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Exacerbation of Antigen-Induced Arthritis in IFN-γ-Deficient Mice As a Result of Unrestricted IL-17 Response

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Proinflammatory Th1 responses are believed to be involved in the induction and perpetuation of rheumatoid arthritis. However, the role of IFN-γ, the major cytokine produced by Th1 cells, is still incompletely defined. In the present study, we investigated the effects of IFN-γ deficiency (IFN-γ−/−) on the course of experimental murine Ag-induced arthritis (AIA). In the acute stage of disease, IFN-γ−/− AIA mice showed significantly increased inflammatory responses compared with wild-type C57BL/6 AIA mice, i.e., exacerbated joint swelling, increased delayed-type hypersensitivity reaction, and increased histopathological scores of arthritis. Intraarticular administration of exogenous IFN-γ at induction of AIA significantly suppressed these acute aggravation effects. Stimulated cells isolated from lymph nodes and spleen of IFN-γ−/− AIA mice showed increased production of IL-2, IL-4, IL-5, IL-6, but most prominently of IL-17. These elevations were paralleled by decreased humoral immune responses, with low serum levels of total and Ag-specific IgG (IgG1, IgG2ab, IgG2b, IgG3). At immunohistology, the knee joints of IFN-γ−/− AIA mice showed massive neutrophil granulocyte infiltration. Treatment with mAbs neutralizing IL-17 diminished the acute inflammation. In vitro, Th cell expansion and production of IL-17 upon restimulation were effectively and dose dependently inhibited by IFN-γ.

H uman rheumatoid arthritis (RA) is a severe autoimmune disease manifesting in inflammation of peripheral joints and progressive destruction of articular cartilage and bone. Disease susceptibility is associated with Ag presentation to T lymphocytes by particular HLA-DR haplotypes. Also, CD4+ T cells infiltrating the RA synovial membrane are predominantly of the Th1 phenotype. According to a current paradigm, a pathogenic role of Th1-type cellular immunity is supposed to prevail over a beneficial Th2 response. IFN-γ, a major effector cytokine produced by Th1 cells, has received much attention concerning its role in autoimmune processes. Nonetheless, the question of the role of IFN-γ in arthritis or other autoimmune diseases has not been sufficiently answered.

On one hand, IFN-γ is known to induce proinflammatory effects, for example macrophage activation (6), MHC class II Ag expression on a variety of cells, and secretion of IgG2a in vivo and in vitro. Consistently with these properties, IFN-γ promotes the severity of proteoglycan-induced arthritis (10, 11). In contrast in collagen-induced arthritis (CIA), IFN-γ has beneficial (12–15) as well as proinflammatory effects (16). In experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis, the CNS of IFN-γ receptor-deficient mice displays neutrophilia with strong up-regulation of the neutrophil-attracting chemokines MIP-2 and T cell activation gene 3 (17).

Ag-induced arthritis (AIA) is a convenient rodent model of RA. In this model, preimmunized C57BL/6 mice receive an intraarticular injection of Ag and develop a monoarticular inflammatory arthritis associated with Ag-specific immune responses. The histopathological features of AIA include hyperplasia of the synovial lining; cellular infiltration of joints and periarticular tissue by neutrophils, macrophages, lymphocytes, and dendritic cells; pannus formation and destruction of cartilage and bone, all features resembling those of human RA. The similarities include also the therapeutic response to some drug treatments and the involvement of CD4+ T cells in the perpetuation of arthritis. AIA mice showed increased production of IL-2, IL-4, IL-5, and IL-17. AIA mice showed increased production of IL-2, IL-4, IL-5, and IL-17. In the present study, we investigated the development of arthritis in IFN-γ-deficient (IFN-γ−/−) mice to determine the role of IFN-γ in AIA. We were able to demonstrate that IFN-γ is not essential for a potent inflammatory response and, surprisingly, its lack even increased the severity of inflammation. Increased arthritis severity in IFN-γ−/− mice was characterized by massive infiltration of neutrophil granulocytes and by prominent elevation of IL-17, a cytokine known to induce granulopoiesis and neutrophil accumulation. Inhibition of IL-17 decreased the severity of AIA in IFN-γ−/− mice, indicating an interaction of IFN-γ and IL-17.

Materials and Methods

Ag-induced arthritis

C57BL/6 mice deficient in IFN-γ (IFN-γ−/−) (The Jackson Laboratory) (21), as well as wild-type control mice, were bred under specific pathogen-free conditions at the animal facility of the University of Jena, Germany. All animal studies were approved by the local commission for animal protection.

Wild-type control and IFN-γ−/− mice, age 7–8 wk, were immunized s.c. at 21 and 14 days before AIA induction with 100 μg of methylated BSA (mBSA; Sigma-Aldrich), dissolved in 50 μl 0.9% NaCl and emulsified with an equal volume of CFA (Sigma-Aldrich), supplemented to 2 mg/ml Mycobacterium tuberculosis (Difco). Additionally to immunization...
fixation in 1% formalin. Tris buffer was used for subsequent washing steps. With biotinylated secondary goat anti-rat IgG Abs (DakoCytomation) and stopped by adding 2NH_2SO_4, and absorbance was measured at 492 nm by anti-CD3 Abs from 145-2C11 hybridoma cell supernatant.

Serum Ab levels were calculated using recombinant cytokines as standard. Microtiter plates (Greiner) were coated with mBSA or Abs directed to mouse IgG, IgG1, IgG2b, IgG3 (all from Southern Biotechnology Associates), IgG2a (Igh-C; haplotype; BD Biosciences), linked to alkaline phosphatase, or for total IgG goat anti-mouse IgG (MP Biomedicals), linked to peroxidase, were used as secondary Abs. Ortho-phenylenediamine (Sigma-Aldrich) with H_2O_2 was used as peroxidase substrate and 4-nitrophenylphosphate disodium salt (Serva) as alkaline phosphatase substrate. Plates were read at 492 or 405 nm in a microplate reader (Tecan). Pooled sera from AIA mice were used as relative reference for Abs specific to mBSA, otherwise myeloma Abs (Sigma-Aldrich) served as standard.

Clinical assessment of AIA

The swelling of the knee joint was recorded during the course of AIA at definite time points as the difference between the right (arthritic) and left (unaffected) knee diameter using an Oditest caliper (Kroepelin). The DTH reaction was evaluated by the increase of ear thickness 24 and 48 h after Ag challenge. For histopathological examination, knee joints were removed, fixed in toto in 4.5% formalin, decalcified in EDTA, and embedded in paraffin. Sections were stained with H&E. The specimens were evaluated in a blinded manner according to a macroscopic scoring system ranging from 0 to 3, with consideration of acute and chronic inflammation in the synovial and periarticular tissues, as well as destruction of cartilage and bone as described (22). Acute inflammation reflects exudation and infiltration of granulocytes, whereas chronic inflammation denotes synovial hyperplasia, infiltration of mononuclear cells, and fibrosis. Acute and chronic inflammation in the joint and periarticular tissue were summed up. Total arthritis score reflects the sum of all parameters.

Immuno histochemical examinations

As previously described (23), serial cryosections of 5-μm thickness from knee joints of IFN-γ−/− mice at the stage of maximum cellular infiltration 1 day after AIA induction were fixed in acetone, blocked with 4% milk/Tris, and stained with primary 10 mg/ml rat mAbs directed against mouse Mac-1 (macrophages and neutrophil granulocytes; Medec), F4/80 (macrophages; Serotec), Gr-1 (neutrophil granulocytes), CD4 (Th cells), or B220 (B cells; all from BD Biosciences) for 1 h at 22°C, followed by incubation with biotinylated secondary goat anti-rat IgG Abs (DakoCytomation) and fixation in 1% formalin. Tris buffer was used for subsequent washing steps. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 0.1 M Na_2HPO_4. Development was done with diaminobenzidine (Sigma-Aldrich) in 0.5% H_2O_2 as substrate for streptavidin-peroxidase-biotin complexes. Mayer’s hematoxylin was used for counterstaining.

Cytokine assays

At day 3 of AIA, single cell suspensions were prepared from lymph nodes (inguinal, popliteal, subaortico) and spleens and cultured (10^6 cells/ml) at 37°C, 5% CO_2 in RPMI 1640 medium containing 10% FCS, 2 mM sodium pyruvate, 10 mM HEPES, 15 mg/ml t-glutamine, 5 μg/ml streptomycin, 5 U/ml penicillin (all from Invitrogen Life Technologies), 5 × 10^5 M 2-ME (Sigma-Aldrich) in the presence of 25 μg of mBSA or 2 μg of plate-bound anti-CD3 Abs from 1452c11 hybridoma cell supernatant.

Supernatants were harvested after 42 h of incubation and analyzed for levels of secreted cytokines using standard sandwich ELISA procedures as previously described (19). Primary and biotin-labeled secondary Abs for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 were purchased from BD Biosciences. Abs directed to IL-17 were from R&D Systems. Washing steps were conducted with 0.05% Tween in PBS. After adding peroxidase-conjugated streptavidin (Dianova), bound complexes were detected by reaction with ortho-phenylenediamine (Sigma-Aldrich) and H_2O_2. The reaction was stopped by adding 2 N H_2SO_4, and absorbance was measured at 492 nm by a microtiter plate reader (Tecan). Cytokine concentrations in the samples were calculated using recombinant cytokines as standard.

For flow cytometric determination of CD4+ cells, lymph node cells were prepared as described and incubated for 20 min with 10 μg/ml FITC-labeled anti-CD4 Abs (BD Biosciences) at 4°C. Frequency of fixed CD4+ cells was measured using a BD LSR II.

Serum Ab levels

Total, mBSA, and autoreactive Ab titers in IFN-γ−/− and wild-type mice were measured 3 days after AIA induction by ELISA as previously described (19). Microtiter plates (Greiner) were coated with mBSA or Abs directed to mouse IgG, IgG1, IgG2b, IgG3 (all from Southern Biotechnology Associates) at 4°C overnight. After 2 h of blocking of nonspecific binding sites with PBS-Tween containing 2% BSA (Sigma-Aldrich), individual serum samples of appropriate dilutions were added to the well and incubated overnight at 4°C. For isotype determination, goat anti-mouse IgG1, IgG2b, IgG3 (Southern Biotechnology Associates), IgG2a (Igh-C; haplootype; BD Biosciences), linked to alkaline phosphatase, or for total IgG goat anti-mouse IgG (MP Biomedicals), linked to peroxidase, were used as secondary Abs. Ortho-phenylenediamine (Sigma-Aldrich) with H_2O_2 was used as peroxidase substrate and 4-nitrophenylphosphate disodium salt (Serva) as alkaline phosphatase substrate. Plates were read at 492 or 405 nm in a microplate reader (Tecan). Pooled sera from AIA mice were used as relative reference for Abs specific to mBSA, otherwise myeloma Abs (Sigma-Aldrich) served as standard.

Treatment with recombinant IFN-γ and IL-17 neutralization

In C57BL/6 wild-type and IFN-γ−/− mice, a single injection was given in the right knee joint cavity containing 100 ng of recombinant murine IFN-γ (Invitrogen Life Technologies) additionally to the Ag at the time of AIA induction. The course of joint swelling was monitored the following days. At day 3 of AIA, mice were sacrificed for histological evaluation of disease as described.

For neutralization of IL-17, 100 μg of monoclonal rat anti-mouse IL-17 Ab (R&D Systems) was i.v. administered 3 days and 5 h before AIA induction. Polyclonal rat IgG, purified from naïve rat serum, was used as control.

Expansion of Th cells and IL-17 secretion

Single cell suspensions from lymph node and spleen cells from IFN-γ−/− or wild-type mice were cultured for 20 min on cell culture dishes coated with rabbit anti-rat IgG Abs (DakoCytomation), cross-reactive to mouse IgM and then incubated with 100 μg per 10^6 cells/ml rat hybridoma Abs directed to CD16/CD32 (24G2), Mac-1 (M1/70), and CD8 (2.4.3).

Sheep Abs against rat IgG labeled with magnetic beads (Dynal Biotech) were used for magnetic negative selection of Th cells. Naive Th cells were isolated by subsequent positive selection of CD62L+ cells with magnetic beads (Miltenyi Biotec) according to manufacturer’s instructions. Purity of CD4+ cells verified by flow cytometry was ~95%. For activation, naive Th cells were cultured for 3 days on plate-bound anti-CD3 and anti-CD28 Abs (2 μg/10^6 cells). After a 4-day resting period, Th cells were restimulated for 4 days with anti-CD3/anti-CD28. During stimulation, recombinant murine IFN-γ (Invitrogen Life Technologies) was added at concentrations between 0 and 50 ng/ml. To determine IL-17 levels, ELISA was used as control. Expansion of Th cells upon stimulation and restimulation was measured by adding [3H]thymidine (0.5 μCi/well; Amersham Biosciences) for the final 18 h. Cells were harvested, and incorporation of radiolabeled thymidine was measured using a microplate scintillation luminescence counter (Canberra-Packard) to assess cell proliferation.

Statistical analysis

Statistical differences between the groups were evaluated using the non-parametric Mann-Whitney U test. Statistical significance was accepted for p < 0.05. All calculations were performed by means of the SPSS software package (v.10.0.5). Data are shown as arithmetic mean and SEM.

Results

Exacerbated inflammatory reaction in IFN-γ−/− mice

To assess the inflammatory response in the absence of IFN-γ-mediated effector mechanisms, AIA was induced in IFN-γ−/− and wild-type mice. The acute inflammatory reaction was significantly exacerbated in IFN-γ−/− mice, as evidenced by a striking increase of joint diameter (+90% in IFN-γ−/− mice vs +30% in the wild-type controls) (Fig. 1A). In the late acute phase of AIA (most evidently at day 7), the extent of joint swelling became comparably low in both groups, reaching congruent levels at day 9 of AIA (Fig. 1A). Histopathological analyses of the arthritic knee joints on day 3 of AIA revealed stronger synovial inflammation in IFN-γ−/− mice than in wild-type controls. The total arthritis score was significantly exacerbated in IFN-γ−/− mice, due in particular to a significant increase of the acute inflammation score, which is based on the degree of neutrophil granulocyte infiltration and exudate (Fig. 1B). The T cell-mediated immune response was assessed by...
measuring the DTH reaction upon intradermal injection of low-dose Ag into the ear on day 7 of AIA. In arthritic IFN-γ−/− mice, ear thickness was increased by ~270%, whereas in the wild-type AIA mice the increase was ~170% (Fig. 1C).

The significant increase of joint swelling, histological score of acute arthritis, and DTH reactivity upon Ag challenge demonstrated an elevation of cellular immune responses in IFN-γ−/− mice. These results concur to indicate a protective role of IFN-γ in the early phase of AIA; also, severe pathological consequences ensue, when IFN-γ-controlled pathways are disrupted.

Constitutive lack of IFN-γ is associated with massive recruitment of neutrophil granulocytes into arthritic joints

In the AIA model, deficiency of IFN-γ led to a massive inflammatory infiltration of the arthritic joint compared with wild-type AIA controls. The infiltration was observed not only in the joint cavity but also in the periarticular tissue, obscuring the normal articular structure (Fig. 2A). In the IFN-γ−/− mice, the cellular infiltrate mainly consisted of polymorphonuclear neutrophil granulocytes (Fig. 2A). An additional interesting feature in these animals was also the frequent occurrence of granulomas in the muscular tissues of upper and lower legs (data not shown).

Immunohistochemical methods were applied to elucidate the nature and frequency of different cell types constituting the cellular infiltrate. The infiltrate consisted mainly of Mac-1+ (CD11b) cells (Fig. 2D), which identify both polymorphonuclear neutrophil granulocytes and monocytes. Further characterization revealed that the cells consisted almost exclusively of Gr-1+ neutrophil granulocytes (Fig. 2D), some F4/80+ macrophages, and a few Th cells (CD4+ and B cells (B220+)) (data not shown).

The increased infiltration of neutrophils in IFN-γ−/− mice suggests that IFN-γ plays a prominent role in regulating the migration of these cells into the site of inflammation.

Reconstitution with exogenous IFN-γ attenuates acute inflammatory response

To assess the effect of IFN-γ reconstitution on the severity of acute AIA in IFN-γ−/− mice, recombinant murine IFN-γ was given locally into the knee joint simultaneously to inducing AIA. Local treatment with recombinant murine IFN-γ significantly reduced joint swelling (~30–40%) at day 1 of AIA (Fig. 3A). At day 3 of AIA, IFN-γ treated and control mice had the same degree of joint swelling. Histological analyses confirmed that the anti-inflammatory effect of recombinant murine IFN-γ substitution in IFN-γ−/− mice was limited to the initial stage of acute AIA (day 1; Fig. 3B).

To determine whether the anti-inflammatory effects of IFN-γ were restricted to the ontogenetic absence of the cytokine, wild-type mice with AIA were also treated with recombinant murine IFN-γ. Notably, AIA was significantly reduced also in the wild-type mice, limiting the importance of genetic predisposition. In contrast to the temporary attenuating effect in the IFN-γ−/− mice,
in wild-type mice a single local recombinant murine IFN-γ application was sufficient to reduce joint inflammation during the complete acute stage of AIA (Fig. 3C). Histological evaluation of acute and chronic inflammation scores at day 3 of AIA revealed a significant reduction of these parameters in the knees of IFN-γ-treated group (Fig. 3D). The local substitution, however, did not produce systemic effects, as shown by unchanged DTH reactivity at day 7 of AIA and unchanged serum Ig levels and cytokine levels in the supernatants of lymph node and spleen cells of treated or untreated animals (whether wild-type or IFN-γ−/−; data not shown). These results demonstrate reducing effects of IFN-γ on the severity of AIA. The duration of the anti-inflammatory effects is presumably affected by consumption and/or dispersal of the cytokine.

To assess capability of wild-type and IFN-γ−/− CD4+ cells to transfer arthritis, cells isolated from immunized mice of appropriate strains were transferred in naive wild-type recipients and Ag was applied. Compared with mice receiving CD4+ cells from wild-type donors, inflammatory reaction (Fig. 3E), histological arthritis score (Fig. 3F) and therefore arthritis severity were increased in mice receiving IFN-γ−/− CD4+ cells.

**Diminished humoral immune response in IFN-γ−/− mice is associated with enhanced cellular response**

Abs play an important role in the pathogenesis of arthritis models, therefore their levels were investigated in view of their possible role in the exacerbation of AIA observed in IFN-γ−/− mice. Interestingly, IFN-γ−/− mice showed significantly lower total IgG and significantly lower IgG1, IgG2b and IgG3 isotypes (Fig. 4). Ag-specific total IgG and IgG2a, IgG2b, and IgG3 were likewise decreased, whereas IgG1 was elevated. Particularly the level of IFN-γ-mediated IgG2a was significantly reduced. Therefore, the massive inflammatory response in IFN-γ−/− mice upon Ag challenge in AIA is unlikely due to an increase of complement activating Abs caused by the constitutive lack of IFN-γ.

To further examine the cytokines IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17 produced in vitro by stimulated lymph node

**FIGURE 3.** Influence of recombinant murine IFN-γ application on AIA severity. A, Substitution of IFN-γ by intraarticular application of 100 ng of recombinant murine IFN-γ (rmIFN-γ) simultaneously to the induction of AIA led to a significant reduction of joint swelling in IFN-γ−/− mice (n = 7). The reduction of swelling was transient, disappearing at day 3 in comparison to untreated mice (n = 6). B, Histological evaluation at day 3 of AIA (d3) revealed no persistent effects in IFN-γ−/− mice. C, In wild-type AIA mice (n = 15 per group), addition of recombinant murine IFN-γ significantly reduced the joint swelling during the whole stage of acute AIA. D, In wild-type AIA mice treated with recombinant murine IFN-γ, the reduction of joint swelling was paralleled by a significant reduction of the scores of acute and chronic inflammation (day 3 of AIA). E, In naive mice transferred CD4+ cells lacking competence to produce IFN-γ evoked increased inflammatory reaction after Ag challenge compared with wild-type IFN-γ-producing CD4+ cells. F, Severity of arthritis was increased in recipients of CD4+ IFN-γ−/− cells (n = 7–8 per group). *, p < 0.05; **, p < 0.01; ***, p < 0.001. Data all represent at least two independent experiments.

**FIGURE 4.** Total and mBSA-specific serum Ig. Ig serum levels were measured in the acute phase of AIA (day 3). Levels of total Ig and subclasses IgG1, IgG2b, and IgG3 were significantly lower in IFN-γ−/− AIA mice (n = 5) than in wild-type AIA mice (n = 8). Levels of mBSA-specific Ig and subclasses IgG2a, IgG2b, and IgG3 were also reduced, especially the IFN-γ-regulated IgG2a, whereas IgG1 was slightly elevated. This pattern reflects a reduced humoral immune response in IFN-γ−/− AIA mice. *, p < 0.05, **, p < 0.01. One of two independent experiments is shown.
and spleen cells from AIA mice (day 3) to analyze general or asymmetric activation of distinct Th subsets. Levels of IL-2, IL-4, IL-5, and IL-17, but as expected not of IFN-γ, were increased in lymph node and spleen cells from IFN-γ−/− mice stimulated with specific Ag (mBSA) or plate-bound anti-CD3 (Fig. 5). IL-5 secretion was below detection limit in supernatants of mBSA-stimulated IFN-γ−/− spleen cells. Levels of IL-10 and IL-6 from lymph node cells were low, whereas both were elevated in anti-CD3-stimulated IFN-γ−/− spleen cell cultures, and IL-6 from spleen cells was elevated upon Ag-specific stimulation. Interestingly, the Th1 cytokine IL-2 was increased, as well as the Th2 cytokines IL-4 and IL-5, which lacked inhibition by Th1-derived IFN-γ. Most strikingly, high amounts of IL-17 were secreted by cells from IFN-γ−/− mice in response to stimulation, indicating a higher level of Th cell activation and therefore enhanced cellular immune responses in IFN-γ−/− mice. Supernatant levels of proinflammatory cytokines IL-1α and TNF-α were not remarkably affected by deficiency of IFN-γ and showed almost no difference between the groups (data not shown).

Because IL-17 is mainly attributed to Th cells, fluorescence staining was applied to measure the lymph node frequency of CD4+ cells in both strains before AIA induction (day 0). Although in IFN-γ−/− mice 26.7 ± 1.3% of cells were CD4+, in the wild-type AIA controls 20.3 ± 0.9% were CD4+ (p = 0.006) (Fig. 6), suggesting inhibiting effects of IFN-γ on Th cell development.

Neutralization of IL-17 reduces AIA severity in IFN-γ−/− mice
The cytokine production of lymph node and spleen cells showed an intensified Th-driven immune response in IFN-γ−/− mice with AIA, characterized by high levels of IL-17. To test whether IL-17 directly contributes to the increased AIA severity in IFN-γ−/− mice, the animals were treated with a neutralizing Ab against IL-17. Anti-IL-17 treatment resulted in reduced joint swelling (Fig. 7A) and reduced arthritis score compared with rat IgG controls (Fig. 7B). Anti-IL-17 treatment was especially effective in reducing infiltration of neutrophil granulocytes and joint destruction.

IFN-γ inhibits expansion and IL-17 production of Th cells
In consequence of the low frequency of mBSA-specific Th cells in AIA, we stimulated in vitro naive IFN-γ−/− Th cells (lacking endogenous cytokine) with anti-CD3 and anti-CD28 Abs to induce a potent Th cell response, necessary to disclose the modulating effects of exogenous IFN-γ on the production of inflammatory IL-17. Cultures were supplemented with defined concentrations of
IFN-γ (0, 2, 10, 50 ng/ml). After the first round of stimulation, Th cells produced low levels of IL-17 (Fig. 8A), which is assigned to Th cells of memory phenotype. The presence of IFN-γ abolished IL-17 secretion. After anti-CD3/anti-CD28 restimulation, IL-17 production was amplified, but significantly decreased by 90% with already 2 ng/ml IFN-γ. Higher amounts of IFN-γ further reduced IL-17 production, confirming the importance of the IFN-γ/IL-17 pathway.

In cell cultures supplemented with IFN-γ, the frequency and size of clusters of proliferating Th cells was reduced. Quantification of this effect by [3H] thymidine incorporation revealed an effective and dose-dependent inhibition of Th cell expansion by addition of IFN-γ to stimulated or restimulated Th cells from IFN-γ−/− mice (Fig. 8B). Stimulation or restimulation of cells with anti-CD3 and anti-CD28 Abs induced strong proliferation of IFN-γ−/− Th cells, which was reduced to the level of wild-type Th cell proliferation by small amounts of added IFN-γ (2 ng/ml). In Th cell cultures isolated from wild-type AIA mice, therefore capable to produce endogenous cytokine, addition of high doses of IFN-γ had no effect compared with samples without additional IFN-γ. Low cytokine doses slightly reduced proliferation when stimulated for the first time, and slightly promoted proliferation when stimulated for the second time. Therefore, the lack of IFN-γ had a strong stimulatory effect on Th cell expansion, abolished by the presence of this cytokine.

**Discussion**

The goal of this study was to investigate the development of AIA in IFN-γ−/− mice and hence to elucidate the function of IFN-γ in the pathogenesis of this arthritis model. Surprisingly, we found that IFN-γ deficiency markedly increases the severity of acute arthritis, demonstrating disease-limiting in vivo effects of IFN-γ in this model.

In our studies, the parameters of inflammation were clearly more pronounced in IFN-γ−/− deficient than in wild-type mice. The swelling of the joint, indicating the intensity of the immune response, was elevated almost 3-fold in IFN-γ−/− mice. The DTH reaction was also significantly increased, even though the lymphocyte recruitment into DTH reactions is described to be driven by IFN-γ (24). Histologically, the knee sections of mice with acute AIA showed a massive inflammatory infiltration, mostly of neutrophils. Despite the inhibiting influences of IFN-γ on osteoclastogenesis, in this early phase of AIA, the degree of destruction of joint cartilage and bone was not very pronounced. T cell cytokines, most notably IL-17, were elevated in the supernatants of lymph node and spleen cells. In contrast, humoral immune responses and Abs to mBSA were decreased, even though Abs are known to be important for disease onset in other arthritis models (25). Particularly the production of IgG2a was affected by the lack of endogenous inflammation role of IL-17 in the acute phase of disease (day 5 of AIA).
IFN-γ. Therefore, enhanced inflammatory response to Ag challenge in the IFN-γ−/− mice is not attributed to augmented Ab production associated with complement activation, but rather to an increase of cellular effector mechanisms. Taken together, these findings clearly demonstrate that in the AIA model, which is mediated by Th cells (19, 20), the absence of the characteristic Th1 cytokine IFN-γ caused an aggravation and not a decline of inflammatory response. This response exemplifies the complexity and importance of IFN-γ in autoimmune diseases. We successfully induced arthritis in naive mice by adoptive transfer experiments with CD4+ cells, underlining the importance of Th cells in pathogenesis of this arthritis model. Interestingly, recipients of CD4+ cells lacking competence to produce IFN-γ developed more arthritis than recipients of wild-type CD4+ cells, demonstrating increased arthritisogenic capabilities of cells unable to produce IFN-γ and therefore the anti-inflammatory effects of this cytokine.

Our findings do not support the established view of a proinflammatory cytokine, but are in line with results from other arthritis or autoimmune disease models. Deletion of the IFN-γ receptor in mouse strains susceptible to CIA, for example, results in an accelerated onset of arthritis compared with wild-type controls, demonstrating that disrupting IFN-γ-mediated pathways has aggravating effects on development of arthritis (14, 15). IFN-γ even has the capability to override limiting genetic constraints, which is a quite unique feature for cytokines. Susceptibility to CIA is restricted to MHC class H-2d and H-2r haplotypes (26), but C57BL/6 (H-2b), BALB/c (H-2d), or 129/Sv (H-2b) mice normally resistant to CIA become highly susceptible when deficient in IFN-γ or IFN-γ receptor (12, 27). Therefore, the capability to generate an IFN-γ response is as effective in preventing experimental disease as the existence of certain haplotypes. Similar effects are known from EAE, another Th1-polarized disease model. EAE severity is more pronounced in IFN-γ−/− mice (28), and nonsusceptible BALB/c and C57BL/6 mouse strains become susceptible to EAE when IFN-γ-mediated pathways are interrupted (17).

Studies with recombinant cytokine or Abs neutralizing IFN-γ have also provided conflicting results. Administration of IFN-γ exacerbates CIA (29, 30) but in contrast, inhibits development of experimental arthritis (31). In rat adjuvant arthritis, the diversity of the effects depends on the stage of arthritis in that administration of IFN-γ before or after CFA causes aggravation or suppression of arthritis, respectively (32). Neutralizing IFN-γ with specific Abs either increases incidence and severity of established CIA (33) or attenuates disease when conducted in early stages of arthritis (34). In EAE, treatment with IFN-γ prevents disease (35). Taken together, these findings illustrate the difficulty to predict the complex effects of this potent immunomodulatory cytokine in the development of autoimmune diseases.

In the present study, exogenous IFN-γ counteracted the severity of arthritis and modulated the inflammatory responses via downregulation of recruitment of inflammatory cells. Treatment with recombinant IFN-γ by a single local application into the knee joint cavity reduced acute joint swelling by ~30–40%. Efficacy was confirmed by a significantly reduced arthritis score in histological analysis of synovial fluid of patients with early RA have found T cell cytokines attributed to the Th2 subtype, but not IFN-γ, negating participation of this cytokine in the initiation of RA (38).

Neutrophilia is a characteristic feature of experimental autoimmune diseases when IFN-γ-mediated pathways are interrupted. This is observed not only in experimental arthritis, but also in EAE, in which numerous neutrophils following expression of chemotactic cytokines are found in the CNS of mice lacking IFN-γ or IFN-γ receptor (17). Cytokine regulation of chemokine and adhesion molecule expression has severe effects on the influx of immunocompetent cells to the site of inflammation, thus affecting magnitude and effectiveness of immune response. IFN-γ, for example, suppresses expression of E- and P-selectins on activated human endothelial cells (39). These adhesion molecules are important for neutrophil and leukocyte infiltration into inflammatory tissue (40) including the migration of Th1 cells into arthritic joints (41, 42). Next to lymphocyte recruitment, IFN-γ also affects inflammatory neutrophil recruitment. Production of neutrophil attracting and activating chemokine granulocyte chemotactic protein-2 by stimulated human fibroblasts and murine macrophage-derived KC and MIP-2 are down-regulated by IFN-γ (43, 44). In vitro studies using RA fibroblasts demonstrated IFN-γ to significantly reduce neutrophil-activating chemokine CXCL8 generation induced by IL-1β or TNF-α, but in contrast increase CCL2 production (45).

One prominent feature of acute AIA was the massive granulocyte infiltration of the affected knee joint. In IFN-γ−/− mice, the mechanisms regulating the influx of these cells were apparently abrogated. These neutrophils, largely predominating over monocytes, are a fundamental source of proteases degrading proteoglycan and other extracellular matrix components (46, 47) and proinflammatory cytokines (48, 49), and may play an essential role in the regulatory interaction between innate resistance and adaptive immunity.

Activation of neutrophils and recruitment to local inflammation is potently affected by IL-17 (50). There is substantial evidence that IL-17-producing Th cells play a particular role in autoimmune disease models (51). This subclass of Th cells is efficient in inducing EAE (52). Also, there is a strong Ag-specific production of IL-17 in peripheral immune organs and in the CNS in acute and chronic EAE (53). Vaccination against IL-17 with virus-like particles conjugated with recombinant murine IL-17 suppresses EAE and CIA (54). IL-17 is an important mediator in Ag-specific flare-ups of experimental arthritis. Blocking IL-17 with polyclonal Abs suppresses joint swelling, inflammation, and cartilage destruction in AIA exacerbations induced in wild-type mice (55).

In the present study, IFN-γ−/− mice with AIA were characterized by the coexistence of T cell activation, massive neutrophil infiltration of arthritic joints, and production of high levels of IL-17. Neutralization of IL-17 potently reduced arthritis severity and in particular neutrophil recruitment to the site of inflammation. We found also that IFN-γ in vitro effectively inhibited the induction of a sizeable pool of IL-17-producing T effector cells from naive T cells, given the in vivo observation of an increased CD4 frequency in lymph nodes of immunized IFN-γ−/− mice.

This coincides with recent findings, showing that IFN-γ antagonizes the IL-23-mediated development of IL-17-producing T cells (56) and also affects Th cell homeostasis, as adoptive transfer experiments with Th1 cells revealed that IFN-γ mediates self-limitation of Th1 effector cells after Ag challenge (57). Regulatory function of Th1 cells in a diabetes model is dependent on IFN-γ and cell contact with target cells. In this model, NO production by APCs is necessary for inhibition of proliferation of pathogenic T cells (58). In CIA, activity of Th regulatory cells is regulated by
endogenous IFN-γ (59), and susceptibility of IFN-γ−/− C57BL/6 mice to disease was found very recently by Chu et al. (60) to be caused by the lack of suppression of Th cell IL-17 production.

The purpose of this study was to investigate the role of IFN-γ in the development of AIA. Our results demonstrate not only that IFN-γ-independent inflammatory pathways exist for this arthritis model, but also that IFN-γ exerts protective effects in the initial stage of disease. Surprisingly, rather IL-17-producing Th cells play a fundamental role in inflammatory pathogenesis of this arthritis model than IFN-γ-producing Th1 cells and IFN-γ-potently inhibited expansion of IL-17-producing Th cells. Because IL-17 is emerging as a possible therapeutic target in RA (50, 53, 54), our results contribute to the elucidation of effector mechanisms in the interaction between IFN-γ, IL-17, and neutrophil recruitment. Regulation of Th cell IL-17 response by IFN-γ may provide a plausible explanation for the proinflammatory effects observed in various autoimmune models when IFN-γ-mediated pathways are disrupted (12–15, 17).

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Disclosures

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References