ARTHRITIS & RHEUMATOLOGY
Vol. 66, No. 5, May 2014, pp 1340–1351
DOI 10.1002/art.38359

Systemic Juvenile Idiopathic Arthritis–like Syndrome in Mice Following Stimulation of the Immune System With Freund's Complete Adjuvant

Regulation by Interferon-γ

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Objective. **Systemic juvenile idiopathic arthritis (JIA) is unique among the rheumatic diseases of childhood, given its distinctive systemic inflammatory character. Inappropriate control of innate immune responses following an initially harmless trigger is thought to account for the excessive inflammatory reaction. The aim of this study was to generate a similar systemic inflammatory syndrome in mice by injecting a relatively innocuous, yet persistent, immune system trigger: Freund's complete adjuvant (CFA), containing heat-killed mycobacteria.**

Methods. **Given the central role of interferon-γ (IFNγ) in immune regulation, we challenged wild-type (WT) and IFNγ-knockout (KO) BALB/c mice with CFA, and analyzed their clinical symptoms and biologic characteristics. The production of cytokines and the effects of anticytokine antibodies were investigated.**

Results. **In WT mice, CFA injection resulted in splenomegaly, lymphadenopathy, neutrophilia, thrombocytosis, and increased cytokine expression. In the absence of IFNγ, these symptoms were more pronounced and were accompanied by weight loss, arthritis, anemia, hemophagocytosis, abundance of immature blood cells, and increased levels of interleukin-6 (IL-6), all of which are reminiscent of the symptoms of systemic JIA. CFA-challenged IFNγ-KO mice showed increased expression of IL-17 by CD4+ T cells and by innate γ/δ T cells. Inflammatory and hematologic changes were prevented by treatment with anti–IL-12/IL-23p40 and anti–IL-17 antibodies.**

Conclusion. **Immune stimulation of IFNγ-KO mice with CFA produces a systemic inflammatory syndrome reflecting the clinical, biologic, and histopathologic picture of systemic JIA. The protective function of IFNγ in preventing anemia and overall systemic inflammation is a striking observation. The finding that both adaptive and innate T cells are important sources of IL-17 may be of relevance in the pathogenesis of systemic JIA.**

Systemic juvenile idiopathic arthritis (JIA) is unique among the chronic arthritides of childhood, in view of its predominant systemic inflammation, consisting of fever and rash, associated with anemia, neutrophilia, and thrombocytosis, especially at disease onset. Hepatosplenomegaly and lymphadenopathy are typically found, whereas arthritis often occurs later during

Supported by grants from the Fund for Scientific Research-Flanders (FWO Vlaanderen), the Regional Government of Flanders (GOA Program), and the Belgian Science Policy Office (Interuniversity Attraction Poles Programme). Ms Avau and Ms K. Put are recipients of fellowships from the FWO Vlaanderen. Ms Brisse is recipient of a fellowship from the Institute for the Promotion of Innovation through Science and Technology Flanders (IWT Vlaanderen).

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Submitted for publication July 23, 2013; accepted in revised form January 9, 2014.

the disease course (1–3). Quantification of cytokines and analyses of gene expression have provided evidence of a predominant role of the innate immune response in its pathogenesis; accordingly, experts consider the disease to be autoinflammatory (2,4). Patients are at risk of developing a macrophage activation syndrome (MAS), a life-threatening complication of childhood inflammatory diseases (5,6), which is characterized by the phagocytosis of blood cells (hemophagocytosis). MAS is classified as a subtype of hemophagocytic lymphohistiocytosis (HLH), a group of histiocyte disorders some of which are caused by defects in genes that control the cytotoxic machinery, resulting in defective cytotoxic lymphocyte activity and sustained immune activation (7,8).

Systemic JIA is thought to be caused by an excessive inflammatory immune reaction to a generally harmless trigger in predisposed children. According to this hypothesis, a defective down-regulation of an initially normal inflammatory response, which may have been triggered by infections, underlies the disease symptoms (2).

In the present study, we tested whether Freund's complete adjuvant (CFA) can elicit a systemic JIA–like syndrome in BALB/c mice. This adjuvant contains heat-killed mycobacteria and represents a relatively innocent, yet persistent, trigger of innate as well as adaptive immunity (9). Interferon-γ (IFNγ)–knockout (KO) mice were included in the study, given the key role of this cytokine in (down)regulating disease symptoms in animal models of autoimmune diseases that rely on the use of CFA in the induction procedure (10,11). We demonstrated that the syndrome developing in CFA-challenged BALB/c mice resembled systemic JIA. While wild-type (WT) mice showed several characteristics of the human disease, IFNγ-KO mice developed more extensive symptoms, with additional features such as anemia, increased numbers of immature blood cells, and increased serum levels of interleukin-6 (IL-6). Furthermore, these mice had defective natural killer (NK) cell cytotoxicity and increased production of IL-17 by CD4+ T cells as well as innate γ/δ T cells. Early treatment with anti–IL-12/IL-23p40 or anti–IL-17 antibodies prohibited the symptoms in CFA-challenged IFNγ-KO mice, highlighting the important proinflammatory role of IL-17 in the syndrome. The possible value and limitations of this experimental system as a model for the study of systemic JIA are discussed.

MATERIALS AND METHODS

Mice and experimental design. The generation and characterization of IFNγ-KO mice on the BALB/c background has previously been described (12). IFNγ-KO and WT mice were bred under specific pathogen–free conditions in the Experimental Animal Centre of Leuven University. Mice ages 6–9 weeks were used and were age- and sex-matched for grouping within each experiment. Experiments were approved by the Ethics Committee of the university.

CFA (Difco) with added heat-killed *Mycobacterium butyricum* (1.5 mg/ml) was emulsified in an equal volume of phosphate buffered saline. A total of 100 μl of the emulsion was injected subcutaneously at the base of the tail of each mouse. The mice were weighed and examined visually. Arthritis scores were recorded as described elsewhere (10). Mice were euthanized on the day on which overt signs of inflammation occurred, between 11 and 39 days after CFA injection. Age-matched noninjected WT and IFNγ-KO mice were included as controls. Pale ears, used as a clinical indicator of anemia, were found to correspond to a red blood cell (RBC) count that was $<4.9 \times 10^6/\mu l$ (2 SD of the mean in the noninjected IFNγ-KO mice).

Peripheral blood analysis. Blood samples obtained by heart puncture were treated with 3.8% trisodium citrate buffer (Sigma-Aldrich). A complete blood cell analysis was performed with a Cell-Dyn 3700 apparatus (Abbott Diagnostics).

Histologic, cytospin, flow cytometric, and cell sorter analyses. For histologic analysis, tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Mouse limbs were decalcified with formic acid. Sections were stained with hematoxylin and eosin (H&E).

For cytospin preparations, single-cell suspensions were spun on a glass slide and then stained with H&E.

For flow cytometry and fluorescence-activated cell sorting, cell suspensions were incubated with anti-CD16/anti-CD32 (Miltenyi Biotec) and stained with the following monoclonal antibodies: fluorescein isothiocyanate-labeled CD71, CD34, CD27, Gr-1, CD3, CD8, and CD25; phycoerythrin (PE)–labeled CD122, CD19, F4/80, γ/δ T cell receptor (TCR), and CD4; allophycocyanin-labeled Ter-119, DX5, CD11b, CD4, and FoxP3; and PE–Cy5–labeled CD3 (all from eBioscience). Flow cytometric analysis was performed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). Propidium iodide (PI) was used to exclude dead cells. Cell sorting was performed on a FACSAria III instrument, as follows: in the spleen, neutrophils (Gr1+CD11b+PI−), macrophages (F4/80+CD11b+PI−), NKT cells (DX5+CD122+CD3+PI−), NK cells (DX5+CD122+CD3−PI−), and γ/δ T cells (CD3+γ/δTCR+CD4−PI−), and in the lymph nodes, B cells (CD19+PI−), CD4+ T cells (CD4+PI−), and CD8+ T cells (CD8+PI−). NK and NKT cells from splenocytes were first enriched by using DX5 magnetic beads (Miltenyi Biotec). Besides cell sorting, γ/δ T cells were also enriched using a TCRγ/δ+ T cell isolation kit (Miltenyi Biotec).

Cell-mediated cytotoxicity assay. A ^{51}Cr-release assay was performed as described previously (13). Target cells were the RMA, RMA-S, RMA-Rae, and YAC-1 cell lines. Effector cells were NK cells enriched from splenocytes by the use of magnetic beads coated with DX5 monoclonal antibody.

Enzyme-linked immunosorbent assay (ELISA). The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-17, and tumor necrosis factor α (TNFα) (all from R&D Systems), IL-13

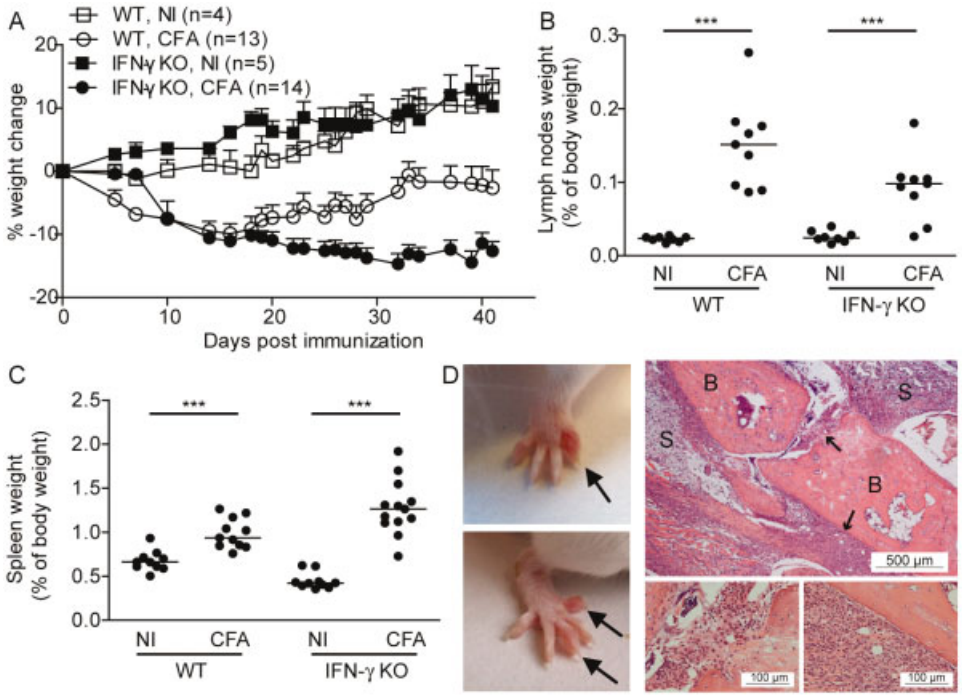

Figure 1. Weight loss, lymphadenopathy, splenomegaly, and arthritis in interferon-γ (IFNγ)–knockout (KO) mice challenged with Freund's complete adjuvant (CFA). Wild-type (WT) mice and IFNγ-KO mice were either not immunized (NI) or were immunized with CFA on day 0, and symptoms of systemic inflammation and arthritis were monitored and scored over the next 6 weeks. **A,** Mean percentage change in body weight in noninjected and CFA-injected mice. Results are from a representative experiment of 5 experiments performed. Values are the mean ± SEM. **B** and **C,** Weights of inguinal lymph nodes (**B**) and spleens (**C**) on days 18–35 postinjection, expressed as a percentage of total body weight. Each symbol represents an individual mouse; horizontal lines show the median results from 2 (**B**) or 4 (**C**) independent experiments. Data are representative of >4 experiments. *** = $P < 0.001$ by Mann-Whitney U test. **D,** Photographs of the front paw (top) and hind paw (bottom) of a CFA-challenged IFNγ-KO mouse obtained on day 20, showing redness and swelling of the toes (**arrows**). Representative photomicrographs of hematoxylin and eosin–stained sections of the ankle joint of a CFA-challenged IFNγ-KO mouse obtained on day 20 postinjection are also shown. Note the synovial hyperplasia and infiltration of mononuclear and polymorphonuclear cells. **S** = synovium; **B** = bone. Regions indicated by the top and bottom **arrows**, respectively, are shown at higher magnification at the bottom, demonstrating pannus formation penetrating into bone (left) and heavily infiltrated synovium (right).

(eBioscience), and IL-18 (MBL) were determined using ELISA kits according to the manufacturers' instructions.

Anti-CD3 stimulation of lymph node cells. Single-cell suspensions of draining lymph nodes were cultured (1×10^6/ml) in RPMI 1640 with L-glutamine (Lonza), supplemented with 10% fetal calf serum, penicillin (100 units/ml; Continental Pharma), streptomycin (100 μg/ml; Continental Pharma), and 50 μ*M* 2-mercaptoethanol (Fluka). Cells were stimulated with anti-CD3/CD28 beads (10 μl/ml; Invitrogen) for 72 hours at 37°C in an atmosphere of 5% CO_2.

Antibody treatment. Monoclonal antibodies against the p40 chain of IL-12/IL-23 were obtained from hybridoma C17.8 (kindly provided by Dr. G. Trinchieri, National Cancer Institute, Frederick, MD). An isotype control antibody was obtained as described elsewhere (14). Monoclonal anti–IL-17A antibody MM17F3 (IgG1κ) was derived as described previously (15). Starting on day −1, mice were treated intraperitoneally with either 200 μg of anti-p40 antibodies once a week, 200 μg of anti–IL-17 antibodies twice a week, or 300 μg of isotype control antibodies once a week.

Detection of cytokine production by quantitative polymerase chain reaction (qPCR). Total RNA was extracted using a PureLink RNA Mini kit (Invitrogen) or an RNeasy Micro kit (purified cells, Qiagen). Complementary DNA was obtained using Superscript II reverse transcriptase and random primers (Invitrogen). Real-time PCR was performed using a TaqMan gene expression assay (Applied Biosystems). Expression levels of IL-1β (assay ID Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439614_m1), IL-17A (Mm00439618_m1), IL-18 (Mm00434225_m1), IFNγ (Mm01168134_m1), and TNFα (Mm00443260_g1) were normalized to the expression of GAPDH RNA (Mm99999915_g1). Relative gene expression was assessed using the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis. Data were analyzed by the nonparametric Mann-Whitney U test. When 3 or more groups were compared, the nonparametric Kruskal-Wallis test was used, followed by Dunn's multiple comparison test. GraphPad Prism software version 5.00 was used.

RESULTS

CFA-induced systemic illness accompanied by mild arthritis in IFNγ-KO mice. BALB/c WT mice and their corresponding IFNγ-KO mice received a single injection of CFA. During the following 6 weeks, mice developed weight loss, which was more chronic in the CFA-treated IFNγ-KO mice (Figure 1A). Lymphadenopathy (Figure 1B) and splenomegaly (Figure 1C) were detected both in WT mice and in IFNγ-KO mice. CFA-injected IFNγ-KO mice, but not their WT counterparts, occasionally showed skin rashes (data not shown). Histologic analysis of the spleen revealed effacement of the normal tissue architecture, with extensive hematopoiesis and myelopoiesis in both subgroups after CFA challenge. Examination of the liver, heart, kidneys, colon, and brain did not reveal any abnormalities (data not shown).

Redness and swelling of the joints in forelimbs and hind limbs were detected predominantly in the IFNγ-KO mice (Figure 1D). The incidence of arthritis was 6% in WT mice (n = 35) versus 44% in IFNγ-KO mice (n = 41). Of note, the incidence varied between experiments, although the difference between groups was consistent. In addition, mice were often euthanized for other purposes before the full development of symptoms. The values given above refer to 4 experiments in which mice were monitored for a long period of time. The mean ± SEM arthritis score was 0.06 ± 0.04 in WT mice and 1.3 ± 0.3 in IFNγ-KO mice. Figure 1D also shows representative images of histologic sections of the inflamed joints of IFNγ-KO mice, illustrating hyperplasia of the synovial tissue, infiltration of the synovium with mononuclear and polymorphonuclear cells, and pannus formation, with cartilage and bone destruction. Although morphologically similar to classic collagen-induced arthritis in DBA/1 mice (a model of rheumatoid arthritis), CFA-induced arthritis in IFNγ-KO BALB/c mice was much less severe, both in terms of its incidence and the scores (10).

Development of thrombocytosis, neutrophilia, and lymphopenia in CFA-challenged WT and IFNγ-KO mice, with anemia in a proportion of the IFNγ-KO mice. We documented the hematologic features of the systemic inflammation. In WT mice, thrombocytosis and neutrophilia developed, starting 2 weeks after CFA injection and lasted throughout the 6-week observation period, with a peak at 3 weeks (Figure 2A). IFNγ-KO mice had a more pronounced increase in the numbers of platelets and neutrophils (0.8-fold and 3.8-fold, respectively) than did the WT mice (0.3-fold and 1.9-fold,

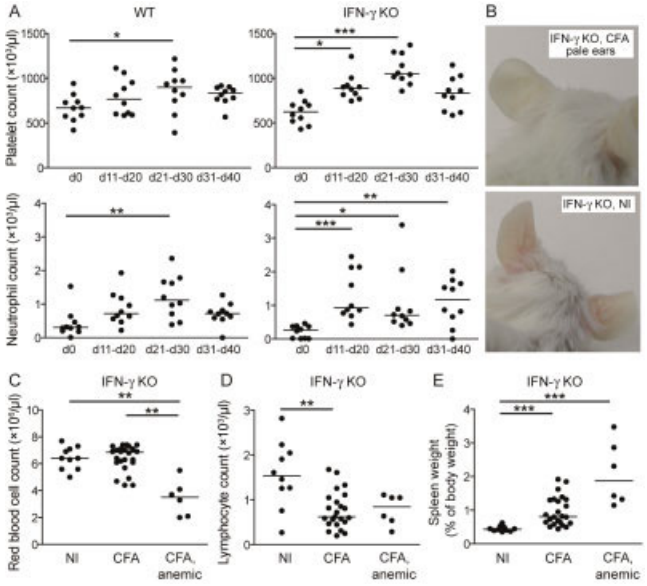

Figure 2. Thrombocytosis, granulocytosis, and anemia in CFA-challenged IFNγ-KO mice. **A,** Platelet counts and neutrophil counts in blood samples taken at the indicated times following CFA injection in WT and IFNγ-KO mice. **B,** Photographs of the ears of a CFA-injected IFNγ-KO mouse and a noninjected IFNγ-KO mouse on day 21 postinjection. Anemia, as indicated by paleness of the ear skin and a lack of visible blood vessels, was observed in ~20% of CFA-challenged IFNγ-KO mice. **C** and **D,** Red blood cell counts (**C**) and lymphocyte counts (**D**) in blood samples from noninjected and CFA-injected IFNγ-KO mice without and with clinically visible anemia. **E,** Weights of spleens on days 11–28 postinjection (when overt signs of inflammation were observed), expressed as a percentage of total body weight. Results are from 12 independent experiments using samples from age-matched mice. In **A**, **C**, **D**, and **E**, each symbol represents an individual mouse; horizontal lines show the median. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ by Kruskal-Wallis test with Dunn's multiple comparison test. See Figure 1 for definitions.

respectively) (day 0 versus days 21–30). Six of 30 IFNγ-KO mice (20%) also developed clinical anemia, as evidenced by pallor and a faded capillary pattern in the ears; Figure 2B shows a representative photograph. The clinically anemic mice displayed a significant reduction in the RBC numbers (Figure 2C) and the hematocrit and hemoglobin levels (data not shown).

The lymphocyte numbers were significantly reduced in CFA-injected WT mice (data not shown) and IFNγ-KO mice, whether anemic or not (Figure 2D). Hematologic derangement was also evident upon examination of the spleen, which in mice, is important for extramedullary myelopoiesis under pathologic conditions. Flow cytometric analysis of splenocytes demonstrated an increase in neutrophils and a decrease in lymphocytes, which was evident in the CD4+ and CD8+ T cell, B cell, and NK cell populations in both subgroups

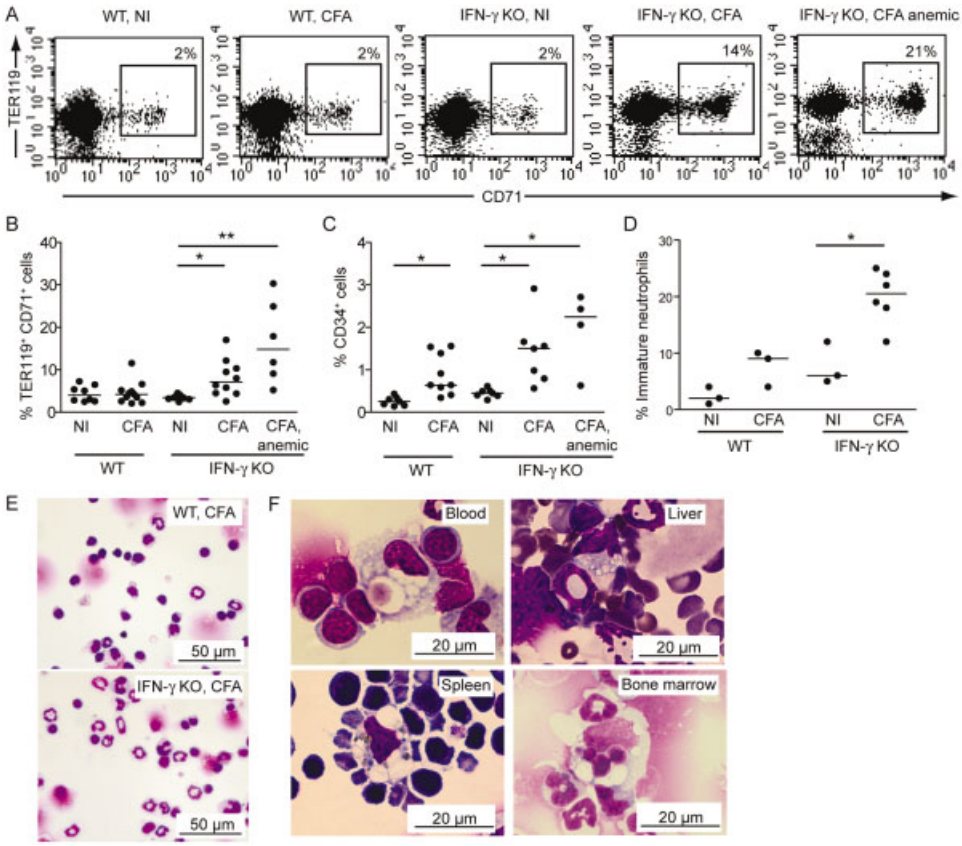

Figure 3. Immature blood cell profile and hemophagocytosis in CFA-challenged IFNγ-KO mice. **A** and **B,** Flow cytometric determination of immature (CD71+Ter-119+) red blood cells (RBCs) in blood samples obtained on day 26 postinjection from noninjected and CFA-injected WT mice and IFNγ-KO mice without and with clinically visible anemia. Representative dot-plots for each group, with counts of double-positive cells expressed as the percentage of total cells (**A**), are shown, as well as CD71+ cells expressed as the percentage of total Ter-119+ blood cells (**B**). **C,** CD34 expression on splenocytes, expressed as the percentage of the total propidium iodide (PI)–negative cells. **D,** Percentages of immature neutrophils in relation to total cells in cytospin preparations of splenocytes in noninjected and CFA-injected WT and IFNγ-KO mice. Results in **B–D** are representative of the findings in individual samples taken on days 14 and 27 and obtained in 2 or 3 independent experiments. Each symbol represents an individual mouse; horizontal lines show the median. * = $P < 0.05$; ** = $P < 0.01$ by Kruskal-Wallis test with Dunn's multiple comparison test (**B** and **C**) or by Mann-Whitney U test (**D**). **E,** Representative photomicrographs of hematoxylin and eosin (H&E)–stained cytospin preparations of spleen cells obtained on day 21 postinjection. Note the numerous immature neutrophils with donut-shaped nuclei in the IFNγ-KO mouse. **F,** Representative photomicrographs of H&E-stained cytospin preparations of blood, spleen, liver, and bone marrow cells obtained on day 28 postinjection from a clinically anemic CFA-injected IFNγ-KO mouse. Images show various lymphocytes and/or RBCs within phagocytes in the different samples. See Figure 1 for other definitions.

(data not shown). Furthermore, CFA-challenged IFNγ-KO anemic mice had grossly enlarged spleens (Figure 2E). The CFA-induced syndrome was associated with a clearly persistent hematologic pattern of inflammation, which was reminiscent of systemic JIA, especially when the clinical manifestations of inflammation and arthritis were taken into account.

Association of systemic inflammation with a drastic increase in immature cell populations in CFA-challenged IFNγ-KO mice. To verify whether anemia in CFA-injected IFNγ-KO mice was caused by decreased production of erythrocytes, flow cytometry was performed on whole blood cells that had been stained for the erythroid-specific Ter-119 marker and the transferrin receptor (CD71), a marker of precursor cells. Representative staining patterns and a summary of all cell counts is shown in Figures 3A and B. CFA-treated WT mice had normal numbers of immature RBCs. However, in IFNγ-KO mice, CFA induced a drastic increase in CD71+Ter-119+ (immature) RBCs, an effect that was more pronounced in anemic animals. These data indicate that anemia in IFNγ-KO mice is not caused by decreased erythropoiesis, but most likely reflects a high turnover of RBCs.

The proportion of hematopoietic progenitor cells, as evidenced by CD34 staining, was also found to be increased in CFA-injected IFNγ-KO mice and less so in WT mice (Figure 3C). Cytospin samples of splenocytes showed increased numbers of immature neutrophils with a typical ring-shaped nucleus. Again, this was more evident in CFA-challenged IFNγ-KO mice than in the corresponding WT mice (Figures 3D and E). The presentation of erythropoiesis and myelopoiesis is consistent with reported observations in systemic JIA patients, where an erythropoiesis gene expression signature (16) and peripheral expansion of CD34+ myelomonocytoid precursors serve as markers of disease activity (17).

Besides increased immature neutrophil counts, cytospin preparations of blood, spleen, liver, and bone marrow cells from CFA-injected IFNγ-KO mice revealed an increased number of macrophages containing ingested erythrocytes, mononuclear leukocytes, and polymorphonuclear leukocytes. Hemophagocytes were particularly observed in anemic mice (Figure 3F). Hemophagocytosis represents excessive activation of macrophages and is a feature that is typical of systemic JIA complicated by MAS (5,18). In addition, up to 50% of patients with systemic JIA—without MAS—have evidence of hemophagocytosis in bone marrow aspirates (2,18).

Association of systemic disease with decreased NK cell cytotoxicity in CFA-challenged IFNγ-KO mice. Systemic JIA patients, especially those with MAS, display a decreased capacity of NK cells to kill target cells (19,20). To measure the cytotoxicity of NK cells in CFA-challenged mice, we enriched DX5+ splenocytes and tested their killing capacity with a ^{51}Cr-release assay on 3 NK cell–sensitive cell lines (RMA-S, RMA-Rae, and YAC-1) compared to a NK cell–insensitive control cell line (RMA). The killing capacity of IFNγ-KO mice was significantly lower than that of WT mice, both in noninjected and in CFA-injected animals (data available upon request from the corresponding author), which corresponds to the known phenotype of IFNγ-KO mice described by Dalton et al (21).

IL-12/IL-23p40– and IL-17–mediated, CFA-induced disease symptoms. We investigated the presence of inflammatory cytokines characteristic of systemic JIA, in particular, IL-1β, IL-6, IL-18, and TNFα, at the mRNA level in blood and lymph node cells and at the protein level in serum. In blood cells, there was a trend, although not significant, toward increased levels of IL-1β and IL-6 in both CFA-challenged WT and IFNγ-KO mice and a significant increase in IL-18 in WT mice relative to the findings in nonchallenged WT mice (Figure 4A). Lymph node cells showed a significant increase in IL-1β and IL-18 levels in both subgroups (Figure 4B). While serum levels of IL-1β, IL-18, and TNFα were undetectable at several time points or were not up-regulated after CFA challenge (data not shown), IL-6 levels were dramatically increased, especially in CFA-challenged IFNγ-KO mice (Figure 4C). Since IL-10 has proven to be important in protecting against hemophagocytosis (22), mRNA expression was also determined in blood and lymph node cells. A comparable increase in IL-10 mRNA expression was evident after CFA injection in both subgroups of mice (data not shown).

T helper cell profiles were analyzed by quantifying the levels of IL-2, IL-4, IL-5, IL-13, and IL-17 in supernatants from lymph nodes cells that had been stimulated with anti-CD3/CD28 beads. IL-2, IL-4, IL-5 and IL-13 were either not detectable or were not up-regulated after CFA challenge (data not shown). Cells from CFA-challenged IFNγ-KO mice produced dramatically more IL-17 than did those from noninjected mice (Figure 4D).

The more prominent production of IL-17 in CFA-challenged IFNγ-KO mice suggested a causal role for this cytokine in the pathogenesis. Therefore, we tested anti–IL-17 antibodies as well as antibodies against p40, a subunit of IL-23, a cytokine important in the production of IL-17 (23). Pretreatment of IFNγ-KO mice with anti-p40 antibodies completely prevented the symptoms: antibody-treated mice did not lose weight, in contrast to isotype control–treated mice (data not shown), nor did they develop arthritis, rash, or other overt signs of inflammation. At necropsy, splenomegaly and lymphadenopathy were absent. Treatment with anti–IL-17 antibody also counteracted weight loss, albeit to a lesser extent than did anti-p40, but nevertheless completely prevented splenomegaly (Figures 4E and F) as well as arthritis and rash (data not shown). Anti-p40 and anti–IL-17 antibody treatment further abolished the induction of neutrophilia, anemia, and thrombocytosis in CFA-challenged IFNγ-KO mice (Figure 4G).

Adaptive and innate immune cells as important sources of IL-17 and IFNγ. Given the apparent causal role of IL-17 in the syndrome and in an effort to unravel the mechanism of inflammation resulting from CFA injection, we investigated sources of IL-17. Besides Th17 cells, γ/δ T cells are known to be potent IL-17–producing cells and were found to be the main source of IL-17 in mycobacterial infections (24). First, percentages and cell numbers of γ/δ T cells, subdivided into a CD27+ IFNγ-producing subset and a CD27– IL-17–producing subset (25), were analyzed in lymph nodes. Upon chal-

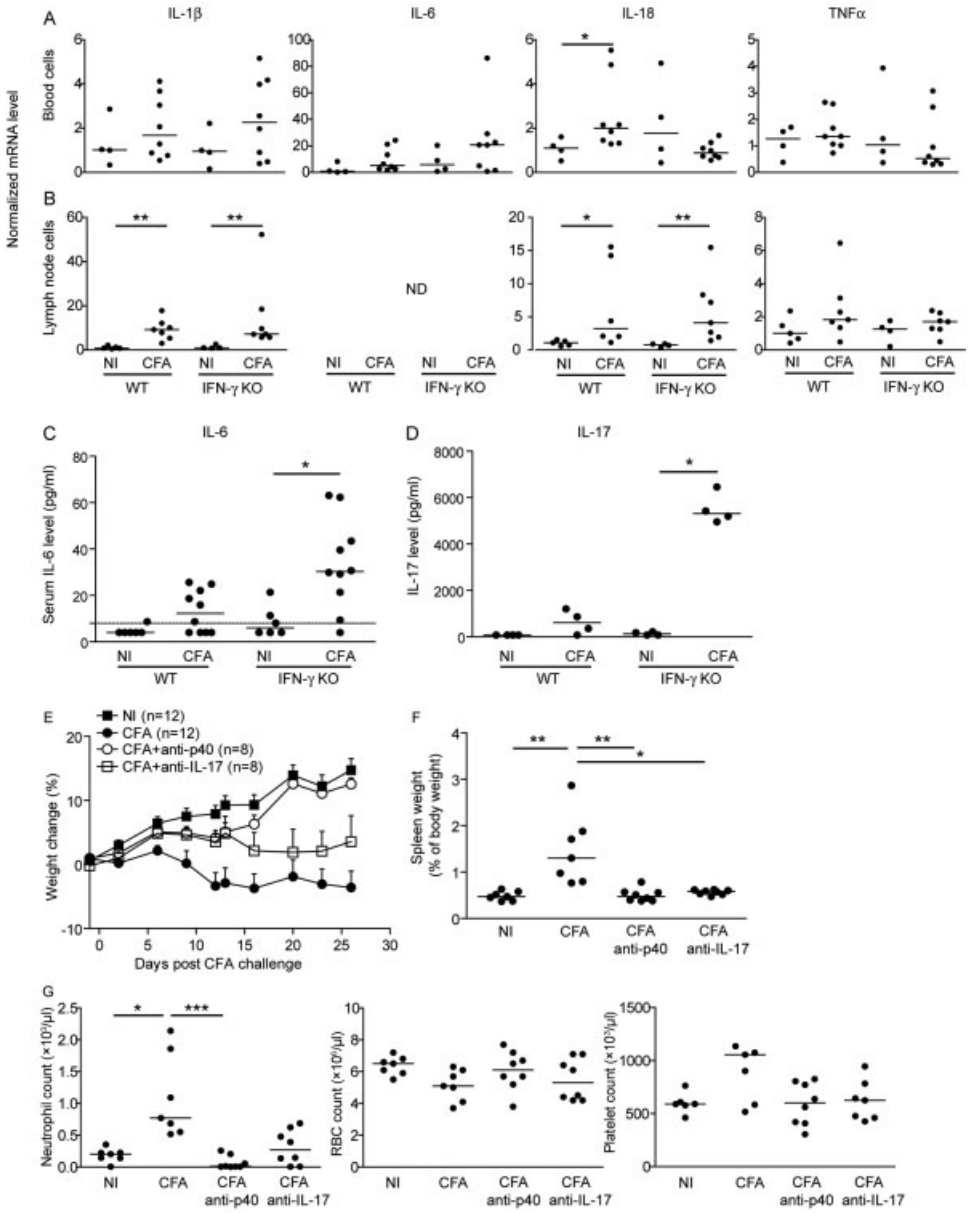

Figure 4. Cytokines in CFA-challenged mice and counteraction of the symptoms by treatment with anti-p40 and anti–IL-17 antibodies. **A** and **B,** Levels of mRNA for the cytokines IL-1β, IL-6, IL-18, and tumor necrosis factor α (TNFα) in blood cells (**A**) and lymph node cells (**B**) obtained on days 25 and 35 postinjection from noninjected and CFA-injected WT and IFNγ-KO mice. Results are from 2 independent experiments. ND = not detected. **C,** Serum IL-6 levels in age-matched mice obtained on days 19, 26, 32, and 39 postinjection. Horizontal line extending from the y-axis represents the limit of detection (8 pg/ml). Results are from 2 independent experiments of 4 experiments performed. **D,** IL-17 levels in supernatants of anti-CD3/CD28–stimulated lymph node cells obtained on day 25 postinjection and cultured for 72 hours. Results are from a single experiment of 3 experiments performed. **E–G,** Percentage change in body weight as compared with day −1 (**E**), weight of spleens, expressed as a ratio of the total body weight (**F**), and blood neutrophil, red blood cells (RBC), and platelet counts (**G**), in noninjected IFNγ-KO mice or in CFA-injected IFNγ-KO mice given weekly intraperitoneal injections of anti-p40 antibodies (200 μg per mouse) or twice-weekly intraperitoneal injections of anti–IL-17 antibodies (200 μg per mouse) starting on day −1. Results are from a single experiment and were confirmed in a separate experiment. Values in **E** are the mean ± SEM. In **A**, **B**, **C**, **D**, **F**, and **G**, each symbol represents an individual mouse; horizontal lines show the median. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$ by Mann-Whitney U test (**A–D**) or by Kruskal-Wallis test with Dunn's multiple comparison test (**F** and **G**). See Figure 1 for other definitions.

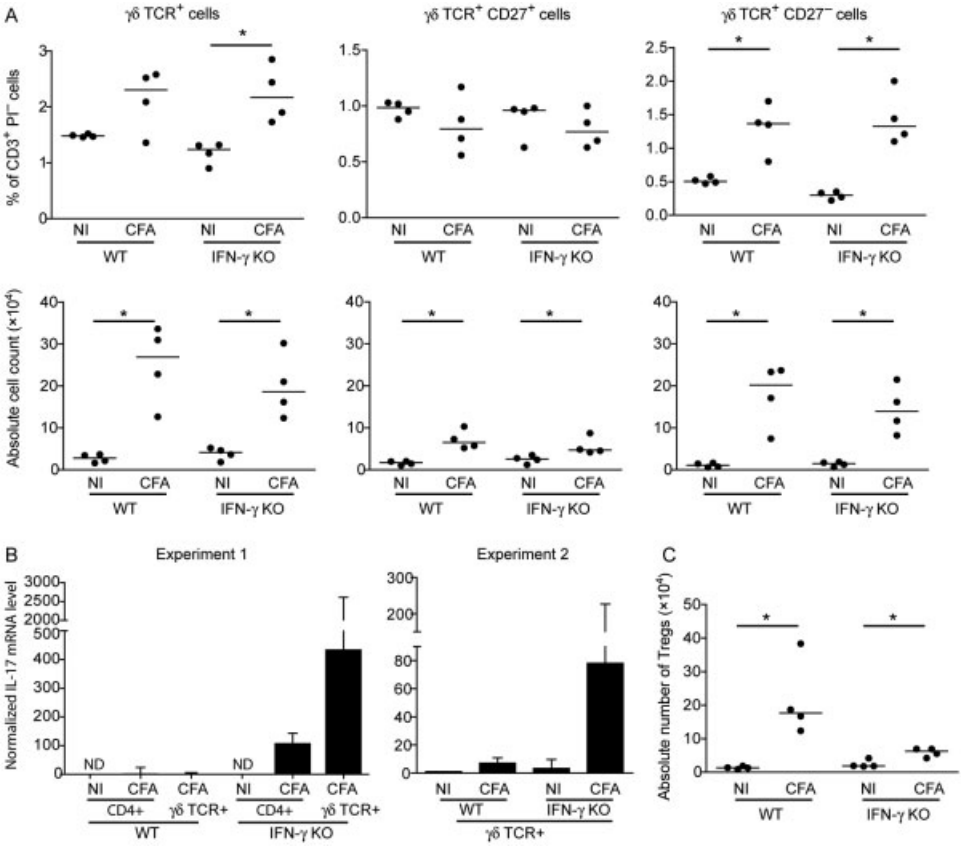

Figure 5. Adaptive CD4+ T cells and innate γ/δ T cells as major sources of interleukin-17 (IL-17). **A,** Percentages of γ/δ T cell receptor (γ/δ TCR)–positive cells, γ/δ TCR+CD27+, and γ/δ TCR+CD27− cells relative to CD3+ propidium iodide (PI)–negative cells (top), as well as the absolute numbers of these cells (bottom) as determined by flow cytometry of lymph node cells from noninjected and CFA-injected WT and IFNγ-KO mice. Results are from a single experiment of 2 experiments performed. **B,** Expression of mRNA for IL-17 in purified CD4+ T cells and γ/δ T cells from noninjected and CFA-injected WT and IFNγ-KO mice. Values are the median and interquartile range of 6 mice per group for the CD4+ cells (results of 2 independent experiments) and 3 mice per group for the γ/δ T cells (results of 1 experiment; confirmed by enrichment of γ/δ T cells from the spleen with magnetic beads, experiment 2). **C,** Absolute numbers of Treg cells (defined as FoxP3+CD4+CD25+) as determined by flow cytometry of lymph node cells from noninjected and CFA-injected WT and IFNγ-KO mice. In **A** and **C**, each symbol represents an individual mouse; horizontal lines show the median. * = $P < 0.05$ by Mann-Whitney U test. See Figure 1 for other definitions.

lenge with CFA, γ/δ T cells were increased in both WT and IFNγ-KO mice, and the increase was more pronounced in the CD27− subpopulation (Figure 5A). Second, we purified neutrophils, macrophages, NKT cells, NK cells, B cells, CD4+ T cells, CD8+ T cells, and γ/δ T cells from CFA-challenged WT and IFNγ-KO mice and analyzed their IL-17 mRNA levels. IL-17 expression was detected in 2 cell populations, CD4+ T cells and γ/δ T cells (Figure 5B), and was more pronounced in the mice without IFNγ. Other cell populations were negative for IL-17 expression (data not shown). Thus, both innate and adaptive responses seem to be important in CFA-induced inflammatory disease.

To further analyze the regulatory role of IFNγ in CFA-challenged WT mice, we investigated the main sources of this cytokine in the purified cell populations of WT mice. Again, innate immune cells turned out to be important producers, as the highest expression of IFNγ was detected in NK cells, γ/δ T cells, and NKT cells (data not shown). Since we previously demonstrated a key role of IFNγ in the stimulation of Treg cells in collagen-induced arthritis (26), we also determined the absolute numbers of Treg cells in WT and IFNγ-KO mice with or without CFA challenge. As can be seen in Figure 5C, CFA resulted in increased Treg cell numbers in both subgroups of mice, but the increase was more pronounced in WT mice (16.5-fold increase in WT mice versus 2.5-fold increase in IFNγ-KO mice).

Table 1. Comparison of findings in CFA-challenged BALB/c mice in the present study with those in systemic JIA patients described in the literature*

	CFA-challenged mice†		Patients with systemic JIA, reference
	WT	IFNγ KO	
Clinical			
Fever	−	−	1–3, 46, 47
Weight loss	±	+	46
Arthritis	±	+‡	1–3, 46, 47
Skin rash	−	+‡	1–3, 46, 47
Splenomegaly	+	+	1–3, 46, 47
Hepatomegaly	−	−	1–3, 46, 47
Lymphadenopathy	+	+	1–3, 46, 47
Hematologic			
Neutrophilia	+	+	1–3, 48
Thrombocytosis	+	+	1–3, 46
Lymphopenia	±	+	49
Anemia	−	+‡	1, 3, 16, 37, 46, 48
Decreased hemoglobin	−	+‡	1, 18, 37,46
Immature red blood cells	−	+	2, 16, 17
Immature white blood cells	+	+	2, 16, 17
Histopathologic			
Hemophagocytosis	−	+‡	1, 2, 18, 46
Immune/inflammatory			
Increased IL-6	±	+	1–3, 16, 17, 37, 46, 48
Increased IL-17/Th17	±	+	2, 35
Increased IL-1β, IL-18	+§	+§	1–3, 20, 46, 48
Impaired NK cell cytotoxicity	−	+	1, 2, 19, 20
Decreased NK cell numbers	+	+	17, 20, 50

* BALB/c wild-type (WT) mice and interferon-γ (IFNγ)–knockout (KO) mice were challenged with a single injection of Freund's complete adjuvant (CFA) at the base of the tail. Clinical, biologic, pathologic, and mechanistic features were analyzed and recorded within 40 days following CFA injection. JIA = juvenile idiopathic arthritis; IL-6 = interleukin-6; NK = natural killer.
† Features were graded as follows: + = present, ± = transient (weight loss), rarely present (arthritis), or only slightly different from noninjected mice, and − = absent.
‡ Present in subsets of mice.
§ Level of mRNA found in lymph node cells.

DISCUSSION

In this study, we demonstrated that injection of CFA into WT BALB/c mice caused a systemic inflammatory reaction, the severity of which was greatly increased in IFNγ-KO mice, resulting in a syndrome reminiscent of systemic JIA in humans. Table 1 provides an overview of the typical systemic JIA symptoms and laboratory characteristics that were investigated in CFA-challenged mice. A number of symptoms, such as skin rash, increased numbers of immature RBCs and neutrophils, anemia, and hemophagocytosis, were found exclusively in the IFNγ-KO mice. We conclude that this experimental system merits further investigation, as it might represent a useful study model in which to unravel the pathogenesis of human systemic JIA. Although some MAS/HLH-associated features were observed, the absence of increased levels of ferritin and liver enzymes (data not shown) indicates that our model does not represent a model for MAS/HLH.

The cause of systemic JIA is not understood; triggers of the disease are unknown, but infectious agents are suspected. Once initiated, a persistent or uncontrolled stimulation of innate immune responses is believed to be important in the pathogenesis (2–4). In our animal model, an emulsion of heat-killed mycobacteria in oil adjuvant (CFA) was chosen to mimic an infectious trigger and ongoing stimulation of the immune response. Killed mycobacteria contain ligands for Toll-like receptors (TLRs), such as peptidoglycans and CpG (9). In a recently described mouse model of MAS, chronic stimulation of TLRs by CpG injection in WT mice was demonstrated to be sufficient to provoke MAS-like symptoms (22). In our model, the mycobacterial component in CFA was found to be crucial, since Freund's incomplete adjuvant was not able to elicit symptoms in the mice (data not shown).

BALB/c mice were chosen because, in contrast to C57BL/6 mice, they are exquisitely susceptible to mycobacterial infection, due to insufficient Th1 immune activation (27). We postulate that the systemic inflammation in IFNγ-KO BALB/c mice results from an uncontrolled immune response to a mycobacterium-containing adjuvant in a mouse strain that, by nature, has difficulties in mounting a potent Th1 response and, moreover, is deficient in IFNγ. Interestingly, IFNγ-KO BALB/c mice infected with *Mycobacterium bovis* (bacillus Calmette-Guérin) developed features similar to those observed in our CFA-challenged mice (28). The similarity in disease pathology obtained in IFNγ-KO mice challenged with either live or heat-killed mycobacteria points to a similar machinery of IFNγ to control inflammation regardless of the infectivity of the stimulus.

IFNγ is a key cytokine in the up-regulation and induction of class I and class II major histocompatibility complex molecules on antigen-presenting cells (APCs) (ref. 26 and confirmed in this study [data not shown]). Therefore, defective clearance of APCs that have ingested microbial particles and, consequently, have prolonged the stimulation of the immune system may contribute to the development of the symptoms. Additionally, IFNγ stimulates Treg cell development and exerts negative feedback control on proinflammatory cytokines (26). Herein, we showed that, upon CFA

injection, Treg cell numbers were significantly increased in WT mice and, to a lesser extent, in IFNγ-KO mice. Furthermore, we present evidence of increased mRNA expression of innate immune cytokines (i.e., IL-1β, IL-6, and IL-18) upon challenge with CFA in both mouse substrains, but at the protein level, only IL-6 was detectable in serum and was found to be significantly increased in the IFNγ-KO mice.

Cytokine secretion in CD3-stimulated lymph node cells from CFA-challenged IFNγ-KO mice was indicative of a T cell profile dominated mainly by IL-17–producing cells. The pathophysiologic relevance of this finding is evident from our observation that systemic inflammation in CFA-challenged IFNγ-KO mice could be blocked by treatment with neutralizing antibodies against IL-17 or against IL-12/IL-23p40. Although p40 is a subunit of both IL-12 and IL-23, the effect of the p40 antibody is most likely due to its neutralizing capacity on IL-23 and, consequently, IL-17, since no differences in IL-12 expression were seen after CFA challenge (data not shown). Besides directly strengthening Th17 cell expansion and pathogenicity (23,26), IL-23 stimulates γ/δ T cells to produce IL-17 and to amplify its production by Th17 cells in vivo (29). It was demonstrated that during mycobacterial infections, γ/δ T cells are increased and are a major source of IL-17, representing a dominant innate response in these chronic infections (24,30). In our model, γ/δ T cell numbers were significantly increased in lymphoid organs, mainly resulting from expansion of the IL-17-producing CD27− subset. Comparison of mRNA levels in purified cell populations from CFA-injected IFNγ-KO mice pointed to an important contribution of innate γ/δ T cells in IL-17 production. Th17 differentiation is strongly inhibited by IFNγ (23,26). The dramatically increased expression of IL-17 by γ/δ T cells in the absence of IFNγ indicates that a similar inhibitory activity applies to γ/δ T cells.

The observation of anemia and hemophagocytosis in the setting of IFNγ deficiency was unexpected in view of 2 reports emphasizing IFNγ as a critical driver of these hematologic changes (31,32). Our data indicate that anemia and hemophagocytosis can occur in the absence of IFNγ. The presence of anemia in our animal model may be caused by the abundance of proinflammatory cytokines, especially IL-6, which is known to block the iron supply to developing erythroid cells (33). CFA-challenged IFNγ-KO mice showed elevated serum levels of IL-6 and an abundance of immature Ter-119+CD71+ RBCs and immature neutrophils. IFNγ is a well-described inhibitor of erythropoiesis and myelopoiesis (32,33), and therefore, the increased immature RBCs and neutrophils may also be seen as a direct consequence of the absence of IFNγ. The presence of hemophagocytosis in the absence of IFNγ corresponds to a study by Milner et al (34), who described an IFNγ-independent pathway of hemophagocytosis and macrophage accumulation in mice exposed to sustained IL-4 stimulation.

The defect in NK cytotoxicity in the absence of IFNγ signaling is consistent with the original description of the phenotype of IFNγ-KO mice (21). The defect may itself account for part of the symptoms of IFNγ-KO mice challenged with CFA. In fact, injection of CFA in NK cell–depleted WT mice only partially resulted in a systemic JIA syndrome (data not shown), which may be explained by the fact that, in addition to their cytotoxic function, NK cells are an important source of potentially harmful cytokines.

Each of these altered inflammatory pathways in CFA-stimulated IFNγ-KO BALB/c mice corresponds to what is known about the pathologic pathways in patients with systemic JIA: 1) an important role of IL-17, as recently indicated by enrichment of both Th1 and Th17 subpopulations in the peripheral blood of systemic JIA patients (35); 2) elevated levels of IL-6 (36) assumed to be among the mechanisms underlying both increased erythropoiesis and anemia (37), and the presence of an erythropoiesis gene expression signature and peripheral expansion of CD34+ myeloid precursor cells (16,17,38); 3) hemophagocytosis present in a substantial number of systemic JIA patients and considered to be a pathognomonic feature of systemic JIA–associated MAS and HLH (2,5,18); and 4) reduced NK cell cytotoxicity characteristically present in MAS and HLH, and more recently also demonstrated in systemic JIA (8,19,20). Thus, not only does the systemic inflammatory syndrome in CFA-challenged BALB/c mice resemble the clinical picture of systemic JIA, the underlying immune and inflammatory pathways, as far as they are established for systemic JIA, likewise show remarkable parallelisms with those in the mouse model.

Does this imply that the IFNγ pathways in systemic JIA patients are in some way defective? Mutations in the IFNγ gene have not been described in systemic JIA, and increased IFNγ levels have been detected in the plasma of systemic JIA patients. However, these plasma levels were found to be 5 times lower in patients with active systemic JIA than in those with active polyarticular JIA, which was unexpected in view of the high levels of IFNγ-inducing IL-18 (39). Interestingly, de Jager et al (20) reported a defective production of

IFNγ by NK cells from systemic JIA patients upon stimulation with IL-18 (20), which is indicative of disturbed IFNγ production. Consistent with these observations, 2 teams of investigators independently reported failure to detect up-regulation of IFNγ-associated genes in freshly isolated peripheral blood mononuclear cells from patients with active systemic JIA (17,40,41). Our data fail to support a major proinflammatory role of IFNγ in the development of systemic JIA. The possibility that IFNγ is somehow prevented from fully exerting its regulatory function remains open.

Although murine models of HLH syndromes point to IFNγ as the dominant causative cytokine (42,43) and the presence of IFNγ-producing lymphocytes was demonstrated in liver biopsy tissues from patients with MAS (44), a direct proinflammatory role of IFNγ in MAS was recently also questioned by Canna et al (45), since symptoms in the CpG mouse model of MAS were independent of IFNγ, with the exception of anemia.

In conclusion, CFA induces a severe systemic inflammatory disease in IFNγ-deficient mice, which can be explained by loss of control over an IL-17–dominated innate and adaptive immune inflammatory response. The inflammatory condition seen in the IFNγ-KO mice is highly reminiscent of the clinical and immunologic features seen in systemic JIA. The model may be of help in the investigation of systemic inflammatory syndromes.

ACKNOWLEDGMENTS

The authors thank Maarten Van Balen for help with preliminary experiments, Thomas Tousseyn for histologic analysis, and Alfons Billiau for critical revision of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Matthys had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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