Involvement of IL-6, Apart from Its Role in Immunity, in Mediating a Chronic Response during Experimental Arthritis

Alfons S. K. de Hooge, Fons A. J. van de Loo,
Onno J. Arntz, and Wim B. van den Berg

From the Rheumatology Research Laboratory, University Medical
Center Nijmegen, Nijmegen, The Netherlands

Interleukin-6 (IL-6) is highly produced during arthritis but its exact function is still unknown. In this study we examined if IL-6, apart from its role in immunity, was involved in the local inflammatory response in experimental arthritis. IL-6 deficient (IL-6^{-/-}) and wild-type mice were first compared in the antigen-induced arthritis model. IL-6 deficiency resulted in a mild, transient inflammation whereas wild-type mice developed a chronic, destructive synovitis. Wild-type mice immunized with one-tenth of the normal antigen dose still developed chronic arthritis despite low antibody levels, excluding reduced humoral immunity in IL-6^{-/-} mice as a crucial phenomenon. In addition, passive immune-complex-induced arthritis did not differ between wild-type and IL-6^{-/-} mice. Another option is reduced levels of Th1 cells in IL-6^{-/-} mice. However, transfer of antigen-specific wild-type lymph node cells to IL-6^{-/-} mice enhanced acute joint inflammation and increased cartilage damage but still could not sustain chronic inflammation, suggesting involvement of nonimmune elements of IL-6 activity in chronicity. In line with this, nonimmunologically mediated zymosan-induced arthritis developed similarly in the first week, but only wild-type mice developed chronic synovitis. These results indicate an important role for IL-6 in propagation of joint inflammation, potentially independent of its role in immunity. (Am J Pathol 2000, 157:2081–2091)

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by a chronic inflammation of the joints. This inflammation finally leads to tissue destruction that disables the patient. Although the exact cause of RA is not yet known pro- and anti-inflammatory cytokines seem to play an important role in the pathology of the disease.¹ Interleukin-6 (IL-6) is a member of the IL-6 family to which leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and IL-11 also belong.^{2,3} Both IL-6 and the agonistic soluble IL-6 receptor are found in large quantities in synovial fluid and serum of RA patients.⁴ The main

producers of IL-6 in the inflamed joint are articular chondrocytes and synovial fibroblasts.^{5,6} Studies on the relation of disease activity and IL-6 concentration have yielded conflicting results.^{7–9} Anti-IL-6 monoclonal antibodies showed transitory clinical improvement in RA patients.¹⁰ Surprisingly, this effect was accompanied by an increase in IL-6 serum levels, which makes it unclear what caused the improvement.

Both pro- and anti-inflammatory properties have been ascribed to IL-6, complicating the establishment of its role in RA. IL-6 plays an important role in the maturation of B cells into antibody-secreting plasma cells,¹¹ differentiation of osteoclasts¹² and macrophages,¹³ generation of an acute-phase response in the liver,^{14–16} and has a co-stimulatory role in T cell activation.^{17,18} On the other hand, IL-6 can induce expression of IL-1 receptor antagonist, soluble tumor necrosis factor (TNF) receptor, and tissue inhibitor of metalloproteinases,^{19,20} which could down-regulate inflammation and reduce connective tissue damage in the inflamed joint. IL-6 also can reduce TNF production.²¹

The dual face of IL-6 as a pro- and anti-inflammatory protein is also reflected by studies in IL-6 gene knockout (IL-6^{-/-}) mice. The local inflammatory response against turpentine was impaired in IL-6^{-/-} mice whereas systemic inflammatory reactions on lipopolysaccharide were not.²² The inflammatory response against *Candida albicans* was also impaired in IL-6^{-/-} mice.²³ Xing et al²⁴ in contrast found increased inflammatory reactions in endotoxic lung or during endotoxemia in IL-6^{-/-} mice and proposed an anti-inflammatory role of IL-6 during acute infection. IL-6^{-/-} mice also had a higher incidence of arthritis after infection with *Borrelia burgdorferi*²⁵ demonstrating an anti-inflammatory role of IL-6. In a previous study we looked into the role of IL-6 in zymosan-induced arthritis (ZIA),²⁶ a nonimmunologically mediated irritant-induced joint inflammation.²⁷ During the first week of ZIA the inflammation developed synchronically in IL-6^{-/-} and wild-type mice. Intriguingly, cartilage damage was in-

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Address reprint requests to Alfons S. K. de Hooge, Rheumatology Research Laboratory, University Medical Center Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands. E-mail: A.deHooge@reuma.azn.nl.

creased in the IL-6^{-/-} mice, pointing at a cartilage protective role for IL-6. A recent study by Ohshima et al²⁸ showed the importance of IL-6 for development of antigen-induced arthritis (AIA), an immunologically mediated model with features of RA such as synovial hyperplasia, influx of inflammatory cells, and cartilage damage.²⁹ Their study focused at the outcome of arthritis at day 14 and differences in the antigen-specific immunity. It remains unclear what caused amelioration of the disease in IL-6^{-/-} mice: the developed, but impaired, antigen-specific immune response or the absence of IL-6 during the inflammation. In the present study we wanted to examine if IL-6, independent of its role in immunity was involved in the inflammatory response in different experimental arthritis models. In these models wild-type and IL-6^{-/-} mice were compared. We confirmed that initial inflammation in IL-6^{-/-} mice did not develop into a chronic inflammatory infiltrate during AIA. Differences in cellular but not humoral immunity had major influence on the onset of AIA. However, transfer of wild-type lymph node cells enhanced the mild inflammatory response in IL-6^{-/-} mice but still did not lead to a chronic infiltrate. In the nonimmunologically mediated ZIA we also found that the acute inflammation of the first week did not develop into a chronic synovial infiltrate in IL-6^{-/-} mice. These results suggest that in both immunologically and nonimmunologically mediated experimental arthritis, there is an important role for IL-6 in propagation of the inflammatory infiltrate.

Materials and Methods

IL-6^{-/-} and Wild-Type Mice

Homozygous IL-6^{-/-} and wild-type (C57Bl/6x129/Sv) F2 mice³⁰ and IL-6^{-/-} mice back-crossed into C57Bl/6 for eight generations (N8) were obtained from Dr. M. Kopf (Basel, Switzerland) and bred in our SPF animal facilities. IL-6 deficiency was routinely checked by polymerase chain reaction (PCR) of genomic DNA. A standard diet and acidified tap water were provided *ad libitum*. At the age of 11 to 13 weeks the animals were used in the experiments. Experiments were performed according to national and institutional regulations for animal use.

AIA

Mice were immunized with 100 μ g of methylated bovine serum albumin (mBSA; Sigma, St. Louis, MO) in Freund's complete adjuvant (Difco, Detroit, MI) divided over the front paws and both flanks. They also received an intraperitoneal injection of 2×10^9 heat-killed *Bordetella pertussis* bacteria (National Institute of Public Health, Bilthoven, The Netherlands) in 1 ml of saline. Seven days later, mice were boosted with 100 μ g of mBSA in Freund's complete adjuvant divided over two places in the neck region. Three weeks after the booster 60 μ g of mBSA in saline (6 μ l total volume) was injected in the knee joint cavity of the right hind leg to induce arthritis.

Immune-Complex-Induced Arthritis

An immune-complex-induced arthritis was elicited in naive mice as described by van Lent et al.³¹ Mice were injected intravenously with 0.2 ml of a polyclonal rabbit anti-lysozyme serum of which the complement had been heat-inactivated. Arthritis was induced 16 hours later by injecting 6 μ l of poly-L-lysine-coupled lysozyme (3 μ g; Sigma) in the joint.

ZIA

ZIA was elicited by intra-articular injection of 180 μ g of zymosan A (*Saccharomyces cerevisiae*) as described before.²⁶

Assessment of Joint Swelling

Mice were injected subcutaneously in the neck with 20 μ Ci of ^{99m}Tc-pertechnetate (^{99m}Tc) in 0.2 ml of physiological saline. After 15 minutes, mice were sedated by intraperitoneal injection of 4.5% chloral hydrate (0.1 ml/10 mg of body weight; Merck, Darmstadt, Germany). Accumulation of the isotope because of increased blood flow and edema in the knee was determined in duplicate by external γ counting using a NaI crystal. The ratio of ^{99m}Tc uptake in the inflamed over the contralateral knee joint was determined and a ratio higher than 1.1 indicated joint swelling.

Histological Evaluation of Knee Joints

Knee joints were dissected, fixed in formalin, decalcified, dehydrated, and embedded in paraffin. Standard frontal sections of 7 μ m were prepared. For assessing cartilage damage, sections were stained with safranin-O and counterstained with fast green. Serial sections were scored in a blind-folded manner by two independent observers. Cartilage depletion was scored from 0 (normal safranin-O staining, no depletion) up to 3 (complete loss of safranin-O staining, complete depletion). Also cellular infiltrate and exudate were scored in a scale from 0 up to 3.

Semiquantitative Reverse Transcriptase (RT)-PCR on Synovial mRNA

Synovial mRNA was isolated and quantitated as described by van Meurs et al.³² Patellae were isolated from knee joints and two pieces of tissue adjacent to the patella were punched out with a 3-mm biopsy punch (Stiefel Laboratorium GmbH, Offenbach am Main, Germany). The tissue was immediately frozen in liquid nitrogen. Tissue samples were homogenized in a freeze mill, thawed in 1 ml of TRIzol reagent, and further processed according to the manufacturers protocol. All reagents for RNA isolation and RT-PCR were from Life Technologies (Breda, The Netherlands). Isolated RNA was treated with DNase before being reverse-transcribed into cDNA with

Moloney murine leukemia virus reverse transcriptase. After increasing numbers of PCR cycles samples were taken and run on an agarose gel. The cycle number at which the PCR product was first detected on the gel was taken as a measure for the amount of specific mRNA originally present in the isolated synovial RNA. PCR for glyceraldehyde-3-phosphate dehydrogenase was performed to verify that equal amounts of cDNA were used. Primers, annealing temperature, and $MgCl_2$ concentration for murine VCAM-1,³³ ICAM-1, E- and P-selectin,³⁴ MCP-1, Mip-1 α and Mip-2 (Blom et al, submitted), TNF- α , IL-1 β , and glyceraldehyde-3-phosphate dehydrogenase³⁵ were as described.

Assessment of mBSA-Specific Antibody Titers

mBSA-specific antibody titers in sera were determined by enzyme-linked immunosorbent assay. Ninety-six-well flat-bottom plates (Costar, Corning, NY) were coated overnight with mBSA (0.1 mg/ml; Sigma), blocked the next day with gelatin (1% in phosphate-buffered saline) and incubated with serial dilutions of the sera. Horseradish peroxidase-labeled secondary antibodies were from Southern Biotechnologies Associates (goat anti-mouse IgG1 or anti-mouse IgG2b; Birmingham, AL) or from Nordic (rat anti-mouse IgG or anti-mouse IgG2a; Tilburg, The Netherlands). Color development after adding substrate (5-aminosalicylic acid, 0.8 mg/ml, 0.8 μ l 30% H_2O_2 /ml in 50 mmol/L of sodium-phosphate, pH 6.0) was monitored in an enzyme-linked immunosorbent assay reader at 450 nm. Dilution curves were plotted and the dilution at optical density 50% was determined. A standard serum of mBSA immunized C57Bl/6 mice was included for intra-assay variation.

Lymphocyte Stimulation Test

Antigen-specific T cell immunity was determined by the proliferative response of T cells to mBSA. Axillary and inguinal lymph nodes were isolated under sterile conditions and disrupted. The suspension was enriched for T cells by nylon wool adherence for 30 minutes. Nonadherent cells were washed from the column and adjusted to 2×10^6 cells/ml in medium (RPMI/penicillin 100 U/ml and streptomycin 100 μ g/ml/1 mmol/L pyruvate/5% fetal calf serum; Life technologies). Cells were plated in a 96-well round-bottom plate (Costar) and antigen-presenting cells were added at 2×10^7 /ml. As antigen-presenting cells irradiated spleen cells from naive mice were used after disruption and lysis of erythrocytes (in 17 mmol/L Tris/144 mmol/L ammonium chloride, pH 7.2). Cells were incubated in three- or sixfold with medium, medium with twofold serial dilutions of mBSA starting at 25 μ g/ml (data shown for 25 μ g/ml) or with concanavalin A (Flow laboratories, Irvine UK) at 1 μ g/ml as an aspecific stimulus. The plates were incubated for 3 days at 37°C and 5% CO_2 . For the last 16 hours the cells were labeled with 0.25 μ Ci 3H -thymidine. After harvesting, incorporation of 3H -thymidine was measured in a β -plate reader and the

increase in counts per minute (cpm) caused by stimulation with mBSA was determined.

Multiplex PCR for T Cell Subsets

T cells were isolated and stimulated as described above. After 1 day RNA was isolated as described above. For RT-PCR analysis of T cell subsets the mouse Th1/Th2 CytoXpress kit was used according to the manufacturers protocol (BioSource, Camarillo, Ca). Products were run on a 2% agarose gel and analyzed with the multianalyst system (BioRad, Richmond, CA).

Cell Transfer Experiments

For cell transfer experiments single-cell preparations of lymph nodes (axillary and inguinal) were made from immunized wild-type or IL-6 $^{-/-}$ mice. To prevent a possible graft-versus-host reaction C57Bl/6 mice were used as donors and C57Bl/6 IL-6 $^{-/-}$ (N8) mice as recipients. Arthritis development did not differ between these mice and the C57Bl/6x129/Sv IL-6 $^{-/-}$ mice (data not shown). Lymph node cells (4×10^7) in 200 μ l of RPMI medium were injected in the tail vein of immunized IL-6 $^{-/-}$ mice before induction of arthritis in the knee joint. As controls IL-6 $^{-/-}$ mice were injected intravenously with lymph node cells from ovalbumin (Sigma) immunized wild-type mice.

IL-6 Measurement

For measuring local IL-6 production in the joint the patella was isolated with surrounding soft tissue (tendon and synovium). The patella was incubated in 200 μ l of serum-free RPMI 1640 for 1 hour. The concentration of IL-6 in these patellar wash-outs and sera was determined by a B9 bioassay as described before.²⁶

Statistical Analysis

Statistical comparison between groups was performed with Student's *t*-test. Values of *P* < 0.05 were considered significant.

Results

Joint Inflammation Subsided after Day 1 in IL-6 $^{-/-}$ Mice

After immunization a mono-articular arthritis was induced by injecting 60 μ g of mBSA in the knee joint cavity of wild-type and IL-6 $^{-/-}$ mice. Joints of both strains became highly swollen at day 1 after injection (Figure 1) but thereafter swelling disappeared rapidly in IL-6 $^{-/-}$ mice, whereas it subsided more gradually and remained significantly higher in wild-type mice. Histological evaluation of the arthritic knee joints showed onset of inflammation in IL-6 $^{-/-}$ mice but the inflammatory infiltrate disappeared rapidly within the first week (Table 1). In both strains the first cells entering the joint were predominantly polymor-

Figure 1. Joint swelling at day 1 and 2 after mBSA injection. Immunized and naive mice were injected with 60 μ g of mBSA. Joint swelling was measured as the ratio of ^{99m}Tc uptake in the arthritic knee (right) over the nonarthritic knee (left). ■, IL-6^{-/-}; □, wild type ($n = 7$; *, $P < 0.05$; **, $P < 0.005$, Student's t -test).

phonuclear cells. At day 2 a synovial infiltrate became apparent although smaller in knee joint cavities of IL-6^{-/-} mice (Figure 2, A and B). By day 7 joints from wild-type mice were highly inflamed whereas the cellular infiltrate and the cartilage proteoglycan depletion had almost disappeared in the IL-6^{-/-} mice (Figure 2, C and D; Table 1). In wild-type mice the inflammation persisted for weeks. Flare-up of the inflammation in wild-type mice by low doses of mBSA, that normally do not induce arthritis, further illustrates reactivity of the chronic synovitis (joint swelling 24 hours after injection on day 21 of AIA, intra-articular saline 1.15 ± 0.09 or intra-articular 2 μ g mBSA 1.58 ± 0.24 , $n = 7$, one of three experiments).

Expression of Adhesion Molecules and Chemokines during Onset of AIA

IL-6 together with its soluble receptor has been shown to stimulate gene expression mediating cellular influx^{36,37} and this could be impaired in joints of IL-6^{-/-} mice during onset of AIA. We therefore compared synovial gene expression in the arthritic joint with basal expression in the contralateral uninjected joint by RT-PCR.³² Both IL-6^{-/-} and wild-type mice

showed increased mRNA expression for the investigated adhesion molecules (ICAM-1, VCAM-1, E-selectin, and P-selectin), chemokines (MCP-1, Mip-1 α , and Mip-2) and cytokines (TNF- α and IL-1 β) in the arthritic joint at 24 (Figure 3A) and 48 hours (Figure 3B) after injection. IL-6 mRNA expression was also highly increased in wild-type mice but undetectable in IL-6^{-/-} mice. For this set of genes IL-6^{-/-} mice responded similarly to wild-type mice at the mRNA level. This suggests that joints of IL-6^{-/-} mice could support a normal influx of inflammatory cells during onset of AIA.

Complement Activation in IL-6^{-/-} Mice

A reduced activation of complement in IL-6^{-/-} mice could contribute to the short-lasting inflammation. To test this possibility we induced an immune-complex-induced arthritis in both strains by administering antigen and antibodies to naive mice. Previously this model was shown to depend on complement activation.³¹ During immune-complex arthritis joint swelling on day 2 did not differ between both strains (IL-6^{-/-}, 1.37 ± 0.16 ; wild-type, 1.34 ± 0.12 ; one of two experiments, $n = 6$). Also the inflammatory infiltrate at day 7 did not differ between IL-6^{-/-} and wild-type mice (IL-6^{-/-}, 1.5 ± 1.0 ; wild-type, 1.2 ± 0.6 ; one of two experiments, $n = 6$). These results indicate that IL-6^{-/-} mice can support a normal complement-mediated joint inflammation.

Differences in Antigen-Specific Antibody Subclasses before Induction of AIA

On the day of arthritis induction comparable mBSA-specific IgG1 and slightly lower IgM levels were found in IL-6^{-/-} mice. In contrast, levels of the complement binding IgG2a and IgG2b subclasses were greatly reduced in the IL-6^{-/-} mice (Figure 4A).

Next, we used an immunization protocol to lower the IgG2a levels in wild-type mice to elucidate their role during onset of inflammation. Wild-type mice were immunized and boosted with 10, 30, or the normal 100 μ g of mBSA. Wild-type mice immunized with 10 μ g of mBSA developed IgG2a titers comparable to normally immunized IL-6^{-/-} mice (Figure 4B), but they still showed significantly higher joint swelling on day 2 (Figure 4C).

Table 1. Development of Joint Inflammation and Cartilage Damage during AIA

	DAY 1 AIA		DAY 2 AIA		DAY 4 AIA		DAY 7 AIA	
	IL-6 ^{-/-} ($n = 6$)	WT ($n = 10$)	IL-6 ^{-/-} ($n = 9$)	WT ($n = 10$)	IL-6 ^{-/-} ($n = 8$)	WT ($n = 13$)	IL-6 ^{-/-} ($n = 8$)	WT ($n = 10$)
Infiltrate	0.5 ± 0.3	0.7 ± 0.3	0.8 ± 0.4	$1.6 \pm 0.4^{\dagger}$	0.4 ± 0.3	$2.3 \pm 0.8^{\dagger}$	0.3 ± 0.2	$2.8 \pm 0.6^{\dagger}$
Exudate	1.5 ± 0.4	$2.1 \pm 0.5^*$	0.7 ± 0.4	$1.9 \pm 1.0^*$	0.1 ± 0.2	$0.6 \pm 0.5^*$	0.1 ± 0.1	$1.5 \pm 0.9^{\dagger}$
Patella	0.2 ± 0.2	0.4 ± 0.4	1.7 ± 0.6	$2.5 \pm 0.5^*$	1.7 ± 0.6	$2.6 \pm 0.7^*$	0.5 ± 0.7	$3.0 \pm 0.0^{\dagger}$
Femur	0.2 ± 0.3	0.7 ± 0.7	1.6 ± 0.9	2.3 ± 0.7	1.6 ± 0.6	$2.7 \pm 0.7^{\dagger}$	0.8 ± 0.8	$3.0 \pm 0.0^{\dagger}$
Medial tibia	nd	nd	1.1 ± 0.6	$2.6 \pm 0.5^{\dagger}$	1.9 ± 0.7	$2.8 \pm 0.4^{\dagger}$	1.3 ± 0.9	$2.6 \pm 0.7^{\dagger}$

Cellular infiltrate, exudate, and cartilage damage as observed in safranin-O-stained sections. Cartilage damage was assessed as loss of red staining observed in patella, femur, or medial tibia. Histology was scored by two independent observers and ranged from 0 (no infiltrate/exudate or no depletion) to 3 (maximal infiltrate/exudate or maximal depletion). Statistical differences between IL-6^{-/-} and wild-type mice are indicated (nd, not determined; *, $P < 0.05$, † , $P < 0.005$; Student's t -test). WT, wild type.

Figure 2. Inflammation and cartilage damage in wild-type (**A** and **B**) and IL-6^{-/-} (**C** and **D**) mice on day 2 (**A** and **C**) and 7 (**B** and **D**) of the AIA. Sections were stained with safranin-O and cartilage damage was observed as loss of red staining. **E:** Position of patella (P), femur (F), synovium (S), growth plate (GP), and cartilage (C) is shown. Original magnification, $\times 200$.

Although there was a dosage effect on joint swelling at day 2, development of arthritis at day 7 was not reduced after immunization with less antigen (infiltrate at day 7 was 2.7 ± 0.4 , 2.6 ± 0.6 , and 2.8 ± 0.3 , respectively, for wild-type mice in the 10-, 30-, and 100- μ g groups). These results suggest that lower antibody titers were not primarily responsible for a mild inflammatory response as observed in IL-6^{-/-} mice.

T Cell Immunity in IL-6^{-/-} Mice

Because even the wild-type mice immunized with the lowest amount of mBSA (10 μ g) did develop a normal arthritis despite reduced antibody levels we chose this group to evaluate T cell immunity. T cells were isolated from lymph nodes of wild-type (10 μ g mBSA)- or IL-6^{-/-} (100 μ g mBSA)-immunized mice and stimulated with

mBSA in the presence of irradiated spleens from naive mice as antigen-presenting cells. IL-6^{-/-} T cells responded to the antigen in the same way as wild-type T cells (Figure 5A). The ratio of mBSA-specific proliferation of IL-6^{-/-} over wild-type T cells (0.90 ± 0.14 from four different experiments) showed that IL-6^{-/-} T cells responded normally to the antigen *in vitro*. Multiplex RT-PCR analysis showed higher IL-4 and IL-5 expression after ConA stimulation of IL-6^{-/-} T cells (+20.0% and +11.4%, respectively; Figure 5B) suggesting a minor shift toward the Th2 type in IL-6^{-/-} mice after immunization.

Transfer of Wild-Type Lymphocytes Restored Joint Swelling on Day 2 in IL-6^{-/-} Mice

To adapt for possible differences in cellular immunity we transferred lymph node cells from immunized wild-type

Figure 3. mRNA expression in synovia from wild-type and IL-6^{-/-} mice on day 1 (**A**) and 2 (**B**) of the AIA. The number of PCR cycles needed to first detect the specific band on an agarose gel was compared between synovia from arthritic and contralateral nonarthritic knee joints. Increased mRNA expression for the investigated gene in arthritic synovia results in a reduced number of PCR cycles to first detect the specific band. Four mice per group were used and equal amounts of cDNA were used as assessed by PCR for glyceraldehyde-3-phosphate dehydrogenase (all appeared at cycle 14). Basal expression in the nonarthritic synovia did not differ between wild-type and IL-6^{-/-} mice. No signal for IL-6 was detected at the end point of the PCR with cDNA from IL-6^{-/-} mice. ■, IL-6^{-/-}; □, wild type.

mice (10 μ g mBSA) to immunized IL-6^{-/-} mice (100 μ g mBSA) at onset of arthritis. Again immunizations yielding comparable IgG2a titers were used.

Transfer of mBSA-specific wild-type lymph node cells completely restored joint swelling in IL-6^{-/-} mice on day 2 of AIA (Figure 6A). Transfer of ovalbumin-specific wild-type or mBSA-specific IL-6^{-/-} lymph node cells in contrast did not restore joint swelling in IL-6^{-/-} mice (Figure 6, A and B). The reverse experiment with transfer of mBSA-specific IL-6^{-/-} lymph node cells to wild-type mice did not influence joint swelling in wild-type mice (Figure 6B).

Although transfer of wild-type cells restored joint swelling on day 2 and led to an increase in cartilage damage it did, however, not sustain joint inflammation at day 7 in IL-6^{-/-} mice (Table 2). At 14 days after transfer of wild-

Figure 4. **A:** mBSA-specific antibody subclasses before onset of AIA. Titers as depicted are the dilution of sera needed to give half the maximal optical density at 450 nm as described in Materials and Methods. ■, IL-6^{-/-}; □, wild type ($n = 7$). **B:** mBSA-specific IgG2a titers in mice immunized and boosted with 100 μ g of mBSA (IL-6^{-/-}) or with 10 or 100 μ g of mBSA (wild type). Each symbol represents one mouse ($P = 0.22$, IL-6^{-/-} (100 μ g) versus wild type, (10 μ g) Student's t -test). Total IgG titers also did not differ (IL-6^{-/-}: 1/720 $\pm$ 194, wild type: 1/734 $\pm$ 180). **C:** Joint swelling in wild-type mice immunized with 10, 30, or 100 μ g of mBSA ($n = 8$). Joint swelling was measured as the ratio of ^{99m}Tc uptake in the arthritic knee (**right**) over the nonarthritic knee (**left**). ■, IL-6^{-/-}; □, wild type (**A** and **C**: *, $P < 0.05$; **, $P < 0.005$, Student's t -test). One of three experiments is shown.

type cells and induction of arthritis, joint inflammation was absent in IL-6^{-/-} mice (data not shown).

ZIA Does Not Become Chronic in IL-6^{-/-} Mice

The results of the cell transfer pointed at an important role for IL-6 in developing a chronic infiltrate. This was confirmed in the nonimmunologically mediated ZIA model.

Figure 5. A: mBSA-specific increase in cpm (cpm mBSA minus cpm medium). Lymph nodes from seven mice per group were pooled and enriched for T cells as described in Materials and Methods. Lymph node cells (2×10^6) together with 2×10^7 irradiated antigen-presenting cells were incubated for 72 hours with mBSA at 25 μ g/ml. Cells were plated in sixfold. Cultures were labeled with 3 H for the last 16 hours. Antigen-presenting cells were used from naive mice of the same strain. IL-6^{-/-} and wild-type mice did not differ in the response to concanavalin A at 1 μ g/ml (IL-6^{-/-}, $28,002 \pm 7,291$ cpm; wild type, $25,436 \pm 1,216$ cpm). One of four experiments is shown. **B:** Th1/Th2 multiplex RT-PCR of IL-6^{-/-} (100 μ g mBSA) or wild-type (10 μ g mBSA) T cells stimulated for 24 hours with conA (1 μ g/ml). C = positive control.

During the first week of ZIA, inflammation did not differ between both strains. By week 3, however, inflammation persisted in wild-type mice whereas it had disappeared in IL-6^{-/-} mice (Table 3). Although the time scale was different in ZIA and AIA, we found in both models that IL-6 was important in either maintaining or turning the acute inflammation into a chronic synovitis.

Discussion

IL-6 is a protein that is highly expressed in joints and serum of arthritic patients, but its exact role during arthritis is not yet known. The generation of IL-6 knockout mice³⁰ made it possible to assess the role of IL-6 in murine arthritis models. In most of these models the role of IL-6 in immune development and the possible role in joint inflammation itself will be intermingled. In our study on AIA we found influence of IL-6 on both humoral and cellular immunity. During onset of arthritis the T cell plays a major role, because transfer of wild-type lymph node cells restored early joint swelling in IL-6^{-/-} mice. The cell transfer, however, also showed that there are two phases in the AIA model in which IL-6 plays an important role. First, IL-6 is necessary for developing a good immune

Figure 6. Joint swelling on day 2 of AIA in IL-6^{-/-} mice after transfer of 4×10^7 lymph node cells derived from mBSA (wild-type/mBSA) or ovalbumin (wild-type/OVA) immunized wild-type mice (**A**) or of mBSA (IL-6^{-/-}/mBSA) immunized IL-6^{-/-} mice (**B**). NT = normal AIA in wild-type mice immunized with 10 μ g of mBSA. Joint swelling was measured as the ratio of ^{99m}Tc uptake in the arthritic knee (**right**) over the nonarthritic knee (**left**) ($n = 6$; *, $P < 0.05$, Student's *t*-test, n.s. = not significant). ■, IL-6^{-/-}, □, wild type. One of three experiments is shown.

response before induction of arthritis. Second, IL-6 is important for developing and maintaining the inflammatory infiltrate.

In a study by Ohshima et al,²⁸ on the immunologically mediated AIA, IL-6^{-/-} mice hardly showed joint inflammation on day 14 of AIA. During the first days of the AIA we observed that IL-6^{-/-} mice do develop an inflammatory infiltrate and show cartilage damage. Joint inflamma-

Table 2. Joint Inflammation and Cartilage Damage at Day 7 of AIA after Transfer of Wild-Type Lymph Node Cells

Acceptor Donor	IL-6 ^{-/-} (n = 6) WT/OVA	IL-6 ^{-/-} (n = 11) WT/mBSA	WT (n = 11) No transfer
Infiltrate	0.2 $\pm$ 0.1	0.8 $\pm$ 0.7	2.7 $\pm$ 0.5 [†]
Exudate	0.0 $\pm$ 0.0*	0.6 $\pm$ 0.6	1.5 $\pm$ 0.6 [†]
Patella	0.3 $\pm$ 0.6*	1.4 $\pm$ 1.1	3.0 $\pm$ 0.0 [†]
Femur	0.8 $\pm$ 0.9 [†]	2.0 $\pm$ 0.7	3.0 $\pm$ 0.0 [†]
Medial tibia	0.8 $\pm$ 0.8*	2.1 $\pm$ 1.0	2.9 $\pm$ 0.3*

Cellular infiltrate, exudate, and cartilage damage at day 7 after cell transfer and induction of AIA was scored as described in Table 1. WT/OVA, transfer of lymph node cells from ovalbumin immunized wild-type mice. WT, wild-type mice immunized with 10 μ g of mBSA. Statistical differences relative to IL-6^{-/-} mice plus WT/mBSA lymph node cells are indicated (*, $P < 0.05$; [†], $P < 0.005$; Student's *t*-test). WT/mBSA, transfer of lymph node cells from mBSA-immunized wild-type mice.

Table 3. Synovial Infiltrate on Day 7 and 21 of Zymosan-Induced Arthritis

Infiltrate	IL-6 ^{-/-}	Wild-type
Day 7 ZIA	1.4 ± 0.6	1.9 ± 1.0 ns
Day 21 ZIA	0.3 ± 0.3	2.2 ± 0.7*

Cellular infiltrate as observed in safranin-O-stained sections. Histology was scored as described in Table 1 (n = 7 per group). One of three experiments is shown. (*, *P* < 0.005, Student's *t*-test; ns = not significant).

tion, however, decreased rapidly and by day 7 almost no inflammatory cells were seen. Another striking observation was the sharp decrease in joint swelling in IL-6^{-/-} mice after day 1. These findings pointed at an important role for IL-6 during the first days of the AIA in development of an inflammatory infiltrate.

The decrease in joint swelling after day 1 could be caused by reduced expression of genes facilitating cellular influx. IL-6 has been reported to stimulate cellular influx.^{36,37} However, when we compared mRNA expression for chemokines, adhesion molecules, and pro-inflammatory cytokines we found small or no differences for the investigated set of genes between wild-type and IL-6^{-/-} mice on day 1 and 2 of the AIA. This indicates that synovial IL-6^{-/-} mice show a normal pro-inflammatory reaction and it seems that the reduced joint swelling and cellular influx cannot be explained by reduced pro-inflammatory gene expression. This is in line with equal synovial mRNA expression for TNF- α and IL-1 β in IL-6^{-/-} and wild-type mice on day 4 of the AIA.²⁸

One of the first cells that enter a site of inflammation is the neutrophil. Romani et al²³ had found that IL-6^{-/-} mice could not mount a peripheral blood neutrophilia in response to infection with *C. albicans*. In our model the number of neutrophils in blood smears did not differ between immunized wild-type and IL-6^{-/-} mice on day 0 and 7 of the arthritis (data not shown). Neutrophils were also the predominant type of cells in the infiltrate during onset of arthritis in both IL-6^{-/-} and wild-type mice, although the total infiltrate in IL-6^{-/-} mice was reduced. Polymorphonuclear cells and macrophages derived from IL-6^{-/-} mice did not exhibit differences in *in vitro* chemotaxis as compared to wild-type cells.³⁷ This finding, the results from our RT-PCR analysis, and equal cell influx during the immune-complex-induced arthritis and the first week of ZIA²⁶ strongly support that neutrophils could enter the arthritic joint in IL-6^{-/-} mice.

Because the developed mBSA-specific immunity is important for inducing arthritis in the mBSA-injected knee joint we compared the immune status of wild-type and IL-6^{-/-} mice. IL-6 plays a role in B cell maturation into antibody-secreting plasma cells.¹¹ Lower mBSA- or collagen-specific total IgG titers have been reported for IL-6^{-/-} mice.^{28,38,39} When we looked into IgG subclasses we found strongly reduced IgG2a and IgG2b titers in IL-6^{-/-} mice, whereas IgG1 titers were not affected. The effect of IL-6 deficiency on antibody subclasses seems to depend in part on the antigen or the type of adjuvant used for immunization.⁴⁰ This seems also true for experimental arthritis because during Lyme

arthritis, in which no previous immunization takes place, IL-6^{-/-} mice developed wild-type levels of IgG1 and IgG2a.²⁵ The reduction in IgG2a and IgG2b subclasses we found in the IL-6^{-/-} mice during AIA seemed not to have a great influence on the primary inflammation. Wild-type mice immunized to generate low IgG2a titers still showed joint swelling at day 2 and developed normal joint inflammation.

Besides antibody levels the activation of complement or complement levels itself could also differ between IL-6^{-/-} and wild-type mice. Kopf et al,⁴¹ had found equal basal levels for complement C3, but IL-6^{-/-} mice failed to increase C3 after immunization. When we compared IL-6^{-/-} and wild-type mice in the passive immune-complex-induced arthritis we did not find differences in joint swelling. Immune-complex arthritis is a passive immunization model that depends on complement activation in response to immune complexes.³¹ Our results showed that complement activation was not impaired in IL-6^{-/-} mice. The above findings showed that the humoral immunity was probably not the main determining factor during onset of AIA. IL-6 also has a role in cellular immunity and a different T cell response against mBSA could occur in IL-6^{-/-} mice during AIA.

Antigen-specific cellular immunity in IL-6^{-/-} mice was compared with that in wild-type mice immunized with one-tenth of the normal amount of mBSA. These wild-type mice still developed AIA although their humoral immunity was reduced to that found in IL-6^{-/-} mice. T cells from wild-type and IL-6^{-/-} mice proliferated to the same extent in response to mBSA. Higher mRNA expression of IL-4 and IL-5 after conA stimulation of IL-6^{-/-} T cells, however, suggested a small shift toward the Th2 type in IL-6^{-/-} mice. *In vitro* results of Ohshima et al,²⁸ also suggested a shift toward the Th2 type in IL-6^{-/-} mice.

The difference in T cell subtypes could influence arthritis development in IL-6^{-/-} mice. To investigate the importance of the T cell type in more detail, we transferred lymph node cells from wild-type to IL-6^{-/-} mice. Previous experiments with C57Bl6 mice had shown that the T cell fraction in lymph-node cell preparations could transfer AIA.⁴² For these experiments wild-type mice immunized with 10 μ g instead of 100 μ g of mBSA were used as donors because they had mBSA-specific IgG2a titers comparable to IL-6^{-/-} mice and still showed higher joint swelling and developed a chronic arthritis. Transfer of lymph node cells from mBSA-immunized but not from ovalbumin-immunized wild-type mice restored joint swelling on day 2 in IL-6^{-/-} mice to wild-type levels. mBSA-specific antibody titers, as assessed by IgG2a, did not increase between day 1 and 2 after transfer and induction of arthritis (data not shown). This suggests an important involvement of the antigen-specific cellular response in joint swelling during onset of arthritis. Transfer of lymph node cells from immunized IL-6^{-/-} mice to immunized IL-6^{-/-} mice did not restore joint swelling. This shows that the restored joint swelling after transfer of mBSA-specific wild-type cells is not caused by increased T cell numbers but instead suggests influence of the T cell subtype on joint swelling.

Despite equal joint swelling, IL-6^{-/-} mice receiving mBSA-immunized wild-type lymph node cells did not develop arthritis as severe as that found in wild-type mice. In synovial washouts on day 1 or 2 after cell transfer no locally produced IL-6 was measured in a B9 bioassay. In joints of wild-type mice, IL-6 is expressed at high levels during AIA.⁴³ The inability to develop a chronic infiltrate by IL-6^{-/-} mice even after transfer of wild-type lymph node cells could be caused by a reduced local immune development. In human rheumatoid synovium, the development of germinal centers has been described.⁴⁴ Interestingly, IL-6^{-/-} mice develop smaller germinal centers compared to wild-type mice⁴¹ and this could also be the case in inflamed synovia.

Transfer of the wild-type lymph node cells 3 days before injection of mBSA showed that they could survive in IL-6^{-/-} mice and remained capable of restoring joint swelling (day 2: 1.68 ± 0.30). Their survival in the inflamed joint, however, could be impaired, as an anti-apoptotic function of IL-6 has been described.^{45,46} IL-6 has been shown to enhance *in vitro* the survival of T cells by inducing the anti-apoptotic protein Bcl-2.⁴⁷ A very recent report showed increased apoptosis of mucosal T cells after inhibition of IL-6 signaling in Crohn's disease.⁴⁸ This further supports an anti-apoptotic role of IL-6 in chronic inflammatory diseases. Increased apoptosis in the joints of IL-6^{-/-} mice could inhibit formation of a normal infiltrate and influence local immune development despite the presence of wild-type T cells. An anti-apoptotic role of IL-6 would also influence survival of other synovial cells such as macrophages and fibroblasts. We currently have started experiments on this issue. A recent report showing development of collagen-induced arthritis in mice lacking functional T and B cells⁴⁹ further stresses the importance of nonimmunological mechanisms in experimental arthritis.

During the nonimmunologically mediated ZIA, inflammation developed normally in IL-6^{-/-} mice during the first week but declined thereafter. Preliminary results showed up-regulation of suppressors of cytokine signaling (SOCS) expression in the inflamed synovia during ZIA. For SOCS3 there was a small increase in expression in both strains. The increase of SOCS1 mRNA expression, however, was much higher for IL-6^{-/-} mice than for wild-type mice at day 14 of the ZIA. This suggests a causal relationship with an early relapse of arthritis in IL-6^{-/-} mice.

SOCS1 belongs to the SOCS family of proteins,^{50,51} an important group of inhibitors of cytokine signaling by the JAK-STAT pathway. Differences in expression of SOCS proteins could affect the inflamed synovium in several ways. First, SOCS proteins inhibit signaling by different pro-inflammatory cytokines such as IL-2,⁵² interferon- γ ,⁵⁰ and members of the IL-6 family.^{50,53} Second, there is evidence that SOCS proteins could influence other pathways besides the JAK-STAT pathway. SOCS1 for example has been shown to inhibit the proliferative signaling of the Kit receptor in hematopoietic and fibroblast cells.⁵⁴ Third, SOCS proteins might inhibit signaling by binding to activated signaling proteins and targeting them for degradation by the proteasome.^{55,56}

Although SOCS proteins function in a negative feedback loop it has also been suggested that they can be expressed independent of cytokine signaling.^{51,57,58} Future research will have to address the regulation of cytokine signaling in inflamed synovia and the role of SOCS proteins in chronicity of arthritis.

In conclusion, we have found in the immunologically mediated AIA and also in the nonimmunologically mediated ZIA that IL-6 plays an important role in progression of initial joint inflammation into a chronic infiltrate. In RA patients, autoimmunity already exists but our finding suggests that anti-IL-6 therapy could still influence maintenance of synovial infiltrates.

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