic effector leukocytes (T cells, macrophages, or fibroblasts) and the resulting tissue damage during the acute phase of AA (Fig. 6).

**DISCUSSION**

The cytokine profiles of the draining LNC of Mtb-immunized Lewis (AA-susceptible) and WKY (AA-resistant) rats revealed important differences. Furthermore, in the case of Lewis rats, the cytokine profiles of LNC were different from those of the synovial tissue from the joints. In Lewis rats, the Inc phase was characterized by high levels of the proinflammatory cytokine IL-17. This is followed by IFN-γ and IL-27 response in that sequence. On the basis of our results, we propose that, in Lewis rats, the cytokine profiles of LNC were different from those of the synovial tissue from the joints.

In Lewis rats, the Inc phase was characterized by high levels of the proinflammatory cytokine IL-17. This is followed by IFN-γ and IL-27 response in that sequence. On the basis of our results, we propose that, in Lewis rats, whose activity in the incubation phase is unopposed by the counter-regulatory IFN-γ and IL-27, initiates the disease process. This inference is supported by the results of an earlier study showing that the in vivo neutralization of IL-17 in the incubation period significantly abrogates the development of AA in susceptible rats, demonstrating the significance of IL-17 in mediating the immune pathology in AA (43). Our results suggest that the IL-17-triggered inflammatory events in the acute
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Figure 4. Cytokine expression in SIC. Total SIC (A) and adherent SIC (B) from the joints of arthritic Lewis rats were restimulated with Bhsp65 (24 h) and Mtb (6 h), respectively. The cytokine mRNA expression in these cells was quantified by qRT-PCR using SYBR Green Master Mix. Results were expressed as ΔmRNA normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). *ova, ovalbumin; *Med, medium.

The regulatory role of IFN-γ and IL-27 in vivo is corroborated by our results showing that (a) the addition of IFN-γ or IL-27 to Bhsp65-primed LNC in vitro suppressed the expression of IL-17; (b) the in vitro neutralization of IFN-γ by anti-IFN-γ antibody in the same experimental system up-regulated IL-17 expression; (c) the addition of IFN-γ and IL-27 to LNC in vitro significantly reduced the expression of the transcription factors (ROR-γt and phosphorylated STAT3) that are critical for the differentiation/activity of Th17 cells; and (d) the in vivo injection of IFN-γ or IL-27 suppressed AA by reducing IL-17 expression. Furthermore, apparently there was a cross-talk between the two IL-17-regulating cytokines so that IFN-γ up-regulated IL-27 expression, displaying a unique aspect of cytokine cross-stimulation. Thus, IFN-γ can directly suppress IL-17 response and, in addition, exert its regulatory effect via increasing IL-27, which in turn can inhibit IL-17 expression.

Recently, the up-regulation of IL-27 by IFN-γ leading to the down-modulation of autoimmune encephalomyelitis in mice has been reported using wild-type and knock-out mice (52). However, our finding is derived from settings of differential outcomes of disease pathology in susceptible and resistant wild-type rat strains. In this regard, the results of these two studies are mutually confirmatory as well as supportive of the physiologic relevance of this novel interplay between IFN-γ and IL-27. The regulatory role of IFN-γ is also supported by the suppression of IL-23 (27). One of our previous studies also supports, albeit indirectly, the regulatory role of IFN-γ in AA (14). According to our results, IL-27 is regulatory in AA, as reported by others in EAE (48).

The pathogenic role of IL-17 has been reported in different experimental models of autoimmunity, including arthritis, experimental autoimmune encephalomyelitis, and systemic lupus erythematosus (49–51). Enigmatically, IL-17 has also been shown to be protective in colitis, diabetes, and uveitis.
The precise experimental conditions that yield a protective versus a pathogenic effect of IL-17 in autoimmunity remain to be fully defined.

Overall, this study has advanced our understanding of the pathogenesis of autoimmune arthritis in the context of newer and established cytokines, as well as the key biochemical mediators of inflammation and tissue damage. It also has provided insights into novel therapeutic targets for controlling autoimmune arthritis.

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