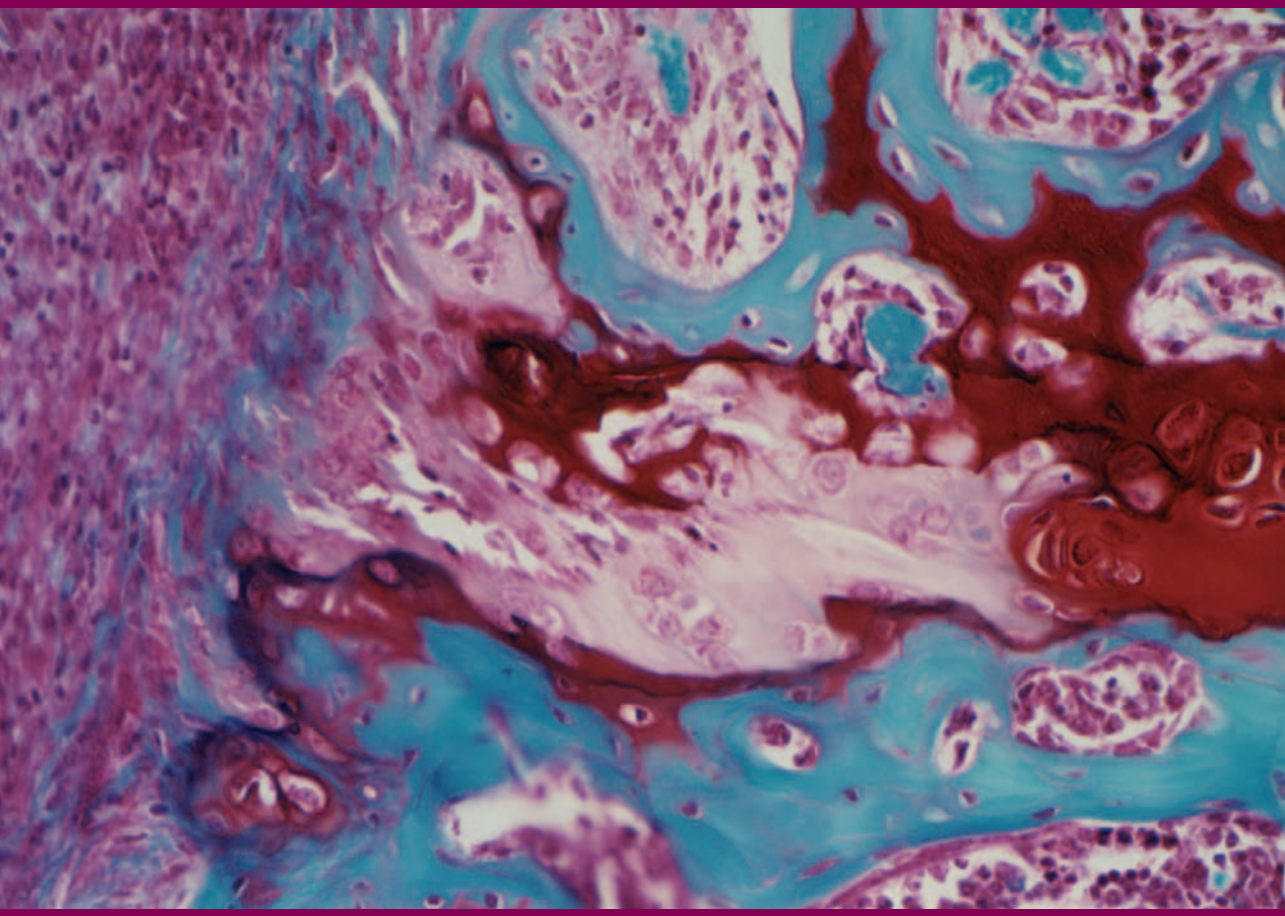
Interleukin-6 and Oncostatin M in experimental joint pathology



Alfons de Hooge

Interleukin-6 and Oncostatin M in experimental joint pathology

Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op dinsdag 2 november 2004
des namiddags om 1.30 precies
door

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geboren op 3 april 1971
te Rotterdam

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Manuscriptcommissie:	Prof. dr. J.W.M. van der Meer (voorzitter) Prof. dr. P.P. Tak, AMC, Amsterdam Dr. P.F.A. Mulders

ISBN 90-9018419-8
Printing: Print Partners Ipskamp, Enschede
Graphic design: Martien Frijns
Photographs cover: Alfons de Hooge

Front cover: Murine growth plate affected by overexpression of Oncostatin M. The normal proteoglycan staining (red) is partly lost just as the normal ordering of chondrocytes in the growth plate.
Back cover: The back cover gives an idea of the other interests of the author and shows a bit of the person behind this scientific work.

The research presented in this thesis was performed at the Laboratory of Experimental Rheumatology and Advanced Therapeutics of the University Medical Center Nijmegen, The Netherlands

Printing of this thesis was financially supported by Het Nationaal Reumafonds (the Dutch Arthritis association).

Voor Piet en Adrie

Contents

CHAPTER 1

Introduction and aim of this thesis

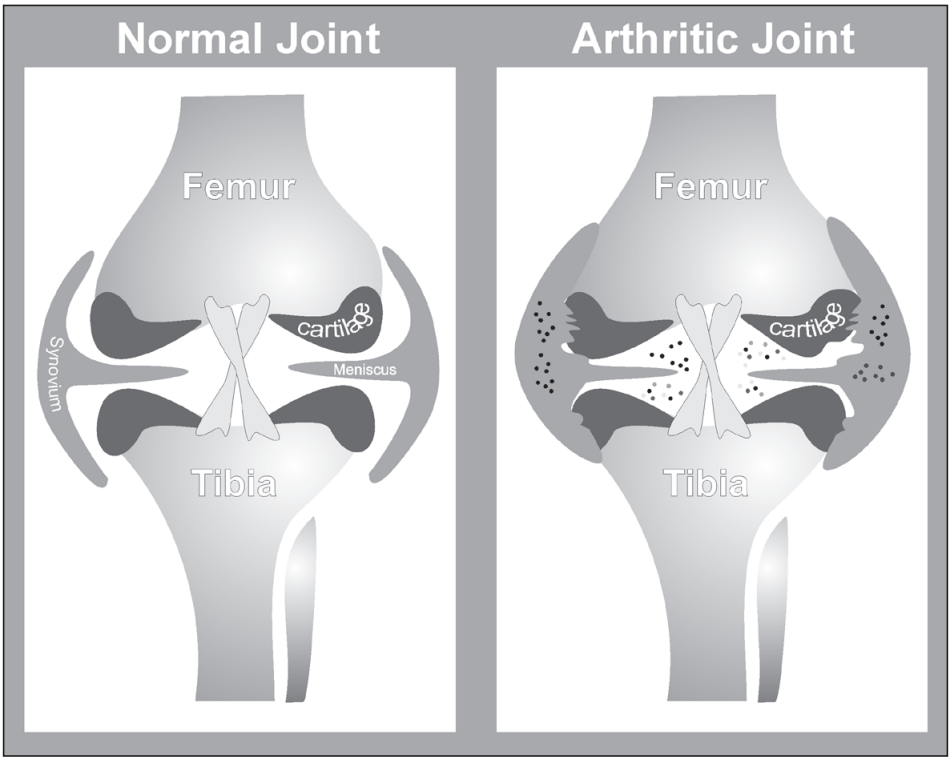

Figure 1 Normal and arthritic joint. During arthritis inflammatory cells enter the joint. These cells (black dots) are present in the joint cavity as an inflammatory exudate and infiltrate the synovium. The synovium furthermore thickens by hypertrophy and hyperplasia of the synovial cells. These synovial changes can lead to the formation of an erosive pannus tissue that can destroy the articular cartilage (shown in right figure).

1.1 Rheumatoid arthritis and osteoarthritis

1.1.1 *Rheumatoid arthritis*

Rheumatoid arthritis (RA) is a systemic and chronic inflammatory disease that affects multiple joints. Approximately 1% of the population will develop RA. Although the disease itself is not lethal, RA patients have a lower quality of life and also a lower life expectancy[1]. Patients suffer from painful, swollen joints and can become disabled by destruction of the articular bone and cartilage. In the chronic phase of RA relatively quiet periods alternate with flare-ups of the disease.

Normal joints are encapsulated by the synovial lining (see figure 1). This thin layer

consists of the so-called synoviocytes, macrophages and fibroblasts-like cells. During RA, the lining becomes thickened by hypertrophy and hyperplasia of the synovial cells and by infiltration with inflammatory cells. These changes in the lining can lead to the formation of pannus: a vascularized granulation tissue that is rich in fibroblasts, lymphocytes and macrophages. The pannus overgrows the weight-bearing surface of the joint and can finally contribute to destruction of the articular surface (see figure 1). Besides inflammatory infiltration of the lining, also an exudate of inflammatory cells can be found in the joint cavity.

The exact cause of RA is still unknown, but it is generally believed to be an autoimmune disease. During the last decades there has been much debate on which cell type is primarily responsible for the joint inflammation and destruction in RA. The presence of T cells in the rheumatoid synovium and the linkage of RA to certain MHC class II molecules have suggested involvement of T cells (reviewed in[2]). The last years, involvement of other cell types has come into focus. Involvement of activated macrophages and fibroblast-like synoviocytes in perpetuation of inflammation and development of joint destruction has been implicated[3;4]. Involvement of B cells came into focus again after development of the KRxN model. Mice that express the transgene encoded KRN T cell receptor and the IAg^7 MHC class II allele develop spontaneous arthritis[5]. These transgenic T cells can respond to IAg^7 MHC class II molecules that are loaded with a peptide fragment from the ubiquitous glucose-6-phosphate isomerase (GPI). Transfer of antibodies against GPI could induce arthritis in recipient mice[6]. Transfer of these antibodies to mast cell deficient mice did not induce arthritis suggesting involvement of mast cells in development of arthritis[7]. In a systemic and complex disease like RA, however, more than one cell type might be responsible for initiating the disease.

Cytokines are proteins that play an important role in the communication between cells. Cytokine stimulation of cells can influence their growth, metabolism or gene expression. In this way cytokines seem to play an important role in RA (reviewed in[8]). The inflamed synovium is a rich source of pro-inflammatory cytokines and other pro-inflammatory and destructive mediators like chemokines and matrix-metalloproteases. IL-1 and TNF-α have been found to be key pro-inflammatory cytokines in RA. The role of both cytokines has been extensively studied in animal RA models[9-11] and inhibitory therapies against IL-1 and TNF-α have made it to the clinic[12;13]. The immuno-modulatory effects of long-term systemic inhibition of cytokines like TNF-α, however, can have its drawbacks as shown by the development of infections after anti- TNF-α therapy[14]. Not only pro-inflammatory and destructive, but also anti-inflammatory cytokines are produced in inflamed joints. Cytokines can even have both pro- as well as anti-inflammatory effects depending on the

cell type that is stimulated. The balance between these pro- and anti-inflammatory signals could therefore contribute to the final outcome in RA.

Another cytokine that is highly produced in inflamed joints of RA patients is Interleukin-6 (IL-6)[15]. Just as for IL-1 and TNF-α, blocking therapies have been developed against IL-6 and the first clinical data show beneficial effects of anti-IL-6 therapy[16]. A more extensive introduction on IL-6 and its role in RA is given in section 1.2 as it is the focus of this thesis.

1.1.2 *Osteoarthritis*

Osteoarthritis (OA) is the most common of all joint disorders. Its incidence increases with age and in people over 60 years old 60 percent have OA in one or more joints. The loss of joint function that accompanies OA makes it a disabling disease. The cause of OA is not known but several mechanisms could be involved[17;18]. Genetic alterations that are found in extracellular matrix components could impair the normal strength and function of the cartilage. These mutations also suggest that OA has a large genetic component[17]. Also biomechanical overloading or an imbalance in synovial homeostasis could contribute to its etiology.

The disease is characterized by breakdown of the cartilage matrix followed by development of fibrillations, fissures and finally complete loss of articular cartilage. Besides the articular cartilage, also the subchondral bone is involved. Thickening of the subchondral bone occurs in OA, leading to increased stiffness and reduced shock absorbing capacity of the bone. Whether changes in the bone cause cartilage pathology or are a consequence of it is still a matter of debate[19]. Another complication related to bone is the formation of osteophytes in OA joints.

A third component of the joint that could be involved is the synovial lining. Joint inflammation can occur in OA, although it is less prominent than in RA. Production of proteases and other destructive mediators by the inflamed synovial lining could contribute to OA pathology.

The production of both anabolic and catabolic factors like cytokines and growth factors by the articular chondrocytes and synovial lining cells could contribute to the coexistense of repair and destructive processes in OA joints (reviewed in[20;21]). Anabolic factors include TGFβ and the bone morphogenetic proteins. Catabolic factors that are produced in the joint include IL-1 and TNFα. The balance between anabolic factors, catabolic factors and their inhibitors could therefore contribute to the final outcome in OA.

1.2 Interleukin-6

1.2.1 *IL-6 family members, their receptors and signal transduction*

Interleukin-6 (IL-6) is a 26 kd protein that gives its name to the IL-6 cytokine family. Also Oncostatin M (OSM), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), IL-11[22;23] and the recently described neurotrophin-1/ B cell stimulating factor-3[24] belong to this family. These cytokines share a similar 3-dimensional structure, can display functional redundancy and stimulate cells through receptor complexes that have the receptor glycoprotein130 (gp130) in common (reviewed in[23;25]).

The receptor complex for the IL-6 family members consists of gp130 and at least one other receptor (Table I) (reviewed in[25;26]). Homo- or heterodimerization of gp130 is necessary for inducing signal transduction. The fact that all IL-6 family members contain gp130 in their receptor complex could at least partly explain the functional redundancy that is observed for these cytokines. Signal transduction by IL-6 starts with binding of IL-6 to gp80, the IL-6 specific receptor. GP80 does not induce signal transduction by itself but instead binds together with IL-6 to the gp130 receptor. Subsequently, a hexameric complex of two IL-6, gp80 and gp130 molecules is formed [27] and signaling proceeds in the cell.

Table I Receptor complexes of the IL-6 family

Only GP130 as signaling receptor		GP130 and LIFR as signaling receptor	
Cytokine	Receptor complex	Cytokine	Receptor complex
IL-6	GP130/GP80	LIF	GP130/LIFR
IL-11	GP130/IL-11R	OSM	GP130/OSMR or LIFR
		CNTF	GP130/LIFR/CNTFR
		CT-1	GP130/LIFR
		CLC/NN-1	GP130/LIFR

The IL-6 family members and their receptors can be divided in two groups. IL-6 and IL-11 only use GP130 for their signal transduction. Receptor complexes for the other members contain a second receptor that can give signal transduction, the LIF receptor. Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), Cardiotrophin-1 (CT-1), Cardiotrophin-like cytokine/novel neurotrophin-1 (CLC/NN-1), receptor (R). Based on Bravo and Heath, 2000 [26].

Not only a membrane-bound form of gp80 exists but also two soluble forms. These can be generated either by differential splicing[28] or by shedding[29]. In contrast to most soluble receptors does the soluble gp80 function as an agonistic instead as an antagonistic

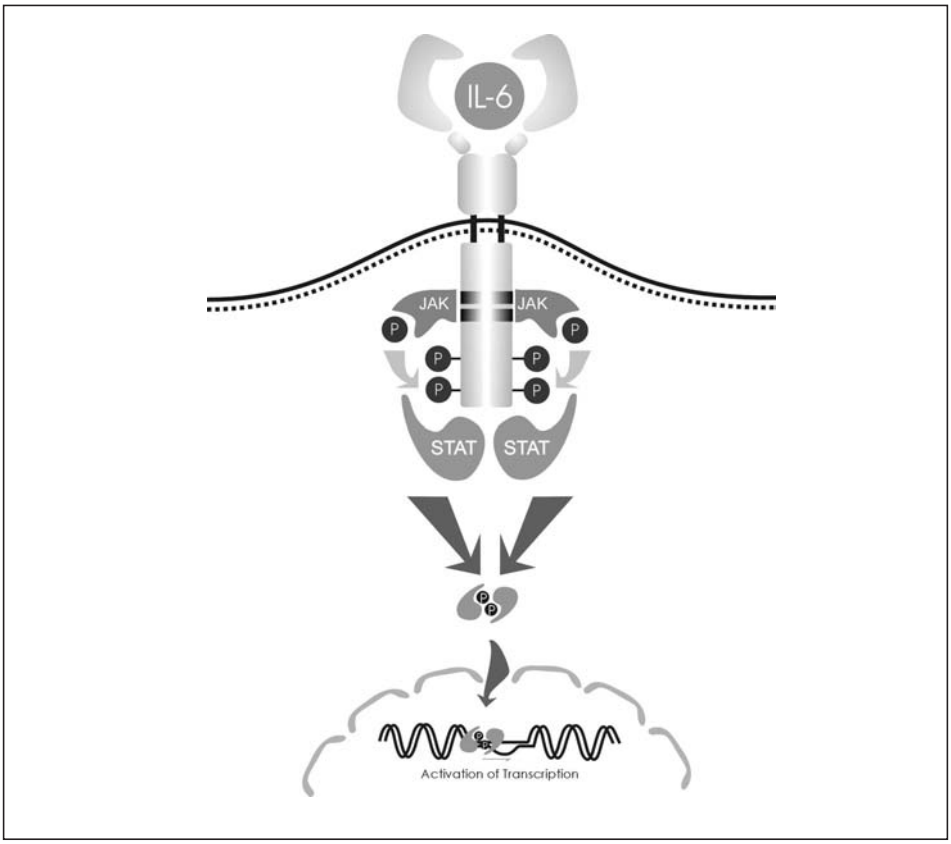

Figure 2 JAK/STAT signaling by IL-6. Upon binding of IL-6 to the gp 80/gp130 receptor complex the GP 130 becomes phosphorylated by Jak kinases. STAT 1 or STAT 3 molecules that are present in the cytoplasm are recruited to the activated GP 130 and become themselves phosphorylated by the JAK kinase. Two activated STAT molecules form a homo- or heterodimer (STAT 3/3, STAT 3/1, STAT 1/1) and translocate to the nucleus. In the nucleus they start transcription of STAT responsive genes. Based on Levy and Darnell (2002)[30].

receptor. Cells that express gp130, but not membrane-bound gp80, can still respond to IL-6 in the presence of soluble gp80. In this way the range of cells that can respond to IL-6 is greatly expanded.

An important pathway for signal transduction of the IL-6 family members is the Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/STAT) pathway (reviewed in[30]). JAK family tyrosine kinases are associated intracellularly with gp130 and become transphosphorylated in response to binding of the cytokine to the receptor complex. The activated JAKs, in turn, phosphorylate specific tyrosine residues in gp130, thereby creating docking sites for STAT proteins. STATs are transcription factors that are present in the cytoplasm in a latent state. They can bind to the phosphorylated STAT

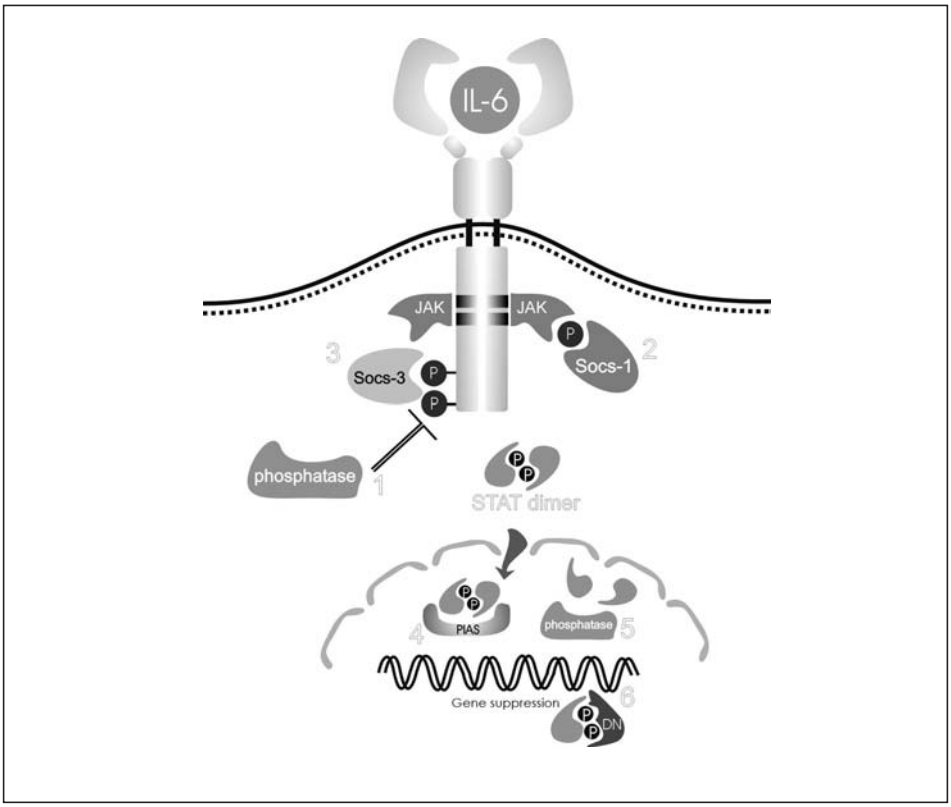

Figure 3 Regulation of JAK/STAT signaling. There are different ways to control and terminate signaling by the JAK/STAT pathway. Phosphatases can remove phosphate groups from GP 130 or from activated STAT proteins. In this way phosphatases can deactivate GP 130 or STAT proteins (1 and 5). SOCS proteins can inhibit signaling by binding to the JAK or to STAT-bindingsites on GP 130 (2 and 3). PIAS proteins can interact with STAT homo- or heterodimers and prevent them for binding to the DNA (4). Naturally occurring short forms of STAT proteins can act as dominant negative regulators by forming inactive dimers with full-length STATs (6). Based on Levy and Darnell (2002)[30].

docking sites and become themselves phosphorylated on specific tyrosine residues by the JAKs. The activated STATs form homo- or heterodimers that translocate to the nucleus where they start transcription of STAT responsive genes. Figure 2 illustrates the JAK/STAT signaling as it is induced by IL-6. In addition to tyrosine phosphorylation, STAT1 and STAT3 can also become phosphorylated on a serine residue and this could increase the transcriptional activity[31].

JAK1 is the main JAK kinase that is involved in signaling by IL-6[32]. STAT3 is the main STAT protein that becomes activated but also STAT1 can become activated in response to IL-6[25;33]. Therefore not only STAT3/STAT3 homodimers but also STAT3/STAT1 het-

erodimers or STAT1/STAT1 homodimers can be found in IL-6 stimulated cells. Pivotal roles for STAT3 in the regulation of cell growth, differentiation and survival have been found (reviewed in[25;30]). STAT1 in contrast is more linked to growth control and apoptosis (reviewed in[34]). STAT5 activation in response to IL-6 has also been reported[35] but its role in the biological effects of IL-6 has been less well studied compared to STAT3 and STAT1.

There exist several ways to control signaling by the JAK/STAT pathway (figure 3). In this way, negative effects of prolonged STAT-induced gene expression are prevented (reviewed in[30;36]). Cytoplasmic tyrosine phosphatases dephosphorylate receptors or kinases and prevent further signaling. Naturally occurring truncated STATs can act as dominant-negative regulators of the full-length STATs and prevent, upon binding to a full-length STAT, the formation of a functional dimer. Another class of inhibitors consists of the Protein Inhibitor of Activated STAT (PIAS) proteins. They block transcriptional activity of STAT dimers in the nucleus. Activated STATs induce expression of members of the Suppressor of Cytokine Signaling (SOCS) protein family. Until now 8 members of this family (SOCS1-7 and CIS1) have been described[37]. These proteins either inhibit the JAK kinase or mask STAT docking sites on the receptor. In this way, a negative feedback loop is created.

IL-6 family members can, besides by the JAK/STAT pathway, also signal through the RAS-MAP kinase pathway[25]. Upon receptor stimulation tyrosine residue 759 of GP130 becomes phosphorylated. This residue is the binding site for the src homology 2 domain-bearing protein tyrosine phosphatase (SHP)-2. This SHP-2 in turn becomes activated and leads to activation of the RAS-MAP kinase pathway. Subsequent ERK activation has been suggested to play a negative regulatory role in STAT3 signal transduction[38].

Several groups have made mice with mutations in the GP130 receptor that affect either the JAK-STAT or RAS-MAP kinase pathway. Ernst *et al*[39] made a mutant mouse with a truncated GP130 ($GP130^{\Delta STAT/\Delta STAT}$). GP130 induced STAT activation but not SHP-2/RAS/ERK activation was inhibited in these mice. These mice showed a reduced humoral and mucosal immune response and a reduced acute phase response in the liver. They also developed gastrointestinal ulcerations and severe joint disease. IL-6 induced SOCS1 mRNA expression was almost completely absent in $GP130^{\Delta STAT/\Delta STAT}$ livers and synovial cells. SOCS3 mRNA expression was also reduced. These results led to the conclusion that the joint disease was a consequence from the disturbance of the balance between GP130 induced JAK/STAT and SHP-2/RAS/ERK signaling.

Researchers from the Hirano laboratory obtained contrasting results. Both STAT3 signal-deficient ($GP130^{FXXQ/FXXQ}$) and GP130 deficient ($GP130^{D/D}$) mice died perinatally.

Mice with a mutated Tyr-759 residue ($GP130^{F759/F759}$) are born normal but develop splenomegaly, lymphadenopathy and show an enhanced acute phase response [40]. An increased GP130 mediated STAT3 activation was observed in these mice. They furthermore found evidence that STAT3 signaling directed T cell development towards the Th1 type. SHP-2 mediated signaling in contrast seemed involved in Th2 development. When the $GP130^{F759/F759}$ mice grow older they develop joint disease in an autoimmune manner that involves T cell immunity[41]. This was linked to augmented STAT3 activation. In contrast to the mice by Ernst *et al.*[39] do these mice only have a point mutation in GP130 and not a large deletion. How this deletion affects the signaling by GP130 and if this could explain the opposite results of Ernst *et al.* and Atsumi *et al.*[41] is not completely clear.

Negative regulation by SOCS proteins could also be involved in the $GP130^{F759/F759}$ mice. Tyr-759 of GP130 is also the binding site for SOCS3[42;43]. SOCS3 mRNA expression was enhanced in the $GP130^{F759/F759}$ mice. Inhibition of STAT3 activation, however, was not possible because of the Tyr-759 mutation. Reduced STAT3 inhibition by SOCS3 could therefore be involved in the observed phenotype of $GP130^{F759/F759}$ mice. An alternative possibility is that the enhanced expression of SOCS3 leads to interference with signaling pathways other than the GP130 induced signaling.

The results with the GP130 mutant mice demonstrate the complexity of establishing the *in vivo* role of the GP130 induced signaling pathways. They furthermore point at an important role for negative regulators like the SOCS proteins in determining the effects of cytokine signaling. The final outcome of IL-6 induced signaling in a given cell might be determined by the expression of the involved signaling molecules and their inhibitors. Furthermore, cross talk between different signaling pathways generated by IL-6 or by other cytokines can either positively or negatively influence the final outcome of stimulation in a given cell type[40;44;45].

1.2.2 *IL-6 and inflammation*

IL-6 was originally named B-cell stimulatory factor 2 because of its ability to induce B cells to produce immunoglobulins[46]. A variety of cells have been shown to produce IL-6. This includes T and B cells, monocytes, endothelial cells and different tumor cells[22]. Since its discovery as a B cell stimulatory factor, IL-6 has been shown to be a pleiotropic cytokine with a wide range of biological activities. After *in vivo* administration, leukocytosis and fever are induced by IL-6[47;48]. IL-6 plays an important role in the maturation of B cells into antibody secreting plasma cells [46], the differentiation of osteoclasts[49] and

macrophages[50], and it has a co-stimulatory role in T cell activation[51;52]. In vitro, IL-6 could rescue resting mouse T cells from apoptosis pointing at an important role for IL-6 in T cell survival[53]. The effect of IL-6 on T cell differentiation has been controversial. IL-6 has been described as an inducer of Th2 differentiation[54]. Other researchers, in contrast, found augmentation of naive, Th1 and Th2 effector CD4 T cell responses by IL-6 but no induction of Th2 differentiation[55]. Furthermore, IL-6 plays a role in differentiation and activity of cytotoxic T cells[56;57] and potentiates the activity of natural killer cells[58]. The influence of IL-6 on T and B cell activity can contribute to a good host response against bacterial and viral infections.

Not only pro-inflammatory, but also anti-inflammatory properties have been found for IL-6. The induction by IL-6 of IL-1 receptor antagonist and soluble TNF receptor expression has been reported[59;60]. Induction of this IL-1 respectively TNF-α inhibitor could down regulate inflammation and reduce tissue damage due to inflammation. IL-6 also can reduce TNF-α production[61]. IL-6 plays an important role in generating an acute-phase response in the liver[62-64]. During this response acute phase proteins are produced that are thought to participate in the defense against tissue damage and infection.

The dual face of IL-6 as a pro- and anti-inflammatory protein is also reflected by studies in IL-6 gene knock out (IL-$6^{-/-}$) mice. IL-$6^{-/-}$ mice are viable and develop normally[65]. They fail, however, to mount a good immune response against vaccinia, *Listeria monocytogenes*[65] and *Candida albicans* infections[66]. Furthermore, the inflammatory acute-phase response after tissue damage or infection was severely compromised[65;67]. The local inflammatory response against turpentine was impaired in IL-$6^{-/-}$ mice while systemic inflammatory reactions on LPS were not[67]. Xing *et al.*[68] in contrast found increased inflammatory reactions in endotoxic lung or during endotoxemia in IL-$6^{-/-}$ mice and proposed an anti-inflammatory role for IL-6 during acute infection. Similarly, enhanced inflammation was found in experimental pancreatitis induced in IL-$6^{-/-}$ mice[69]. Together these results demonstrate that the effect of IL-6 deficiency at least partly depends on the localization and type of inflammatory stimuli.

1.2.3 *IL-6 and arthritis*

Increased levels of IL-6 have been found in serum and synovial fluid of RA patients. Especially in the synovial fluid several 1000 pg/ml of IL-6 can be measured[15;70] and synovial fluid levels correlated with clinical parameters of inflammation[71]. Also in systemic Juvenile Idiopathic Arthritis are the serum and synovial fluid levels of IL-6 significantly

enhanced[72]. Many cell types, including T and B cells, fibroblasts, macrophages and chondrocytes can produce IL-6[70;73]. There have been significant correlations found between serum levels of IL-6 and acute phase proteins[74] and between synovial IL-6, IgG and Rheumatoid Factor levels[75;76]. The positive role of IL-6 in developing antibody-secreting plasma cells could contribute to the latter correlation.

Through its role in activation and survival of T cells, IL-6 could contribute to T cell activity in RA and other autoimmune diseases. Anti-IL-6 receptor antibodies induced apoptosis of T cells in a murine colitis model and ameliorated this disease[77]. IL-6 could also contribute to development of auto-immunity in other ways. High IL-6 levels can lead to the processing and presentation of cryptic epitopes by dendritic cells[78]. In this way IL-6 might contribute to development of T cell immunity against self-antigens. Recently, IL-6 was found to play a major role in the control of T regulatory cells[79]. High IL-6 concentrations could inhibit T regulatory cells and this might contribute to the generation of auto-reactive T cell immunity.

Other cell types in the joint, besides T and B cells, could be influenced by IL-6. The combination of IL-6 and the soluble IL-6 receptor can activate endothelial cells and hence stimulate the inflammatory response of these cells[80]. IL-6 has also been reported to induce synovial fibroblast proliferation[81;82] but the opposite result, inhibition, has also been found[83]. The combination of IL-6 with its soluble receptor can induce osteoclast formation[49;84] and, in this way, IL-6 could contribute to bone erosion in RA.

Table II The negative and positive roles that IL-6 could play during RA.

Negative role by	Positive role by
Maturation plasma cells	Induction IL-1 Ra
Activation and survival T cells	Induction soluble TNF receptor
Differentiation T cells	Induction TIMP's
Differentiation osteoclasts	Reduction TNF production
Systemic acute phase response	Local acute phase response

Negative and positive properties that have been described for IL-6 and that make its role in RA unclear. For references see 1.2.2 and 1.2.3.

IL-6 could not only have negative but also positive effects for RA as is shown in table II. Induction of IL-1 receptor antagonist and soluble TNF receptor expression by IL-6 [59;60] could downregulate the inflammatory and destructive effects of IL-1 and TNF-α. Several reports suggest a protective role for IL-6 in cartilage pathology. In our laboratory we have found increased cartilage proteoglycan depletion in IL-6 deficient mice in the early phase of zymosan-induced arthritis (ZIA)[85]. During this phase inflammation did not differ

between wildtype and IL-6 deficient mice. Intra-articular injection of IL-6 ameliorated the cartilage destruction in IL-6 deficient mice. Addition of IL-6 to human chondrocytes induced expression of the proteinase inhibitor α_1-antitrypsin suggesting induction of a protective acute phase response in these cells[86]. Murine anti-human IL-6 antibodies showed a transient clinical improvement in RA patients[87]. Surprisingly, serum IL-6 levels increased in these patients making it unclear what exactly caused the improvement. The exact role of IL-6 in RA therefore needs to be investigated further as well as the possibility to separate the positive from the negative effects of IL-6 in joint pathology.

1.2.4 *IL-6 and osteoarthritis*

Increased expression of IL-6 in the joint is not only found in RA but also in OA. IL-6 production has been found in chondrocytes from OA cartilage and IL-1 was found to be an important inducer of IL-6 in these cells[88]. The exact function of IL-6 in OA is not clear. Different effects of IL-6 on chondrocytes have been described. Recombinant human IL-6 did not induce proliferation of bovine chondrocytes neither did it influence proteoglycan production[89]. Proliferation and proteoglycan synthesis in rabbit articular chondrocytes were, however, both inhibited by human IL-6[90]. Most likely species differences are related to these opposing results. Guerne *et al.*[91] have reported that the combination of IL-6 and soluble gp80 inhibits proteoglycan synthesis in human articular chondrocytes. Proteoglycan synthesis, however, was measured with an Elisa that detected soluble proteoglycans. It can therefore not be excluded that proteoglycan breakdown but not synthesis was influenced by IL-6. The induction of α_1-antitrypsin[86] and TIMP-1[92] in human chondrocytes suggests a protective role for IL-6 against cartilage pathology.

IL-6 has also been studied in experimental or spontaneous OA models in animals. Synovial IL-6 levels increased after naturally acquired or experimentally induced cranial cruciate ligament rupture in dogs[93]. In this study lower IL-6 concentrations were found in dogs with higher radiographic OA scores. Sectioning the anterior cruciate ligament in dogs likewise increased IL-6 levels[94]. In these dogs IL-6 expression correlated positively with increased PG synthesis.

Increased IL-6 mRNA expression was found in cartilage of C57 black mice with early stages of spontaneous OA[95]. IL-6 expression, however, did not correlate with histological changes. Male STR/ort mice develop osteoarthritic lesions of the knee joint by 35 weeks of age. In situ hybridisation studies showed increased IL-6 mRNA expression in cartilage at the site of the lesions[96]. It is, however, not clear if this IL-6 expression plays a role in developing cartilage damage in mice.

1.3 Oncostatin M and arthritis

Oncostatin M (OSM) is a 28 kd glycoprotein that belongs to the IL-6 family[97]. Like the other IL-6 family members, OSM was found to be a multifunctional cytokine. OSM can for example stimulate an acute-phase response in liver cells[98] and enhance expression of tissue inhibitor of metalloproteinase 1[99] and of adhesion molecules like ICAM-1[100]. Just as for IL-6, both pro- and anti-inflammatory effects of OSM have been described[100;101].

Elevated levels of OSM (up to several 100 pg/ml) can be detected in the synovial fluid of RA patients and synovial macrophages are the source of this OSM[102;103]. Unlike its family member IL-6, OSM was not detected in the serum of RA patients[102]. This suggests a local rather than a systemic role for OSM in RA. Circumstantial evidence for a local role is the positive correlation found between concentrations of OSM and cartilage degradation markers in synovial fluid[104]. Injection of recombinant human OSM in the joints of goats induced inflammation[105]. Similarly, an adenoviral vector expressing murine OSM induced joint inflammation in mice[106]. These results further support a local and pro-inflammatory role for OSM in joint pathology. Recently, intraperitoneal administration of blocking antibodies to OSM ameliorated experimental arthritis in mice[107], demonstrating a pro-inflammatory role for OSM in murine arthritis. A systemic effect of blocking OSM can, however, not be excluded under these experimental conditions.

OSM is a strong inducer of IL-6 and could contribute to the local IL-6 expression in RA. OSM furthermore has been shown to synergize with or enhance the activity of other cytokines. OSM was the first cytokine that, in combination with IL-1β, could induce collagen release from human cartilage[103]. A similar synergy in collagen breakdown was found for the combination of OSM and IL-17 on explants of bovine nasal cartilage[108]. OSM could therefore contribute to joint pathology by enhancing the effects of other pro-inflammatory and destructive cytokines.

OSM was detected at low frequency (4/32) and at low concentrations (mean of 7.9 pg/ml) in synovial fluid of OA patients[103]. By in situ hybridisation, OSM mRNA was detected in OA femoral heads[109]. Alone or in combination with other cytokines, OSM could influence cartilage and bone metabolism and integrity. It is therefore possible that OSM contributes to OA pathology but further investigation on this is beyond the scope of this thesis.

Aim of this thesis

The basis for the research described in this thesis was the interesting finding that IL-6 had a cartilage protective role during the acute phase of zymosan-induced arthritis. Although IL-6 is generally seen as a pro-inflammatory cytokine, these results demonstrated that IL-6 might also exert positive effects at the inflammatory site in RA patients. This ambiguity on the involvement of IL-6 in RA is also reflected in the title of this research project: Role of IL-6 in chronic arthritis: good or bad guy? In the first part of this thesis the good and bad roles of IL-6 will be further investigated in murine models for RA and for osteoarthritis (OA), another major joint disease.

Elevated levels of IL-6 have been found in synovial fluid of affected joints from OA patients. Based on the cartilage protective role of IL-6 found in experimental arthritis (the good guy) we extended this study to murine models of osteoarthritis. In **chapter 2,** knee joints of old wildtype (WT) and IL-6$^{-/-}$ mice were compared histologically for signs of OA-like pathology. Old IL-6$^{-/-}$ male mice showed a higher incidence of major OA-like changes as proteoglycan deposition and bone formation in the ligaments and complete cartilage erosion. Cartilage PG production and breakdown were reduced in old IL-6$^{-/-}$ male mice compared to old WT mice. Similar to its protective role in experimental RA, these results suggest a protective role against connective tissue damage for IL-6 in OA.

IL-6 is seen as a bad guy in RA because of its pro-inflammatory properties. The influence of IL-6 on development of immunity is well known. IL-6 could therefore play a role in development of auto-immunity during RA. The pro-inflammatory role of IL-6 could, however, not only rely on immune development because IL-6 can also directly stimulate resident cells of the synovium like synovial fibroblasts and endothelial cells. Previous studies showed reduced cellular and humoral immunity in IL-6$^{-/-}$ mice during experimental arthritis but failed to discriminate between immune and non-immune effects of IL-6. In **chapter 3** the role of immune and non-immune mechanisms in the pro-inflammatory properties of IL-6 is investigated. WT and IL-6$^{-/-}$ mice were compared in different arthritis models: antigen-, immune-complex - and zymosan-induced arthritis. These experiments confirmed the importance of IL-6 for development of antigen-specific humoral and cellular immunity. However, transfer of WT lymphocytes to IL-6$^{-/-}$ mice could enhance the acute inflammatory phase of the AIA but did not lead to a chronic arthritis in these mice. Similarly, IL-6$^{-/-}$ mice developed an acute inflammation during the ZIA but failed to develop into a chronic synovitis. Together these results indicate an important role for IL-6 in propagation of joint inflammation, potentially independent of its role in immunity.

STAT proteins play an important role in the signaling by IL-6. STAT3 and, to a lesser extend, STAT1 become activated in response to stimulation by IL-6. The relation between STAT activation, IL-6 and development of chronic synovitis is investigated in **chapter 4**. STAT3 activation in the synovial tissue occurred directly at the onset of the arthritis and continued during the chronic phase of ZIA. This activation was IL-6 dependent. STAT1 became only activated during chronic arthritis. Severe enhancement of inflammation, but not of cartilage damage, in STAT1$^{-/-}$ mice suggests that STAT1-regulated genes protect or control the inflammation in arthritis. STAT3 activation was not impaired in these mice. Manipulation of the IL-6 induced signal transduction in the synovium could become a way to separate the pro-inflammatory effects of IL-6 from its cartilage protective properties.

In the second part of this thesis, the role of the IL-6 family member Oncostatin M (OSM) in joint pathology was investigated. OSM is produced in inflamed joints, is a strong inducer of IL-6 and can activate both STAT3 and STAT1. Adenoviral overexpression of murine OSM induces joint inflammation in both WT and IL-6$^{-/-}$ mice as shown in **chapter 5**. During this inflammation, the periosteum became activated and bone apposition occurred. In vitro experiments showed that OSM enhanced the bone morphogenetic protein-2 induced differentiation of C2C12 cells towards the osteoblastic lineage as measured by increased alkaline phosphatase activity. This suggests that enhancement of BMP-2 induced osteoblast differentiation or activity by OSM could be a mechanism that contributed to the observed bone apposition *in vivo*.

In **chapter 6** we show that OSM overexpression also leads to cartilage damage. This cartilage damage was mediated in part by IL-1 but was not counteracted by IL-6. Also the growth plate became affected by OSM. Expression of OSM during juvenile idiopathic arthritis could possibly be involved in growth plate damage during this disease. We found that OSM is expressed in most of the joints of JIA patients that we examined. Together chapter 5 and 6 show that both OSM and IL-6 have inflammatory properties but differ in their effects on bone and cartilage, respectively.

In the third part of this thesis we describe a spin off from our IL-6 research. The chapters 3 and 4 showed an important role for IL-6 in development of chronic arthritis. Furthermore, IL-6 is a general marker of disease activity. In **chapter 7** we describe an inflammation-inducible adenoviral expression system based on the IL-6 promoter. This expression system made it possible to achieve repeatable and disease-dependent gene expression *in vivo*. This system was not dependent on the presence of IL-6 and might

therefore become a useful tool to obtain disease-regulated expression of biologicals (e.g. IL-6 inhibitors) to tackle arthritis locally by gene-therapy.

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CHAPTER 2

Male IL-6 gene knock out mice developed more advanced osteoarthritis upon aging

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Conditionally accepted by Osteoarthritis and Cartilage

Abstract

Objective: Interleukin-6 (IL-6) is expressed in osteoarthritic joints but its function in osteoarthritis (OA) is unknown. To study this, spontaneous and experimental OA were evaluated in IL-6 deficient (IL-$6^{-/-}$) mice.

Design: Histology of knees of 18-23 months old wild type (wt) and IL-$6^{-/-}$ mice was compared for signs of OA. Cartilage proteoglycan (PG) density was measured by image analysis on safranin-O stained whole knee sections. Chondrocyte PG synthesis was measured *ex vivo* by ^{35}S-sulfate incorporation. Knee bone mineral density (BMD) was measured by dual x-ray absorptiometry (DEXA). In young mice (3 months), OA was induced by intra-articular injection of collagenase.

Results: The incidence of extensive cartilage loss at both lateral - and medial sides was markedly higher in old IL-$6^{-/-}$ males, but not females, as compared to their wild-type controls. Compared to age-matched wt mice, reduced *ex vivo* PG synthesis was found during aging in IL-$6^{-/-}$ males, without affecting their cartilage PG density. IL-$6^{-/-}$ males showed more extensive extracellular matrix deposition in the collateral ligaments and subchondral bone sclerosis, predominantly at the medial side. Total knee BMD decreased more in IL-$6^{-/-}$ (-23%) than in wt (-10%) males during aging. Collagenase-induced OA showed a similar degree of joint pathology in both strains, implying that OA susceptibility was not different at younger age.

Conclusions: Upon aging IL-$6^{-/-}$ male mice developed more severe spontaneous OA. Reduced PG synthesis and BMD values might be indicative for an impaired repair response in IL-$6^{-/-}$ mice. This suggests a protective role for IL-6 in age-related OA.

Introduction

Osteoarthritis, the most common of all joint disorders, is a disabling disease whose incidence increases with age. The cause of OA is unknown but several mechanisms could be involved[1] including genetic/age-related alterations in extracellular matrix components, biomechanical stress or an imbalance in synovial homeostasis. The disease is characterized by breakdown of the cartilage matrix followed by development of fibrillations, fissures and this ultimately can lead to complete loss of articular cartilage. Another characteristic of OA is hypertrophy of the bone. Thickening of the subchondral bone can lead to increased stiffness and reduced shock absorbing capacity of the bone. Another complication related to bone is the formation of osteophytes in OA joints. Whether changes in the bone cause cartilage pathology or vice versa is still a matter of debate[2;3].

The production of both anabolic and catabolic cytokines and growth factors by the articular chondrocytes and synovial lining cells could contribute to the coexistense of repair and destructive processes in OA joints reviewed in van der Kraan and van den Berg, 2000[4]; van den Berg, 2001[5]. Increased experimental OA in IL-1β deficient mice suggested recently that catabolic cytokines could also have beneficial effects and could contribute to joint homeostasis in OA[6]. One other cytokine whose expression is increased in affected joints of OA patients is the multifunctional cytokine interleukin-6 (IL-6). IL-6 production has been found in chondrocytes from OA cartilage[7] but its exact function in this disease is not clear. IL-6 could reduce PG synthesis in vitro[8] but this was investigated with normal and not with OA cartilage. IL-6, in contrast, had no effect on PG metabolism itself but depressed IL-1β induced PG breakdown in OA chondrocytes *in vitro*[9]. The induction by IL-6 of α1-antitrypsin[10] and Timp-1[11] in human chondrocytes also suggests a protective role against cartilage pathology. Previously we had found a cartilage protective role for IL-6 during the onset of zymosan-induced arthritis (ZIA) in mice[12]. IL-6 deficient mice had higher articular cartilage PG loss during onset of ZIA. This PG loss could be normalized by intra-articular injection of IL-6. Furthermore, IL-6 injections in naive mice could stimulate PG synthesis moderately[13].

IL-6 also has properties that could have a negative effect in the joint e.g. in the presence of the soluble IL-6 receptor IL-6 contributes to osteoclast development[14], and this links it to bone erosion. Furthermore, IL-6 plays a role in development of chronic joint inflammation[15;16], and might therefore be involved in osteoarthritis-associated joint inflammation.

Investigations on the role of IL-6 in murine OA are limited. Increased IL-6 mRNA expression was found in cartilage of C57 black mice with early stages of spontaneous OA[17].

IL-6 expression, however, did not correlate with histological changes. Male STR/ort mice develop osteoarthritic lesions of the knee joint by 35 weeks of age. In situ hybridisation studies showed increased IL-6 mRNA expression in cartilage at the site of the lesions[18]. It is, however, not clear if this IL-6 expression plays a role in developing cartilage damage in mice. Recently, it was shown that young STR/ort females had a higher expression of IL-6 in their cartilage than males[19]. Because in the STR/ort strain female mice develop less OA than males, it was of interest to include both male and female mice in our study.

In the present study we have investigated both spontaneously developed age-related OA as well as experimentally induced OA in wt and IL-6 deficient mice.

Materials and methods

Spontaneous and experimental osteoarthritis

Wildtype C57Bl6 (Charles River, Sulzfeld, Germany) and IL-6 deficient mice[20], backcrossed for 8 times into C57Bl6, were used in the experiments. Breeding pairs of the IL-6 deficient mice were a kind gift of dr. Manfred Kopf. (Basel, Switzerland). During a period of four years, groups of healthy mice were followed for the spontaneous development of OA. Knee joints of male and female mice were isolated when they had reached the age of 18 months or older. The mean age in months ± sd for the different groups was 19.8 ± 0.6 wt male, 20.0 ± 2.1 IL-$6^{-/-}$ male, 19.6 ± 2.2 wt female and 19.6 ± 1.7 IL-$6^{-/-}$ female. Isolated knee joints were fixed in formalin and processed for histological evaluation. Male C57Bl6 wt and IL-$6^{-/-}$ mice were used for experimental OA at the age of 3-4 months. Experimental OA was induced by injecting 6 µl of physiological saline containing 1 unit of collagenase (from Clostridium histolyticum, type VII, Sigma, St. Louise, MO) in the knee joint of mice. The injection was repeated once at day 2 after the first injection. At day 42 after the start of the experiment, knee joints were isolated, formalin fixed and processed for histological evaluation. All mice were housed in filter-top cages under standard pathogen free conditions and a standard diet and water were provided *ad libitum*. Experiments were performed according to national and institutional regulations for animal use.

Histological evaluation of knee joints

Histological signs of OA were scored in a blindfolded manner on 5 semi-serial sections of the joint. Cartilage erosion was scored on a scale from 0-3: 0 no cartilage erosion, 1 superficial ruffling, 2 surface erosion and/or fissures, 3 complete loss of cartilage. Proteoglycan deposition (red color in saf O stained sections) and bone formation in the ligaments were scored on a scale from 0-3: 0 no changes, 1 moderate red staining, 2 extensive red staining, 3 extensive red staining and bone formation in the ligament. Incidence of mice with erosion and/or PG deposition was expressed as a percentage of the total number of mice in that group. The incidence of mice with the highest score (3) is used as a marker for development of severe OA in the different groups. The joint sections were furthermore evaluated for the presence or absence of dislocation of the patella, subchondral bone sclerosis, joint inflammation, osteophyte formation and bone apposition.

Chondrocyte PG synthesis

Proteoglycan synthesis was assessed by ^{35}S-sulfate incorporation in patellar cartilage. Patellae were dissected, with a minimum amount of surrounding synovium, under sterile conditions. The *ex vivo* synthesis assays were performed in RPMI/penicillin 100 U/ml and streptomycin 100 μg/ml/ 1 mmol/l pyruvate/ 5% fetal calf serum (Life Technologies, Breda, The Netherlands). The patellae were placed separately in 200 μl medium containing 4 μCi ^{35}S-sulfate and incubated for three hours at 37°C and 5% CO_2. After labelling, the patellae were washed twice with physiological saline and fixed overnight in 100% ethanol. Patellae were decalcified in 5% formic acid for 4 hours at room temperature. Thereafter, the articular cartilage was stripped from the underlying bone and dissolved O/N in 0,25 ml lumasolve at 60°C (Lumac, Groningen, The Netherlands). After addition of 1 ml lipoluma (Lumac, Groningen, The Netherlands), the ^{35}S-sulfate content of each patella was measured by liquid scintillation counting in a Trilux 1450 MicroBeta (Perkin-Elmer Wallac, Turku, Finland).

Cartilage PG breakdown

Patellae were isolated and labelled with ^{35}S-sulfate as described for the ex vivo PG synthesis. Part of the patellae was fixed after labelling (t = 0). The rest was, after washing, further incubated for 48 hours at 37°C and 5% CO_2 in medium without ^{35}S-sulfate. Medium was changed after the first 24 hours. After 48 hours incubation the patellae were washed, fixed and processed as described for the *ex vivo* synthesis.

Assessment of PG density in patellar cartilage

Patellar PG density was quantified by image analysis on histological slides as described previously by van der Kraan *et al*, 1994[21]. Images of safranin-O-stained sections were captured using a JVC 3-CCD color video camera (Victor Company of Japan Ltd., Tokyo, Japan) and displayed on a computer monitor. Patellar cartilage was selected and the amount of red staining was measured using the Qwin image analysis system (Leica Imaging Systems Ltd., Cambridge, UK). Of each mouse three sections were measured.

Measurement of knee bone mineral density

Bone mineral density (BMD) of murine knee joints was determined by dual energy x-ray absorptiometry (DEXA). Knee joints were scanned with a Norland bone densitometer XR-46 (Norland Medical Systems, Inc. fort Atkinson, WI). Calibration of the densitometer was performed with a calibration phantom before measurement. Scans were performed at a speed of 60 mm/s with a resolution of 0,5 x 0,5 mm and included the femoral condyles, patella and tibial plateau. The BMD is expressed as mg/cm^2

Statistical analysis

Statistical analysis between groups was performed with Student's t-test. Distribution of maximal OA scores was analyzed by a Chi-square test with a 95% confidence interval. Values of $p<0.05$ were considered significant in both tests.

Results

Osteoarthritis related pathology in old mice

Knee joints were isolated from wt and IL-6$^{-/-}$ mice of both sexes at the age of 18 months or older. At the femoral-tibial junction, cartilage surface became affected varying from superficial ruffling to complete erosion of the cartilage layer. PG deposition and, in more advanced cases, bone apposition occurred in the collateral ligaments. Subchondral bone sclerosis was also frequently observed. Inflammation was not observed in females, 1 out of 36 wt males had an inflamed joint while 3 out of 39 IL-6$^{-/-}$ males showed signs of previous joint inflammation. Dislocation of the patella was not observed in these old mice.

Table I Spontaneous cartilage erosion in old wt and IL-6$^{-/-}$ mice

Strain	Mice	Incidence	No of mice per score [a]				Side[b]	
	n =	%	0	1	2	3	M	L
wt female	24	63	9	13	1	1 (4%)	2 (0)	14 (1)
IL-6$^{-/-}$ female	27	70	8	14	2	3 [ns] (11%)	4 (1)	17 (3)
wt male	36	66	12	15	5	4 (11%)	12 (4)	18 (1)
IL-6$^{-/-}$ male	39	87	5	15	5	14[*] (35%)	16(11)	26 (8)

Cartilage erosion of old wt and IL-6$^{-/-}$ mice (18-23 months). Scoring ranged from 0-3 as described in materials and methods. For each mouse the maximum score, either medial or lateral or equal on both sides, is indicated. This means that the other side could have lower or no signs of cartilage erosion. Incidence is calculated as the percentage of mice with a positive score of 1, 2 or 3. [a] The number between brackets represents the percentage of mice showing the highest score. [b] Distribution of the maximal score (either 1, 2 or 3) for cartilage damage over the medial (M) and lateral (L) side of the knee joint. In case the score is equal for both sides than that joint is counted for medial as well as lateral. The number between brackets indicates the number of joints with maximal damage score 3 at that side. Distribution of maximal cartilage erosion scores as compared to wt male or female mice was analyzed by a Chi-square test with a 95% confidence interval. *$p<0.05$, ** $p<0.005$, ns not significant.

Higher incidence and more substantiated cartilage damage found in old IL-6$^{-/-}$ males

Female mice of both strains had only moderate signs of OA with mild cartilage erosion and only in exceptional cases complete cartilage loss (Table 1, Figure 1A and B). Male wt mice also developed OA at the same incidence and with comparable degree of OA pathology as the females. However, significantly more IL-6$^{-/-}$ male mice showed severe OA with complete cartilage erosion at both medial and lateral side (Table1, Figure 1 C and D). Further experiments were therefore focused on male mice.

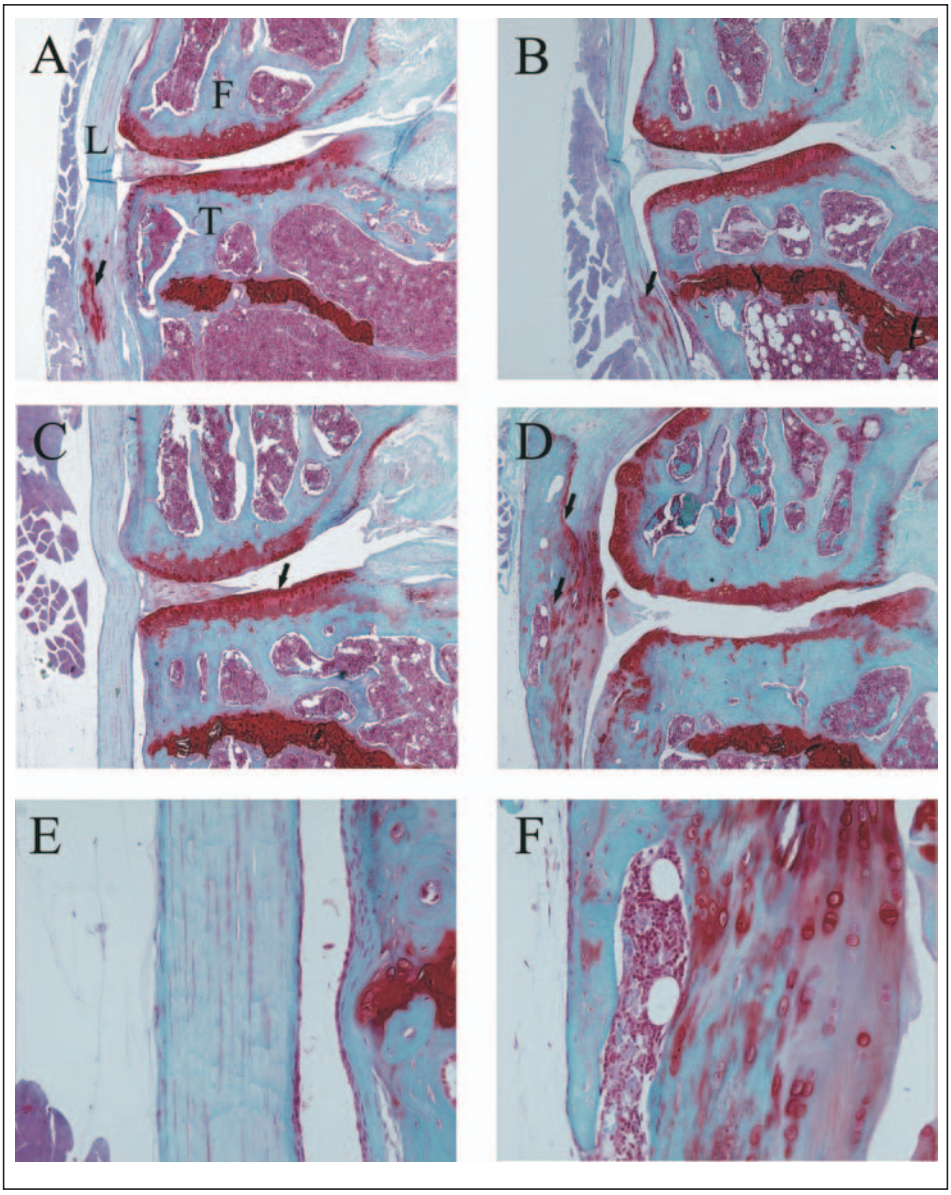

Fig. 1. Spontaneous OA in old wt and IL-6$^{-/-}$ mice.
WT (A, 23 months) and IL-6$^{-/-}$ (B, 22 months) female mice with PG deposition in the collateral ligament (arrow in A and B) but without cartilage erosion. Mild surface erosion (arrow) in the tibial cartilage of an old wt male (C, 20 months). Severe OA as seen in an old IL-6$^{-/-}$ male (D, 19 months). Severe cartilage erosion occurs in both femur and tibia. Bone sclerosis is seen under the affected cartilage. Bone apposition (arrows) and PG deposition occur in the collateral ligament. The normal ligament structure as seen in the wt male (E) and bone formation in the ligament of the IL-6$^{-/-}$ male (F) are shown in more detail. F = femur, L = ligament, T = tibia. Safranin-O staining. Original magnification A-D 50x, E and F 500x.

Table 2 Ex vivo cartilage PG synthesis and breakdown in 4 and 12 months old male wt and IL-6$^{-/-}$ mice

Mice	age (mo)	Cartilage 35S content		
		t = 0 (cpm)	t = 48h (cpm)	% loss (48h-0h)
wt	4	1351 ± 225	369 ± 163	73
IL-6$^{-/-}$	4	1361 ± 273^{ns}	422 ± 62	69
wt	12	1349 ± 365	261 ± 58	81
IL-6$^{-/-}$	12	882 ± 62 **	228 ± 51	74

Ex vivo PG synthesis and breakdown in patellae from 4 and 12 months old wt and IL-6$^{-/-}$ mice. Ex vivo PG synthesis is shown by the ^{35}S incorporation directly after labeling (t = 0). PG breakdown was determined by comparing incorporation at t = 0 and the amount of ^{35}S that is still present in the cartilage after 48h incubation in ^{35}S-free medium. n = 6 per group. One of two experiments with similar results is shown. mean ± sd, ** $p < 0.005$ IL-6$^{-/-}$ mice compared to wt mice of the same age. Student's t-test. ns not significant.

The cartilage erosion occurred in absence of cartilage PG depletion. Image analysis performed on safranin-O stained histological sections showed no significant difference (Student's t-test) between the wt and IL-6$^{-/-}$ male mice in the PG density of patellar cartilage (260+/-116 (n=16) respectively 246+/-95 (n =14) mean ± sd). However, already at 12 months a marked reduction was found in patellar chondrocyte PG synthesis in IL-6$^{-/-}$ males in comparison to age-matched wt mice (Table 2). This difference in PG synthesis with aging does not lead to reduced PG density but could be indicative for diminished chondrocyte function. As a consequence the cartilage may become more vulnerable and the repair response against osteoarthritic insults may be hampered in IL-6$^{-/-}$ mice. This is illustrated by increased inhibition of PG synthesis when patellae of 12-13 months old mice were incubated *in vitro* for 24 hours with 1ng/ml IL-1β (wt 42% inhibition, IL-6$^{-/-}$ 57% inhibition, n = 6).

Increased subchondral bone sclerosis in old IL-6$^{-/-}$ males

Differences in the bone mineral density (BMD) between OA patients and healthy age-matched controls have been reported. An increase in BMD is thought to cause stiffness of bone, which leads to increased pressure and ultimately damage to the overlaying cartilage. The BMD of murine knee joints was measured by the DEXA technique. Young male mice (3 months) of both strains did not differ in their BMD value (IL-6$^{-/-}$ 63.2 +/- 7.7 mg/cm^2, n = 12; wt 68.9 +/- 10.2 mg/cm^2, n = 13. mean ± sd). IL-6$^{-/-}$ males at 20 months of age, in contrast, had a significantly reduced BMD value as compared to age-

Table 3 Bone sclerosis in old wt and IL-6$^{-/-}$ mice

	n =	Incidence[a]	Medial[b]	Lateral
wt female	24	21%	5	0
IL-6$^{-/-}$ female	27	26%	6	2
wt male	36	19%	7	0
IL-6$^{-/-}$ male	39	49%	18	2

Distribution of subchondral bone sclerosis over the medial and lateral side of the joint in old wt and IL-6$^{-/-}$ mice. [a] The incidence is calculated as the percentage of mice that have bone sclerosis either medial, lateral or at both sides. [b] Distribution of bone sclerosis over the medial and lateral side of the knee joint. In case bone sclerosis is present at both sides than that joint is counted for medial as well as lateral.

matched wt mice (48.7 +/- 5.3 mg/cm^2 (n = 13) respectively 61.8 +/- 6.1 mg/cm^2 (n = 12), mean ± sd, $p < 0.001$, Student's t-test). The reduction in the mean BMD was also greater for IL-6$^{-/-}$ males as for wt males (-23% versus -10%) in the period from 3 to 20 months. Histology, however, showed subchondral bone sclerosis in the tibial plateaus, which occurred more frequently at the medial side and had the highest incidence (49%) in old IL-6$^{-/-}$ males (Table 3, Figure 1D). Although bone sclerosis was found in 11 of the 14 IL-6$^{-/-}$ males with complete cartilage loss, it did not co-localize with cartilage erosion in general (compare Table 1 and 3).

Osteophyte formation, another bone related change during OA, was observed less frequently in both females (wt 2 of 24; IL-6$^{-/-}$ 4 of 27) and males (wt 1 of 36; IL-6$^{-/-}$ 7 of 39).

Enhanced PG and bone apposition in collateral ligaments of old IL-6$^{-/-}$ male mice

Changes in collateral ligaments may jeopardize joint stability and are implicated in the osteoarthritic process. In both sexes of each strain, the apposition of PG and bone in the collateral ligaments occurred mainly at the medial side (Table 4, figure 1A and B). A significant higher incidence and more severe PG and bone deposition in the collateral ligaments was found in the IL-6$^{-/-}$ males as compared to their wt controls (Table 4, figure 1 C-F). In 9 of the 10 IL-6$^{-/-}$ males with maximal apposition in the ligament (score 3), cartilage erosion was also maximal at the same medial side.

Table 4 Spontaneous extracellular matrix deposition in ligaments of old wt and IL-6$^{-/-}$ mice

Strain	Mice	Incidence	No of mice per score [a]					Side[b]	
	n =	%	0	1	2	3		M	L
wt female	24	33	16	4	4	0	(0%)	6	2
IL-6$^{-/-}$ female	27	48	14	5	5	3 [ns]	(11%)	11 (2)	2 (1)
wt male	36	11	32	0	1	3	(8%)	4 (3)	0 (0)
IL-6$^{-/-}$ male	39	51	19	4	6	10[**]	(26%)	18(10)	2 (0)

PG and bone deposition (extracellular matrix, EM) in the ligaments of old wt and IL-6$^{-/-}$ mice (18-23 months). Scoring ranged from 0-3 as described in materials and methods. For each mouse the maximum score, either medial or lateral or equal on both sides, is indicated. This means that the other side could have lower or no signs of EM deposition. Incidence is calculated as the percentage of mice with a positive score of 1, 2 or 3. [a] The number between brackets represents the percentage of mice showing the highest score. [b] Distribution of the maximal score (either 1, 2 or 3) for ligament PG deposition/bone formation over the medial (M) and lateral (L) side of the knee joint. In case the score is equal for both sides than that joint is counted for medial as well as lateral. The number between brackets indicates the number of joints with maximal damage score 3 at that side.
Distribution of maximal EM deposition scores as compared to wt male or female mice was analyzed by a Chi-square test with a 95% confidence interval. *p<0.05, ** p<0.005, ns not significant.

Similar pathology of experimental OA in wt and IL-6$^{-/-}$ mice at young age

Analysis of the spontaneous OA showed a clear difference between old wt and IL-6$^{-/-}$ male mice. To investigate if IL-6$^{-/-}$ mice are already at younger age more prone to develop OA-like changes we compared young (3 months) wt and IL-6$^{-/-}$ male mice in the collagenase induced OA. Ligaments and cartilage were severely affected in both strains (fig 2A and B, table 5). PG deposition and bone formation in the ligaments was mostly observed at the medial side in both strains. Cartilage damage occurred mostly on the medial side. Subchondral bone sclerosis was scarce and was found only at the medial part in 3 mice of each group. Patellar dislocation was observed in four wt mice but not in IL-6$^{-/-}$ mice. Bone apposition and osteophyte formation, in contrast, were observed frequently in both groups (9 of 12 in wt and 9 of 11 in IL-6$^{-/-}$ mice).

These results suggest that during aging IL-6$^{-/-}$ male mice start to differ from their wt counterparts in the osteoarthritic response. Intriguingly, chondrocyte PG synthesis (Table 2) and BMD also did not differ between young (3-4 months) wt and IL-6$^{-/-}$ mice.

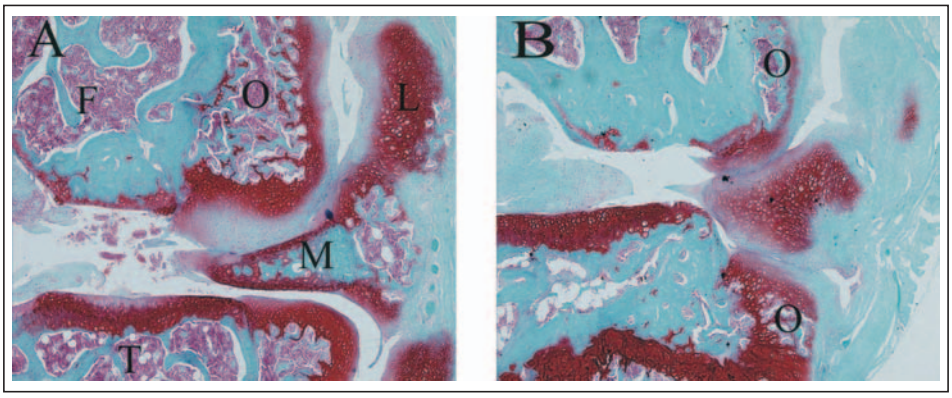

Figure 2 Collagenase induced OA in wt and IL-6$^{-/-}$ male mice
Severe OA at day 42 after the first collagenase injection in both wt (A) and IL-6$^{-/-}$ (B) male mice. Complete cartilage erosion is seen at the femur. Osteophytes are formed at the tibia and femur. PG deposition is seen in the collateral ligament. F = femur, L = ligament, T = tibia, O = osteophyte, M = meniscus. Safranin-O staining. Original magnification 50x.

Table 5 Cartilage erosion and PG deposition in the ligaments of male mice with collagenase induced osteoarthritis

A.		Cartilage erosion						
Strain	Mice	Incidence	No of mice per score [a]				Side [b]	
	n =	%	0	1	2	3	M	L
wt	12	92	1	3	3	5 (42%)	10 (4)	3 (3)
IL-6$^{-/-}$	11	91	1	1	3	6 (55%)	7 (4)	5 (3)
B.		Extracellular matrix deposition in ligaments						
Strain	Mice	Incidence	No of mice per score [a]				Side [b]	
	n =	%	0	1	2	3	M	L
wt	12	92	1	1	1	9 (75%)	10 (9)	2 (1)
IL-6$^{-/-}$	11	91	1	0	0	10 (91%)	10 (10)	1 (1)

A) Cartilage erosion and B) PG and bone deposition in the ligaments was scored on day 42 after the first injection with collagenase. [a, b] Score for cartilage and ligament damage and medial/lateral distribution are presented as in table 1 and 4. Distribution of maximal OA scores was analyzed by a Chi-square test with a 95% confidence interval but did not differ significantly between wt and IL-6$^{-/-}$ mice.

Discussion

Expression of the pleiotropic cytokine interleukin-6 is increased in the joints of OA patients. Chondrocytes, osteoblasts, osteoclasts as well as synoviocytes can respond to IL-6 and the final outcome of IL-6 expression in OA is therefore unclear. Our present study on spontaneous OA in IL6$^{-/-}$ and wt mice suggests that especially in males IL-6 could have a beneficial effect on joint pathology during OA.

Depending on the affected joint, human OA occurs either more in females or is equally present in both sexes. In our study wt male and female mice did not differ significantly in cartilage erosion and ligament PG deposition. A gender difference, in contrast, was seen in the IL6$^{-/-}$ mice where males, but not females, developed significantly more severe OA when compared to age-matched wt mice. Androgens have been shown to reduce IL-6 production and androgen receptors have been found in bone[22] and ligaments[23]. Dihydrotestosterone reduced *in vitro* IL-6 production in human osteoblasts and, in combination with mechanical strain, shifted bone metabolism from high turnover into an osteoanabolic state[24]. It is therefore possible that under influence of androgens, joint metabolism in response to mechanical strain or damage is switched into an enhanced anabolic mode in our IL6$^{-/-}$ males. This could then contribute to development of subchondral bone sclerosis and ligament ossification.

Future studies should address the relation between IL-6 expression, OA development and sex hormones in mice of different age. Our present study showed that young IL-6$^{-/-}$ male mice were not yet more susceptible to experimental OA suggesting IL-6 involvement only upon aging. We can, however, not rule out that presence or absence of IL-6 expression at younger age already has an effect on development of OA.

Based on the increase of severe cartilage damage in old IL-6 deficient male mice, our present study suggests a modifying or protective role for IL-6 against cartilage pathology in OA. Proteoglycans form an important component of the cartilage matrix and, by retaining water, contribute to the shock absorbing capacity of the cartilage. These PG's are continuously synthesized and degraded. In line with previous results[25], young IL6$^{-/-}$ male mice did not differ from wt mice in ex vivo cartilage PG synthesis. When these mice got older (12 months), however, PG synthesis decreased in IL6$^{-/-}$ but not in wt mice. This suggests a positive relation between IL-6 and PG synthesis similar to the positive correlation between IL-6 and cartilage PG synthesis in dogs with experimentally induced OA[26]. Preliminary results showed that 24-hour incubation of 12 months old IL6$^{-/-}$ patellae in IGF supplemented medium restored PG synthesis to wt levels (data not shown). This suggests presence of lower levels of chondrocyte stimulating factors in older IL6$^{-/-}$ mice, which should be subject of further investigation. Histological measurement of cartilage PG's revealed no difference in net PG density of old IL6$^{-/-}$ and wt patellar cartilage. This will most likely be caused by decreased PG turnover in IL6$^{-/-}$ mice. Although a reduced PG synthesis in old IL6$^{-/-}$ mice does not affect the patella, it might impair the recovery or repair of cartilage and, in this way, could make it more prone to develop OA lesions.

The collateral and cruciate ligaments contribute to stability of the joint. We had previously found a positive relation between ligament damage and cartilage loss at the medi-

al side in wt mice with collagenase-induced OA[27]. A similar positive relation between calcification and ossification of the medial collateral ligament (MCL) and the development of OA lesions has been found in STR/ort mice[28]. In our present study we found more intense PG deposition and bone formation in the MCL of old IL6$^{-/-}$ male mice. Deposition of this so-called fibrocartilage and subsequent ligament stiffening could contribute to cartilage damage and bone sclerosis by altering the load distribution in the joint. Increased stiffness of the MCLs is also found in OA patients when compared to age-matched healthy people[29]. Heterotopic osteogenesis of human ligaments has been most studied in the spine where ligament fibroblasts can differentiate into chondrocytes in response to bone morphogenetic protein-2 (BMP-2)[30]. IL-6 can inhibit BMP-2 mRNA expression[31] and it is therefore possible that increased expression of BMP-2 or related growth factors contributes to the ligament damage in the IL-6$^{-/-}$ males.

Alternatively to being a cause, fibrocartilage deposition in and stiffening of ligaments might also be a consequence of other remodeling processes that occurred earlier in the joint. Increased collagen remodeling was measured in the anterior cruciate ligament (ACL) of STR/ort mice before radiological signs of OA were detected and the ACL was also weaker than in control mice[32]. Similarly, a reduced strength and stiffness of the ACL was found in people over 50 years of age when compared to young adults[33]. Weaker ligaments could contribute to greater joint instability and in this way might increase the risk of development and progression of knee OA[34]. Ligament lesions in either cruciate or collateral ligaments are frequently found in patients with advanced OA[35]. During progression of OA and as a response to increased joint laxity, the ligaments might subsequently become stiffer by development of fibrocartilage.

In vitro cultures of pig MCL fibroblasts showed that collagen production is positively correlated with IL-6 production[36]. This suggests that absence of IL-6 in our study might reduce the MCL collagen content, which might lead to a weaker ligament or impaired response to damage. Experimental ligament damage was induced by collagenase injection. The equal OA development that was observed in wt and IL-6$^{-/-}$ mice with collagenase-induced OA could indicate that during aging under normal conditions IL-6 plays an important role in maintaining ligament function.

Subchondral bone sclerosis can occur in human OA and was also in our study the most prominent bone-related change. Subchondral bone sclerosis could lead to a reduced shock absorbing capacity that finally leads to cartilage erosion. *In vitro* IL-6 expression could divide human OA osteoblasts in normal and high IL-6 producers[37]. This study, however, could not relate IL-6 expression to pathological findings. In osteophytes, IL-6 mRNA has been detected in active osteoblasts[38]. In trabecular OA bone,

decreased IL-6 mRNA expression was associated with enhanced mRNA expression for the bone formation marker osteocalcin[39]. The increased bone sclerosis in the old IL6$^{-/-}$ males could be in line with these last results.

Although the resolution of the bone densitometer did not allow us to evaluate the subchondral bone in detail due to the small size of the murine joint, DEXA measurements revealed a reduced BMD in knees of old, but not young, IL-6$^{-/-}$ male mice when compared to wt mice. Our data, therefore, suggest a BMD preserving role for IL-6. The reduced BMD might seem conflicting with the increased sclerosis in the IL-6$^{-/-}$ males but studies on human OA suggest it is not. Although bone volumes can increase in OA, the subchondral bone of these patients is less mineralized than normal bone (reviewed in Hunter and Spector, 2003[40]). Different reports showed a reduced BMD in OA subchondral bone[38;41;42]. Longitudinal studies relating BMD and OA development showed that high BMD at non-joint sites is associated with increased risk of OA. Once people have OA, in contrast, a low BMD and high bone turnover appear associated with enhanced progression of the disease (reviewed in Hunter and Spector, 2003[40]). This could explain the coexistence of reduced BMD, bone sclerosis and enhanced OA development in IL-6$^{-/-}$ males.

In our old wt and IL-6$^{-/-}$ males bone sclerosis, ligament ossification and complete cartilage erosion coincided in the most severe cases but only at the medial side. Bone sclerosis and ligament ossification were almost completely restricted to the medial side in all groups. Cartilage erosion, in contrast, occurred at both sides and had the highest incidence of all pathological changes. Together with the reduced PG synthesis that started already at younger age this suggests onset of OA in the cartilage. Histological and functional studies with mice at a younger age might identify the location of the first pathological change. Independent of the disease etiology, our present data clearly showed more severe cartilage erosion and ligament ossification in IL-6$^{-/-}$ male mice.

Acknowledgement

We would like to thank Fieke Mooren for performing the histological image analysis.

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CHAPTER 3

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

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American Journal of Pathology 2000, 157:2081-2091

Abstract

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 wildtype 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.

Introduction

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 osteoclasts[12] 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 al*[24] 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 increased in the IL-$6^{-/-}$ mice,

pointing at a cartilage protective role for IL-6. A recent study by Ohshima *et al*[28] 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 mice[30] and IL-6$^{-/-}$ mice backcrossed 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 μg 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 $2x10^9$ heat-killed *Bordetella pertussis* bacteria (National Institute of Public Health, Bilthoven, The Netherlands) in 1 ml of saline. Seven days later, mice were boostered with 100 μg of mBSA in Freund's complete adjuvant divided over two places in the neck region. Three weeks after the booster 60 μg of mBSA in saline (6 μl 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 μl of poly-L-lysine-coupled lysozyme (3 μg; Sigma) in the joint.

ZIA

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

Assessment of Joint Swelling

Mice were injected subcutaneously in the neck with 20 μCi of ^{99m}Technetium 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 μm 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-1α and Mip-2 (Blom *et al*, submitted), TNF-α, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase[35] 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 flatbottom 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-aminosalicylzuur, 0.8 mg/ml, 0.8 μl 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 $2x10^6$ cells/ml in medium (RPMI/penicillin 100 U/ml and streptomycin 100 μg/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 $2x10^7$/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 μg/ml (data shown for 25 μg/ml) or with concanavalin A (Flow laboratories, Irvine UK) at 1 μg/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 μCi ^{3}H-thymidine. After harvesting, incorporation of ^{3}H-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 (4x10^{7})in 200 μl 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 μl of serumfree 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 μg 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

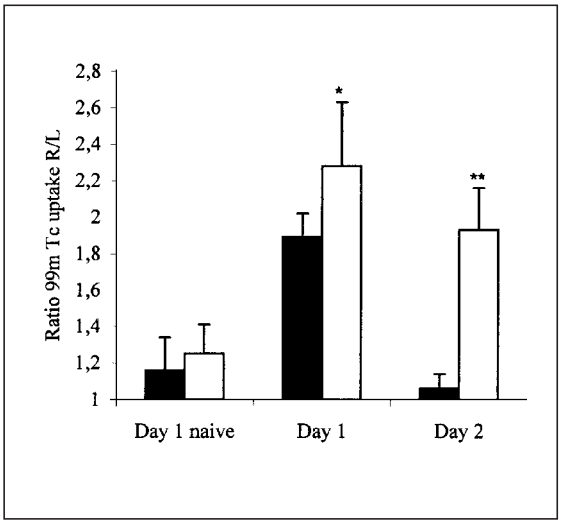

Figure 1 Joint swelling at day 1 and 2 after mBSA injection. Immunized and naive mice were injected with 60 μg of mBSA. Joint swelling was measured as the ratio of ^{99m}Tc uptake in the arthritic knee (**right**) over the nonarthritic knee (**left**). ■, IL-6$^{-/-}$; □, wild type ($n = 7$; *, $P <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

Table 1 Development of Joint Inflammation and Cartilage Damage during AIA

	DAY1 AIA		DAY2 AIA		DAY4 AIA		DAY7 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 ± 0.4 †	0.4 ± 0.3	2.3 ± 0.8 †	0.3 ± 0.2	2.8 ± 0.6 †
Exudate	1.5 ± 0.4	2.1 ± 0.5*	0.7 ± 0.4	1.9 ± 1.0*	0.1 ± 0.2	0.6 ± 0.5*	0.1 ± 0.1	1.5 ± 0.9 †
Patella	0.2 ± 0.2	0.4 ± 0.4	1.7 ± 0.6	2.5 ± 0.5*	1.7 ± 0.6	2.6 ± 0.7*	0.5 ± 0.7	3.0 ± 0.0 †
Femur	0.2 ± 0.3	0.7 ± 0.7	1.6 ± 0.9	2.3 ± 0.7	1.6 ± 0.6	2.7 ± 0.7 †	0.8 ± 0.8	3.0 ± 0.0 †
Medial tibia	nd	nd	1.1 ± 0.6	2.6 ± 0.5 †	1.9 ± 0.7	2.8 ± 0.4 †	1.3 ± 0.9	2.6 ± 0.7 †

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.

the first week (Table 1). In both strains the first cells entering the joint were predominantly polymorphonuclear cells. At day 2 a synovial infiltrate became apparent although smaller in knee joint cavities of IL-6$^{-/-}$ mice (Figure 2, A and C). 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, B 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 μg mBSA 1.58 ± 0.24, $n = 7$, one of three experiments).

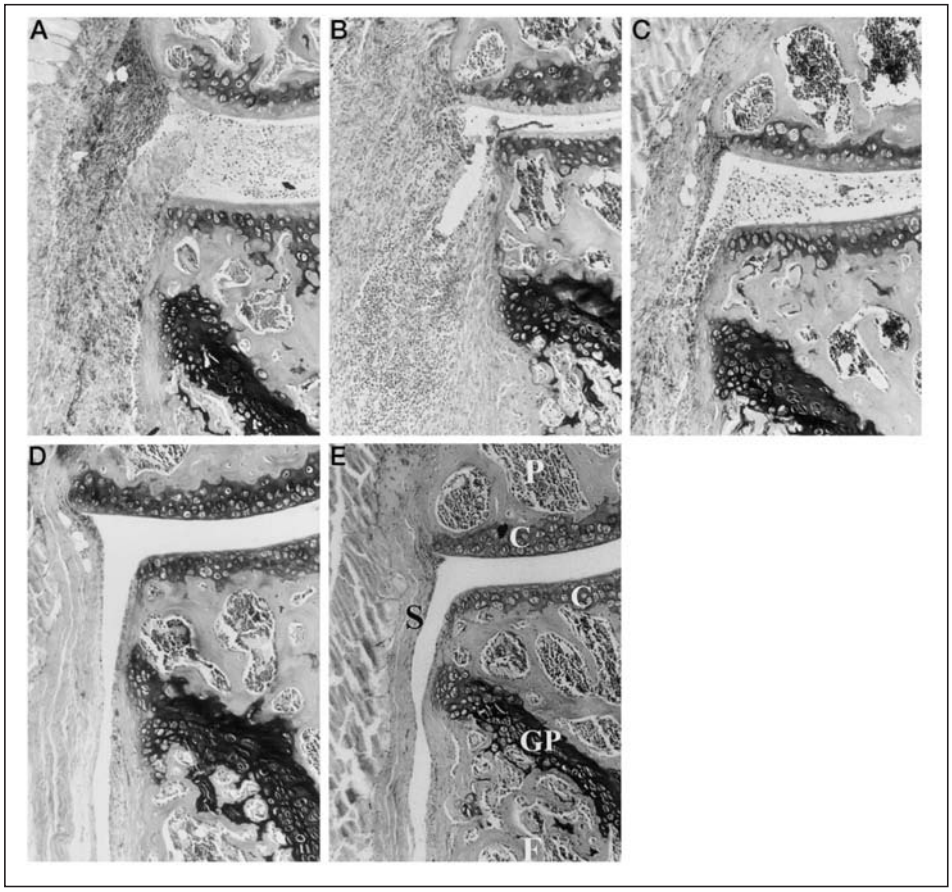

Figure 2. Inflammation and cartilage damage in wild-type (**A** and **B**) and IL-6$^{-/-}$ (**C** and **D**) mice on day 2 (**A** and **C**) and 7 (**B** and **D**) of the AIA. Sections were stained with safranin-O and cartilage damage was observed as loss of red staining. **E:** Position of patella (P), femur (F), synovium (S), growth plate (GP), and cartilage (C) is shown. Original magnification, x200.

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-1α, and Mip-2) and cytokines (TNF-α and IL-1β) in the arthritic joint at 24 (Figure 3A) and 48

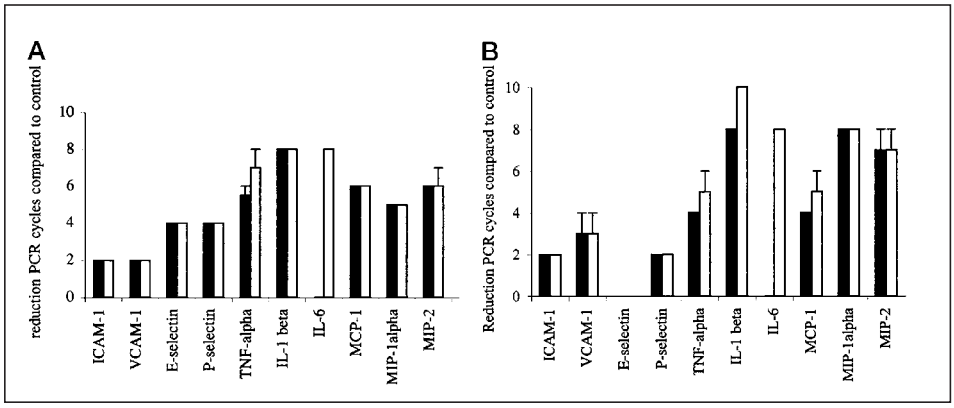

Figure 3 mRNA expression in synovia from wild-type and IL-6$^{-/-}$ mice on day1(**A**) and 2 (**B**) of the AIA. The number of PCR cycles needed to first detect the specific band on an agarose gel was compared between synovia from arthritic and contralateral nonarthritic knee joints. Increased mRNA 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.

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 administrating 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; wildtype, 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.

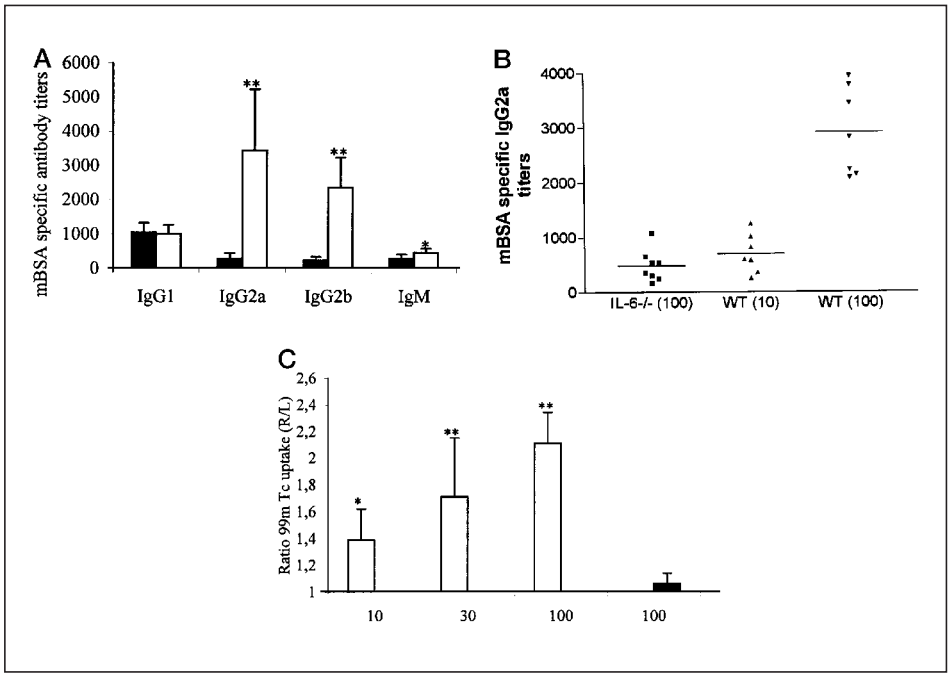

Figure 4 A: mBSA-specific antibody subclasses before onset of AIA. Titers as depicted are the dilution of sera needed to give half the maximal optical density at 450 nm as described in Materials and Methods. ■, IL-6$^{-/-}$; □, wild type ($n = 7$). **B:** mBSA-specific IgG2a titers in mice immunized and boostered with 100 μg of mBSA (IL-6$^{-/-}$) or with 10 or 100 μg of mBSA (wild type). Each symbol represents one mouse ($P = 0.22$, IL-6$^{-/-}$ (100 μg) *versus* wild type, (10 μg) Student's *t*-test). Total IgG titers also did not differ (IL-6$^{-/-}$: 1/720 ± 194, wild type: 1/734 ± 180). **C:** Joint swelling in wild-type mice immunized with 10, 30, or 100 μg 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.

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 boostered with 10, 30, or the normal 100 μg of mBSA. Wild-type mice immunized with 10 μg 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). 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-μg groups). These results suggest that lower antibody titers were not primarily responsible for a mild inflammatory response as observed in IL-6$^{-/-}$ mice.

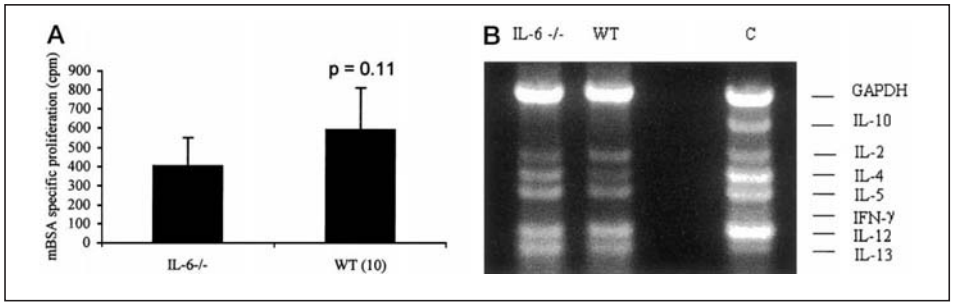

Figure 5 **A:** mBSA-specific increase in cpm (cpm mBSA minus cpm medium). Lymph nodes from seven mice per group were pooled and enriched for T cells as described in Materials and Methods. Lymph node cells ($2x10^6$) together with $2x10^7$irradiated antigen-presenting cells were incubated for 72 hours with mBSA at 25 μg/ml. Cells were plated in sixfold. Cultures were labeled with ^3H 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 μg/ml (IL-6$^{-/-}$, 28,002 ± 7,291 cpm; wild type, 25,436 ± 1,216 cpm) One of four experiments is shown. **B:** Th1/Th2 multiplex RT-PCR of IL-6$^{-/-}$ (100 μg mBSA) or wild-type (10 μg mBSA) T cells stimulated for 24 hours with conA (1 μg/ml). C = positive control.

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

Because even the wild-type mice immunized with the lowest amount of mBSA (10 μg) 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 μg mBSA)-or IL-6$^{-/-}$ (100 μg 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.

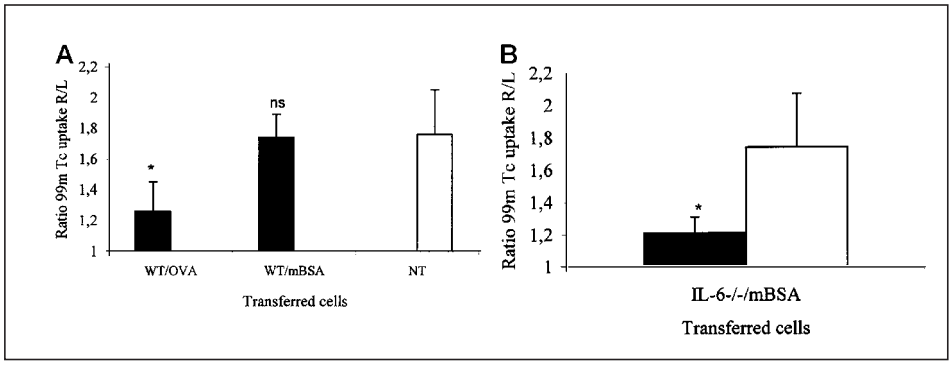

Figure 6 Joint swelling on day 2 of AIA in IL-6$^{-/-}$ mice after transfer of 4x10^{7}lymph node cells derived from mBSA (wild-type/mBSA) or ovalbumin (wild-type/OVA) immunized wild-type mice (**A**) or of mBSA (IL-6$^{-/-}$/ mBSA) immunized IL-6$^{-/-}$ mice (**B**). NT = normal AIA in wild-type mice immunized with 10 μg 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.

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 ± 0.1	0.8 ± 0.7	2.7 ± 0.5 †
Exudate	0.0 ± 0.0*	0.6 ± 0.6	1.5 ± 0.6 †
Patella	0.3 ± 0.6*	1.4 ± 1.1	3.0 ± 0.0 †
Femur	0.8 ± 0.9 †	2.0 ± 0.7	3.0 ± 0.0 †
Medial tibia	0.8 ± 0.8*	2.1 ± 1.0	2.9 ± 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 wildtype mice. WT, wild-type mice immunized with 10 μg 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 wildtype mice.

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 mice (10 μg mBSA) to immunized IL-6$^{-/-}$ mice (100 μg 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

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

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 ± 1.0ns
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).

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. 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 mice[30] 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 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 inflammation, 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 synovia of 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-in-flammatory gene expression. This is in line with equal synovial mRNA expression for TNF-α and IL-1β 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 al*[23] 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 ZIA[26] 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. Wildtype 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 μg instead of 100 μg 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 mice[41] 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 cells[49] 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 remission 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.

Acknowledgments

We thank Dr. M. Kopf (Basel) for providing the IL-$6^{-/-}$ strains and Theo van den Ing (Animal Laboratory) for help with the cell transfers.

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CHAPTER 4

Local Activation of STAT-1 and STAT-3 in the Inflamed Synovium During Zymosan-Induced Arthritis

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Arthritis and Rheumatism 2004, 50:2014-2023

Abstract

Objective. STAT proteins play an important role in cytokine signaling. Some investigators have reported preferential activation of STAT-1, and others have reported preferential activation of STAT-3, in response to endogenous interleukin-6 (IL-6), in patients with rheumatoid arthritis. The present study was undertaken to investigate synovial STAT-1 and STAT-3 activation in an experimental animal model of arthritis.

Methods. Zymosan was injected intraarticularly into naive wild-type (WT), IL-6$^{-/-}$, and STAT-1$^{-/-}$ mice to induce arthritis. Western blots of synovial lysates were probed with phosphospecific antibodies to detect STAT-1/STAT-3 activation. Inflammation was assessed histologically. Synovial gene expression of the STAT-induced feedback inhibitors suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 in WT and STAT-1$^{-/-}$ mice was investigated by reverse transcriptase- polymerase chain reaction.

Results. STAT-3 was activated in inflamed synovium of WT mice throughout the course of disease, whereas activated STAT-1 was observed only during the chronic phase. In IL-6$^{-/-}$mice, STAT activation was limited to STAT-3 on day 1. Although macrophage influx was not inhibited, disease went into remission after day 7 in IL-6$^{-/-}$ mice. STAT-1 deficiency resulted in exacerbation of chronic joint inflammation and granuloma formation. In STAT-1$^{-/-}$mice, STAT-3 activation in the inflamed joints was unaltered as compared with WT mice. However, synovial SOCS-1, but not SOCS-3, gene expression was markedly reduced in STAT-1$^{-/-}$ mice.

Conclusion. The results in the IL-6$^{-/-}$ mice suggest that STAT-3 is involved in the chronicity of ZIA. Exacerbation of arthritis in STAT-1$^{-/-}$ mice suggests an opposing effect of STAT-1, i.e., suppression of joint inflammation. The expression of SOCS-1 could be the underlying mechanism by which STAT-1 controls joint inflammation.

Introduction

The JAK/STAT pathway plays an important role in cytokine and growth factor-induced signal transduction. Signaling through this pathway is mediated by phosphorylation of STAT proteins (1). Upon activation the STATs dimerize, translocate to the nucleus, and initiate transcription of STAT-responsive genes. Seven different STATs (STATs 1-4, 5a, 5b, and 6) that can form both STAT homo-and STAT heterodimers have been described to date. During recent years it has become clear that STATs play key roles in developmental processes, growth control, immune system development, and resistance to infection (2). Dysregulated STAT function contributes to human disease, as has been studied most extensively in cancer development (3).

Rheumatoid arthritis (RA) is characterized by chronic inflammation of the joint. Although its exact cause is still unknown, pro-and antiinflammatory cytokines seem to play an important role in RA pathology (4). Studies on activation of STATs in RA synovial fluid (SF) cells have yielded conflicting results. Activated STAT-3 has been found in SF cells from RA patients (5). Furthermore, SF from RA patients has been shown to induce STAT-3 activation in monocytes (6); this activity could be blocked by neutralization of IL-6. Yokota *et al*, in contrast, observed activation of STAT-1, and not STAT-3, in RA SF cells (7); this activation could also be prevented by incubation with anti-IL-6 antibodies. The reason for these differences regarding STAT-1 and STAT-3 activation is not yet clear.

IL-6 is a multifunctional cytokine that is found in large quantities in the SF and serum of patients with RA (8). Amelioration of collagen-induced arthritis (9,10) as well as antigen-induced arthritis (AIA) (11,12) in IL-$6^{-/-}$ mice has been reported, which indicated an important proinflammatory role for IL-6. In these models, IL-6 deficiency led to reduced antigen-specific immunity prior to actual joint inflammation. We recently reported that transfer of wild-type (WT) lymph node cells could enhance the acute inflammation and also the cartilage damage in IL-$6^{-/-}$ mice with AIA. Chronic synovitis, however, still did not develop in IL-$6^{-/-}$ mice (12). This suggests an important role for IL-6 in chronicity of arthritis, which is possibly independent of its role in immunity. Studies of IL-6 blocking in RA patients have demonstrated a significant therapeutic effect on clinical and laboratory parameters (13). To date, the effect of anti-IL-6 treatment on STAT activation has not been investigated.

Different biologic effects of STAT-1 and STAT-3 have been described, and it remains to be determined which subtype contributes to the proinflammatory effect of IL-6 in RA. In the present study we compared synovial STAT-1 and STAT-3 activation during the

course of experimental arthritis in WT and IL-6$^{-/-}$mice. To avoid differences caused by immunizations, we compared these mice during the acute and chronic phases of non-immunologically mediated zymosan-induced arthritis (ZIA) (14). To further investigate STAT-1 involvement, we induced ZIA in STAT-1$^{-/-}$ mice and studied arthritis development in these animals.

Materials and methods

Animals

Breeding pairs of homozygous IL-6$^{-/-}$ mice, WT (C57BL/6 x129Sv)F2 mice (15), and IL-6$^{-/-}$ mice backcrossed onto C57BL/6 mice for 8 generations were obtained from Dr. M. Kopf (Basel, Switzerland). C57BL/6 mice were obtained from Charles River Deutschland (Sulzfeld, Germany). All mice were housed in filtertop cages in our specific pathogen-free animal facilities. A standard diet and acidified tap water were provided ad libitum. The animals were studied at the age of 8-10 weeks. STAT-1$^{-/-}$ mice were originally developed by Dr. D. Levy (New York, NY) (16). Breeding pairs of STAT-1$^{-/-}$ mice and their WT controls were kept at the Institute for Agrobiotechnology. Experiments with STAT-1$^{-/-}$ mice were performed in isolators at the animal facilities of the University of Nijmegen. Sterile food and water were provided ad libitum. Experiments were conducted according to national and institutional regulations for animal use.

Zymosan-induced arthritis

A 30-mg/ml suspension of zymosan A (*Saccharomyces cerevisiae*) was made in endotoxinfree saline. The suspension was autoclaved and arthritis was induced by intraarticular injection of 180 μg of zymosan A into the knee joint cavity. The contralateral knee joint served as a within-animal control. Physiologic saline was used for control injections.

Histologic study of knee joints

Knee joints were dissected, fixed in formalin, decalcified, dehydrated, and embedded in

paraffin. Standard 7-μm frontal sections were prepared and stained with hematoxylin and eosin or Safranin O. Serial sections were scored for inflammation on a scale of 0-3 by 2 independent observers, in a blinded manner. For differences in inflammation score, *P* values less than < 0.05 (by rank sum test) were considered significant.

Isolation of inflamed synovium for protein determinations

Synovial tissue was isolated in a standardized manner as described previously (17). Surrounding muscle, patellar ligament, and patella were removed before isolation of the synovial tissue. Care was taken not to damage large blood vessels. The tissue was immediately frozen in liquid nitrogen. Tissue samples were homogenized in a freeze mill and lysed on ice for 15 minutes in lysis buffer containing 0.5% Nonidet P40, 10 m*M* Tris HCl (pH 7.4), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* Na3VO4 (pH 10.0), and 1x protease inhibitor cocktail (PharMingen, Woerden, The Netherlands). Insoluble material was removed by centrifugation. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL).

Western blotting

For Western blotting, 30 μg of synovial cell lysate was run on a sodium dodecyl sulfate-7.5% polyacrylamide gel. Cell lysates from HeLa cells (Cell Signaling Technology, Beverly, MA) with or without interferon-α (IFNα) treatment served as positive and negative controls for STAT-1/STAT-3 activation. Proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Hybond P; Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were blocked with Tris buffered saline-Tween/1% bovine serum albumin (BSA; Sigma, St. Louis, MO)/1% nonfat dry milk (Campina, Eindhoven, The Netherlands). After incubation with first and secondary antibodies, the membranes were developed with an enhanced chemiluminescence detection system (ECL+Plus; Amersham Pharmacia Biotech). For reprobing, the membranes were stripped in 0.2*M* glycine (pH 2.5)/0.05% Tween 20 at 80°C for 20 minutes.

Antibodies

Rabbit anti-mouse antibodies specific for STAT-1, STAT-3, phosphorylated STAT-1 (Tyr^{701}), and phosphorylated STAT-3 (Tyr^{705}) were obtained from Cell Signaling Technology. Goat anti-mouse actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control for protein loading. Horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit IgG and donkey anti-goat IgG) were from Santa Cruz Biotechnology.

NIMP-R14 and F4/80 staining

Knee joint sections were stained for the presence of the NIMP-R14 epitope (18), which is mainly present on neutrophils, and for the F4/80 cell surface glycoprotein, which is expressed on macrophages. Sections were deparaffinized and, after antigen retrieval and preincubation for 15 minutes with 20% normal rabbit serum, were incubated for 1 hour with anti-NIMP-R14 antibodies (a kind gift from Dr. M. Strath, London, UK), anti F4/80 (Instruchemie, Hilversum, The Netherlands), or normal rat Ig. After incubation for 30 minutes with peroxidase-labeled rabbit anti-rat secondary antibody in 5% normal mouse serum/ phosphate buffered saline, the sections were incubated for 10 minutes with diaminobenzidine (1 mg/ml in 50 m*M* Tris HCl [pH 7.6], 0.001% H2O2). Sections were counterstained with hematoxylin for 30 seconds.

Synovial RNA isolation

Synovial tissue was isolated in a standardized manner and immediately frozen in liquid nitrogen. The tissue was homogenized in a freeze mill, thawed in 1 ml of TRIzol reagent, and further processed according to the protocol recommended by the manufacturer (Life Technologies Breda, The Netherlands). Isolated RNA was treated with DNase I before being reverse transcribed into complementary DNA (cDNA) with Moloney murine leukemia virus reverse transcriptase.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for SOCS-1 and SOCS-3

Primers were designed with Primer3 software (19) and manufactured at Eurogentec (Seraing, Belgium). The following primers were used: for SOCS-1, forward 5'-CTTAACCCGGTACTCCGTGA-3', reverse 5'-GAGGTCTCCAGCCAGAAGTG-3'; for SOCS-3, forward 5'-ATTCACCCAGGTGGCTACAG-3', reverse 5'-AACACCGGACCAGTTCC AGG-3'. Primers were annealed at 55°C. The reaction mixture contained 2 μl of cDNA, 1 x PCR buffer, 20 pmoles of each primer, 0.2 m*M* of each dNTP, 1m*M* MgCl2, and 2 units *Taq* DNA polymerase. All PCR reagents were from Life Technologies.

Samples were taken after increasing numbers of PCR cycles as described previously (17) and run on a 1.6% agarose gel. PCR products were stained with ethidium bromide and visualized under ultraviolet light. The cycle number at which the PCR product was first detected on the gel was taken as a measure for the amount of specific messenger RNA (mRNA) originally present in the isolated synovial RNA. PCR for GAPDH was performed to verify that equal amounts of cDNA were used. PCR for IL-6 confirmed IL-6 deficiency in IL-$6^{-/-}$ mice.

RESULTS

Zymosan-induced arthritis and STAT activation in WT mice

Injection of zymosan into the knee joints of naive WT mice elicited a local inflammation that could be divided into 2 phases. Acute joint swelling and an influx of polymorphonuclear granulocytes (PMNs) characterized the first 7 days (Figure 1A). Synovial hyperplasia and infiltration of mononuclear cells dominated the second phase, between week 2 and week 4 (Figure 1B). Protein lysates of synovia from inflamed and contralateral uninjected knee joints were made. We first studied synovial STAT-3 activation with antibodies specific for Tyr^{705}-phosphorylated STAT-3. In WT mice, STAT-3 became activated during both the acute and the chronic phase of ZIA (Figure 2). We observed STAT-3 activation until the end of the experiments (at week 4 of arthritis). No synovial STAT-3 activation was observed on day 1 or day 6 after injection of saline (results not shown), excluding the possibility of a side effect of the injection. Activation of STAT-1 was investigated with antibodies specific for Tyr^{701}-phosphorylated STAT-1. No STAT-1, activated or not, could be detected in inflamed synovia of WT mice during

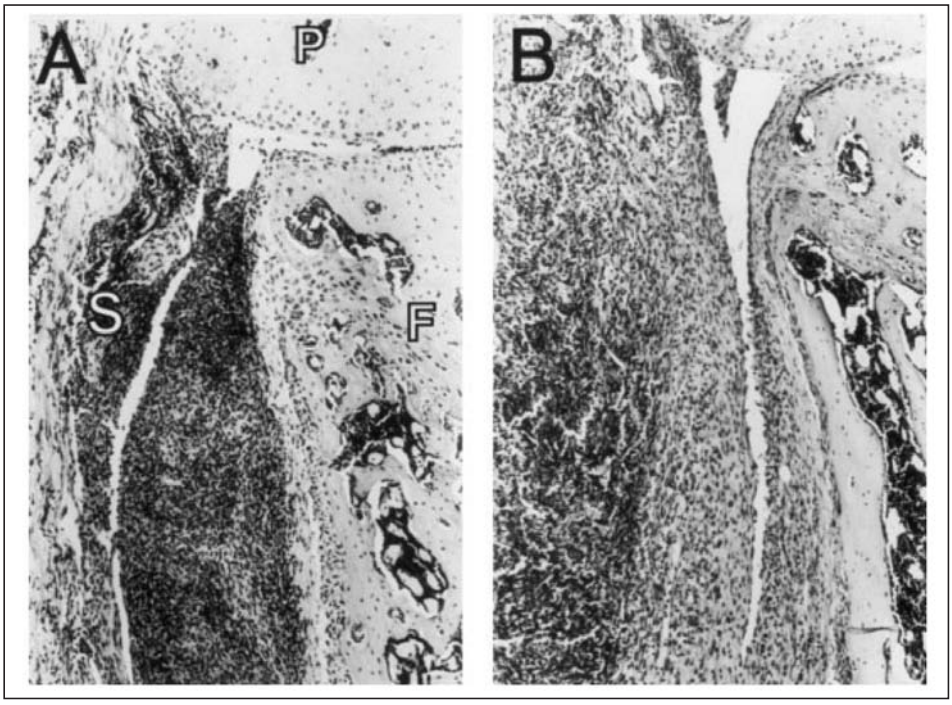

Figure 1 Joint inflammation induced by intraarticular injection of zymosan into wild-type mice. **A,** Day 7 of zymosan-induced arthritis (ZIA). Inflammation is evident. **B,** Day 21 of ZIA. The inflammation has progressed to chronic synovitis. Similar results were obtained in 3 experiments with 7 mice per group. **P** = patella; **F** = femur; **S** = inflamed synovium. (Hematoxylin and eosin stained; original magnification x 155.)

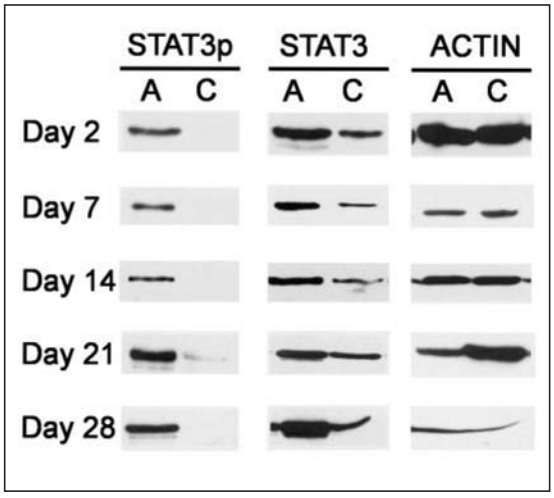

Figure 2 Continuous STAT-3 activation during zymosan-induced arthritis (ZIA) in wild-type (WT) mice. Activated STAT-3 was detected in the inflamed arthritic synovium (A) of WT mice during both the acute phase (days 1-7) and the chronic phase (after day 7) of ZIA. Although STAT-3 was expressed, no STAT-3 activation was detected in synovium from uninjected contralateral knee joints (C) of the same mice. Similar results were obtained in at least 2 independent experiments with 3-4 mice per time point. STAT-3p = phosphorylated STAT-3.

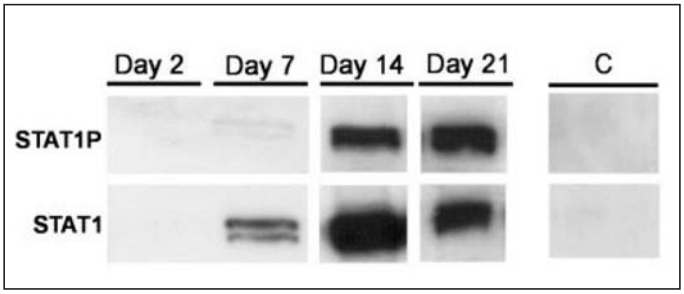

Figure 3 STAT-1 activation in the inflamed synovium of WT mice. Activated STAT-1 was observed in the arthritic synovium of WT mice during the chronic phase of ZIA (days 14 and 21). No activation was observed during the acute phase of ZIA (day 2). No or very limited STAT-1 activation was observed on day 7. At no time was STAT-1 activation observed in contralateral knee joints (C; day 21 shown as an example); similarly, it was not induced by injection of physiologic saline. Similar results were obtained in at least 2 independent experiments with 3-4 mice per time point. See Figure 2 for definitions.

the time of ZIA onset. Minor STAT-1 activation started in some mice on day 7. Thereafter, the total amount of STAT-1 protein in the synovium increased and STAT-1 activation became clearly detectable (days 14 and 21 of ZIA) (Figure 3).

Zymosan-induced arthritis and STAT activation in IL-6$^{-/-}$ mice.

Injection of zymosan induced an acute inflammation in IL-6$^{-/-}$mice (Figures 4A and C). In contrast to ZIA in WT mice, joint inflammation in IL-6$^{-/-}$ mice did not became chronic after the first week and was completely resolved by day 21 (Figure 4B).

In the original description of the ZIA model, 2 inflammatory phases could be distinguished: an acute phase characterized by PMN influx and a chronic phase with mononuclear cells as the main infiltrating cell type. This latter phase was characterized by synovial hypertrophy and pannus formation, features of chronic arthritis. The transition took place between day 7 and day 14. At no time were lymphoid aggregates or plasma cells observed during ZIA (14). Immunostaining with NIMPR14 confirmed the presence of PMNs during the acute inflammation and their decline in the chronic phase of ZIA in WT mice (Figures 5A and E). In IL-6$^{-/-}$ mice, PMNs were also the dominant cell type in the acute phase but, in contrast to WT mice, they were completely absent when the inflammation went into remission (Figures 5C and G). Macrophages (F4/80-positive cells) were absent during the acute phase in WT mice (Figure 5B), started to enter during the transition phase, and were clearly present during the chronic phase (Figure 5F). In IL-6$^{-/-}$ mice, in contrast, macrophages were present in both the acute and the remission phases (Figures 5D and H).

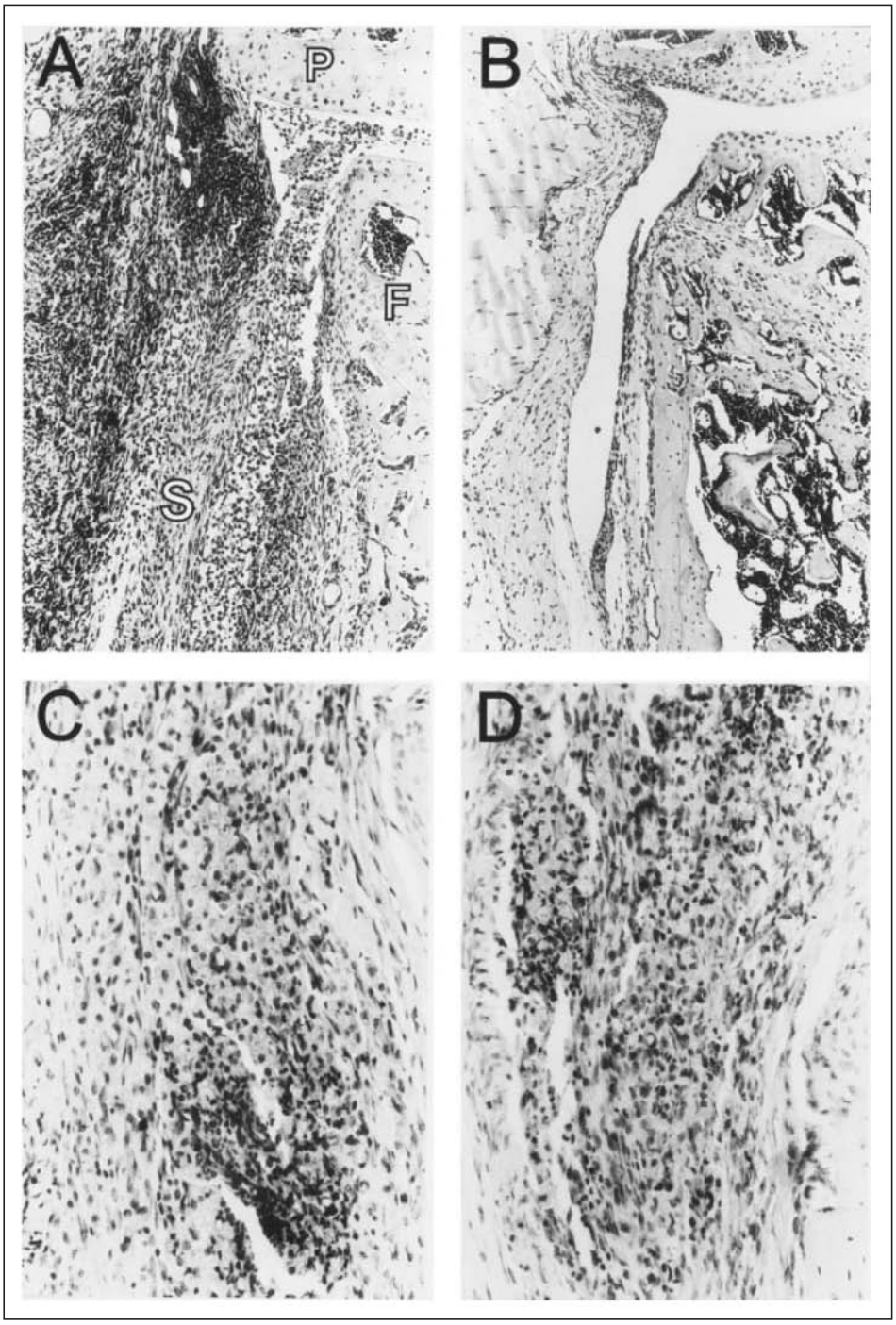

Figure 4 Impaired inflammation in interleukin-6$^{-/-}$ (IL-6$^{-/-}$) mice. **A,** Acute inflammation in an IL-6$^{-/-}$ mouse on day 7. **B,** Absence of chronic synovitis in an IL-6$^{-/-}$ mouse on day 21. **C** and **D,** Detail of inflamed synovium on day 7 of ZIA in an IL-6$^{-/-}$ and a WT mouse, respectively. Similar results were obtained in 3 experiments with 7 mice per group. **P** = patella; **F** = femur; **S** = inflamed synovium (see Figure 2 for other definitions). (Hematoxylin and eosin stained; original magnification x 155 in **A** and **B**; x 400 in **C** and **D.**)

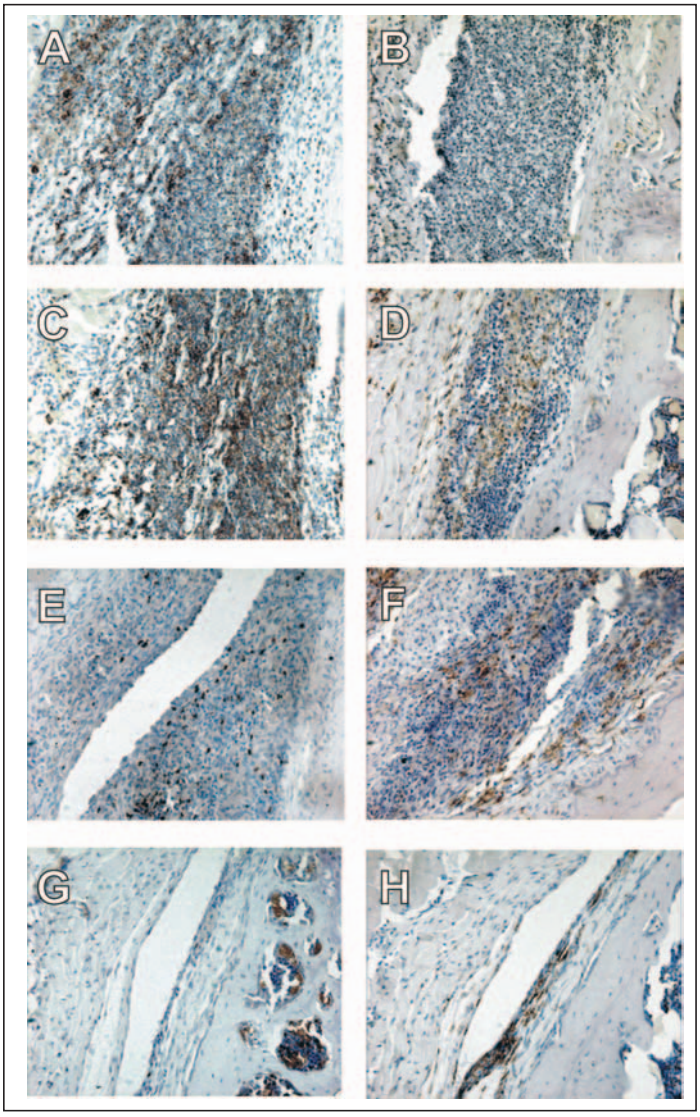

Figure 5 Immunodetection of polymorphonuclear cells (PMNs) and macrophages in inflamed synovia of WT and interleukin-$6^{-/-}$ (IL-$6^{-/-}$) mice. PMNs were detected with NIMP-R14 staining and macrophages with F4/80 staining, as described in Materials and Methods. **A** and **B,** Staining with NIMP-R14 and F4/80, respectively, in WT mice on day 2 of ZIA. **C** and **D,** Staining with NIMP-R14 and F4/80, respectively, in IL-$6^{-/-}$ mice on day 2 of ZIA. **E** and **F,** Staining with NIMP-R14 and F4/80, respectively, in WT mice on day 14 of ZIA. **G** and **H,** Staining with NIMP-R14 and F4/80, respectively, in IL-$6^{-/-}$ mice on day 14 of ZIA. Normal rat Ig did not produce staining on sections from either strain (results not shown). See Figure 2 for other definitions. (Original magnification x 200.)

Synovial cell lysates from arthritic IL-$6^{-/-}$ mice showed activation of STAT-3 on the first day of ZIA (Figure 6A). After day 1, STAT-3 activation was no longer detectable in synovia of IL-$6^{-/-}$ mice (Figure 6B), even though the joint remained inflamed during the first week. STAT-1 activation was completely absent in synovia of IL-$6^{-/-}$ mice (Figure 6C). This lack of STAT activation does not seem to reflect absence of inflammatory cells since both PMN and macrophage influx occurred in the IL-$6^{-/-}$ mice as demonstrated by immunohistochemistry analysis.

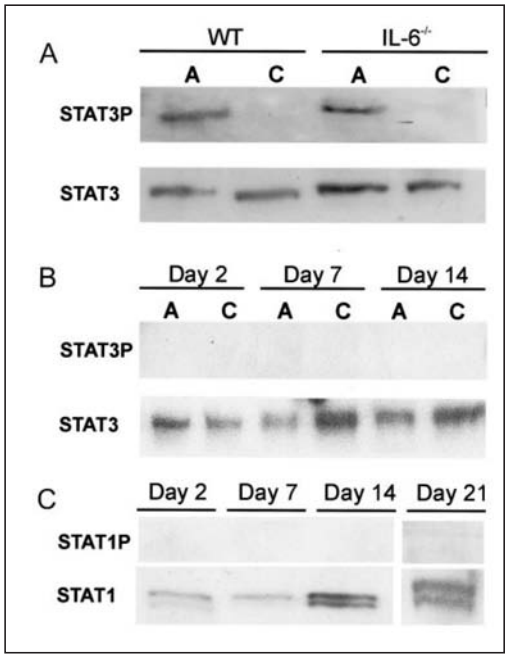

Figure 6 Impaired STAT-3 and STAT-1 activation in interleukin-6 $^{-/-}$ (IL-6$^{-/-}$) mice. **A,** As in WT mice, STAT-3 became activated in the synovium of IL-6$^{-/-}$ mice on day 1 after intraarticular injection of zymosan. **B,** No STAT-3 activation was observed in the synovium of IL-6$^{-/-}$ mice on day 2, 7, or 14 after intraarticular injection. **C,** No STAT-1 activation was observed in the synovium of IL-6$^{-/-}$ mice after zymosan injection. In the studies of STAT-3 activation on days 2, 7, and 14 and of STAT-1 activation on days 2, 7, 14, and 21, a positive signal was observed for the positive control that was present on the same blot (results not shown). Similar results were obtained in at least 2 independent experiments with 3-4 mice per time point. See Figure 2 for other definitions.

Exacerbation of ZIA in STAT-1$^{-/-}$ mice

Activation of STAT-1 was detectable only during the chronic phase of ZIA. To investigate whether activated STAT-1 plays a role in the chronicity of arthritis, we injected zymosan into the knee joints of STAT-1$^{-/-}$ mice. Histologic analysis (Figures 7A and B) showed that inflammation in these mice was significantly increased, to a degree that caused joint immobilization (mean ± SD inflammation score 2.9 ± 0.4 in STAT-1$^{-/-}$ mice versus 1.75 ± 0.3 in WT mice; n = 8 per group) ($p < 0.001$ by rank sum test). Such joint immobilization was not observed in WT mice with ZIA. The inflamed synovium of STAT-1$^{-/-}$ mice was highly infiltrated with PMNs that partly were concentrated in granuloma-like structures (Figure 7C). Although more severe than in WT mice, the inflammation remained restricted to the injected joints, and no other signs of disease were observed in the STAT-1$^{-/-}$ mice. Injection of saline did not induce joint inflammation in STAT-1$^{-/-}$ mice (Figure 7D). Proteoglycan depletion in the articular cartilage was observed in both WT and STAT-1$^{-/-}$ mice (Figures 7E and F). The increased inflammation, however, did not lead to enhanced cartilage or bone erosion in the STAT-1$^{-/-}$ mice. This suggests that STAT-1 predominantly plays a role in regulating the inflammation during arthritis. Western blot analyses of synovial lysates showed that STAT-3 becomes activated during ZIA in STAT-1$^{-/-}$ mice (Figure 8).

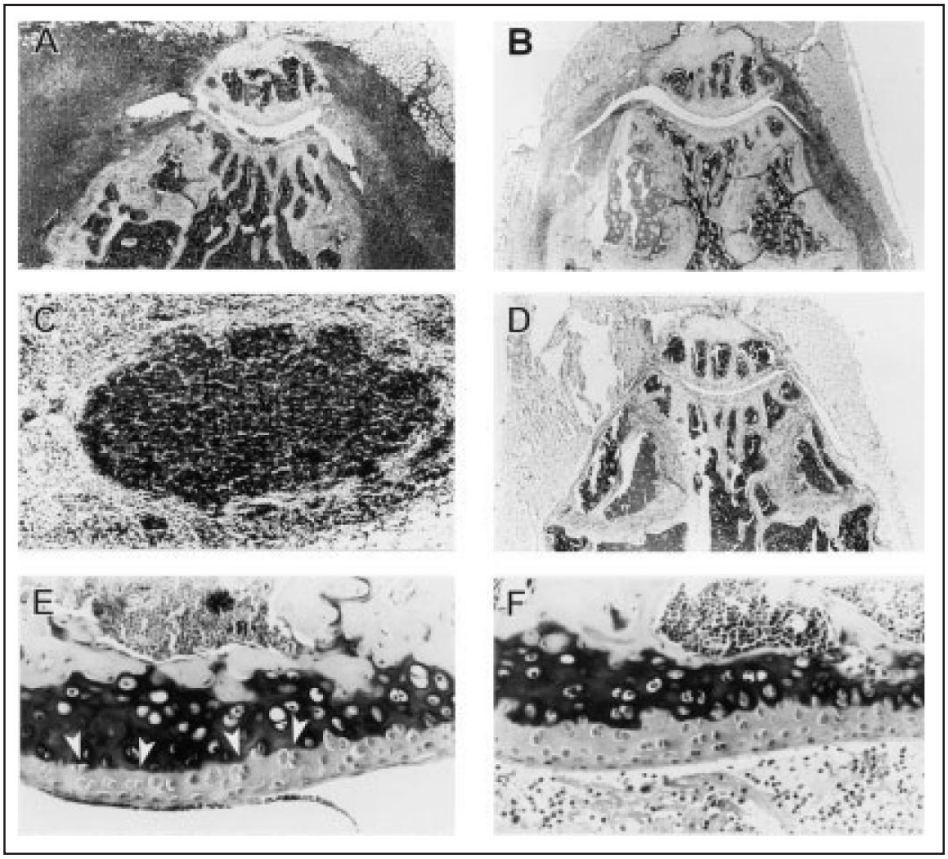

Figure 7 Exacerbated inflammation in STAT-1$^{-/-}$ mice. **A,** Exacerbation of joint inflammation in a STAT-1$^{-/-}$ mouse on day 14 of ZIA. The enhanced inflammation led to joint fixation in these mice; this was observed in 3 different experiments with 6-8 mice per group (hematoxylin and eosin stained). **B,** Conventional ZIA in a WT mouse (hematoxylin and eosin stained). **C,** Granuloma-like structures in the inflamed synovium of a STAT-1$^{-/-}$mouse. NIMP-R14 staining showed these structures to be rich in polymorphonuclear cells (diaminobenzidine stained [dark dots] and hematoxylin counterstained). **D,** Lack of inflammation in the joint of a STAT-1$^{-/-}$ mouse injected with physiologic saline (hematoxylin and eosin stained). **E** and **F,** Proteoglycan (PG) loss but no cartilage erosion in both STAT-1$^{-/-}$ (**E**) and WT (**F**) mice on day 14 of ZIA. In both strains, PG loss is shown in the upper cartilage layers by reduced staining with Safranin O. **Arrowheads** in **E** indicate the tidemark that separates the PG-depleted upper cartilage layer (gray) from the nondepleted deeper layers (dark). **P** = patella; **F** = femur; **S** = synovium (see Figure 2 for other definitions). (Original magnification x 50 in **A, B,** and **D;** x 200 in **C;** x 400 in **E** and **F.**)

SOCS-1 and SOCS-3 expression in inflamed synovia

The SOCS family proteins are important regulators of STAT activation; these proteins are inhibitors that are themselves induced by activated STATs. By semiquantitative RT-PCR it was shown that SOCS-1 gene expression was enhanced in inflamed joints on day

14 of ZIA in WT mice (Table 1). This coincided with the activation of STAT-1 in this inflamed synovial tissue. SOCS-3 gene expression, in contrast, was enhanced on both day 7 and day 14 of ZIA, and this coincided with the activation of STAT-3.

The exacerbation of arthritis in STAT-1$^{-/-}$ mice could be caused by a derailment of SOCS regulation. We therefore compared synovial SOCS-1 and -3 gene expression between WT and STAT-1$^{-/-}$ mice. Reduced SOCS-1 expression was found in inflamed synovia of STAT-1$^{-/-}$ mice during ZIA (Table 2). SOCS-3 expression, in contrast, was not reduced in STAT-1$^{-/-}$ mice.

Taken together, the present results demonstrate local activation of both STAT-1 and STAT-3 during chronic ZIA. Furthermore, they suggest an important role of STAT-1 in controlling the inflammation. The reduced SOCS-1 expression in STAT-1$^{-/-}$ mice makes SOCS-1 a candidate gene that could contribute to the control of arthritis.

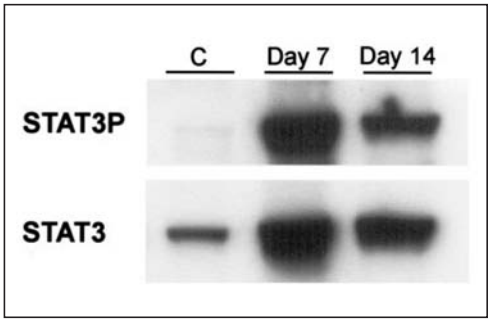

Figure 8 STAT-3 activation in the inflamed joints of STAT-1$^{-/-}$ mice. Activated STAT-3 was detected in the inflamed synovium of STAT-1$^{-/-}$ mice on days 7 and 14 of ZIA. No STAT-3 activation was detected in the contralateral knee joint. Inflamed synovia from 4 STAT-1$^{-/-}$ mice were examined, with similar results. See Figure 2 for definitions.

Table 1 Expression of SOCS-1 and SOCS-3 mRNA and of phosphorylated STAT-1 and STAT-3 in the synovium of wild-type mice with zymosan-induced arthritis (ZIA)

	RT-PCR*		Westernblot †	
	Cycle, SOCS-1	Cycle, SOCS-3	Phosphorylated STAT-1	Phosphorylated STAT-3
No arthritis	12 ± 2	11 ± 1	–	–
ZIA day 7	12	7 ± 1	–/+	++
ZIA day 14	6	6	++	++

*Expression of mRNA for suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 was determined by semiquantitative reverse transcriptase- polymerase chain reaction (RT-PCR) as described in Materials and Methods. For each sample, the cycle number at which GAPDH, SOCS-1, and SOCS-3 were first detected was determined. The cycle numbers for SOCS-1 and SOCS-3 have been corrected for GAPDH expression by presenting the number of cycles after the first detection of GAPDH. A lower cycle number indicates that the product is expressed at higher levels. At each time point, synovial tissue from 6 mice was examined and PCR was performed twice. Values are the mean or the mean ± SD for 1 of 2 independent experiments with similar results (where only the mean is shown, the SD was 0). †- = no activation in inflamed synovium; -/+ = no or minimal activation in inflamed synovium; ++ = marked activation in inflamed synovium.

Table 2 Expression of SOCS-1 and SOCS-3 mRNA in the synovium of wild-type WT and STAT1$^{-/-}$ mice with ZIA*

	Cycle, SOCS-1		Cycle, SOCS-3	
	Wild-type mice	STAT1 $^{-/-}$ mice	Wild-type mice	STAT1 $^{-/-}$ mice
PBS day 7	14 ± 1	14 ± 1	13 ± 1	12 ± 1.5
ZIA day 7	5 ± 1.5	8 ± 1	5 ± 1.5	4 ± 1
PBS day 14	13 ± 0.5	13 ± 1.5	11 ± 1	11 ± 1.5
ZIA day 14	5 ± 2	8	6.5 ± 0.5	5

*Expression of mRNA for SOCS-1 and SOCS-3 was determined by semiquantitative RT-PCR as described in Materials and Methods. For each sample, the cycle number at which GAPDH, SOCS-1, and SOCS-3 were first detected was determined. The cycle numbers for SOCS-1 and SOCS-3 have been corrected for GAPDH expression by presenting the number of cycles after the first detection of GAPDH. A lower cycle number indicates that the product is expressed at higher levels. The experiments were performed once on day 7 and twice on day 14. At each time point, synovial tissue from 6 mice was examined and PCR was performed twice. Values are the mean or the mean ± SD (where only the mean is shown, the SD was 0). PBS = phosphate buffered saline (see Table 1 for other definitions).

Discussion

In the present study, temporal differences in STAT activation were found. STAT-1 expression and activation were restricted to the chronic phase of ZIA (days 14-21). In IL-6$^{-/-}$ mice, continuous STAT-3 activation was not observed even though these animals exhibited the expected acute inflammation. Our results provide evidence that without STAT-1 activation, mice develop a chronic granulomatous synovitis.

Zymosan-induced arthritis is characterized by an acute (days 1-7) influx of PMNs (observed with NIMP-R14 immunostaining) followed by a gradual increase (days 7-21) of mononuclear cell infiltration into the synovium (observed with F4/80 immunostaining). In IL-6$^{-/-}$mice, the acute joint inflammation did not develop into a chronic phase. Previous in vitro studies have demonstrated a direct role of IL-6 in the recruitment of PMNs and monocytes. Endothelial cells lack the IL-6 receptor (gp80), and only in combination with the soluble IL-6 receptor α subunit (sIL-6Rα) can IL-6 induce the PMN-binding proteins E-selectin and intercellular adhesion molecule 1 and the monocyte binding protein vascular cell adhesion molecule 1 (20). This role of IL-6 in PMN influx was not confirmed in this study or in a previous study showing that IL-6 deficiency did not affect the acute phase of antigen-induced joint inflammation or the gene expression of adhesion molecules E-selectin and P-selectin (12). In studies of zymosan-induced peritonitis, however, the acute inflammation was reduced in IL-6$^{-/-}$ mice (21).

This discrepancy in the IL-6 dependency of inflammation could be related to the site of inflammation. It was previously shown, in experiments using zymosan to induce inflammation, that acute peritonitis is dependent on the integrins lymphocyte function-associated antigen 1 and Mac-1, whereas these integrins are not important in joint inflammation (22). Likewise, zymosan can elicit joint inflammation via complement activation (23), but complement is minimally involved in zymosan-induced peritonitis (24). It is therefore possible that, in contrast to the situation in peritonitis, IL-6, though present during the onset of ZIA, does not play a decisive role during this phase of the disease and is overruled by the action of other cytokines.

In this study we identified a major role of IL-6 in the transition from the acute to the chronic phase of arthritis. The transition from PMN to mononuclear cell infiltration can be explained by enhanced clearance of PMNs and the induction of the mononuclear cell chemokine monocyte chemotactic protein 1 by the endothelium after stimulation with the IL-6/sIL-6R α complex (25,26). However, after the first week of joint inflammation, PMNs were still present in WT mice but absent in IL-6$^{-/-}$ mice, and this suggests a protective effect of IL-6 on PMN survival. There is compelling evidence that STAT-3 is involved in the survival of PMNs (27,28), possibly by prevention of apoptosis. The role of IL-6 in the clearance of PMNs could, however, be far more complex, with IFNγ contributing to the IL-6/sIL-6R-mediated clearance of PMNs from the site of inflammation (29). IFNγ largely signals through STAT-1 activation, and STAT-1 could therefore be involved in PMN resolution. This is consistent with the finding of exaggerated and prolonged PMN infiltration in the STAT-1$^{-/-}$ mice in the present study.

We showed that during ZIA, synovial SOCS-1 and -3 mRNA expression coincided with the activation of STAT-1 and STAT-3, respectively (Table 1). In recent studies using genetically modified mice and gene therapy approaches, it has been demonstrated that STAT-1/3 activation and SOCS expression are important regulators of experimental arthritis. Mice with a point mutation in the SH2-binding site of gp130 (Y759), the docking site of SOCS-3, develop autoimmune-mediated joint inflammation, probably due to prolonged STAT-3 activation in their T cells (30). Furthermore, periarticular adenoviral overexpression of SOCS-3 is effective in attenuating collagen-induced arthritis in mice (31). SOCS proteins, however, might redirect signaling pathways in a concentration-dependent manner (32-34). In the absence of SOCS-3, prolonged STAT-3 and STAT-1 activation has been observed in vitro and *in vivo* after stimulation with IL-6. The prolonged STAT-1 activation most likely contributed to the IFNγ-like gene expression in response to IL-6 (33,34).

We found that SOCS-1 expression was diminished in the STAT-1$^{-/-}$ mice. In studies

of mice lacking the SOCS-1 gene, it has been shown that SOCS-1 is an important negative regulator of acute arthritis in the methylated BSA/IL-1 arthritis model (35). In experiments using SOCS-1$^{-/-}$ macrophages, Kinjyo *et al* demonstrated that SOCS-1 could negatively regulate lipopolysaccharide-induced cytokine production by inhibiting Toll-like receptor 4 signaling (36). This inhibition was mediated through interference with IL-1R- associated kinase-mediated NF-κB activation and demonstrates that SOCS proteins can also directly inhibit other signaling pathways besides JAK-STAT. The experiments performed in the STAT-1$^{-/-}$ mice clearly showed that SOCS-1 gene expression was decreased, and this might have caused unrestricted proinflammatory cytokine signaling and exacerbation of arthritis.

Microarray analysis of synovial biopsy specimens from 21 patients showed that the RA population could be divided into 2 groups with different levels of STAT-1 expression, and with higher levels of STAT-1 indicative of an immunologically mediated inflammatory process (37). That study, however, did not include an investigation of either STAT-1 activation or the relationship between STAT-1 expression and disease parameters. Whether STAT-1 plays a pro-or an antiinflammatory role in human RA requires further examination. This division on the basis of STAT-1 expression and the reported differences in STAT activation (5,7) could be reflective of different phases of RA, as was suggested by the temporal expression and activation of STAT-1 in the ZIA model.

Besides the JAK/STAT pathway, IL-6 can also activate mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways. These pathways could also be involved in RA (38). Future studies should address their induction by IL-6 and the interplay between these different pathways in arthritis.

Our study adds further data on the involvement of STAT proteins in the progression of arthritis and elucidates a possible role of STAT-1 in the downregulation of arthritis and of STAT-3 in the perpetuation of joint inflammation. STAT-1 involvement is not limited to the arthritis induced by the irritant zymosan, since preliminary experiments showed that STAT-1 deficiency also exacerbates immune complex-induced arthritis. Opposing effects of activated STAT-1 and STAT-3 have been described for different diseases (39,40), and our study demonstrates that this could be true for RA as well (41). The present results also show that STAT-1 and STAT-3 are differentially regulated during the acute and chronic phases of arthritis. The findings reported herein suggest that selective manipulation of STAT activation or SOCS expression may have therapeutic efficacy in RA. This warrants further investigation.

Acknowledgments

We would like to thank Dr. D. Levy (New York, NY) for the breeding pairs of STAT-1$^{-/-}$ mice, Dr. M. Kopf (Basel, Switzerland) for the breeding pairs of IL-6$^{-/-}$ and wild-type control mice, Windy Smallegoor (Nijmegen, The Netherlands) for participating in the investigation of STAT-1 activation during ZIA, Ruben Smeets (Nijmegen, The Netherlands) for help in preparing the figures, and Matthias Müller (Vienna, Austria) for critically reading the manuscript.

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CHAPTER 5

Adenoviral Transfer of Murine Oncostatin M Elicits Periosteal Bone Apposition in Knee Joints of Mice, Despite Synovial Inflammation and Up-Regulated Expression of Interleukin-6 and Receptor Activator of Nuclear Factor-κB Ligand

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American Journal of Pathology 2002, 160:1733-1743

Abstract

Oncostatin M (OSM) has been described as a bone-remodeling factor either stimulating osteoblast activity or osteoclast formation *in vitro*. To elucidate the *in vivo* effect of OSM on bone remodeling, we injected an adenoviral vector encoding murine OSM in knee joints of mice. OSM strongly induced interleukin (IL)-6 gene expression, a known mediator of osteoclast development. We investigated the OSM effect in wild-type and IL-6-deficient mice and found a similar degree of OSM-induced joint inflammation. Within the first week of inflammation, the periosteum along the femur and tibia increased in cell number and stained positive for the osteoblast marker alkaline phosphatase. At these sites bone apposition occurred in both strains as demonstrated by Goldner and Von Kossa staining. *In vitro* OSM enhanced the effect of bone morphogenetic protein-2 on osteoblast differentiation. Immunohistochemistry demonstrated expression of receptor activator of nuclear factor-κB ligand (RANKL) and its receptor , receptor activator of nuclear factor-κB (RANK), in the periosteum but osteoclasts were not detected at sites of bone apposition. Induced mRNA expression for the receptor activator of nuclear factor-κB ligand inhibitor osteoprotegerin probably controlled osteoclast development during OSM overexpression. Our results show that OSM favors bone apposition at periosteal sites instead of resorption *in vivo*. This effect was not dependent on or inhibited by IL-6.

Introduction

Oncostatin M (OSM) is a 28-kd glycoprotein that belongs to the interleukin (IL)-6 family.[1] It was originally discovered by its ability to inhibit the growth of the melanoma cell line A375.[2] Subsequently, more effects were discovered and OSM was found to be a multifunctional cytokine like the other IL-6 family members. OSM can for example stimulate an acute-phase response in liver cells[3] and enhance expression of tissue inhibitor of metalloproteinase-1[4] and adhesion molecules such as ICAM-1.[5] Macrophages and activated T cells have been shown to produce OSM.[2,6]

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is accompanied by destruction of joints. Elevated levels of OSM can be detected in the synovial fluid of RA patients and synovial macrophages are the source of this OSM.[7,8] Unlike its family member IL-6, OSM was not detected in the serum of RA patients.[7] This suggests a local rather than a systemic role for OSM in RA. Circumstantial evidence for a local role is the positive correlation found between concentrations of OSM and cartilage degradation markers in synovial fluid.[9] Injection of recombinant human OSM in the joints of goats induced inflammation,[10] further supporting a local and pro-inflammatory role for OSM in joint pathology. Recently, intraperitoneal administration of blocking antibodies to OSM ameliorated experimental arthritis in mice,[11] demonstrating a pro-inflammatory role for OSM in murine arthritis. A systemic effect of blocking OSM can, however, not be excluded under these experimental conditions.

The receptor complex for OSM consists of the glycoprotein gp130 that is used by all of the IL-6 family members, and a second receptor. This second receptor in the complex is either the leukemia inhibitory factor receptor-β or the OSM receptor-β.[12] The isolation of the murine OSM receptor-β showed that the use of these receptor complexes differed between species.[13] Human OSM utilizes both receptor complexes on human cells but only the gp130/leukemia inhibitory factor receptor-β complex on murine cells. Murine OSM in contrast only binds to the gp130/OSM receptor-β complex on murine cells. At present it is not known if this differential use of receptor complexes is also true for other combinations of species. The conflicting pro-inflammatory[5] and anti-inflammatory[14] effects of human OSM in mice, however, stress the need to use species-specific OSM in experimental disease models.

Injection of an adenoviral vector expressing murine OSM (AdmuOSM) in murine knee joints led to inflammation and synovial cell proliferation.[15] OSM has been shown to be a potent inducer of IL-6 gene expression. IL-6 plays a pivotal role in development and chronicity of experimental arthritis.[16-18] In the present study we have injected this

same vector in the knee joints of wild-type and IL-6-deficient mice to elucidate a role for IL-6 in the AdmuOSM-induced pathology.

Bone erosion can take place in patients with RA[19] and IL-6 family members are also implicated to play a role in this process. IL-6 together with the soluble IL-6 receptor has been shown to induce the formation of osteoclast-like cells.[20] IL-6 also has been shown to enhance the resorbing activity of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells.[21] The role of OSM during pathological bone remodeling is at present unclear. OSM can influence differentiation and proliferation of osteoblasts[22] relating it to bone development. On the other hand can OSM also induce the formation of osteoclast-like cells[20,23] relating it to bone erosion. Interestingly, we discovered apposition of new bone tissue after injection of the AdmuOSM vector. This new bone apposition took place under inflamed conditions and was not dependent on nor inhibited by IL-6.

Materials and Methods

Animals

For this study C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). IL-6-deficient mice[24] backcrossed for eight generations into C57BL/6 mice were obtained from Dr. M. Kopf (Basel, Switzerland) and breeding colonies were kept at the Central Animal Facilities of the Catholic University of Nijmegen. Male animals were used between 11 and 13 weeks of age. All mice were housed in filter-top cages under specific pathogen-free conditions and a standard diet and water were provided *ad libitum*. The mice were housed in isolators after adenoviral injection. Experiments were performed according to national and institutional regulations for animal use.

Adenoviral Vectors and Intra-Articular Injection

The construction of the replication-deficient E1-deleted AdmuOSM was described before.[25] As a control vector Addl70-3 (No. 5), a vector without insert, was used. For *in vivo* experiments the virus was diluted in physiological saline and 2.10^6-plaque-forming units (pfu) in a total volume of 6 μl were injected in the knee joint cavity.

Histological Evaluation of Knee Joints

Knee joints were dissected, fixed in formalin, decalcified, dehydrated, and embedded in paraffin. Standard frontal sections of 7 μm were prepared and stained with safranin-O and counterstained with fast green. Synovial inflammation was scored on five semiserial sections of the joint. Scoring on a scale from 0 up to 3 was performed in a blindfolded manner by two independent observers.

Isolation of Synovial RNA and Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Synovial mRNA was isolated and quantitated as described by van Meurs and colleagues.[26] Patellae with surrounding synovium 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 manufacturer's protocol. All reagents for RNA isolation and RT-PCR were from Life Technologies (Breda, The Netherlands). Isolated RNA was treated with DNase I before being reverse-transcribed into cDNA with MMLV 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 for osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL)[27] were used as described before. For IL-6 the following primers were used: IL-6 forward 5'TCT-GCA-AGA-GAC-TTC-CAT-CCA and reverse 5'GCA-AGT-GCA-TCA-TCG-TTG-TTC (55°C, 1 mmol/L $MgCl_2$).

Alkaline Phosphatase (ALP) Staining

Cryostat sections of knee joints (7 μm) were stained for ALP activity with the naphthol AS-BI (Sigma, St. Louis, MO) method.[28]

Von Kossa and Goldner's Trichrome Staining

Whole formalin-fixed knee joints were embedded in plastic and 7-μm thick sections were cut. Sections were stained with von Kossa staining[29] to identify calcified bone and with a Goldner's trichrome staining[30] to demonstrate the presence and maturation of newly formed bone.

Image Analysis of Newly Formed Bone

The surface area of the newly formed bone in wild-type and IL-6-deficient mice was measured using the Qwin image analysis system (Leica Imaging Systems Ltd., Cambridge, UK). Images of safranin-O-stained sections were captured using a JVC 3-CCD color video camera (Victor Company of Japan Ltd., Tokyo, Japan) and displayed on a computer monitor. Per joint four measurements of bone apposition on the femur were performed in a standardized manner. Both the length of the original cortical bone (marked by a precipitation line in the staining) and the area of the newly formed bone were measured. The amount of newly formed bone is expressed as μm^2 new bone/10 μm cortical bone.

TRAP Staining

Whole formalin-fixed knee joints were decalcified in 10% ethylenediaminetetraacetic acid (Titriplex III; Merck, Darmstadt, Germany)/1 mmol/L Tris-HCl (pH 7.4) for 2 weeks at 4°C. Decalcified knee joints were processed for paraffin embedding and 7-μm thick tissue sections were prepared. These sections were stained for TRAP with the leukocyte acid phosphatase kit (Sigma, St. Louis, MO) according to the manufacturer's protocol.

Immunohistochemistry of RANKL and RANK

Tissue sections (7 μm) of paraffin-embedded whole knee joints were treated for 15 minutes with 3% H_2O_2/methanol at room temperature. After antigen retrieval (2 hours in 10 mmol/L of citrate, pH 6.0, at room temperature) sections were incubated with the primary antibody or normal serum for 1 hour. Antibodies used were rabbit anti-RANK (H300) at 2 μg/ml, goat anti-RANKL (N19) at 1 μg/ml, goat IgG at 1 μg/ml (all from

Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit IgG at 2 μg/ml (DAKO, Glostrup, Denmark). After rinsing, sections were blocked for 20 minutes at room temperature with 4% normal mouse serum for RANKL and normal goat serum for RANK. Thereafter, sections for RANKL were incubated for 30 minutes with biotinylated mouse anti-goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and detected by biotin-streptavidin/peroxidase staining (Elite kit; Vector Laboratories, Burlingame, CA). Sections for RANK were incubated for 30 minutes with horseradish peroxidase-conjugated goat anti-rabbit IgG (DAKO). Development of the peroxidase staining was done with 3',3'diaminobenzidine. Sections were counterstained with hematoxylin.

ALP Assay on C2C12 Cells

C2C12 cells were obtained from the American Tissue Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium/10% newborn calf serum/100 U/ml penicillin/100 μg/ml streptomycin at 37°C in a humidified atmosphere at 7.5% CO_2. The cells were seeded at 6.7 x 10^3 cells per well in a 96-well round-bottom plate. The next day the cells were washed with phosphate-buffered saline (PBS). Subsequently, the cells were stimulated for 3 days in medium containing 5% newborn calf serum and different combinations of the following recombinant proteins: recombinant murine OSM, recombinant human bone morphogenetic protein-2 (BMP-2), recombinant human IL-6, and recombinant soluble IL-6 receptor (R&D Systems, Minneapolis, MN). IL-6 and soluble IL-6 receptors were used in a 1:1 ratio. After 3 days the cells were washed with cold Hank's buffer and fixed for 10 minutes with 4% formalin on ice. Thereafter the cells were washed with cold PBS and incubated with substrate (1 mol/L diethanolamine, 1 mmol/L $MgCl_2$, and 5.26 mg/ml *p*-nitrophenylphosphate). The plate was placed at 37°C in the dark. The reaction was stopped after 5 minutes with 0.5 mol/L of NaOH and the OD 405 nm was measured on a Ceres UV 900C spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT). The effect of recombinant proteins on the number of cells was measured by Neutral Red staining[31] and measured at 550 nm.

Statistical Analysis

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

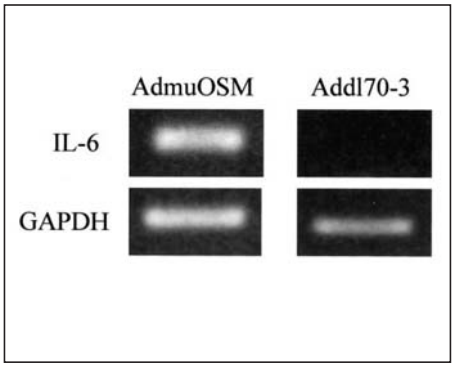

Figure 1 IL-6 gene expression in AdmuOSM-injected knee joints. Semiquantitative RT-PCR showed enhanced IL-6 gene expression (data shown for 30 cycles) in the AdmuOSM-injected knee joint. IL-6 gene expression was not detected after Addl70-3 injection (not even after 40 PCR cycles). RNA was isolated from synovia of three mice on day 3 after injection of the adenoviral vectors. PCR for glyceraldehyde-3-phosphate dehydrogenase was performed to assess the amount of cDNA used (data shown for 18 cycles). PCR samples were taken during the PCR and analyzed on a 1.6% agarose gel as described in Materials and Methods. IL-6 mRNA expression in AdmuOSM-injected knee joints was first detected after 27 PCR cycles.

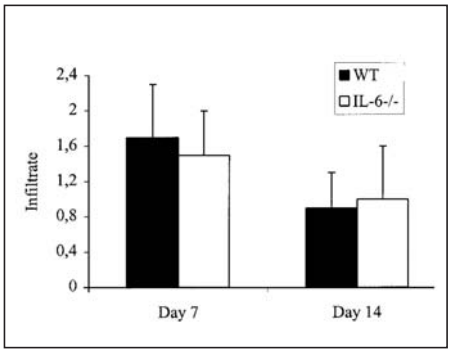

Figure 2 Histological scoring for the joint inflammation at days 7 and 14 after AdmuOSM injection. ■, Wild-type mice; □, IL-6-deficient mice. Per time point six mice per group were evaluated. Both strains did not differ in the inflammation ($P > 0.05$, Student's *t*-test).

Results

Adenoviral Overexpression of OSM Induces Joint Inflammation in Wild-Type and IL-6-Deficient Mice

Intra-articular injection of 2.10^6-pfu AdmuOSM, but not of the control vector Addl70-3, in the joint of wild-type mice induced an inflammation that was characterized by influx of mononuclear and polymorphonuclear cells and synovial hyperplasia. Synovitis lasted at least until week 4 after injection, the last time point studied. No signs of inflammation were macroscopically observed in the ankle or foot of the leg receiving AdmuOSM in the knee joint, indicating that OSM induced a localized joint inflammation.

OSM is a strong inducer of IL-6 gene expression and semiquantitative RT-PCR of injected knee joints showed that AdmuOSM induced IL-6 expression (Figure 1). To elucidate the role of IL-6 in the AdmuOSM-induced inflammation, we injected this vector in knee joints of IL-6-deficient mice. Histological scoring of joint inflammation showed no difference between both strains indicating that the inflammatory effect of OSM did not depend directly on IL-6 (Figure 2).

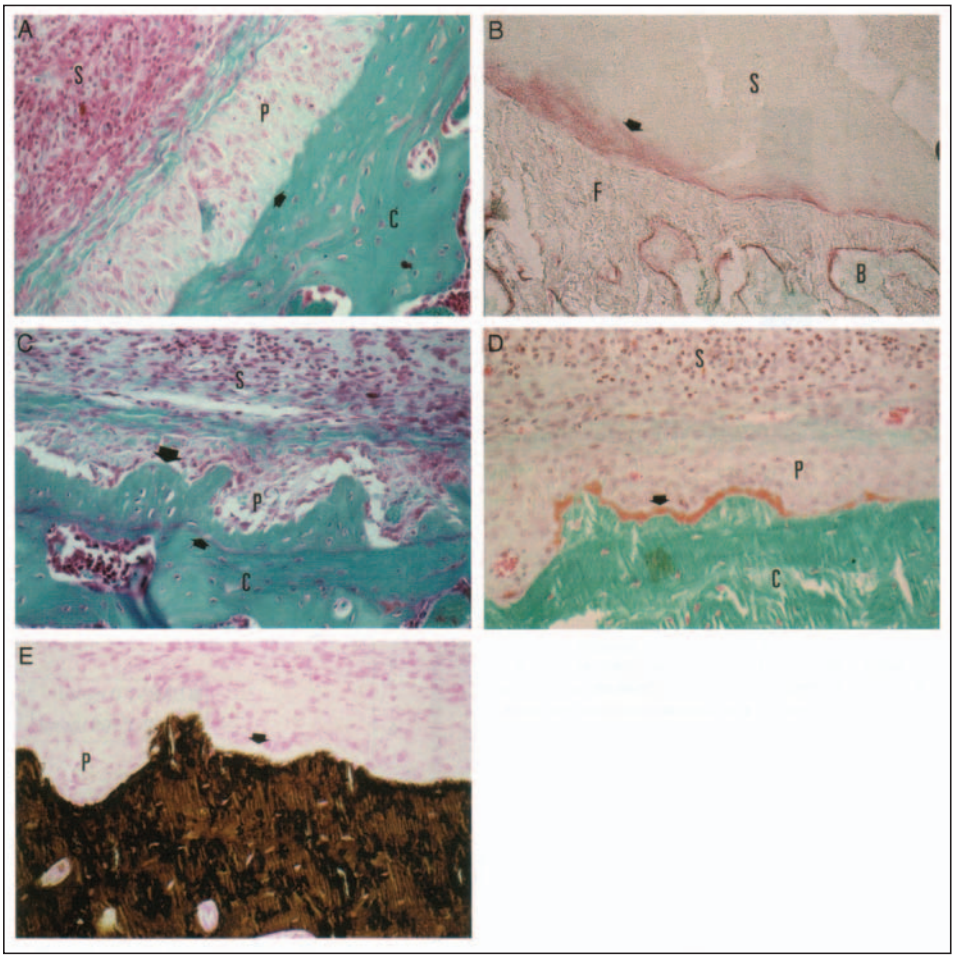

Figure 3 A: Activated periosteum of the femur 7 days after injection of AdmuOSM. Stained by Safranin-O. An **arrow** indicates the surface of the femoral cortical bone. **B:** Positive staining of the activated periost for ALP activity (red) is indicated with an **arrow**. Bone and inflammation is stained in green with methyl green. **C:** New bone formed on the femur of a wild-type mouse 14 days after injection of AdmuOSM (**large arrow**). A **small arrow** indicates the former surface of the bone. **D:** Goldner staining of the newly formed bone. The latest formed and still unmineralized bone is stained in orange and is indicated with an **arrow**. The mineralized bone is stained in green. **E:** Von Kossa staining of a section adjacent to the section of **D**. The mineralized bone is stained in brown. The newly formed and unmineralized bone is not stained by the Von Kossa staining (white layer indicated by an **arrow**). B, Bone marrow; C, cortical bone of the femur; F, femur; P, periosteum, S, inflamed synovium. Original magnifications: x400 (**A**, **C-E**); x180 (**B**).

Bone Formation in AdmuOSM-Injected Knee Joints

OSM can influence both cell types that are involved in bone remodeling, the osteoblast and the osteoclast. Its family member IL-6, in contrast, is generally described as an erosive cytokine through the induction of osteoclast development.[20] It was therefore of

interest to compare the effect of OSM and the OSM-induced inflammation on the articular bone in both wild-type and IL-6-deficient mice.

Already at day 3 of the inflammation, we observed the formation of several layers of periosteal cells along the femur and tibia of the AdmuOSM-injected knee joint (Figure 3A). Positive staining of these layers for ALP activity showed the osteoblast-like nature of these cells (Figure 3B). During the second week of inflammation apposition of new bone occurred next to this thickened periosteal cell layer as shown for day 14 after adenoviral injection (Figure 3C). A Goldner staining demonstrated the most recently formed and still not completely mineralized new bone closest to the periosteal cell layer in orange (Figure 3D). This layer could not be stained by the Von Kossa technique (Figure 3E) further demonstrating that mineralization of this bone is still not completed. The mineralization of new bone that was already completed was demonstrated by both stainings. This clearly shows that new bone is formed in AdmuOSM-injected knee joints and that the process of bone formation is still continuing at day 14 after injection. Injection of the control vector Addl70-3 did not lead to new bone formation. Periosteal bone apposition and joint inflammation were also observed with AdmuOSM when the contralateral knee joint was not injected (Dr. C. D. Richards, McMaster University, Hamilton, Ontario, Canada, unpublished observation).

The formation of layers of osteoblast-like cells and the deposition of new bone occurred in both wild-type and IL-6-deficient mice. The IL-6-deficient mice did not differ from wild-type mice in the amount of newly formed bone (Figure 4). Adenoviral expression of murine IL-6 did not induce apposition of new bone in the knee joint (data not shown). This strengthens our results in the IL-6-deficient mice showing that the observed OSM effect on the articular bone is not mediated by IL-6.

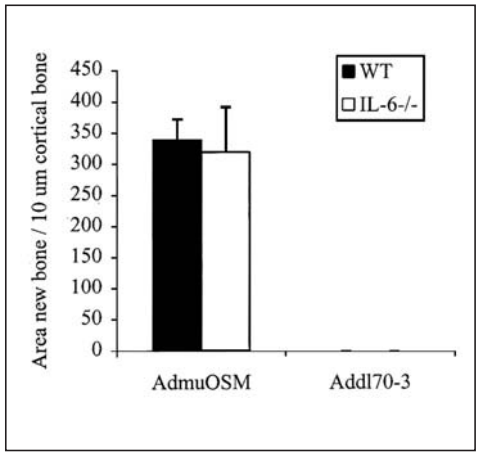

Figure 4 Quantification of newly formed bone in wild-type and IL-6-deficient mice. The surface area of newly formed bone was measured as described in Materials and Methods and was expressed as μm^2 new bone/10 μm cortical bone. No significant difference was found between wild-type ($n = 10$) and IL-6-deficient mice ($n = 8$) ($P = 0.469$, Student's t-test). Injection of the control vector Addl70-3 did not lead to bone apposition. Data shown are for day 14 after adenoviral injection. ■, Wild-type mice; □, IL-6-deficient mice.

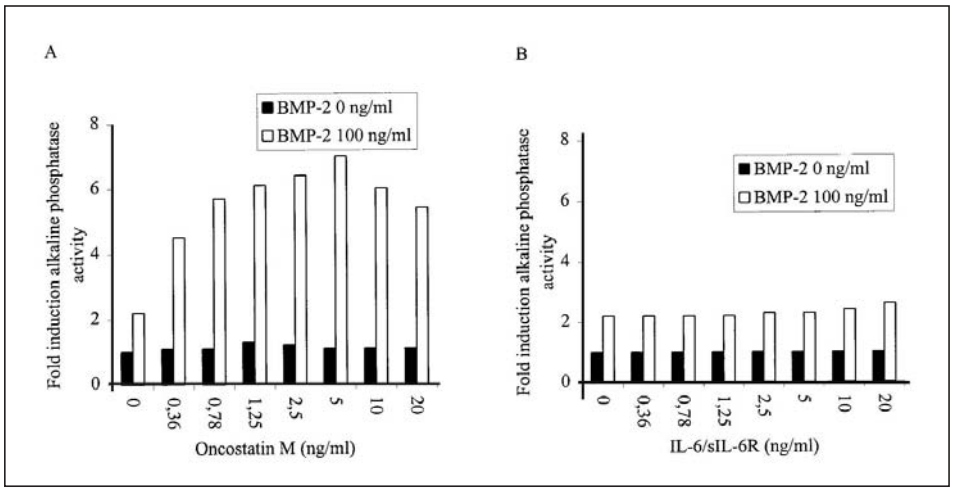

Figure 5 OSM enhances the BMP-2-induced ALP activity in C2C12 cells. **A:** C2C12 cells were incubated for 3 days with variable concentrations of recombinant murine OSM in the presence or absence of 100 ng/ml of recombinant human BMP-2. ALP activity was measured as described in Materials and Methods. The OD 405-nm value for cells without OSM or BMP-2 added to the medium was set at 1 and used for calculating the effect of BMP-2, OSM, and the combination of both proteins. OSM alone did not induce ALP activity in these cells even at concentrations up to 500 ng/ml (data not shown). BMP-2 alone gave a twofold induction of ALP activity. This BMP-2-induced ALP activity was greatly enhanced by OSM. This same effect of OSM was also found when 300 ng/ml of BMP-2 was used (data not shown). **B:** C2C12 cells were incubated for 3 days with variable concentrations of recombinant IL-6 and the soluble IL-6 receptor in the presence or absence of 100 ng/ml of recombinant human BMP-2. In contrast to OSM the IL-6/sIL-6R combination did not enhance the BMP-2-induced ALP activity. IL-6/sIL-6R alone did also not induce ALP activity in the C2C12 cells. The experiments of Figure 5, A and B, were performed in duplicate for at least three times with similar results. One representative experiment is shown. The SD in ALP activity was less than 5% between duplicate measurements.

OSM Enhances BMP-2-Induced ALP Activity in Vitro

A possible direct effect of OSM on development of the ALP-positive cell layer was further investigated *in vitro*. For these experiments we used pluripotent murine C2C12 cells that can differentiate toward the osteoblast lineage[32] and measured ALP activity after 3 days of culture. Recombinant murine OSM alone was, in contrast to a bone-forming factor such as BMP-2, not able to induce ALP activity in these cells. Addition of OSM to a constant BMP-2 concentration, however, had a strong enhancing effect on the BMP-2-induced ALP activity (Figure 5A). The mean induction of three experiments was 2.1 ± 0.2 for 100 ng/ml BMP-2, 3.7 ± 1.0 for 100 ng/ml BMP-2 + 0.36 ng/ml OSM ($P < 0.05$), and 5.8 ± 1.1 for 100 ng/ml BMP-2 + 5 ng/ml OSM ($P < 0.005$) (Student's *t*-test, $n = 6$). The other concentrations of OSM tested in combination with BMP-2 also differ significantly from incubations with BMP-2 alone. Equal neutral red staining for C2C12 cells

treated with BMP-2 or BMP-2 and OSM excluded an effect on cell proliferation (data not shown). The enhanced ALP activity does not seem to depend directly or indirectly on IL-6 because it was not observed after incubation with the combination of BMP-2, IL-6, and the soluble IL-6 receptor (Figure 5B). These results suggest that the *in vivo* observed effect of OSM is dependent on cooperation of OSM with other bone-forming factors such as, for example, BMP-2.

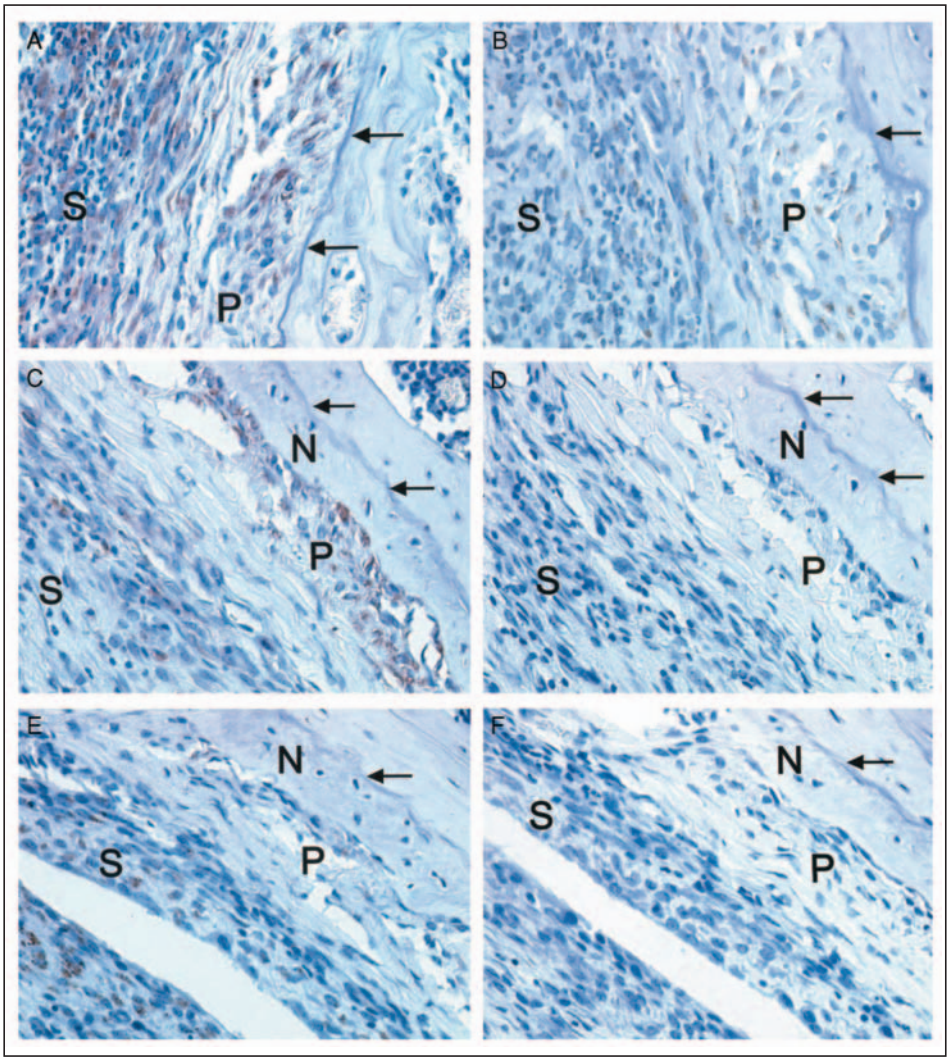

Figure 6 Immunohistochemistry for RANK and RANKL expression in AdmuOSM-injected knee joints. RANK (**A**) and RANKL (**B**) are detected in the periosteum and the inflamed synovium 7 days after injection of AdmuOSM. Continued expression of RANK (**C**) and RANKL (**E**) 14 days after AdmuOSM injection. Control sera for the RANK (**D**) or RANKL (**F**) antisera did not show staining of the periosteum or the inflamed synovium. Data shown are for day 14 after AdmuOSM injection. **Arrows** indicate the surface of the cortical bone of the femur before injection of AdmuOSM. N, Newly formed bone; P, periosteum; and S, inflamed synovium. Original magnifications, x400.

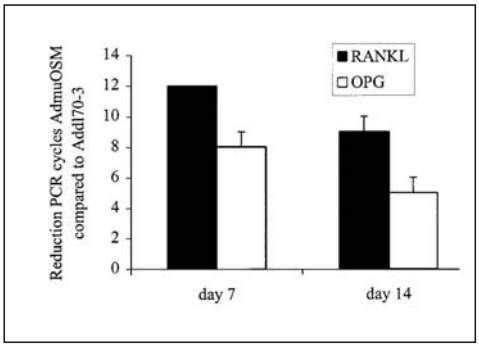

Figure 7 Enhanced synovial mRNA expression for RANKL and OPG at days 7 and 14 of the AdmuOSM-induced inflammation. Gene expressions for RANKL and OPG were compared between synovia from AdmuOSM-injected and contralateral Addl70-3-injected knee joints as described in Materials and Methods. Six mice per group were used and equal amounts of cDNA were used as assessed by PCR for glyceraldehyde-3-phosphate dehydrogenase. RT-PCR reactions were performed at least twice. ■, RANKL; □, OPG.

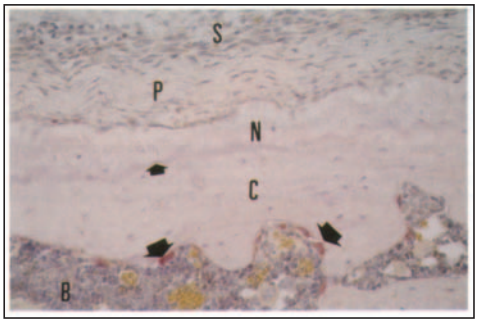

Figure 8 Trap staining of a wild-type knee joint 14 days after injection of AdmuOSM. TRAP-positive cells were observed in the bone marrow but not in the inflammation or on the new bone. A joint from a mouse with collagen-induced arthritis served as a positive control for the staining (data not shown). A **small arrow** indicates the former surface of the bone. The **large arrows** indicate TRAP-positive cells in the bone marrow. B, Bone marrow, C, cortical bone of the femur; N, newly formed bone; P, periosteum; S, inflamed synovium. Original magnification, x200.

Expression of RANKL, RANK, and OPG in the Inflamed Joint

The new deposited bone had an irregular border facing the joint cavity in wild-type mice as well as in the IL-6-deficient mice. Because *in vitro* results have implicated that OSM could induce osteoclast formation,[23] it was possible that OSM not only induced bone formation but also bone resorption in the inflamed joint. A key molecule involved in osteoclastogenesis is RANKL. Because IL-6-type cytokines have been shown to enhance RANKL mRNA levels,[33] we studied expression of RANKL and its receptor RANK in the AdmuOSM-induced inflammation. Both RANKL and RANK were detected immunohistochemically in the inflamed synovium and the periosteal cell layers (day 7; Figure 6, A and B) and expression continued after apposition of the new bone (day 14; Figure 6, C and E). No RANK or RANKL could be demonstrated in Addl70-3-injected knee joints (data not shown). A similar expression pattern for RANKL and RANK was detected in joint sections from AdmuOSMinjected wild-type and IL-6-deficient mice.

The RANK/RANKL system and the development of osteoclasts can be controlled by the RANKL antagonist OPG. Semiquantitative RT-PCR on synovial mRNA showed that besides expression for RANKL, OPG expression also was up-regulated in the AdmuOSM-induced inflammation (Figure 7). Induction of OPG occurred in both wild-

type and IL-6-deficient mice. Gene induction for OPG could provide a counterbalance against the observed RANKL expression. TRAP staining of AdmuOSM-injected knee joints did not identify osteoclasts in the inflamed synovium or at sites of new bone formation (Figure 8). TRAP-positive multinucleated cells were also not detected on the layer of osteoblast-like cells earlier during inflammation on day 7 (data not shown). These results indicate that the OSM-induced inflammation does not create an environment favoring osteoclast development. Our results show that OSM can induce bone formation *in vivo*, an effect found to be independent of IL-6.

Discussion

OSM is a member of the IL-6 family of cytokines. Experiments with recombinant OSM have led to conflicting results as both pro-and anti-inflammatory properties have been published.[5,14] Most reports, however, describe OSM to be pro-inflammatory. These conflicting results could be related to the different models used. Human and murine IL-6 use the same receptor complex on murine cells. Human and murine OSM, in contrast, make use of different receptor complexes on murine cells.[13] This finding could be another complicating factor and murine OSM should therefore be used to establish its role in experimental inflammatory models in mice.

OSM can be detected in the synovial fluid but not in the serum of RA patients.[7] This suggests a local role for OSM in the inflammation and joint damage during RA. Recently, it was shown that murine OSM stimulated anchorage-independent growth of murine synovial fibroblasts *in vitro* and that AdmuOSM induced joint inflammation in the knee joints of mice.[15] This was not observed with a control vector. Our *in vivo* results confirm these data and support a local pro-inflammatory role for OSM during RA.

Adenoviral vectors have been used to express proteins of interest in the knee joint.[34] Advantages of these vectors are that they can infect both dividing and nondividing cells and can express a protein for days in the knee joint. Adenoviral vectors, however, can by themselves also cause inflammation. In our hands no inflammation is induced in naive knee joints injected with 1.10^7-pfu empty control virus or virus-expressing luciferase or β-galactosidase marker genes. In the present study we have used five times less virus and we did not observe inflammation in the contralateral knee joint injected with control virus.

The fact that no sensitive test is available specific for murine OSM makes is difficult to address the production by the AdmuOSM vector. We have tried to set up a bio-assay by incubating the murine B9 cell line with wash-outs of AdmuOSM injected IL-6 defi-

cient knee joints. This IL-6 dependent cell line can also respond to OSM, although at higher concentration.[37] The detection limit for recombinant murine OSM was 2 ng/ml. This is relatively high compared to the several 100 pg/ml that we generally observe with 1.10^7 PFU of vectors expressing other transgenes. The AdmuOSM wash-outs did not show a production above the detection limit. The production of OSM is therefore less than 2 ng/ml but a precise measurement awaits the development of a more sensitive specific assay.

In our study we have found a high induction of gene expression for IL-6 after injection of AdmuOSM in naive wt mice. IL-6 plays a very important role during experimental arthritis.[16-18] IL-6 gene expression was, however, not necessary for development of the AdmuOSM induced inflammation. This was demonstrated by a similar degree of inflammation when we injected the AdmuOSM vector in the joints of wt and IL-6 deficient mice. Injection of an adenoviral vector expressing murine IL-6 did not lead to inflammation even in a tenfold higher concentration.[15] This observation was confirmed by results in our laboratory (unpublished data). Together these data suggest that locally produced OSM plays by itself a pro-inflammatory role during joint inflammation. Secondly, it suggests that members of the IL-6 family play different roles during joint inflammation and that they cannot simply be substituted for by other family members.

Under nonpathological circumstances there is a continuous synthesis of new bone by osteoblasts and breakdown of bone by osteoclasts. This balance can be disrupted as is shown by the occurrence of bone erosion during RA. Osteoclasts have been identified as the main bone resorbing cells in RA.[38] The members of the IL-6 family can influence differentiation and activation of both osteoblasts and osteoclasts and hence influence bone homeostasis.[22] Unraveling the precise involvement of these cytokines in bone remodeling and homeostasis could not only be important for RA but also for other osteolytic diseases such as Paget's disease and giant cell tumors. IL-6 is generally described to stimulate bone resorption. OSM can influence both osteoblasts and osteoclasts and its role is less clear. The results observed after injecting the AdmuOSM vector in the knee joints of naive mice showed apposition of new bone in the periosteum of the inflamed joint. This phenomenon was observed in all knee joints injected with AdmuOSM in four different experiments. We studied the role that both osteoblasts and osteoclasts could play in this phenomenon.

Expression of OSM by the AdmuOSM vector not only caused inflammation but also activated the periosteal cells along the femur and tibia. These cells were positive for ALP activity, a marker for osteoblasts and osteoblast-like cells. In all wild-type and IL-6-deficient mice tested bone apposition occurred at these sites with a thickened layer of

periosteal cells. The formation of this ALP-positive cell layer pointed at an important influence of OSM on osteoblastic cells *in vivo*. Most experiments on the relation between OSM and osteoblast function have been performed *in vitro* with human OSM. Recombinant human OSM has been found to activate murine osteoblasts and to inhibit basal bone resorption *in vitro*.[39] Also, human OSM did prevent apoptosis in both murine and human osteoblastic cell lines.[40] As described above the use of human or murine OSM on murine cells might lead to different results. To obtain additional information on the role of OSM in osteoblast differentiation and activation we have performed *in vitro* experiments with recombinant murine OSM on the murine C2C12 cell line. These cells can differentiate toward the osteoblast lineage and express osteoblastic markers such as ALP, osteocalcin, and Cbfa1.[32] They also showed mRNA expression of the specific OSM receptor-β (data not shown).

When ALP activity was measured in the C2C12 cells, we found that murine OSM by itself did not differentiate these cells toward the osteoblast lineage. Similarly, it was found that human OSM did not differentiate murine embryonic fibroblasts toward the osteoblast lineage.[41] The ALP activity of C2C12 cells in response to BMP-2, however, was clearly enhanced by addition of OSM and not by IL-6. This suggests that the *in vivo*-observed ALP activity might be an effect of OSM co-operating with a bone-forming factor. We previously reported that an intraarticular injection of recombinant BMP-2 protein induced chondrogenesis in the murine knee joint.[42] Uusitalo and colleagues[43] found that adenoviral transfection of the periosteum with BMP-2 also caused cartilage callus tissue formation and that endochondral ossification replaced most of this callus cartilage by bone. Previous studies using crude extracts of BMPs showed direct periosteal bone induction that was not preceded by chondrogenesis.[44] Further research is needed to determine whether OSM has stimulatory or (re)directing effects on BMP-2 or other bone-forming factors *in vivo*.

The observed bone formation could also be a direct effect of OSM on mature osteoblast-like cells *in vivo*. OSM can activate the signal transducer and activator of transcription (STAT)-3 in osteoblasts.[45] Activation of STAT-3 by OSM can induce expression of c-Fos as shown in the hepatoma cell line HepG2.[46] Fos proteins form together with the Jun proteins, the activator protein-1 (AP-1) transcription complex. Recently, it was shown that overexpression of the fos proteins FRA-1[47] or ΔFosB[48] led to a progressive increase in overall bone mass in an as yet unknown way. Further research on the signal transduction and gene expression of osteoblasts under influence of OSM is therefore needed to determine whether the observed new bone formation is regulated by AP-1. Another factor that could play a role in the AdmuOSM-induced bone formation is

Cbfa1, the first osteoblast-specific transcription factor. It controls the rate of bone formation by differentiated osteoblasts.[49] A relation between Cbfa1 and OSM, however, has not yet been investigated.

Bone formation is besides locally also systemically controlled.[50] An effect of OSM on the endocrine system during the AdmuOSM-induced inflammation seems, however, unlikely because no activation of osteoblasts or bone formation was observed in the contralateral knee joints injected with Addl70-3.

Consistent with our findings using the adenoviral vector, excessive bone growth was observed in the femur of a transgenic mouse expressing bovine OSM.[51] However, no overt inflammation was detected surrounding this new bone (Dr. C. Clegg, personal communication). This suggests that the effect of OSM on bone formation does not depend directly on the observed pro-inflammatory properties of OSM.

The other cell type involved in bone remodeling and homeostasis, the osteoclast, can also be influenced by OSM. Recombinant murine OSM increased *in vitro* the formation of osteoclasts in co-cultures of murine bone marrow and calvaria cells.[23] TRAP-positive cells were, however, not detected in the inflamed synovium of AdmuOSM-injected knee joints. Only very rarely were they detected on the newly formed bone. Langdon and colleagues[15] showed with this same virus that in some joints the inflammation protruded through the bone marrow. We also observed this occasionally in our experiments, but not at sites of bone apposition. The existence of channels between the bone marrow and the synovium has been described.[52] It is possible that cell trafficking between the inflamed synovium and the bone marrow occurs through these channels. This could be accompanied by widening of these channels, although a mechanism behind this is still unknown. TRAP staining of AdmuOSM-injected knee joints did not detect osteoclasts in these channels. This makes it unlikely that in our experimental conditions osteoclasts are involved in widening of the channels. Aggressive fibroblasts could be involved in this process. Synovial fibroblasts with a transformed phenotype are present in synovial pannus tissue and their presence has been related to joint damage.[53-55] This would be in line with the observed positive effect of murine OSM on the anchorage-independent growth of mouse synovial fibroblasts.[15]

OSM has been shown to induce expression of RANKL in stromal/osteoblastic cells.[33] This RANKL can interact with the receptor RANK on the precursors of osteoclasts. The RANK/RANKL system and the interaction between osteoblasts and osteoclast precursor cells are crucial for the development of osteoclasts.[56] Semiquantitative RT-PCR analysis showed increased expression of RANKL in the inflamed synovium. Immunohistochemistry showed expression of RANKL and RANK proteins in the syn-

ovium and in the activated osteoblast-like cell layers. At present, we do not have an assay to demonstrate presence of the soluble RANKL inhibitor OPG on histological sections but increased mRNA expression for OPG was found in the synovium. The ratio between RANKL and OPG expression has been implicated as a determining factor in bone resorption during RA.[57] In our experiments expression of RANKL and RANK did not induce osteoclast development *in vivo*. It could be that the balance between RANKL and OPG favors bone formation during the AdmuOSM-induced inflammation but this needs further experimental research. Another option is expression of an inhibitor or repression of an osteoclast activator that as yet has not been related to OSM.

We have observed formation of new bone from the periosteum in both the antigen-and the zymosan-induced arthritis in mice.[58,59] Because of the irregular shape of the newly formed periosteal bone it will not always have been recognized as bone apposition. The results of the Goldner's trichrome staining and the absence of TRAP-positive cells in the periosteum clearly demonstrates that in our model with the AdmuOSM vector bone apposition and not erosion takes place. OSM could also play a role in bone apposition in experimental arthritis models but further research on this is needed. Expression of the pro-inflammatory cytokines IL-1 or IL-17 did induce inflammation and bone erosion but no bone apposition in naive knee joints (manuscript in preparation). This indicates that inflammation is not always accompanied by bone apposition. The cytokine environment could influence the effect of a given cytokine on bone remodeling. The effects of OSM on the periosteum, but not its pro-inflammatory properties, could be negatively influenced by other cytokines in RA. During RA, bone erosion instead of apposition occurs. In other human arthropathies the cytokine environment could have a different effect and bone apposition and erosion can even occur at different parts of the joint. Periosteal bone apposition for example takes place in diseases such as Reiter disease,[60] juvenile chronic arthritis,[61] erosive os-teoarthritis,[62] and hypertrophic osteoarthropathy.[63] OSM could have a role in these diseases, as suggested by our results with the OSM adenoviral vector. Future therapies for these diseases, as well as RA, might therefore be targeted at this cytokine.

Acknowledgments

We thank Natasja Lieuwes and Dinie Versleyen for Von Kossa and Goldner stainings, Birgitte Oppers for the RANKL and RANK immunohistochemistry, Dr. C. Clegg (Seattle, WA) for unpublished data, and Dr. M. Kopf (Basel, Switzerland) for the IL-6-deficient mice.

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CHAPTER 6

Growth Plate Damage, a Feature of Juvenile Idiopathic Arthritis, Can Be Induced by Adenoviral Gene Transfer of Oncostatin M: A Comparative Study in Gene-Deficient Mice

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Arthritis and Rheumatism 2003, 48:1750-1761

Abstract

Objective. To investigate the involvement of proinflammatory and destructive mediators in oncostatin M (OSM)-induced joint pathology, using gene-deficient mice.

Methods. An adenoviral vector expressing murine OSM was injected into the joints of naive wild-type mice and mice deficient for interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNFα), or inducible nitric oxide synthase (iNOS). Reverse transcription-polymerase chain reaction was used to study gene expression. Inflammation and cartilage proteoglycan (PG) depletion were assessed by histology. OSM and IL-1 levels in synovial fluid from patients with juvenile idiopathic arthritis (JIA) were measured by enzyme-linked immunosorbent assay.

Results. Adenoviral expression of murine OSM led to joint inflammation, bone apposition, chondrophyte formation, articular cartilage PG depletion, and VDIPEN neoepitope expression in wild-type mice. A unique and consistent observation was the focal PG depletion and disorganization of the growth plate cartilage during the first week of inflammation. Synovial IL-1β, IL-6, TNFα, and iNOS gene expression was strongly induced. Of these factors, only deficiency in IL-1 markedly reduced inflammation and PG depletion and completely prevented growth plate damage. In addition, this is the first study in which OSM was detected in JIA synovial fluid. Most samples were also IL-1β positive.

Conclusion. IL-1, but not IL-6, TNFα, or iNOS, plays an important role in joint disease induced by intraarticular gene transfer of OSM in mice. The effect of OSM on murine connective tissue and the presence of OSM in human synovial fluid make involvement of OSM in human arthropathies very likely.

Introduction

Oncostatin M (OSM) is a multifunctional cytokine that belongs to the interleukin-6 (IL-6) family (1). Elevated levels of OSM can be detected in the synovial fluid, but not in the serum, of patients with rheumatoid arthritis (RA) (2). Immunohistochemical analysis of RA synovial tissue showed that synovial macrophages are the source of OSM in the inflamed joint (3). A pathologic role for OSM in RA is suspected, because OSM by itself can induce joint inflammation in animals. Injecting recombinant human OSM into the joints of goats induced the influx of polymorphonuclear cells (PMNs), followed by cells of the macrophage/monocyte lineage (4). Joint inflammation was also induced by adenoviral expression of murine OSM in mice (5,6). The enhanced expression of adhesion molecules such as E-selectin and P-selectin (7,8), CXC chemokines (8), and the CC chemokine monocyte chemotactic protein 1 (9) by OSM could contribute to the influx of inflammatory cells. Furthermore, synovial fibroblasts displayed a transformed phenotype under the influence of OSM (5), suggesting involvement of OSM in pannus formation.

Besides chronic joint inflammation, RA is also characterized by destruction of articular cartilage and bone. Cartilage consists of a framework of collagen fibers in which proteoglycans (PGs) are entrapped. These PGs can retain water, which enables the cartilage to resist compressive forces. Proinflammatory cytokines such as IL-1 (10,11) are involved in cartilage degradation. Results from experiments in recent years suggest a similarly important role for OSM in the cartilage degradation of RA. OSM was shown to induce collagen release from bovine cartilage in vitro (12). It also stimulated PG release and suppressed PG synthesis in porcine articular cartilage explants (13). Injecting OSM into the joints of goats (4) decreased the cartilage PG content. In humans, OSM concentrations in synovial fluid correlate positively with levels of cartilage degradation markers (14). OSM was also the first cytokine that, in combination with IL-1α, was demonstrated to induce collagen release from human cartilage (3).

The development of joint inflammation and cartilage damage in experimental arthritis can be greatly influenced by the expression of proinflammatory cytokines and other mediators. We previously demonstrated that blocking of IL-1 could prevent inhibition of PG synthesis in experimental arthritis (15,16). The formation of nitric oxide (NO) was shown to be involved in IL-1-induced inhibition of PG synthesis in vitro (17), and PG loss was reduced in experimental arthritis in mice deficient for the inducible NO synthase (iNOS) gene (18). Studies entailing blocking of tumor necrosis factor α (TNFα) showed involvement of TNFα in the early phase of joint inflammation (19,20), while studies of experimental arthritis in IL-6-deficient mice showed involvement of IL-6 in the chronicity of

arthritis (21). In the present study, we investigated the involvement of these proinflammatory mediators in OSM-induced joint disease. We injected an adenoviral vector expressing murine OSM into the joints of mice deficient for IL-1, IL-6, TNFα, or iNOS and studied the effects of these gene deletions on OSM-induced joint pathology.

Ubiquitous transgenic overexpression of bovine OSM has been found to be lethal for newborn mice. One mouse survived and developed growth plate disorganization, with enhanced growth of the hind legs (22). Growth plate damage (23,24) as well as localized growth abnormalities (25) are characteristic features of juvenile idiopathic arthritis (JIA). Therefore, we studied the effects of murine OSM gene transfer not only on development of inflammation and articular cartilage damage, but also on the growth plate. Furthermore, we studied expression of OSM in the synovial fluid of patients with JIA.

Patients and methods

Animals

For this study, male mice deficient for the following genes were used: IL-1α and IL-1β (26), IL-6 (27), TNFα (28), and iNOS (29). C57BL/6 and C57BL/6 x 129Sv mice were used as wild-type controls. Breeding colonies were kept at the Central Animal Facilities of the University of Nijmegen. Animals used in the experiments were between 11 and 13 weeks of age. All mice were housed in filter-top cages under specific pathogen-free conditions. A standard diet and water were provided ad libitum. After injection of the adenoviral vector, the mice were housed in isolators. Experiments were performed according to national and institutional regulations for animal use.

Adenoviral vectors and intraarticular injection

The construction of adenoviral vectors expressing murine OSM (AdMuOSM) or murine IL-17 (AdmIL-17) has been described previously (30,31). AdDL70-3, a vector without insert, was used as a control vector. For *in vivo* experiments, the virus was diluted in physiologic saline, and 2 x 10^6 plaque-forming units (PFU) in a total volume of 6 μl were injected into the knee joint cavity. Construction of NIH3T3 cells overexpressing human IL-1β will be described elsewhere (Joosten L: unpublished observations). A total of 2.5 x 10^4 cells were injected into the knee joint.

Histologic evaluation of knee joints

Knee joints were dissected, fixed in formalin, decalcified, dehydrated, and embedded in paraffin. Standard 7-μm frontal sections were prepared. Sections were stained with Safranin O and counterstained with fast green for assessing cartilage damage. Histopathologic findings were scored on 5 semi-serial sections of the joint. Scoring was performed in a blinded manner by 2 independent observers. Cartilage depletion was scored from 0 (normal Safranin O staining; no depletion) to 3 (complete loss of Safranin O staining; complete depletion). Joint inflammation was also scored on a 0-3-point scale.

NIMP-R14 staining

The influx of PMNs was assessed by staining knee joint sections for the presence of the NIMP-R14 epitope (32), which is present mainly on neutrophils. Sections were deparaffinized, treated with 0.1% trypsin in 0.1% $CaCl_2$, pH 7.8 and preincubated for 15 minutes with 20% normal rabbit serum before incubation for 1 hour with anti- NIMP-R14 antibodies (a kind gift from Dr. M. Strath, London, UK). After incubation with a peroxidase-labeled rabbit anti-rat secondary antibody in 5% normal mouse serum/phosphate buffered saline (PBS) for 30 minutes, the sections were incubated with diaminobenzidine (1 mg/ml in 50 m*M* Tris HCl, pH 7.6, 0.001% H_2O_2) for 10 minutes. Sections were counterstained with hematoxylin for 30 seconds. Normal rat immunoglobulin was used as a negative control.

Image analysis of newly formed bone

The area of newly formed bone was measured using the QWin image analysis system (Leica, Cambridge, UK). Images of Safranin O-stained sections were captured using a JVC 3-CCD color video camera and displayed on a computer monitor. For each joint, 4 measurements of the length of the original cortical bone (marked by a precipitation line in the staining) and the area of newly formed bone on the femur were performed in a standardized manner. The amount of newly formed bone is expressed as μm^2 of new bone/10 μm of cortical bone.

Isolation of synovial RNA and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Synovial messenger RNA (mRNA) was isolated and quantitated as described previously (33). The patellae (with surrounding synovium) were isolated from the knee joints, and 2 pieces of tissue adjacent to the patella were punched out with a 3-mm biopsy punch (Stiefel, Wächters Bach, 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 manufacturer's protocol. All reagents for RNA isolation and RT-PCR were obtained from Life Technologies (Breda, The Netherlands). Isolated RNA was treated with DNase I before being reverse transcribed into complementary DNA (cDNA) with Moloney murine leukemia virus reverse transcriptase.

After increasing numbers of PCR cycles, samples were obtained and run on an agarose gel. The cycle number at which the PCR product was first detected on the gel was obtained as a measure for the amount of specific mRNA originally present in the isolated synovial RNA. PCR for GAPDH was performed to verify that equal amounts of cDNA were used. Primers for IL-1β, TNFα, GAPDH (34), and IL-6 (6) were used as described previously. The iNOS primers used (at 55°C, 1m*M* $MgCl_2$) were as follows: forward CCC-TAA-GAG-TCA-CCA-AAA-TGG, reverse CTA-CAG-TTC-CGA-GCG-TCA-AA. The OSM primers used (at 55°C, 1 m*M* $MgCl_2$) were as follows: forward CTT-GGA-GCC-CTA-TAT-CCG-CC, re-verse GTG-TGG-AGC-CAT-CGT-CCC-ATT-C. Primers were designed using Oligo 4.0 and Primer software (Molecular Biology Insights, Cascade, CO).

Ex vivo PG synthesis

PG synthesis was assessed by ^{35}S-sulfate incorporation in patellar cartilage. Patellae from knee joints injected with adenoviral vectors and from the uninjected contralateral knee joints were dissected, with a minimum amount of surrounding synovium, under sterile conditions. The ex vivo synthesis assays were performed with RPMI supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mmole/liter pyruvate, and 5% fetal calf serum (Life Technologies). The patellae were placed separately in 200 μl of medium containing 4 μCi ^{35}S-sulfate and incubated for 3 hours at 37°C in a humidified atmosphere of 5% CO_2. After labeling, the patellae were washed with physiologic saline and fixed overnight in 4% formalin. Fixed patellae were decalcified in 5% formic acid for 4 hours, dissected, and dissolved in 0.25 ml Lumasolve (Omnilabo, Breda, The

Netherlands). After addition of 1 ml of Lipoluma (Omnilabo), the ^{35}S-sulfate content of each patella was measured by liquid scintillation counting in a TriLux 1450 MicroBeta (Perkin-Elmer Wallac, Turku, Finland). Data for joints injected with adenoviral vectors are presented as a percentage of the normal chondrocyte PG synthesis in the contralateral, uninjected knee joint.

VDIPEN neoepitope staining

Irreversible PG damage was assessed by immunohistochemistry for the VDIPEN neoepitope. Joint sections were deparaffinized, rehydrated, and digested for 1 hour at 37°C in 0.25 units/ml of chondroitinase ABC in 0.1*M* Tris HCl, pH 8.0 (Sigma, Zwijndrecht, The Netherlands) to remove chondroitin sulfate from the PGs. Sections were treated with 1% H_2O_2 in methanol for 20 minutes, followed by treatment with 0.1% Triton X-100 in PBS for 5 minutes. After incubation with 1.5% normal goat serum for 20 minutes, sections were incubated overnight at 4°C with affinity-purified rabbit anti-VDIPEN IgG (a kind gift from Dr. I. Singer, Rahway, NJ) or with normal rabbit IgG. The next day, the sections were incubated with biotinylated goat anti-rabbit IgG followed by labeling with avidin-peroxidase (Elite kit; Vector, Burlingame, CA). Peroxidase development was performed using nickel enhancement to increase sensitivity. Sections were counterstained with 2% orange G for 5 minutes.

Synovial fluid from patients with JIA

Synovial fluid was collected from children with JIA who attended the University Medical Center Nijmegen or the St. Maartens Clinic for intraarticular glucocorticosteroid injection. The samples were collected in accordance with local ethics legislation and were made available anonymously to the authors after informed consent was received from the parents. Diagnosis according to the revised International League of Associations for Rheumatology criteria (35) is described in Table 1. The synovial fluid was centrifuged for 10 minutes at 3,000 revolutions per minute, aliquoted, and stored at -70°C.

Table 1 OSM and IL-1β in synovial fluid of patients with JIA*

Patient	Age, years/sex	JIA subtype	OSM, pg/ml	IL-1 β, pg/ml
1	6/F	Oligoarthritis	18.4 ± 4.1	5.2 ± 0.8
2	10.9/M	Oligoarthritis	ND	ND
3	10.2/F	RF – polyarthritis	12.7 ± 0.5	5.1 ± 1.0
4	7.6/F	Oligoarthritis	17.5 ± 1.4	ND
5(left knee)	9.1/F	Oligoarthritis	10.1 ± 0.5	5.4 ± 0.8
5(right knee)	9.1/F	Oligoarthritis	13.7 ± 0.4	ND
6	2.6/F	Oligoarthritis	ND	NM
7	15.4/F	RF – polyarthritis	ND	11.3 ± 2.7
8	13/F	RF – polyarthritis	9.6 ± 1.6	2.2 ± 0.5
9	5.7/F	Systemic	23.1 ± 0.2	4.2 ± 1.1
10	6.2/F	Oligoarthritis	27.4 ± 0.9	56.7 ± 5.2
11	11.3/F	Oligoarthritis	4.1 ± 0.5	ND
12	4.4/F	Oligoarthritis	16.3 ± 2.5	11.7 ± 2.7

* All samples were obtained from knee joints, except sample 10, which was obtained from an ankle joint. In patients 2, 4, and 6, the arthritic leg was longer than the unaffected leg. Bony overgrowth of the knee occurred in patients 1, 6, and 10. Oncostatin M (OSM) and interleukin-1β (IL-1β) were determined by enzyme-linked immunosorbent assay, performed twice, in duplicate. Values are the mean ± SD. JIA = juvenile idiopathic arthritis; ND = not detectable; RF = rheumatoid factor; NM = not measured.

Cytokine measurement in synovial fluid

The amount of OSM and IL-1β in synovial fluid was determined by enzyme-linked immunosorbent assay (ELISA), using the Quantikine human OSM immunoassay or the Quantikine human IL-1β immunoassay (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol.

Statistical analysis

The rank sum test was used for statistical comparison between groups. *P* values less than 0.05 were considered significant.

Results

Joint pathology after OSM gene transfer

In wild-type mice, intraarticular injection of AdMuOSM induced joint inflammation (Figures 1A and B) that was characterized by the influx of PMNs (Figure 1C) and mononuclear cells as well as by synovial hyperplasia. The AdMuOSM-induced inflammation led to cartilage PG loss in the patella and femur, as demonstrated by loss of red staining in the Safranin O-stained knee joint sections (Figures 1A and B). Both inflammation and cartilage PG loss lasted for at least 4 weeks. The periosteum became activat-

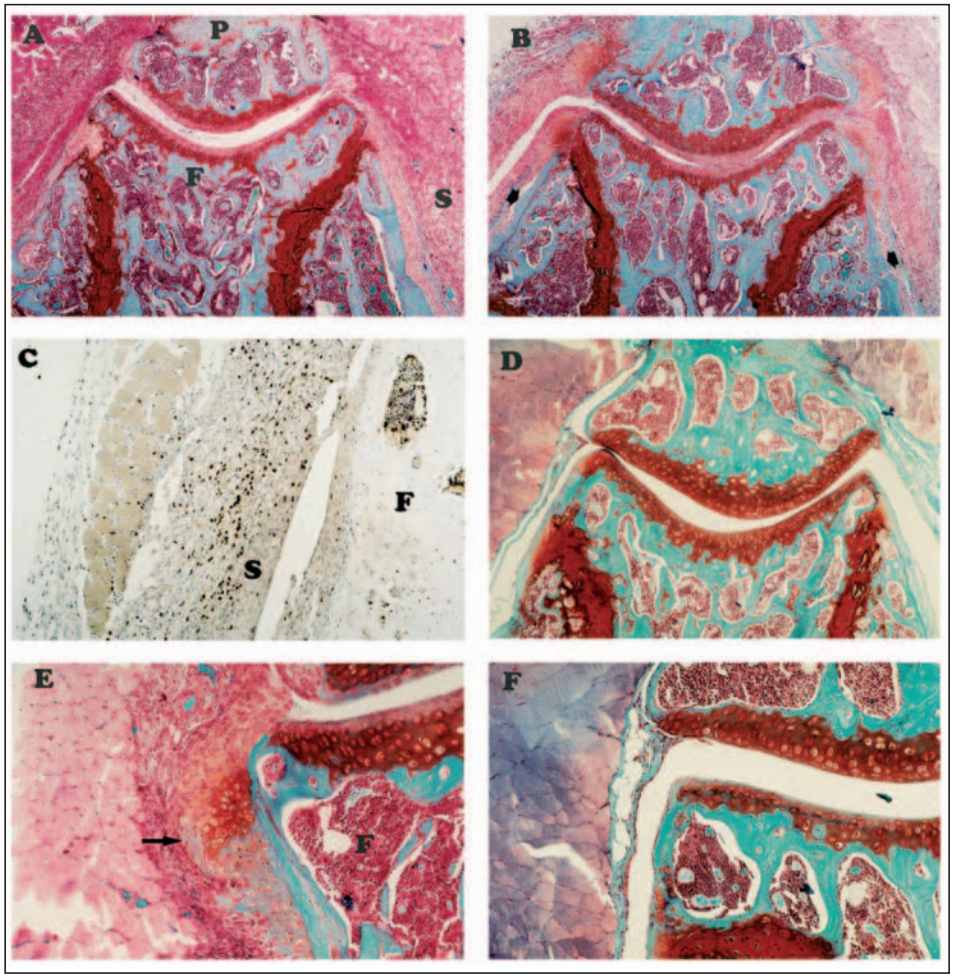

Figure 1 Joint pathology induced by the adenoviral murine oncostatin M vector (AdMuOSM) in wild-type mice. Inflammation and cartilage proteoglycan (PG) depletion on **A,** day 7 and **B,** day 14 after injection of AdMuOSM into the knee joint of wild-type mice. PG depletion is shown by reduced red staining of the upper cartilage layer of the patella and femur. In **B, arrows** indicate periosteal bone apposition. **C,** NIMPR-14 staining of polymorphonuclear cells in the inflamed synovium on day 7 after AdMuOSM injection. **D,** Normal joint histology on day 14 after injection of the control vector AdDL70-3. Similar histology was observed on day 7 after injection (results not shown). **E,** Chondrophyte formation (**arrow**) at the femoral head on day 14 after AdMuOSM injection. **F,** No chondrophyte formation is observed at the femoral head on day 14 after AdDL70-3 injection. **F** = femur; **P** = patella; **S** = inflamed synovium. (Safranin O stained [except in **D**]; original magnification x 50 in **A, B**, and **D;** x 200 in **C, E**, and **F.**)

ed (Figure 1A), and apposition of new bone occurred at this site (Figure 1B). No additional apposition or remodeling of the new bone occurred after day 14. Chondrophytes, abnormal cartilaginous masses that can develop on the articular surface of bone, were formed on the patella and femur (Figure 1E) and increased in size until at least week 4. In contrast, injection of 2 x 10^6 PFU of the control vector AdDL70-3 did not induce joint inflammation, PG loss, bone apposition, or chondrophyte formation (Figures 1D and F).

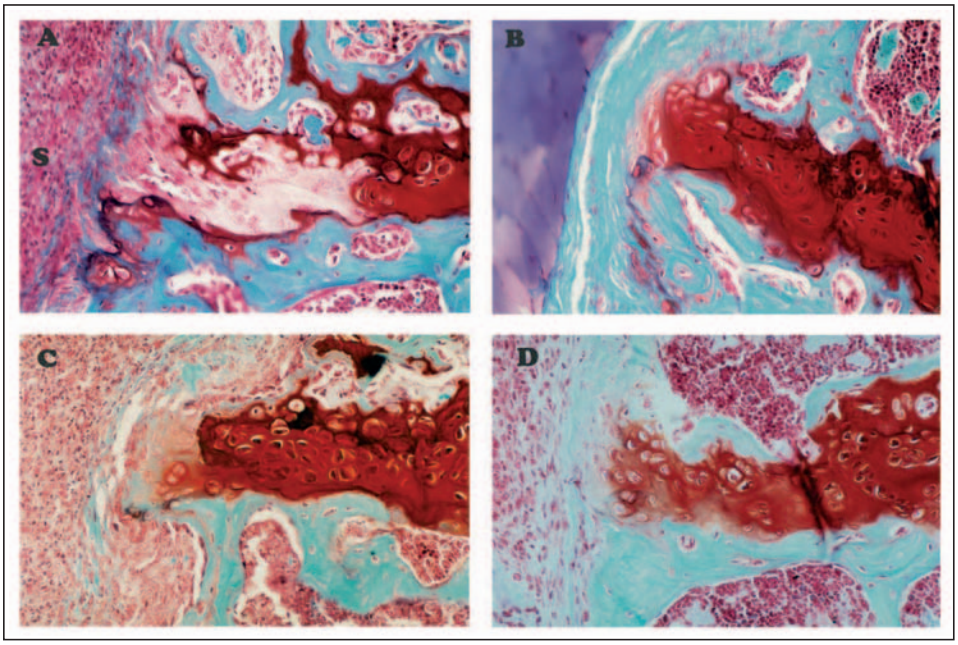

Figure 2 Growth plate damage induced by the adenoviral murine oncostatin M vector (AdMuOSM) in wild-type mice. **A,** Proteoglycan depletion and disorganization of the growth plate on day 7 after injection of AdMuOSM. **B,** Normal growth plate on day 7 after injection of AdDL70-3. Note also the absence of inflammation after AdDL70-3 injection. **C,** Normal growth plate after injection of 2.5 x 10^4 NIH3T3 cells expressing human interleukin-1β. **D,** Normal growth plate after injection of the adenoviral murine interleukin-17 vector, S = inflamed synovium. (Safranin O stained; original magnification x 200.)

Growth plate damage after OSM gene transfer

A unique feature of OSM gene transfer that has not been previously reported is that the AdMuOSM vector also caused damage to the growth plate cartilage. In all wild-type mice studied, we observed PG depletion and loss of matrix integrity in the growth plates (Figure 2A) adjacent to the periosteum. The PG content in the growth plates recovered after the first week of inflammation, but the matrix integrity and the normal arrange-

ment of chondrocytes were not restored. This growth plate damage was not observed after injection of the control vector AdDL70-3 (Figure 2B). It also was not observed after local gene transfer of IL-1 or IL-17 (Figures 2C and D), although both induced joint inflammation and articular cartilage PG depletion (Joosten LAB, Koenders MI: unpublished observations). These observations exclude the possibility that the damage occurred as a general consequence of local proinflammatory cytokine overexpression.

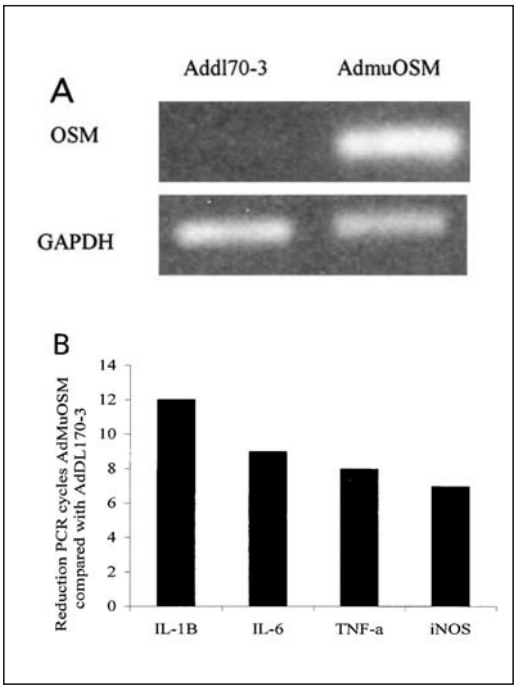

Figure 3 Oncostatin M (OSM) and cytokine gene expression during adenoviral murine OSM (AdMuOSM)-induced inflammation. **A,** OSM gene expression in synovium 3 days after injection of AdMu-OSM, as detected by semiquantitative reverse transcription- polymerase chain reaction (RT-PCR). Results represent the linear part of the PCR reaction (26 PCR cycles for OSM and 22 cycles for GAPDH). **B**, Enhanced gene expression for interleukin-1β (IL-1β), IL-6, tumor necrosis factor α (TNFα), and inducible nitric oxide synthase (iNOS) on day 3 after injection of the adenoviral vector. Gene expression was compared between synovia from AdMuOSM-injected and contralateral AdDL70-3-injected knee joints in 4 mice, as described in Patients and Methods. The control vector in the contralateral knee joint served as a within-animal control. Gene expression was examined in 2 groups of 2 pooled synovia. Equal amounts of complementary DNA were used, as assessed by PCR for GAPDH. Semiquantitative RT-PCR was performed at least twice.

Cytokine dependence of OSM-induced joint pathology

RT-PCR revealed synovial expression of OSM gene in the knee joint injected with AdMuOSM, but not in that injected with AdDL70-3 (Figure 3A). On day 3, the first OSM PCR product was detected 2 cycles after detection of the first GAPDH PCR product. On day 7, it was detected a mean (± SD) of 5 ± 1 cycles after detection of the first GAPDH PCR product, indicating a decrease in OSM gene expression. OSM can induce expression of IL-6 (36) and can enhance the effects of other proinflammatory cytokines such as IL-1 and TNFα (3). Semiquantitative RT-PCR analysis showed upregulated expression of mRNA for IL-1β, IL-6, and TNFα associated with the AdMuOSM-induced

inflammation (Figure 3B). Because these cytokines can have great influence on joint inflammation, chondrocyte metabolism, and cartilage damage, we determined their role in the AdMuOSM-induced joint disease by injecting 2 x 10^6 PFU AdMuOSM into the knee joints of mice deficient for IL-1, IL-6, or TNFα. Mice deficient for the iNOS gene also received the injection. This factor (iNOS) plays a role in the suppression of cartilage PG synthesis and was also up-regulated at the mRNA level (Figure 3B).

Table 2 AdMuOSM-induced joint pathology in wild-type and cytokine-deficient mice*

	Inflammation†		PG depletion†		Relative PG synthesis, %,	Chondrophyte incidence,	Bone apposition, μm^2 of new bone/ 10 μm of cortical bone,	Growth plate damage incidence,
Mice	Day7	Day14	Day7	Day14	day 4‡	day14§	day 14¶	day 7§
Wild-type	1.7 ± 0.6	0.9 ± 0.4	1.8 ± 0.6	1.9 ± 0.6	142 ± 34.8	88	338 ± 47	100
IL-1 $\alpha/\beta^{-/-}$	1.0 ± 0.2¶	0.7 ± 0.4	0.5 ± 0.3#	2.0 ± 0.7	167 ± 10.3	100	317 ± 41	0
TNF $\alpha^{-/-}$	1.0 ± 0.5	1.0 ± 0.6	1.4 ± 0.5	1.5 ± 0.4	NM	100	374 ± 74¶	100
IL-6 $^{-/-}$	1.5 ± 0.5	1.0 ± 0.6	2.3 ± 0.5	2.4 ± 0.4	NM	100	312 ± 76	100
iNOS $^{-/-}$	1.8 ± 0.7	1.1 ± 0.4	1.6 ± 1.1	2.2 ± 1.2	NM	80	355 ± 62	100

* Data shown are for 1 representative experiment with 5-9 mice per group. Except where indicated otherwise, values are the mean ± SD. IL-1α/β = interleukin-1α/β; TNFα = tumor necrosis factor α; iNOS = inducible nitric oxide synthase.
† Scored on a 0-3-point scale. Data for proteoglycan (PG) depletion are for patellar cartilage; similar results (not shown) were obtained for femoral cartilage.
‡ Ex vivo patellar PG synthesis was compared with synthesis in the patella of the uninjected contralateral knee joint. Values >100% indicate increased PG synthesis in the patella from the adenoviral murine oncostatin M (AdMuOSM)-injected knee joint. Injection of AdDL70-3 induced only a slight increase of PG synthesis (mean ± SD 111 ± 14.9%). Six to 8 patellae per group were used, and PG synthesis was measured twice.
§ Percentage of mice.
¶ $P < 0.05$ versus wild-type mice, by rank sum test.
# $P < 0.005$ versus wild-type mice, by rank sum test.

IL-1 was observed to play an important role during the first week of AdMuOSM-induced joint pathology. Histologic scoring showed a reduced synovial infiltrate on day 7 after injection of AdMuOSM in IL-1α/β-deficient mice (Table 2). The influence of IL-1 on joint inflammation decreased after day 7, and by day 14, inflammation in wild-type and IL-1α/β-deficient mice did not differ (Table 2). Deficiency for TNFα, IL-6, and iNOS did not affect AdMuOSM-induced joint inflammation. Inflammation in the TNFα-deficient mice tended to be reduced on day 7 after injection, but this difference versus wild-type mice did not reach statistical significance ($P = 0.05$).

Cartilage PG depletion was also significantly reduced in the IL-1α/β-deficient mice during the first week of inflammation (Table 2). Ex vivo PG synthesis was increased after injection of AdMuOSM into wildtype mice (Table 2). This suggests that the observed PG depletion in wild-type mice is caused by enhanced PG breakdown. The fact that the ex

vivo PG synthesis increased further in IL-1 α/β-deficient mice suggests that endogenous IL-1 can at least partly counteract OSM-induced stimulation of PG synthesis. Deficiency for TNFα, IL-6, and iNOS did not affect the AdMuOSM-induced PG loss on days 7 and 14 after injection of the vector.

By day 14, PG loss in the IL-1α/β-deficient mice had developed to the same extent as that in the wild-type mice (Table 2), with marked expression of the matrix metalloproteinase (MMP)-generated VDIPEN neoepitope (Figures 4A and C). In contrast, the AdDL70-3 control vector did not lead to generation of the VDIPEN neoepitope (Figure 4B).

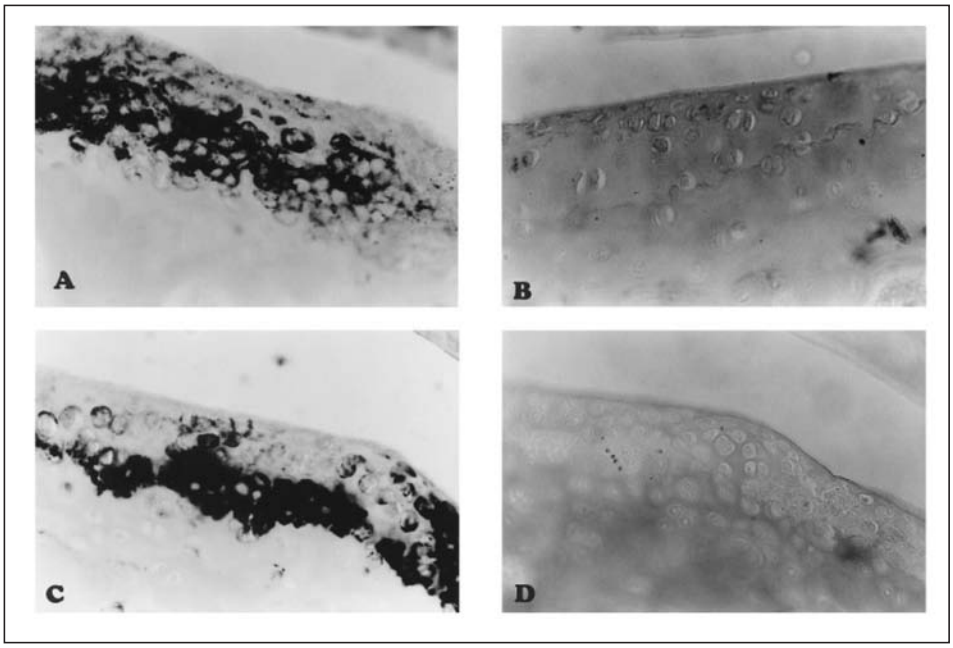

Figure 4 VDIPEN neoepitope staining in cartilage after injection of AdMuOSM. **A,** Positive VDIPEN staining in the cartilage of a wild-type mouse 14 days after AdMuOSM injection. Staining is detected in the matrix surrounding the chondrocytes at the surface and in the deeper cartilage layers. **B,** Negative VDIPEN staining in a wild-type mouse 14 days after injection with AdDL70-3. **C**, Positive VDIPEN staining in an interleukin-1α/β - deficient mouse 14 days after injection of AdMuOSM. **D**, Negative staining in a wild-type mouse 14 days after injection of AdMuOSM, when preimmune serum was used. (Original magnification x 760.)

Cytokine dependence of OSM-induced growth plate damage

The endogenous role of IL-1 was even more prominent in the AdMuOSM-induced growth plate damage. No loss of PGs or disruption of the matrix integrity was found in the growth plates of AdMuOSM-treated IL-1α/β -deficient mice (Table 2). In contrast to

the IL-1α/β -deficient mice, growth plate damage did develop in all mice deficient for TNFα, IL-6, or iNOS (Table 2). Similar to the situation in wild-type mice, the PG content of the affected growth plate was restored on day 14 of inflammation in mice deficient for TNFα, IL-6, and iNOS.

OSM in synovial fluid of patients with JIA

The OSM-induced joint disease in the mice resembled the arthritic changes that are observed in human arthropathies such as RA and JIA. For this reason, we were interested in determining whether OSM could be detected in JIA synovial fluid, as was already demonstrated for RA synovial fluid (2,3). Indeed, we could measure OSM in 77% of the JIA synovial fluid samples that were examined by ELISA (Table 1). Furthermore, 70% of the OSM-positive samples were also positive for IL-1β (Table 1).

Discussion

OSM is produced in the inflamed joints of patients with RA (3), and results of several in vitro experiments suggest that OSM could play an important role in cartilage damage in RA. OSM induced collagen release from bovine cartilage explants (12) and, in combination with IL-1, from human cartilage explants (3). Furthermore, OSM stimulated PG release and suppressed PG synthesis in porcine articular cartilage (13). In the present study, we used an adenoviral vector expressing murine OSM to investigate *in vivo* the influence of proinflammatory mediators, which are important for RA, on OSM-induced joint pathology.

The AdMuOSM vector induced inflammation, cartilage PG depletion, periosteal bone apposition, and chondrophyte formation in the joints of naive mice. Semiquantitative RT-PCR analysis showed increased expression of mRNA for IL-6, TNFα, IL-1β, and iNOS in the AdMuOSM-injected knee joint. A relationship with OSM-induced pathology was investigated in mice deficient for these proinflammatory factors.

OSM is a strong inducer of IL-6 gene expression. We had previously observed that AdMuOSM-induced inflammation was not inhibited by IL-6 deficiency (6). The present results demonstrate that cartilage PG depletion is also not affected in these mice. In contrast to the important role of IL-6 in experimental arthritis (21,37), the present results do not indicate such a role for IL-6 in AdMuOSM-induced joint disease. A positive correla-

tion between TNFα and OSM concentrations has been demonstrated in synovial fluid obtained from patients with RA (14). However, whether there is a direct relationship between these cytokines was, until now, not clear. Results of the present study show that cartilage PG depletion induced by AdMuOSM was not affected by TNFα deficiency. Although inflammation in TNFα-deficient mice tended to be reduced on day 7, by day 14 inflammation in these mice did not differ from that in wild-type mice. This is consistent with reports showing that TNFα is important during disease onset, but that TNFα deficiency or inhibition did not prevent development of severe arthritis in experimental models (38,39). Taken together, our results do not suggest an important role for TNFα in OSM-induced joint pathology.

Nitric oxide is produced in the inflamed joints of patients with RA (40) and can contribute to IL-1- induced inhibition of PG synthesis (16,41). We previously demonstrated that cartilage PG depletion, but not joint inflammation, is significantly reduced in iNOS deficient mice with zymosan-induced arthritis (18). In the present experiments, PG depletion did not differ between iNOS-deficient and wild-type mice, indicating that NO formation is not essential for AdMuOSM-induced cartilage damage.

Joint inflammation and cartilage PG depletion in IL-1α/β-deficient mice were significantly reduced on day 7. This shows an important role for IL-1 in AdMuOSM-induced joint pathology. IL-1 has been shown to be a key factor in the development of experimental arthritis (10,42). Both inhibition of PG synthesis and stimulation of PG breakdown can induce PG depletion in arthritis, and IL-1 can be involved in both processes (11,43). In our experiments, ex vivo PG synthesis in patellae from AdMuOSM-injected joints was not inhibited, but rather was increased. This excess in PG synthesis, however, could not prevent articular cartilage PG loss. Breakdown of PGs would, therefore, be the main cause of the observed PG loss in wild-type mice. In the IL-1α/β-deficient mice, ex vivo PG synthesis was even further increased, and this could (at least in part) contribute to the reduced PG loss in these mice. The increased PG synthesis in the IL-1α/β-deficient mice furthermore indicates that in wild-type mice, IL-1 will partly inhibit the elevation of PG synthesis. We previously observed that blocking of IL-1 in experimental arthritis completely prevented inhibition of PG synthesis but did not influence inflammation-induced PG breakdown (15).

Bell *et al* (4) reported that coinjecting human OSM with recombinant human IL-1 receptor antagonist (IL-1Ra) into the joints of goats could not attenuate OSM-induced cartilage PG depletion. In a previous study, we observed that prolonged high concentrations of IL-1Ra were necessary to prevent IL-1-induced inhibition of PG synthesis in antigen-induced arthritis. These concentrations could be achieved with mini- osmotic

pumps but not by bolus injection of IL-1Ra (15). The negative results described by Bell *et al* could therefore be attributable to poor pharmacokinetics of IL-1Ra in the joint. In the present study, we used IL-1α/β-deficient mice to circumvent these problems and observed clear involvement of IL-1 in the OSM-induced PG loss that occurred during the first week of inflammation.

We have previously shown that repeated injections of IL-1 induce inflammation and cartilage damage in the murine knee joint (11). In that study, the polymorphonuclear cell was the predominant cell type in the inflammatory infiltrate. A role for PMNs in cartilage damage has been shown in vitro (44) and *in vivo* (45). Recently, OSM was shown to selectively recruit PMNs in an in vitro flow chamber assay (46). Using NIMP-R14 staining, we could detect PMNs in the inflamed synovium of both wild-type and IL-1α/β-deficient mice (results not shown), suggesting that IL-1 is not necessary for OSM-induced PMN influx. This, however, does not exclude a relationship between IL-1 and PMNs in the observed PG depletion. Activation of PMNs might differ between wild-type and IL-1α/β-deficient mice; this requires further investigation.

During AdMuOSM-induced inflammation, irreversible damage to the PG network occurred, as demonstrated by the presence of the MMP-induced VDIPEN neoepitope. Expression of VDIPEN was shown to correlate with severe cartilage damage in murine arthritis (47). The VDIPEN neoepitope was also detected in cartilage from IL-1α/β-deficient mice, indicating that irreversible PG damage can occur independent of IL-1. Future research is needed to identify the enzyme that is responsible for this irreversible damage and its relationship to OSM.

Periosteal bone apposition and chondrophyte formation were induced in both wild-type and gene-deficient mice. Periosteal bone apposition can occur in the short tubular bones of the phalanges, metacarpals, and metatarsals, and also in the long bones during JIA (48,49). To our knowledge, little is known about the significance of periosteal bone apposition in JIA. Bone apposition was not induced by overexpression of IL-1 or IL-17 (data not shown), which excludes the possibility that bone apposition was a general consequence of inflammation. We previously had observed that OSM could enhance in vitro the bone morphogenetic protein 2-induced differentiation of C2C12 cells toward the osteoblastic lineage (6). This suggests that OSM could play a positive regulatory role during bone formation by enhancing the activity of bone-forming factors. Chondrophyte and osteophyte formation is common in osteoarthritis (OA). Osteophytes can also develop in RA and JIA with secondary OA, but this happens less frequently.

In general, adenovirally mediated gene transfer to the joint results in a transient transgene expression (50,51) lasting from 1 to 2 weeks. We observed that most of the changes

in the murine knee joint had already developed during the first week, when OSM gene expression was demonstrated. The involvement of IL-1 provides circumstantial evidence for a relationship between transgene expression and the observed joint pathology. This was evident on day 7 but not on day 14. After day 7, OSM-induced inflammation subsided, and the growth plate PG content returned to normal levels. During the first week, periosteal activation, leading to bone apposition on day 14, also took place. Thereafter, the process of new bone formation did not proceed. Surprisingly, articular cartilage PG depletion continued after day 7. This is probably not directly related to OSM activity but could be a result of irreversible cartilage damage, delayed repair mechanisms, or morphologic changes (e.g., chondrophyte formation), which could influence cartilage integrity.

A unique finding associated with injection of AdMuOSM is that the growth plate became damaged. This was not observed with vectors expressing either IL-1 or IL-17, although both induced articular cartilage damage. Such growth plate damage has not been previously observed in experimental arthritis in mice of the same age. This process was demonstrated to be dependent on endogenous IL-1. In growth plates, there is a balance between cartilage matrix degradation, proliferation, matrix formation, and hypertrophy. Expression of IL-1 mRNA has been detected in the growth plate of developing bones in mice (52). In vitro results of studies using growth plate chondrocytes from the rat suggested that IL-1 induces resting growth cells to acquire a phenotype of growth zone cells in an autocrine manner (53). Furthermore, IL-1 could play a role in the bone and cartilage resorption processes that occur in the growth plate during the formation of new bone. OSM could either enhance or modify the autocrine effects of IL-1 on growth plate chondrocytes, thereby leading to growth plate PG loss, disorganization, and finally growth abnormalities.

Growth plate changes in patients with JIA have been reported. Magnetic resonance imaging studies have shown epiphyseal cartilage loss in the knees of patients with JIA (23), and in unilateral juvenile arthritis the femoral epiphysis of the arthritic side was observed to be enlarged (24). Although IL-6 is found in elevated concentrations in serum and synovial fluid in JIA (54), the presence of OSM has, as far as we know, not been investigated in these patients. Using ELISA techniques, we detected OSM in synovial fluid of most of the examined children, and we could also detect IL-1β in most of our OSM-positive samples. Our experiments in the cytokine-deficient mice indicated that OSM, in the presence of IL-1, could cause serious risks to the integrity of growth plate cartilage. It is possible that the combination of these cytokines is similarly involved in growth plate damage in JIA.

Both increased growth and growth retardation occur frequently in JIA (25,55). Most of our synovial fluid samples were obtained from patients with oligoarthritis who had involvement of the knee joint. A study by Simon *et al* (56) showed a relationship between age at disease onset, involvement of the knee, and localized growth abnormalities in oligoarthritis (formerly called monoarticular and pauciarticular RA). In patients in whom JIA began before age 9 years, the involved side was the longer one. Disease onset after this age led to rapid premature closure of the growth plate and shortening of the involved side. Among the positive samples in our study, the highest concentrations of OSM were found in those obtained from the younger children, which could implicate a role for OSM in increased growth of the involved side. This is further supported by the finding that a mouse transgenic for bovine OSM had enlarged hind limbs (22). We recently began collaborations in order to increase the number of synovial fluid samples available for study from patients with the different forms of JIA. We hope that this will also enable us to further characterize OSM expression during the time course of the disease.

In conclusion, our results demonstrate an important role for endogenous IL-1 in AdMuOSM-induced joint pathology, but no involvement of TNFα, IL-6, or iNOS. The induction of growth plate damage in mice adds a newly recognized pathologic consequence of OSM expression that would be particularly relevant in JIA. The AdMuOSM vector provides a useful tool to further investigate this process in more detail. Our results in the cytokine-deficient mice and the detection of OSM in synovial fluid of patients with JIA suggest that the proinflammatory and cartilage-damaging effects of OSM are relevant in human arthropathies such as RA and JIA.

Acknowledgments

We thank Astrid Holthuysen for the VDIPEN staining and Dr. P. Schwarzenberger (New Orleans, LA) for the AdmIL-17 vector.

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CHAPTER 7

An inflammation-inducible adenoviral expression system for local treatment of the arthritic joint

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Gene Therapy 2004, 11:581- 590

Abstract

To achieve a disease-regulated transgene expression for physiologically responsive gene therapy of arthritis, a hybrid promoter was constructed. The human IL-1b enhancer region (-3690 to -2720) upstream of the human IL-6 promoter region (-163 to +12) was essential in mounting a robust response in HIG-82 synovial fibroblasts and in RAW 264,7 macrophages. A replication-deficient adenovirus was engineered with luciferase (Luc) controlled by the IL-1/IL-6 promoter (Ad5.IL-1/IL-6-Luc). LPS caused a 23- and 4.6-fold induction of Luc. activity in RAW cells transfected with Ad5.IL-1/IL-6-Luc or the conventional Ad5.CMV-Luc construct, respectively. Next, adenoviruses (10^6 ffu) were injected into the knees of C57Bl/6 mice. An intra-articular injection of zymosan, 3 days after Ad5.IL-1/IL-6-Luc, increased Luc. activity by 39-fold but had no effect in the Ad5.CMV-Luc joints. The constitutive CMV promoter was rapidly silenced and could not be reactivated *in vivo*. In contrast, the IL-1/IL-6 promoter could be reactivated by Streptococcal cell wall (SCW)-induced arthritis up to 21 days after infection. Next the IL-1/IL-6 promoter was compared to the C3-Tat/HIV-LTR two-component system in wild-type, IL-$6^{-/-}$ and IL-$1^{-/-}$ gene knockout mice. Both systems responded well to LPS-, zymosan- and SCW-induced arthritis. However, the basal activity of the IL-1/IL-6 promoter was lower and IL-6 independent. This study showed that the IL-1/IL-6 promoter is feasible to achieve disease-regulated transgene expression for treatment of arthritis.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with unknown etiology. The clinical course of RA is characterized by a variable disease activity of spontaneous remissions and exacerbations (Flare-ups) of the chronic inflammatory joint process. In females patients, remissions are often associated with pregnancy and exacerbations occur postpartum.[1,2] These flare-ups of the joint of the rheumatoid joint disease have often the characteristics of an acute type of inflammation superimposed on the smouldering joint inflammation already present.

In experimental animals, flare-up reactions of joint inflammation have been studied predominantly in immunologically mediated arthritis models. Flare-ups can be induced by a rechallenge of non-arthritogenic dosages of antigen, growth factors, for example, colony-stimulating factors, and cytokines like IL-1.[3-5] Resident macrophage-like synovial lining cells play an important role in the antigen-induced flare-up reaction,[6] and both TNFα and IL-1 were markedly produced at the time of flare-ups. We demonstrated that selective blocking of IL-1 during the antigen-induced flare-up clearly reduced joint swelling and cartilage proteoglycan loss.[7]

The feasibility of local or systemic gene therapy for RA has been demonstrated in numerous animal experiments.[8] Two phase I clinical trials are undertaken with the objective to block IL-1 by local IL-1Ra overexpression in future therapies.[9,10] Constant high levels of antiinflammatory proteins could be undesirable; they might increase the risk of infection as now observed with anti- TNF and anti-IL-1 treatment of patients with RA.[11-14] Furthermore, the homeostatic balance might adapt to the constant high concentrations of transgene protein so as to reduce its therapeutic efficacy (tachyphylaxis). Ideally, treatment of RA must parallel the intermittent course of the disease to meet the variable physiological demands and prevent undesirable exposure of the system. A number of vectors with drug-regulatable promoters for achieving regulatable transgene expression have been described.[15] A drawback is the necessity of constant disease monitoring to achieve optimal efficacy in RA, which is further complicated by the unpredictable, relapsing clinical course. The major challenge is a disease-regulated expression of a recombinant protein to meet the variable demands during RA: high during a relapse and low during remission of the disease. Currently the cytomegalovirus (CMV) is the most frequently used promoter to express the transgene, but this constitutive promoter when used in adenoviral vectors is rapidly silenced giving short-lasting expression *in vivo*. Varley *et al*[16] have developed a two-component expression system that responds to inflammatory stimuli *in vivo*. In their system, the complement factor 3 regulates pro-

duction of the HIV transactivator of transcription (Tat) protein, which regulates the HIV long terminal repeat (LTR) promoter for transgene expression. The feasibility of this two-component system as an inflammation-inducible promoter for autoregulated expression of IL-10 and IL-1Ra was recently demonstrated in two different experimental arthritis models.[17,18] However, this promoter may not be suitable for RA as the Tat is a foreign immunogenic protein and may have undesirable effects by transactivating host genes, and Tat immunogenicity has been implicated in CNS disorder AIDS dementia due to its toxicity. For this we constructed a hybrid promoter of the enhancer region of the human IL-1 promoter with the human IL-6 promoter, which does not possess the gene-silencing CpG regions that are present in most viral promoters (eg CMV promoters). From many animal studies, others and we demonstrated that both IL-1 and IL-6 belong to the early responsive genes, which are upregulated at the primary onset and secondary flare-ups of experimental arthritis.[7,19] In this study, we demonstrated that the IL-1/IL-6 promoter fulfills the criteria of a disease-inducible promoter - low basal activity and high during the acute inflammatory response - showing that this promoter might be feasible for autoregulated protein drug treatment of RA.

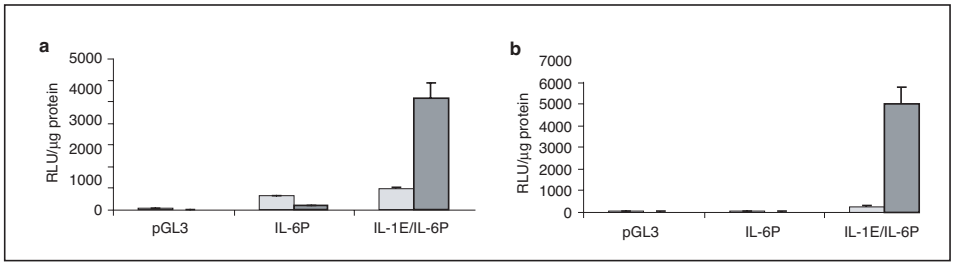

Figure 1 The IL-1 enhance sequence enhances the IL-6 promoter activity. HIG-82 cells (a) or RAW 264,7 cells (b) were transiently transfected with the pGL3 luciferase reporter vector without a promoter or the IL-6 promoter (-162 to +12) alone or in combination of the IL-1 enhancer sequence (-360 to -2720). Cells were either not stimulated ■ or stimulated with LPS (20 µg/ml)+PMA (10 ng/ml)+dbcAMP (100 nM) ■. After 18 h, the luciferase activity in the cell lysate was measured and expressed as relative light units and corrected for the amount of protein extracted (RLU/µg).

Results

IL-1 enhancer is critical for the inducible character of the IL-1/IL-6 hybrid promoter

The murine RAW 264,7 macrophage cell line and the rabbit synovial fibroblast cells (HIG-82) were transiently transfected with the pGL3 luciferase reporter vector (Figure 1). Cells

were stimulated with lipopolysaccharide (LPS, 20 μg/ml)+phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) + 8-bromo-cAMP (dbcAMP, 100 nM) for broad-range gene activation by NF-κB, PKC and PKA activated protein kinases (AP1), respectively. The human IL-6 promoter region (-163 to +12) alone did not respond to this cocktail; however, when the human IL-1 beta enhancer region (-3690 to -2720) was placed upstream, a pronounced induction of 5.3-fold in HIG82 cells and 18-fold in RAW cells was observed.

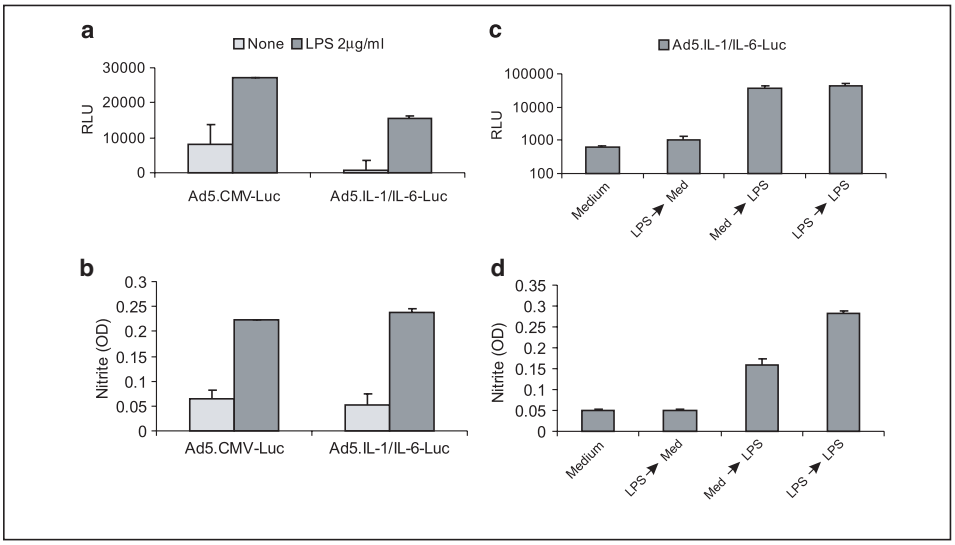

Figure 2 LPS-induced activation of the IL-1/IL-6 promoter in an adenoviral reporter vector. RAW 264,7 (10^5 cells) were transfected with Ad5.CMV-Luc or Ad5.IL-1/IL-6-Luc at an MOI of 10 (a, b) or 1 (c, d). After 4 h of infection, the medium was replaced and cells were stimulated with LPS. At 1 day after LPS (2 μg/ml) stimulation, the luciferase content of the cells (a) and the nitrite concentration in the culture supernatant (b) were measured. In a second experiment, RAW cells were once stimulated with LPS (0.2 g/ml) 6 h after Ad5.IL-1/IL-6.Luc infection (LPS>med) or after 56 h (Med>LPS) or twice at both 6 and 56 h (LPS>LPS). Luciferase activity (c, RLU) and nitrite concentration (d, $OD_{540\ nm}$) were measured 72 h after infection.

In vitro characterization of the IL-1/IL-6 promoter using a first-generation adenoviral Luc reporter vector

RAW macrophages were infected with Ad5.IL-1/IL-6-Luc or the control virus Ad5.CMV-Luc with an MOI of 10 and in the presence of an Fc-CAR fusion protein to enhance infection efficiency (Figure 2). Transfected cells were challenged with LPS (0.2-2 μg/ml) and this stimulated Luc expression 3.7-5.5-fold (range of four separate experiments) using the CMV promoter and 20-51.4-fold using the IL-1/IL-6 hybrid promoter

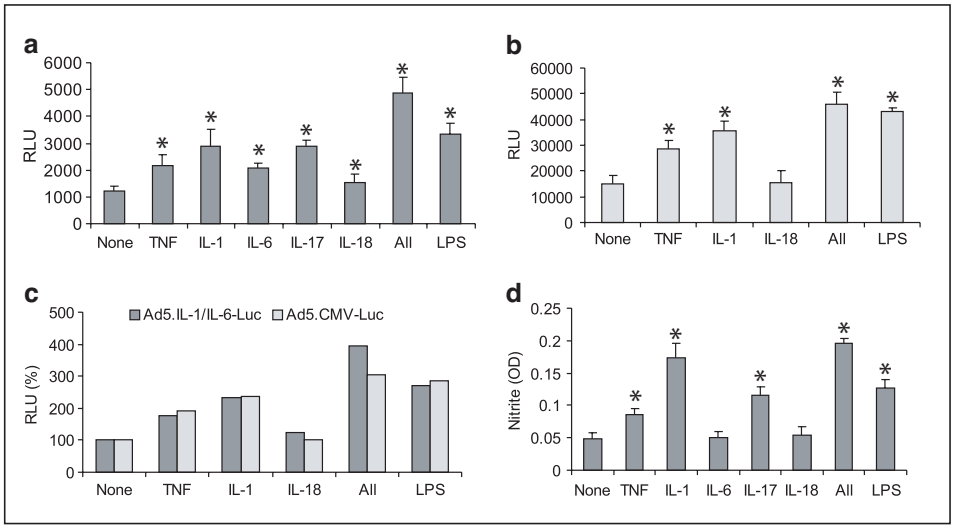

Figure 3 Cytokine response profile of the IL-1/IL-6 (a) and the CMV promoter (b) in a murine chondrocyte cell line. The murine immortalized chondrocyte cell line (H4) were transfected and 4 h later the culture medium was changed and cells were stimulated with murine recombinant IL-1α, IL-18 (both at 10 ng/ml), IL-6, IL-17, TNFα (all three at 100 ng/ml), or the combination of TNF, IL-1 and IL-18 (All), or LPS (5 mg/ml). After 18 h, the luciferase activity in the cell lysates (a, b) and the nitrite concentration in the conditioned culture supernatant (d) were measured. Levelling the basal Luc. activity showed that both promoters had the same cytokine induction index (c). None, no stimulation; RLU, relative light units. *Statistically significant using the Student's t-test with P-values <0.0001 as compared to the nonstimulated group.

(Figure 2a). LPS-induced nitrite production by Ad5.CMV-Luc and Ad5.IL-1/IL-6-Luc-transfected cells was enhanced 3.2-4.7-fold, independent of the virus used (Figure 2b), indicating that the cellular LPS triggering was not different between both adenoviral vectors. In another experiment we investigated whether the IL-1/IL-6 promoter could be reactivated by a second LPS challenge. RAW cells were transfected with Ad5.IL-1/IL-6-Luc and challenged with LPS. Pulse-chase experiments showed that the Luc. activity in the cells transfected with Ad5.IL-1/IL-6-Luc returned to almost background levels 48 h after the LPS stimulation. A second LPS rechallenge at this time point could still enhance the Luc. activity to the same extent as the first LPS challenge, indicating that the IL-1/IL-6 promoter remains responsive also after the first activation period. The cytokine responsiveness of the IL-1/IL-6 hybrid promoter was tested on an immortalized murine chondrocyte cell line. These cells were infected at an MOI of 50 and stimulated with different murine recombinant proteins or LPS. A significant upregulation of luciferase activity was found after stimulation of the cells with IL-1a, TNFα, IL-6, IL-17 and IL-18, and a combination of the cytokines; TNF, IL-1 and IL-18 showed additive effect on the IL-1/IL-6 pro-

moter (Figure 3a). The cytokine response of the IL-1/IL-6 promoter was different from the endogenous iNOS gene, as both IL-6 and IL-18 failed to induce nitric oxide (NO) production in these cells under the same condition (Figure 3d). The CMV promoter also showed a high background activity in these cells as compared to the IL-1/IL-6 promoter. Nonetheless, the CMV promoter showed the same cytokine responsiveness as the IL-1/IL-6 promoter, although the effect of IL-18 did not reach significance (Figure 3b and c).

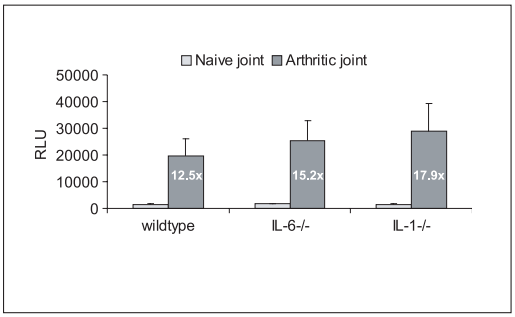

Figure 4 In vivo response of the IL-1/IL-6 promoter in gene knockout mice. Wild-type, IL-6$^{-/-}$ and IL-1a/b$^{-/-}$ double knockout mice were injected intra-articularly with 10^6 ffu Ad5.IL-1/IL-6-Luc virus into both knee joints. After 3 days, the right knees received 180 mg of zymosan material by an intra-articular injection (black bars). At 1 day thereafter, patellae with adjacent synovial tissue were isolated from both left and right knee joints, partially digested, and the luciferase activity was measured in the lysate. A significant induction (white numbers in the black bars) of luciferase expression ($P<0.001$) was observed in all mouse strains.

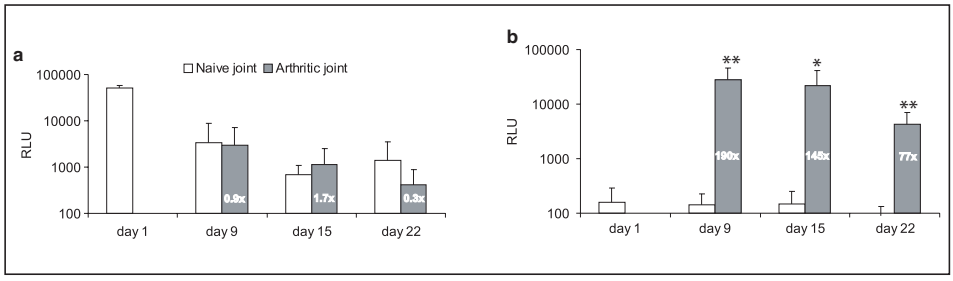

Figure 5 In vivo comparison of the CMV with the IL-1/IL-6 promoter responsiveness to an arthritic stimulus. Mice were injected intraarticularly with 10^6 ffu of Ad5.CMV-Luc (a) or Ad5.IL-1/IL-6-Luc (b) virus into both knee joints. After 1 day, the Luc. activity was measured in the synovial tissue. At days 8, 14 and 21 after infection, the right knee joints (black bars) received 25 μg SCW material as an arthritogenic trigger. After 24 h, the Luc. activity was measured in the synovial tissue obtained from both naive and SCW-injected knees. Luciferase activity reached statistically significant differences compared to the naive knee joints with P-values <0.05 (*) and <0.001 (**) using the Student's t-test. The fold inductions of Luc. activities are depicted in white numbers. Note that the CMV promoter caused a high initial luciferase expression.

In vivo characterization of the IL-1/IL-6 promoter using

A first-generation adenoviral Luc reporter vector Ad5.IL-1/IL-6-Luc (10^6 ffu) was injected into both murine knee joint cavities of wild-type, IL-6$^{-/-}$ and IL-1a/b$^{-/-}$ double knockout mice. After 3 days, zymosan (180 μg) was injected into the right knee joint cavities

only. After 24 h, Luc. activity was 12.5 increased in wild-type, 15 in IL-$6^{-/-}$ and 18 in IL-$1\alpha\beta^{-/-}$ mice as compared to their contralateral noninflamed knee joint (Figure 4). The CMV promoter is rapidly silenced in the murine knee joint and could not be activated by an intraarticular injection of the arthritogenic Streptococcal cell wall (SCW) material (Figure 5a). In contrast to the CMV promoter, the IL-1/IL-6 promoter is not constitutively active (day 1 after infection) but could be activated even up to 21 days after infection by an intra-articular injection of SCW material (Figure 5b). Hirt extraction and quantification of PCR products for viral DNA showed that the loss of adenoviral DNA was approximately 60% between days 1 and 15 after injection and not significantly different for both the CMV and IL-1/IL-6 constructs (Figure 6). The loss of viral DNA may be responsible for the short expression kinetics of Luc using the CMV promoter construct. As the viral DNA levels are reduced in both groups, an enhanced immune response (cytotoxic T cells) against the Ad5.CMV-Luc-infected cells is highly unlikely and cannot explain the differences found in responsiveness with the Ad5.IL-1/IL-6-Luc-injected knee joints.

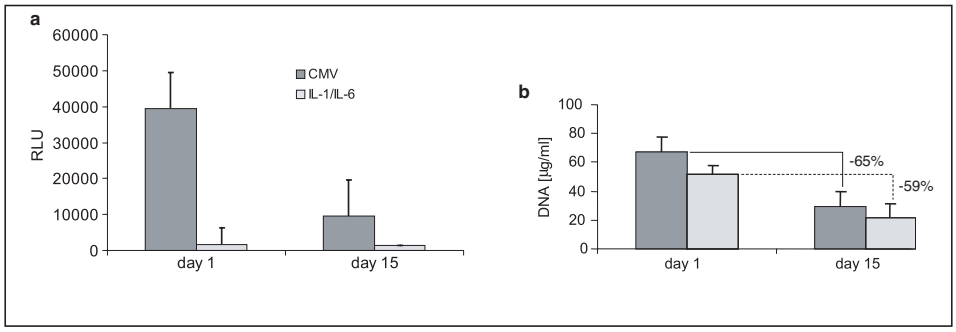

Figure 6 Loss of viral DNA in vivo. Mice (n=5 per group) were injected intra-articularly with 10^6 ffu of Ad5.CMV-Luc or Ad5.IL-1/IL-6-Luc virus into both knee joints. At days 1 and 15, synovial tissue was taken from the infected joints and viral DNA was extracted by Hirt extraction. The luciferase content in the extract (Hirt extraction without SDS) was measured (a) and in the same sample the viral DNA was amplified by PCR and quantified as described in Materials and methods (b). Note the same reduction (%) in the viral DNA content of both groups.

Comparative study of IL-1/IL-6 hybrid system with the C3-Tat/Hiv two-component system

Varley *et al*[16] developed a disease-inducible two-component expression system, which is efficacious in animal models of arthritis.[17,18] We compared the expression characteris-

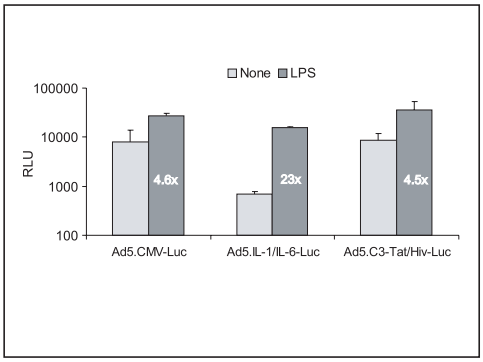

Figure 7 Comparative in vitro study of the LPS response in three different promoter systems. RAW macrophages (10^5 cells) were transfected with Ad5.CMV-Luc, Ad5.IL-1/IL-6-Luc or Ad5.C3-Tat/HIV-Luc at an MOI of 10. After 24 h, cells were stimulated with LPS at a concentration of 2 μg/ml or not stimulated. The luciferase expression was measured 24 h later and was expressed as RLU. The fold induction by LPS as compared to the unstimulated cells is depicted as white numbers in the black bars. The LPS-induced stimulation of Luc. activity was statistically significant with all three promoter systems.

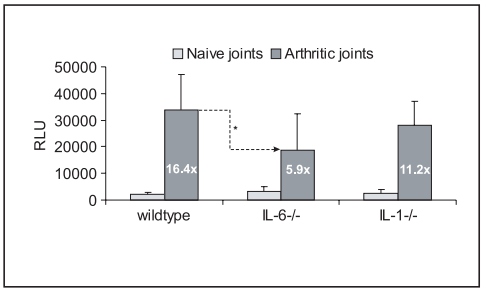

Figure 8 In vivo response of the C3-Tat/HIV two-component expression system in gene knockout mice. Wild-type, IL-$6^{-/-}$ and IL-1a/$b^{-/-}$ double knockout mice were injected intra-articularly with 10^6 ffu Ad5.C3-Tat/HIV-Luc virus into both knee joints. After 3 days, the right knees received 180 μg of zymosan material by an intra-articular injection (black bars). At 1 day thereafter, the luciferase activity was measured in the synovial tissue lysates. A significant induction ($P<0.001$) of luciferase expression (white numbers in the black bars) by the zymosan injection was observed in all mouse strains. Note the significant (*$P<0.001$) reduction in the zymosan-induced luciferase expression in the IL-6 gene knockout mice.

tics of this two-component system with the IL-1/IL-6 hybrid promoter construct. The C3-Tat/Hiv two-component system in RAW cells showed the same high background activity and LPS induction comparable to the conventional CMV promoter (Figure 7). The IL-1/IL-6 promoter had a 10-fold lower background activity and the LPS-induced increment of Luc. activity (23-fold) exceeded that of the two other expression systems (4.5-fold). Next, the *in vivo* performance of the C3-Tat/Hiv two-component system was compared to the IL-1/IL-6 hybrid promoter system. For this, the same amounts (10^6 ffu) of adenoviral luciferase reported vectors were injected into the knee joint of wild-type, IL-$6^{-/-}$ and IL-1α/$\beta^{-/-}$ gene knockout mice. After 3 days, zymosan (180 μg) was injected intra-articularly and 24 h later the Luc. activity was measured. The zymosan-induced luciferase activity was similar between the Ad5.IL-1/IL-6-Luc- and Ad5.C3-Tat/Hiv-Luc-injected knee joints (Figures 4 and 8). However, a significant reduction of zymosan-induced luciferase activity (45%, $P<0.0001$) was found in the IL-6-deficient mice with the Ad5.C3-Tat/Hiv-Luc vector, whereas the activity was slightly increased in the Ad5.IL-1/IL-6-Luc-injected joints. No differences between induction of luciferase was found

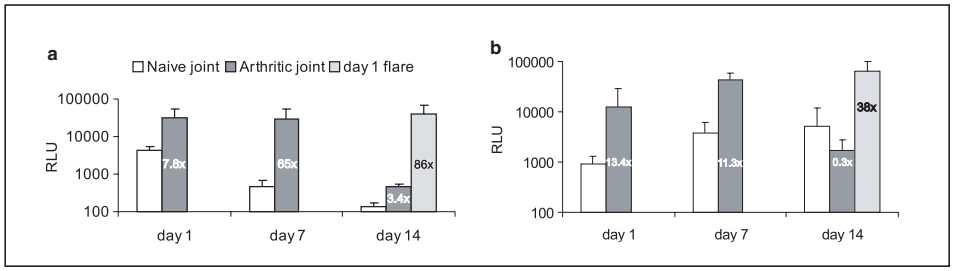

Figure 9 Transgene expression during the course of SCW arthritis. Mice received an intra-articular injection with 10^6 ffu of either Ad5.IL-1/IL-6- Luc (a) or Ad5.C3-Tat/HIV-Luc (b) into both knee joints. After 1, arthritis was induced in the right knee joint by an intra-articular injection of 25 μg SCW material; the left knee served as a within-animal control of the background transgene expression. At days 1, 7 and 14, histology was taken and joint inflammation (infiltration into synovium and exudation of neutrophils into joint cavity on a 0-3 scale) was 1.5±0.1, 0.7±0.3 and 0.3±0.1, respectively. A second SCW injection caused a flare-up of the inflammation resulting in a score of 1.2±0.3. The luciferase activity in the synovial tissue was assessed at days 1, 7 and 14 of arthritis and is expressed as RLU. At day 13, one group of animals received a second intraarticular injection of 25 μg SCW material to reactivate the smouldering inflammation and the transgene promoter. The fold induction of luciferase activity by arthritis is depicted in the bars.

between both disease-regulatable promoter systems in the IL-1α/β-deficient mice. Next, the expression of the C3-Tat/HIV and IL-1/IL-6 promoters was studied during the course of SCW arthritis. Intra-articular injection of SCW material caused a transient acute joint inflammation, which lasted for 7 days.[20] Adenoviruses were injected into both knee joints 1 day before induction of arthritis in the right knee joint, and the left naive joint served as a within-animal control of basal transgene expression. The expression of Luc was higher in the naive joints infected with Ad5.IL-1/IL-6-Luc at day 1 of arthritis, but much lower at days 7 and 14 as compared to the Ad5.C3-Tat/Hiv-Luc-infected naive joints (Figure 9a and b). Injection of SCW material resulted in an acute joint inflammation, with infiltration and exudation of neutrophils. This immediately resulted in upregulation of the IL-1/IL-6 (Figure 9a) and C3-Tat/HIV (Figure 9b) promoter activity. At day 14, the acute joint inflammation had subsided and a second SCW injection immediately exacerbated the smouldering joint inflammation (inflammation flared from 0.3±0.1 up to 1.2±0.3). The second SCW injection upregulated both promoters, resulting in approximately the same luciferase expression (Figure 9). This suggests that the adenoviral DNA was neither lost nor silenced during the 2-week *in vivo* period. Because the background level of the IL-1/IL-6 promoter was lower, a higher stimulation index was found at days 7 and 14 of SCW arthritis.

Discussion

Several groups have shown the feasibility of viral gene transfer of the inflamed joint for treatment of arthritis in a large number of publications. The major challenge is to obtain a tailor-made expression of therapeutic genes following the variable disease activity of spontaneous remissions and exacerbations in RA. For this, we developed a hybrid promoter system consisting of the human IL-1 enhancer sequence in front of the human IL-6 promoter to allow autoregulation of the transgene during arthritis.

The IL-6 promoter region (-163 to +12) alone showed little to no responsiveness in the absence of the IL-1 enhancer region (-3690 to -2720) (Figure 1). We found that in the murine macrophage cell line RAW 264,7, the LPS-induced upregulation of luciferase was higher with the IL-1/IL-6 promoter than with the CMV promoter (Figure 2). Blocking IL-1 with either IL-1 receptor antagonist protein or using neutralizing rabbit anti-IL-1 antibodies had no effect on the LPS-induced promoter activation although it blocked the LPS-induced NO production completely (data not shown). Murine recombinant IL-1a or IL-1b had no effect on the IL-1/IL-6 promoter nor did it induce NO release in these cells (data not shown). Kim and Son[21] showed that neither IL-1, IL-2, IL-6 nor TNFα could induce NO in RAW cells, but that NO was highly upregulated by LPS in the presence of IFNγ. Our results suggest that for full activation by recombinant proteins these RAW cells probably additionally need IFNγ. In the murine immortalized chondrocyte cell line (H4), the hybrid promoter, however, responded to a wide range of cell stimuli as LPS and the cytokines IL-1, IL-6, IL-17, IL-18, and TNFα (Figure 3a). Unexpectedly, the conventional CMV promoter also showed the same responsiveness for IL-1a, TNFα and LPS (Figure 3b and c). Moreover, the CMV immediate-early promoter has previously been shown to be regulated by various cytokines. It has been shown in monocytic and endothelial cells that the human CMV promoter can be induced by PGE2, TNFα and IL-1β,[22-24] probably in part via NF-κB activation.[25,26] TNFα has also been shown to negatively regulate the CMV immediate-early promoter depending on the cell type studied.[27,28] Based on our in vitro studies and that of others, the human CMV immediate-early promoter may behave like the IL-1/IL-6 enhancer-promoter construct. The difference found between the IL-1/IL-6 hybrid and CMV promoter in response to LPS in RAW cells suggests that we cannot exclude that both promoters may respond differently in other target cells. We showed that the endogenous iNOS gene was differently regulated than the IL-1/IL-6 promoter or the CMV promoter in our chondrocyte cell line (Figure 3).

Our study confirmed the general problem encountered with the conventional CMV promoter: high basal activity, transient and soon after nonresponsive probably due to

promoter silencing *in vivo* (Figures 2, 3 and 5). The high background activity of CMV was evident in both in vitro and *in vivo* situations. Furthermore, the amount of adenoviruses injected into the knee joint cavity only elicited a marginal joint inflammation. Therefore, a significant contribution of viral-related inflammation on the basal CMV activity was not likely to occur. The cytokines, interferon-gamma and TNFα, can inhibit transgene expression controlled by most viral promoter/enhancers delivered by adenoviral, retroviral or plasmids in vitro.[28] A specific transcriptional block as a result of DNA methylation or chromatin condensation can silence viral promoters. Evidence is emerging that the methyl-CpG-binding protein 2 (MeCP2) links DNA methylation with histone deacetylases to form a transcriptional repression multiprotein complex.[29,30] The CMV-ie promoter has five palindromic CpG motifs that are potential sites of DNA methylation and promoter silencing.[31] We found that the histone deacetylase inhibitor trichostatin A (TSA) can only partly reactivate the silenced CMV promoter in the synovial tissue *ex vivo* (manuscript in preparation). This showed that the silencing of the CMV promoter is probably far more complex than CpG methylation alone.

The most important difference between the conventional CMV promoter and our IL-1/IL-6 promoter is that the latter promoter was not silenced in vitro nor *in vivo* (Figures 2d, 5 and 9). This was illustrated by the rapid induction of the IL-1/IL-6-regulated Luc expression by evoking SCW-mediated joint inflammation for a long period after transduction Figure 5). Furthermore, Figures 2d and 9 show that the IL-1/IL-6 promoter was not silenced irreversibly by an initial activation period and remained at least as responsive for a second activation trigger. Only three short (6 bp sequence) palindromic CpG-like sequences are present in our IL-1/IL-6 hybrid promoter, but either they did not become methylated or are in a nonrelevant area of the promoter as we found no evidence of promoter silencing. The reduced response at day 21 after infection with the Ad5.IL-1/IL-6.Luc was related to the 80% loss of viral DNA.

It is possible that elimination of the adenoviral vector/ transfected cells contributed to the loss of CMV-regulated transgene expression in mice. Recent studies showed that TNFα plays an important role in the onset of an inflammatory response against adenoviruses and in eliciting an immune response against adenoviral and transgene products.[32-34] Inhibition of TNFα in mice markedly prolonged adenoviral-mediated transgene expression in mice.[34,35] The CMV promoter gives a high constitutive expression, and this might initiate an antiluciferase response thus eliminating the Ad5.CMV-Luc transfected cells. This is less likely to happen with Ad5.IL-1/IL-6-Luc, as this promoter exerts a low basal luciferase expression without stimulation. We found that at day 15 the adenoviral DNA content in the murine knee joint was reduced to 40% of that measured

at 1 day after infection, whereas the luciferase activity declined to 22% over that same time period (Figure 6). The loss of viral DNA could be the result of immune response against the transfected cells, but this cannot explain the complete unresponsiveness of the CMV promoter *in vivo* (Figure 5). Unexpectedly, the CMV promoter seemed to perform quite well in the LPS-induced arthritis model in the rat.[36,37] In these studies, the rat synovium was stably transfected with an adeno-associated viral (AAV) vector. The CMV promoter in AAV-transfected liver was, however, poorly inducible by LPS.[38] As the AAV genome integrates into host genome, it is possible that it undergoes position effect and that enhancer elements from nearby promoter sites in the host genome crosstalk with the CMV promoter in the AAV construct. So far, the evidence that the CMV promoter in an AAV vector can be used for physiologically responsive gene therapy in other more relevant arthritis models is lacking.

We previously achieved a disease-inducible transgene expression in experimental arthritis using the two-component system described by Varley *et al.*[16] This system was developed to treat acute inflammatory diseases (eg sepsis) and was designed to give low basal and induced short-term transgene expression.[39] In this system, the viral HIV-LTR promoter is also vulnerable to silencing by CpG methylation, but the transactivator of transcription (Tat) protein can overcome the HIV-LTR promoter inactivation although it was unable to demethylate both CpG sites in this promoter.[40] In comparative studies, we found that the IL-1/IL-6 hybrid promoter performed equally well as the C3-Tat/HIVLTR two-component system and both systems responded to intra-articular injections of LPS, SCW and zymosan (Figures 7-9). However, only the two-component system was strongly dependent on the endogenous IL-6 activity *in vivo* (Figure 8). This was expected as the complement C3 upregulation in response to an immunogen was completely absent in the IL-6$^{-/-}$ mice[41,42] and was probably regulated at the level of complement C3 promoter activation.[43,44] It is known that the TAT protein can transactivate inflammatory host genes,[45] including the IL-6 gene.[46] It is plausible that the transactivation of IL-6 gene causes activation of the C3 promoter, which can explain the higher background levels found with the C3-Tat/HIV two-component system as compared to our IL-1/IL-6 construct (Figures 7 and 9). It must be emphasized that the basal transgene levels and the fold induction using the C3-Tat/HIV two-component system were far better in adenoviral-transfected HepG2 cells and in primary rat synovial fibroblasts.[16,18] A major difference was the 24-h serum (0.5% FBS) starvation used in these studies whereas we performed our experiments in 5% FCS. Different batches of fetal calf serum may contain varying amounts of growth factors, cytokines (IL-6) or traces of endotoxin, and the C3-Tat/HIV two-component promoter system is extremely responsive to stimula-

tion. In a recent pilot experiment, serum starvation of RAWcells (in an identical experimental setup) did not affect both basal or the fold induction of Luc. activity by LPS.

Another important difference between both disease-inducible promoters is that the Tat protein in the C3-Tat/ HIV-LTR system is a foreign immunogenic protein. Both the IL-6 dependency and foreign Tat protein make the C3-Tat/HIV-LTR system in comparison to the IL-1/IL-6 hybrid promoter less suitable for in the therapeutic approach of gene transfer in RA.

There is circumstantial evidence (Figure 9) that with the IL-1/IL-6 hybrid promoter but not the C3-Tat/HIVLTR system we experienced a contralateral effect on the activation of the promoter by the arthritic joint. The 'contralateral effect', that is the administration of the expression vector in one joint to suppress arthritis in distal or contralateral joints, has been reported by many laboratories.[47-52] This study, which shows activation of the IL-1/IL-6 enhancer-promoter in the contralateral joints by signals derived from the arthritic joint, suggests that there is crosstalk between joints. Treatment of one joint may interfere with this crosstalk and by this suppresses arthritis in the other contralateral joint.

We demonstrated that our IL-1/IL-6 hybrid promoter upregulates transgene expression in response to a wide range of cytokines *in vitro* and during an acute joint inflammation. The latter was induced by either zymosan (heat-inactivated yeast) or SCW (Gram-positive bacterial cell walls) material, and both stimuli activate cells via dimerization of toll-like receptors (TLR)-2 and TLR-6.[53] The TLR-2/6 and TLR-4 (LPS receptor) all can induce NF-κB activation and by this directly stimulate the IL-1/IL-6 receptor. For this, we also investigated the IL-1/IL-6 promoter in an immune-mediated inflammation, that of collagen-induced arthritis (CIA), and found that the IL-1/IL-6 promoter was upregulated at the onset of CIA and that the amount of transgene (luciferase) correlated with the severity of the joint inflammation (preliminary data).

In this study, we demonstrated that the IL-1/IL-6 promoter fulfills the criteria of a disease-inducible promoter: low basal activity and high during the acute inflammatory response. This promoter might be feasible for auto-regulated protein drug treatment of RA using adenoviral gene therapy.

Materials and methods

Animals

For this study, C57Bl/6 male mice were obtained from Charles River (Sulzfeld, Germany). Breeding pairs of IL-1α/β-deficient mice[54] (a kind gift of Dr Y Iwakura, Center of Experimental Medicine, University of Tokyo, Japan, who studied in conjunction with Dr MG Netea, University Medical Center Nijmegen, The Netherlands) and IL-6 gene knock-out[55] (a kind gift of Dr M Kopf, Molecular Biomedicine, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland) were backcrossed into C57Bl/6 mice. The mice were housed in filter-top cages. Mice were kept in low-pressure isolators and used between 10 and 12 weeks of age. They were fed a standard diet and tap water *ad libitum*. All *in vivo* studies complied with national legislation and were approved by the local authorities according to the 'Care of Use of Animals', with related codes of practice.

Construction of IL-1/IL-6 hybrid promoter

The human IL-6 promoter sequence (-179/+12) was excised out of the PGL2 luciferase reporter construct (kindly provided by Kishimoto[56]) and inserted into the *Sac*I/*Xho*I restriction sites of the PGL3 basic luciferase reporter vector (Promega). The human IL-1 gene region (-3690/ -2720) with marked promoter enhancer activities[57] was prepared by the PCR method using human genomic DNA as a template and the oligonucleotide primers (Eurogentec) — human IL-1β forward primer 5'-CTA CCC GCC ATC CAA GAG GGA GAA GAA-3', and the reverse primer 5"-TAC CAT GGC TCG AGG GAA ATT TTG GA-3' — and blunt ligated into the Srf I site of the pCR-Script™-SK(+) cloning kit (Stratagene). The IL-1 enhancer sequence was inserted in the K*pn*I/*Sac*I sites upstream of the IL-6 promoter in the PGL3 basic luciferase reporter vector.

Adenoviral vectors and transduction

The IL-1/IL6 luciferase in PGL3 basic luciferase reporter vector was excised with *Sal*I and ligated into pShuttle (AdEasy system). Replication-deficient adenoviruses (E1 and E3 deleted) were prepared as described.[58] Viral particles (v.p.) were measured spectrophotometrically by the method of Mittereded *et al.*[59] To determine the titer of infectious

viral particles (ffu), the vector stock was titrated on 911 cells (kind gift of Hoeben, LUMC, the Netherlands[60]) and the viral capsid was detected 20 h after transduction by immunohistochemistry.[61] The v.p. and ffu per ml of the adenoviral vector stocks were Ad5.CMV-Luc 1.5 x 10^{12} v.p., 1.2 x 10^{10} ffu, Ad5.IL-1/IL-6-Luc 5.4 x 10^{11} v.p., 9 x 10^{9} ffu and Ad5.C3-Tat/HIV-Luc 2.9 x 10^{11} v.p., 6 x 10^{9} ffu (kind gift of Munford, UT Southwestern Medical Center, Dallas, TX, USA[16]).

In vitro transduction experiments

HIG-82 (rabbit synoviocyte cell line, ATCC), RAW 264,7 (murine macrophage cell line, ATCC) and H4 (murine chondrocyte cell line[62]) were used in the transduction experiments. Cells (5 x 10^{5}) were plated on a six-well culture plate (Costar), and transfected with Lipofectamin 2000 (Invitrogene). After 24 h, cells were washed and thereafter stimulated for 18 h. Cell culture supernatant was kept for nitrite measurement and cells were harvested and cell lysate was obtained by osmotic and mechanical disruption. Protein content and luciferase activity were assessed as described below.

Cells were plated in 96-well flat-bottom plates (1 x 10^{5} cells/well) and 24 h later transfected with adenoviruses at different MOI as indicated in the figures. In case of the RAW cells, a CARex-Fc fusion protein (kindly provided by Hemmi, University of Z rich, Switzerland) was used during infection (50 ng/10^{7} ffu) for selective targeting of the adenovirus to the Fc-γ receptor type I (CD64), which will enhance the transduction up to 250-fold.[63] After 2-3 h, cells were rinsed to reduce the number of free adenoviruses in culture, and acclimated. After 20 h, cells were stimulated and their luciferase content was measured 24 h later. Conditioned culture supernatants were stored at -80°C until further analysis for their nitrite content.

In vivo transduction experiments

Endotoxin-free stocks of the adenoviral vectors were diluted in physiological saline and 10^{5}-10^{7} ffu in 6 ml was injected into the knee joint cavity.

ZIA model

A homogeneous suspension of 300 μg of zymosan A (from Saccharomyces cerevisiae; Sigma, St Louis, MO, USA), dissolved in 10 ml of endotoxin-free saline, was obtained by repeated boiling, followed by sonic emulsification. The suspension was autoclaved and stored in 0.5 ml aliquots at -20°C. Arthritis was induced by intraarticular injection of 180 μg of zymosan through the suprapatellar ligament into the joint cavity. In all, 10^7 ffu of the adenoviral vectors was intra-articularly injected 4 weeks after ZIA induction.

Streptococcus cell wall model

Cell walls from Streptococcus pyogenes were prepared as described previously.[64] Unilateral arthritis was induced by intra-articular injection of 24 μg SCW in 6 μl PBS into the right knee joint of C57/Bl6 mice.

Luciferase measurements

In vitro transduction experiments. To cell lysates in bidest Bright Glo (Bright Glot luciferase assay system, Promega) was added and luciferase activity was measured according to the manufacturer's protocol.

In vivo transduction experiments. Patellae with surrounding tissue were dissected, put in 250 μl cell culture lysis buffer (Promega) and snap frozen in liquid nitrogen. Samples were thawed, vortexed for 2 min, and centrifuged for 1 min at 10 000 g. We measured luciferase in 10 μl of the supernatant with the luciferase assay system (Promega) according to the manufacturer's protocol in a luminometer (Polarstar galaxy, BMG, Germany). Total protein concentration in the supernatant was determined with the Coomassie Protein Assay Reagent (Pierce, Illinois, USA). The luciferase activity was expressed as relative light units (RLU) and normalized to total protein content of the cell/tissue extracts.

Nitrite measurements

The medium concentration of NO_2 (a stable breakdown product of NO) was determined by Griess reaction using $NaNO_2$ standards. The Griess reagent consisted of 0.1% naphthylethylene diamine dihydrochloride, 1:1 diluted with 1.0% sulfanilamide in 5% H_3PO_4. Briefly, 100 μl of the conditioned medium was mixed with 100 μl Griess reagent in a flat-bottom microtiter plate, and adsorbance was read at 545 nm using an ELISA plate reader.

Assessment of intra-articular adenoviral DNA

To determine the adenoviral DNA content in the synovial tissue, adenoviral DNA was isolated by Hirt extraction, detected by PCR and analyzed on ethidium bromide-stained agarose gel.[65] Briefly, the synovial tissue was grinded in a mortar at 4°C in TE buffer. Proteinase K was added and incubated for 1 h at 37°C. NaCl (5 M) was added and the suspension was incubated overnight at 4°C and centrifuged at 4°C for 15 min at 15 000 g. In later experiments, sodium dodecyl sulfate (SDS) was used to inactivate proteins. The adenoviral DNA in the supernatant was detected by PCR using the forward primer (H1) 5'-GCC GCA GTG GTC TTA CAT GCA CAT C-3' and the reverse primer (H2) 5'-CAG CAC GCC GCG GAT GTC AAA GT-3'.[66] Samples (5 μl) were taken at increasing cycle numbers at five cycle intervals. The PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide. DNA was isolated from the gel and measured with picogreen.

Statistical analysis

Means ± sd of the various groups were determined and potential differences between the groups were tested using the Mann-Whitney rank-sum test for all data.

Acknowledgements

We thank M Netea (Internal Medicine, UMCN, Nijmegen, The Netherlands) for the collaboration on the IL-1α/β gene knockout mice of which the breeding pairs were kindly

provided by Y Iwakura (Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Japan). We also thank M Kopf (Molecular Biomedicine, Department of Environmental Sciences, Swiss Federal Institute of Technology, Zurich, Switzerland) for providing us with the IL-6 gene knockout mice breeding pairs. Furthermore, we express our gratitude to T Kishimoto (Department of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan) for kindly providing us the human IL-6 promoter vector. The CARex-Fc fusion protein for optimal adenoviral infection of macrophages was kindly provided by S Hemmi (Institute of Molecular Biology, University of Z rich, Z rich, Switzerland).

This research was supported by grants from the Dutch Arthritis Association (941,304) and the Dutch Organization for Scientific Research (902-27-218).

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CHAPTER 8

Summary and final considerations

Joint disorders like osteoarthritis and rheumatoid arthritis can have great and long lasting effects on patients suffering from these diseases. In the affected joints elevated levels of cytokines have been found. Depending on the type of cytokine these molecules can have either negative or positive influences on disease progression and joint integrity. One of the cytokines that is found to be elevated in joints of RA en OA patients is interleukin-6. The existence of animal models for RA and OA and modern techniques like gene knock out mice and viral overexpression systems have contributed to a huge amount of knowledge on the role of cytokines in joint diseases. In the present thesis the role of IL6 in murine arthritis and osteoarthritis was investigated.

In **chapter 2** we started with a comparison of wild type and IL6 deficient mice in spontaneous and experimental osteoarthritis. Just as in humans, mice developed signs of OA upon aging. In our study we found more spontaneous OA in old male IL6$^{-/-}$ mice than in the wt controls. Old IL6$^{-/-}$ male mice showed the most severe cartilage loss, subchondral bone sclerosis and proteoglycan (PG) deposition/bone formation in the ligaments. OA development was studied in more detail in male mice. Cartilage PG synthesis and breakdown were reduced in one year old IL6$^{-/-}$ mice. Total cartilage PG content, however, was not affected in old IL-6$^{-/-}$ mice. Also the bones were affected by IL6 deficiency as we measured a lower bone mineral density of the total knee joint in old IL6$^{-/-}$ mice. Collagenase injections in the knee joint led to severe signs of OA in both strains. This suggests that one way of protection by IL-6 could be in regulating joint instability. Together our results suggest a protective role for IL6 in OA and do not make it a likely therapeutic target in this disease.

In **chapter 3** wt and IL6$^{-/-}$ mice were compared in different arthritis models. Equal inflammation in both strains was found in the short lasting immune-complex induced arthritis. In the antigen-induced arthritis (AIA) we found that IL6$^{-/-}$ mice developed an acute inflammatory reaction but the cellular infiltrate declined rapidly and IL6$^{-/-}$ joints appeared non -inflamed by day 7 of AIA. WT mice, in contrast, developed a chronic arthritis that lasted for more than 3 weeks. Gene expression for several important pro-inflammatory cytokines, chemokines and adhesion-molecules were upregulated in the synovial tissue during the first two days of the AIA and were indistinguishable between the IL6$^{-/-}$ and wt mice. Immunized IL6$^{-/-}$ mice had lower antigen-specific IgG2a and IgG2b titres as wt mice, but equal IgG1 titres. Lowering the amount of antigen during immunization in wt mice reduced the IgG2A titres to levels that were found in IL6$^{-/-}$ mice sensitised with the normal high amount of antigen. This, however, did not prevent normal development of inflammation in these wt mice. Comparison of cytokine expres-

sion in T cells indicated a shift to the Th1 subtype in wt mice and to the Th2 subtype in IL6$^{-/-}$ mice. Transfer of antigen-specific wt lymph node cells to IL6$^{-/-}$ mice increased the acute inflammation and cartilage damage but did not lead to development of a chronic inflammation. Similar to our results in the AIA, we found that IL6$^{-/-}$ mice developed an acute inflammation in the irritant induced zymosan-induced arthritis (ZIA) that failed to proceed to the chronic phase. Taken together these different arthritis models showed that IL6 played an important role in propagation of joint inflammation, potentially independent of its role in immunity.

Signal Transducer and Activator of Transcription (STAT) molecules play an important role in cytokine signaling. IL-6 can signal through activated STAT1 and STAT3. Intra-cellular inhibition of signaling molecules in the synovium could be a good way to inhibit the pro-inflammatory effects of IL-6 but to preserve the beneficial effects in cartilage. Therefore, in **chapter 4,** activation of STAT1 and STAT3 was investigated in the joints of wt and IL6$^{-/-}$ mice during the acute and chronic phase of ZIA. STAT 3 was activated continuously during both acute and chronic inflammation in wt mice and this was IL6 dependent. STAT 1, in contrast, became only activated in wt mice during the chronic phase. The influx of PMN- and macrophages occurred in both strains and this excluded absence of these cells to be the cause of the impaired STAT activation in IL-6$^{-/-}$ mice. Expression of the STAT-inhibitors SOCS-1 and -3 showed increased SOCS 3 expression during both phases and increased SOCS 1 expression only during the chronic phase. In STAT1$^{-/-}$ mice exacerbation of chronic joint inflammation occurred. In these mice STAT 3 was activated and SOCS 3 expression increased to the same extent as in the wt mice. The expression of SOCS 1, however, was markedly reduced in the STAT1$^{-/-}$ mice. Together these results suggest that IL6 could play a role in chronic joint inflammation through STAT 3 activation, making it a likely target for inhibition. An opposed inflammation-controlling role is proposed for STAT 1.

The IL-6 family member Oncostatin M (OSM) is also expressed in the inflamed joints of RA patients and might become an additional therapeutic target for STAT inhibition because it also signals through the JAK-STAT pathway. Similar to IL-6, OSM has also been described as a cytokine with both pro- and anti-inflammatory properties. Therefore, the effects of OSM on the different components of the joint need to be investigated. In **chapter 5** we investigated murine Oncostatin M induced joint pathology, with an emphasis on inflammation and bone pathology, in wt and IL6$^{-/-}$ mice. Adenoviral OSM gene transfer in the knee induced a pronounced joint inflammation that developed also in the absence of IL6. In both strains OSM overexpression resulted in activation of the periosteum leading to periosteal bone apposition. OSM, but not IL-6, could enhance

the BMP-2 induced alkaline phosphatase expression/activity in C2C12 cells *in vitro*. This suggests that OSM can stimulate bone formation by enhancing the activity of bone forming factors. Bone formation occurred *in vivo* despite the expression of osteoclast activating RANKL and its receptor RANK in the synovium and periosteum. Expression of the RANKL antagonist osteoprotegerin (OPG) could shift the balance towards bone formation instead of resorption during the OSM-induced joint inflammation.

In **chapter 6** OSM-induced joint pathology was further investigated in mice deficient in IL1α/β, IL6, TNFα or INOS. At day 7, joint inflammation and cartilage PG depletion were significantly reduced in IL1α/β$^{-/-}$ but not in the other mouse strains. By day 14, inflammation, PG depletion and bone apposition did not differ between these strains. OSM overexpression was found to affect growth plate integrity and PG content during the first week in all except the IL1α/β$^{-/-}$ mice. Growth plate damage can occur in juvenile idiopathic arthritis (JIA) and we found that OSM protein is present in the synovial fluid of children with JIA. Together these results show IL-1 dependence of OSM-induced joint pathology and suggest that OSM could not only be important for joint pathology in RA but also in JIA.

In **chapter 7** we made use of the data acquired from previous studies on the cytokine expression profiles in our murine models of arthritis for the design of a disease-inducible transgene expression cassette. An adenoviral vector was constructed containing the human IL-6 promoter and the human IL1β enhancer region. Expression of the luciferase reporter gene was rapidly silenced *in vivo* when the constitutively active viral CMV promoter was used. In sharp contrast, the hybrid IL-1/IL-6 promoter showed a low basal activity but high activity during the acute phase of arthritis. Furthermore, it could be reactivated by a flare-up of the disease. By this study we showed feasibility of the IL-1/IL-6 promoter construct to achieve disease regulated transgene expression that can be used for tailor-made treatment of arthritis reducing the risk of side-effects that is expected with the conventional uncontrolled expression systems.

Final considerations

IL-6, good or bad guy in RA? With this question, the research described in this thesis started. Both pro- and anti-inflammatory properties had been described for IL-6. Previous research in our lab had shown that IL-6 had a cartilage protective effect during onset of zymosan-induced arthritis (ZIA) [1]. Our present results in murine osteoarthritis (OA) (**chapter 2**) further point towards this cartilage protective role for IL-6.

The role of IL-6 has been less studied in OA than in RA. In our study, cartilage loss was increased in IL-6$^{-/-}$ mice. Also *ex vivo* PG synthesis and breakdown were reduced in these mice. These effects of IL-6 deficiency could not only be important in OA but also in RA. Although the net cartilage PG density was not affected, old IL-6$^{-/-}$ mice might be more vulnerable to arthritic cartilage damage due to their reduced PG synthesis. This could mean that previous studies, using young mice, might have underestimated the cartilage protective role of IL-6. Future experimental arthritis studies in older IL-6$^{-/-}$ mice would therefore be very interesting. Based on our present and previous results IL-6 plays, with regard to cartilage damage, a role as good guy in joint pathologies like RA and OA.

A multifunctional cytokine like IL-6, however, also has properties that put it in a bad perspective for RA. Previous studie[2-4] mainly pointed at reduced antigen-specific immunity in IL-6$^{-/-}$ mice during experimental RA. Our present results confirmed these data but also showed that not only reduced immunity is involved in preventing development of chronic arthritis in these mice (**chapter 3**). Similarly, Nowell *et al.* recently found that injection of a IL-6/ soluble IL-6 receptor fusion protein enhanced joint inflammation in IL-6$^{-/-}$ mice to wt levels at day 3 of AIA without enhancing antigen-specific immunity[5].

What are the roles that IL-6 plays in the arthritic joint? Circumstantial evidence shows that IL-6 plays a role in orchestrating the switch from PMN- to monocyte/macrophage-influx. In this way IL-6 could then contribute to development of chronic arthritis. Most evidence was obtained using a peritonitis model in IL-6$^{-/-}$ mice[6]. Our present results suggest that IL-6 plays a different role in joint inflammation. Synovial gene expression for chemokines, adhesion-molecules and other pro-inflammatory cytokines was equally induced in wt and IL-6$^{-/-}$ mice during onset of antigen-induced arthritis (AIA) (**chapter 3**). Immunohistochemistry of ZIA even showed that macrophages could enter the joint in IL-6$^{-/-}$ mice (**chapter 4**). IL-6 therefore does not seem to be necessary for monocyte/macrophage influx in this model and might be involved in other aspects of joint inflammation.

Our results on STAT activation during ZIA could point at a different role for IL-6. Lack of STAT1/3 phosphorylation despite presence of PMN's, macrophages and synoviocytes in the joints of IL-6$^{-/-}$ mice (**chapter 4**) could mean that these cells are not activated properly or that certain pro-inflammatory pathways are blocked in these mice. Possible examples could be pathways related to apoptosis/anti-apoptosis, cell cycling and proliferation. One way to address this could be large-scale gene comparison between wt and IL-6$^{-/-}$ mice with for example micro-arrays.

Based on our results one could say that IL-6 plays a protective role with regard to cartilage but a pro-inflammatory role in the synovium. Future anti-IL-6 therapies should be

developed in a way that they inhibit its actions in the synovium but preserve them in cartilage. There are several possibilities to inhibit IL-6 in RA. Administrating antibodies against IL-6 or the IL-6 receptor is an option that is now showing positive effects in first clinical trials[7]. Alternatives are biologicals like soluble GP130, mutated IL-6 or soluble IL-6 receptor molecules[5;8]. It remains, however, to be determined if the IL-6 produced in the cartilage itself is enough to have a protective effect or that additional IL-6 from the synovium is necessary. This would have consequences for IL-6 inhibitory therapies in the synovium. Based on our results in **chapter 4**, selective inhibition of STAT3 signaling could be a way to restrict anti-IL-6 therapies to the synovium. Either introducing or enhancing expression of dominant-negative STATs or of other inhibitors like the SOCS and PIAS proteins in the synovium could be a way to accomplish this selective inhibition. Alternatively, enhancement of STAT1 signaling could also have anti-inflammatory effects.

Gene therapy would be an option to achieve overexpression of e.g. SOCS proteins in the synovium. Previously Shouda *et al.*[9] showed that adenoviral gene transfer of SOCS3 could inhibit collagen-induced arthritis (CIA). In their study, however, the virus was injected peri-articular in the ankle. Intra-articular gene delivery in contrast is more difficult. Intra-articular injection of an adenoviral SOCS3 vector in the knee joint did not inhibit CIA in our hands (manuscript in preparation). We showed previously that standard adenoviral vectors were limited in their way to penetrate the synovium and that modifications of the adenoviral fiberknob could enhance gene transfection in this tissue[10]. Further development of these vectors must therefore take place to make gene therapy a practical option for treatment of RA.

One such improvement is shown in **chapter 7**. The IL-6 promoter was used to construct an adenoviral vector with a disease-inducible promoter. Expression of a therapeutic gene cannot only be locally but also temporally restricted with this vector. Controlled expression of anti-IL-6 molecules would be desirable since long term cytokine blocking could lead to problems with infections. This occurs, for example, in part of the patients on anti-TNF-α therapy[11]. Furthermore, complete and long-term suppression of IL-6 could have adverse effects for cartilage or bone as shown in **chapter 2**. Our IL-1/IL-6 promoter construct could become a good tool towards local, disease-inducible anti-IL-6 therapy in the synovium.

Another member of the IL-6 family that is expressed in the inflamed joints of RA patients is Oncostatin M. In **chapters 5 and 6** we show that overexpression of murine OSM can induce joint inflammation in naive mice. This was independent of IL-6 but dependent on IL-1. OSM, like IL-6, can activate STAT3. Therapies aimed at inhibiting

signaling by the GP130/JAK/STAT route could therefore have a broader range than just inhibition of IL-6. The observed periosteal bone apposition in the inflamed joints (**chapter 5**) illustrates that OSM, like IL-6, can both be a good (anabolic factor for bone) and a bad guy (inducing joint inflammation and cartilage damage) during joint pathology.

One interesting effect of OSM overexpression was that the growth plates became affected. This raised our interest to investigate OSM expression in juvenile idiopathic arthritis (JIA). We could indeed measure OSM expression in most of the synovial JIA fluids that we tested. Research is under way to screen a larger JIA patient population for OSM expression. OSM expression in our JIA group was lower than the expression that is reported for RA[12]. For possible anti-OSM therapies in JIA this could be an advantage. Its synergistic effect on other proinflammatory cytokines might lead to an even broader therapeutic effect of anti-OSM treatment. This synergistic effect on other cytokines has for example been demonstrated for IL-1[12] and was also found by us for BMP-2.

To generally conclude this thesis we propose that IL-6 plays a protective role in osteoarthritis and should not be inhibited in this disease. With regard to arthritis, IL-6 is a good guy related to cartilage but a bad guy related to inflammation of the synovium. We furthermore propose that specific inhibition of IL-6 signaling in the synovium might be a way to prevent this pro-inflammatory role of IL-6.

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Samenvatting

Reumatoïde artritis (RA) is de bekendste vorm van reumatische gewrichtsaandoeningen. Het is een immunologische ziekte waarbij de eigen afweercellen zich richten tegen lichaamseigen structuren in de gewrichten. Dit leidt tot een chronische ontsteking die het kraakbeen en bot aantast. In het ontstoken gewricht worden veel verschillende cytokines gemaakt. Dit zijn zgn. boodschappereiwitten die een rol spelen bij de communicatie tussen cellen. Deze cytokines kunnen positieve als ook negatieve effecten hebben op de cellen in het ontstoken gewricht. Een cytokine dat veel gemaakt wordt in ontstoken gewrichten, is interleukine-6 (IL-6). IL-6 heeft eigenschappen die het zowel gunstig als ongunstig maken voor de reuma patiënt. In dit proefschrift is de rol van IL-6 in gewrichtsaandoeningen nader onderzocht in muizenmodellen met normale (IL-$6^{+/+}$) en IL-6 knock-out (IL-$6^{-/-}$) muizen. Deze laatste stam mist het IL-6 gen en maakt daardoor geen IL-6.

Eerder onderzoek op ons lab had aangetoond dat IL-6 een beschermende rol heeft voor het gewrichtskraakbeen tijdens het begin van de ontsteking. De vraag of IL-6 onafhankelijk van een ontsteking kraakbeen kan beschermen is niet alleen relevant voor artritis maar ook voor artrose (osteoarthritis of OA), een zeer veel voorkomende gewrichtsziekte waarbij kraakbeenschade optreedt maar waarbij ontsteking een minder belangrijke rol speelt. In hoofdstuk 2 is de rol van IL-6 in relatie tot kraakbeenschade nader onderzocht in oude $IL6^{+/+}$ en IL-$6^{-/-}$ muizen. Hierbij werd gevonden dat oude IL-$6^{-/-}$ mannetjes muizen meer en ernstiger artrose hadden dan oude IL-$6^{+/+}$ mannetjes. Naast meer verlies van kraakbeen hadden de oude IL-$6^{-/-}$ mannetjes meer verbening van de kniebanden en meer verdichting van het bot onder het kraakbeen. Tijdens de veroudering nam tevens de synthese van bepaalde kraakbeen componenten af en verloor het bot meer mineralen in IL-$6^{-/-}$ mannetjes. Dit suggereert een belangrijke beschermende rol voor IL-6 tijdens artrose. Tevens versterkt deze uitkomst de kraakbeenbeschermende rol in het gewricht die wij eerder vonden tijdens arthritis.

In hoofdstuk 3 en 4 werd de rol van IL-6 in het ontstekingsproces nader onderzocht. In hoofdstuk 3 werden IL-$6^{+/+}$ en $IL6^{-/-}$ muizen vergeleken in verschillende reuma modellen. In al deze modellen ontwikkelen IL-$6^{-/-}$ muizen wel een acute ontsteking. Deze gaat echter niet over in een chronische ontsteking. In de immunologische modellen vonden wij dat IL-6 een rol speelt bij de productie van zowel antilichamen als T cellen die specifiek gericht zijn tegen een eiwit waarmee de ontsteking werd uitgelokt. Hierbij speelde een verandering in het type T cellen dat gemaakt werd in de $IL6^{-/-}$ muizen de grootste rol. Transplantatie van normale T cellen maakte de ontsteking in IL-$6^{-/-}$ muizen echter niet chronisch. Aangezien de ontsteking ook niet chronisch werd bij een niet-immunologisch model sug-

gereert dit dat IL-6 belangrijk is voor immuniteit maar ook op een andere manier een belangrijke rol speelt bij het ontstaan van chronische ontsteking in gewrichten.

In hoofdstuk 4 is gekeken naar de signaaloverdracht van IL-6. IL-6 bindt aan specifieke receptoren aan de buitenkant van de cel. Hierna worden in de cel zgn. STAT eiwitten geactiveerd. Dit zijn STAT1 en STAT3. Na activatie kunnen deze STAT's aan elkaar binden en naar de celkern verhuizen. In de celkern kunnen zij specifiek bepaalde genen in het DNA aanzetten. In het niet-immunologische zymosan model (gistdeeltjes inspuiten in de knie) vonden wij continue activatie van STAT3 in het ontstoken gewricht. STAT1 activatie kwam alleen tijdens de chronische fase van de ontsteking voor. Deze STAT activatie ontbrak in IL-$6^{-/-}$ muizen. De betekenis van de STAT1 activatie werd onderzocht in STAT$1^{-/-}$ muizen. Deze muizen lieten een verergering van de ontsteking zien. STAT3 activatie was normaal in deze muizen. Dit suggereert dat IL-6 via STAT3 een pro-inflammatoire rol speelt en dat STAT1 ontstekingremmend werkt.

In hoofdstuk 5 en 6 is de rol van een familielid van IL-6, oncostatine M (OSM), in gewrichtsontsteking onderzocht. In hoofdstuk 5 werd een adenovirus gebruikt als drager om het muizen OSM gen in de knie van IL-$6^{+/+}$ en IL-$6^{-/-}$ muizen te brengen en actief te maken. OSM kan in de knie een ontsteking starten, ook zonder IL-6. OSM productie leidde echter ondanks de ontsteking ook tot vorming van nieuw bot. Dit laatste zou een positief effect kunnen zijn van OSM. In hoofdstuk 6 is de afhankelijkheid van de OSM-geïnduceerde ontsteking van andere cytokines onderzocht. Naast ontsteking en botvorming treedt ook kraakbeenschade op onafhankelijk van IL-6 en TNF-a. De afwezigheid van IL-1 daarentegen had wel een remmend effect op schade en ontsteking. Opvallend was dat OSM ook de groeischijf aantastte. Groeischijven zijn nog actief bij kinderen en OSM zou daarom een rol kunnen spelen bij schade aan de groeischijf bij jeugdreuma. In vocht van ontstoken gewrichten van kinderen met jeugdreuma konden wij productie van OSM aantonen.

De voorafgaande hoofdstukken toonden aan dat IL-6 en OSM zowel positieve als negatieve effecten hebben in ontstoken gewrichten. Vooral in het ontstoken gewrichtskapsel zou het goed zijn om IL-6 te remmen. Met remmers van STAT3 (hoofdstuk 4) zou men daar lokaal het effect van IL-6 kunnen remmen terwijl de positieve werking op kraakbeen behouden blijft. In hoofdstuk 7 beschrijven wij een adenovirus dat gebruikt kan worden om zulke remmers of andere therapeutische eiwitten lokaal te laten maken in het gewricht. In dit virus is de productie van het therapeutische eiwit afhankelijk van prikkels die de IL-6 productie stimuleren. Deze zgn. ontstekingsafhankelijke vector kan op momenten van actieve ziekte de productie van therapeutische eiwitten sterk verhogen. Hiermee zou het in de toekomst mogelijk moeten worden om in het gewricht en afhankelijk van de ziekte-activiteit IL-6 en andere pro-inflammatoire cytokines te remmen.

Acknowledgements

In modern science cooperation with other researchers has become very important. In this part of my thesis I would like to thank our foreign collaborators who have made a significant contribution to the research on IL-6 and Oncostatin M. The research on IL-6 started in Nijmegen with the kind gift of breeder pairs of the IL-6 deficient mice and their wild type controls by dr. Manfred Kopf (Basel, Switzerland). These mice have been used in all the chapters of this thesis and have revealed important new information on the role of IL-6 in joint pathology. I would like to thank dr. Carl Richards (Hamilton, Canada) for the kind gift of adenoviral vectors that expressed IL-6 or murine Oncostatin M. Our experiments with the OSM-vector have resulted in two papers that demonstrated that Oncostatin M is, like IL-6, a cytokine with both good and bad properties. I would like to thank dr. Thomas Kolbe (Tulln, Austria) and dr. Matthias Müller (Vienna, Austria) for the STAT1 deficient mice. Breeding pairs of these mice were originally a kind gift of dr. David Levy (New York, USA). STAT proteins have become a hot item in research on inflammation. The STAT1 deficient mice enabled us, as far as we know, to present the first data on the role of STAT1 in experimental arthritis suggesting opposite roles of STAT1 and STAT3 in arthritis. To conclude this chapter I would like to thank dr. Alan Varley (Dallas, USA) for the C3-TAT/HIV two component adenoviral system that was used in chapter 7.

Dankwoord

Vijf jaar heb ik met veel plezier op het lab reumatische ziekten gewerkt. Tot slot van dit proefschrift wil ik dan ook iedereen bedanken die aan het onderzoek en aan de arbeidsvreugde heeft bijgedragen. In tegenstelling tot mijn werkbesprekingen zal ik proberen mij in te houden. Waardering geef je elkaar tenslotte op de werkvloer en niet pas als je alweer elders werkt.

Allereerst wil ik mijn promotor Wim van den Berg en mijn co-promotor en begeleider Fons van de Loo bedanken voor de goede begeleiding. Ons onderlinge contact was, altijd in een ontspannen sfeer, gericht om zo goed mogelijk ons onderzoek te doen. Dit laatste is zeker gelukt en ik ben dan ook best trots op het eindresultaat dat wij gezamenlijk hebben bereikt. Tegen mensen van buiten Nijmegen kan ik zeggen dat het gehele lab een creatieve en zeer productieve plek is om te werken. Wat ik verder vooral ook gewaardeerd heb, was de vrijheid die ik kreeg om zelf onderzoekslijntjes uit te zetten. Soms misschien iets te vaak (volgens Fons) maar uiteindelijk kwam daar meestal toch een leuk resultaat uit.

Tijdens mijn onderzoek heb ik veel steun gehad aan Miranda Bennink en Onno Arntz. Zonder hun analytische ondersteuning was het nooit mogelijk geweest om zoveel verschillende onderzoeken te doen. Naast jullie inzet en vaardigheden wil ik vooral ons goede onderlinge contact noemen, waarin naast veel plezier ook tijd was voor serieuze gesprekken over werk en privé. Apart wil ik Miranda bedanken voor haar hulp toen mij het typen bemoeilijkt werd door een blessure.

Tijdens mijn project hebben drie studentes bij mij stage gelopen. Ik wil Annet Westerhof, Marije Koenders en Windy Smallegoor bedanken voor het werk dat zij gedaan hebben en de prettige samenwerking.

In het groepje van Fons heb ik gezelschap gehad van twee mede-AIO's, Andrew Bakker en Ruben Smeets. Naast voor de goede en gezellige samenwerking wil ik hen ook bedanken voor de hulp die ik kreeg bij het onder de knie krijgen van bepaalde computerprogramma's. Zo zijn de figuren uit de intro bijvoorbeeld van de hand van Ruben. Verder mag ook de gedeelde "interesse" van Ruben en mij voor Schotse single malts hier wel genoemd worden.

Het gehele lab wil ik bedanken voor de prettige samenwerking en de gezellige sfeer op het werk en tijdens onze uitjes.

Tijdens mijn project heb ik ook samengewerkt met een aantal andere onderzoekers en analisten van buiten het reuma lab. Allereerst wil ik dr. Theo Fieselier (UMC St. Radboud, afd. Kindergeneeskunde) en dr. Marcel Franssen (St. Maartenskliniek Nijmegen) bedanken voor synoviaal vocht van jeugdreumapatiëntjes. Ik vond het ook interessant en leerzaam dat ik een keer bij zo'n afname aanwezig mocht zijn. Natasja Liewes en Dinie Versleyen van de Orthopedie (UMC St. Radboud) wil ik bedanken voor de Goldner en Von Kossa kleuringen uit hoofdstuk 5. Diana de Jong en Wilma Steegenga (Toegepaste Biologie, KUN) wil ik bedanken voor de samenwerking toen ik bij jullie op het lab proeven kon doen met jullie C_2C_{12}-cellen in combinatie met OSM (hoofdstuk 5).

Helaas heeft niet alles wat ik gedaan heb dit proefschrift gehaald. Toch gaat ook mijn dank uit naar Tineke Coenen-de Roo (Organon, Oss) en Gerjon Hannink (Orthopedie, UMC St.Radboud) voor onderzoek dat wij gezamenlijk hebben gedaan aan de oude IL-6 knock-outs.

De medewerkers van de CDL wil ik bedanken voor hun goede zorg voor de muizen.

Tot slot wil ik mijn familie bedanken voor hun steun en de interesse die zij toonden in mijn werk. Dit resulteerde onder andere in een samenwerking met mijn vader (hoofdstuk 2).

Wanneer straks het hora est geklonken heeft, komt er voor mij ook echt een einde aan mijn werk aan Interleukine-6. Het aantal mensen dat in de wereld specifiek aan IL-6 werkt is beperkt. IL-6 is wel een oud cytokine genoemd en op het lab werden wel eens grappen gemaakt dat ik de laatste was die nog aan IL-6 werkte. Toch denk ik dat IL-6 en de IL-6 familie nog interessant genoeg zijn om verder te onderzoeken. Doordat het multifunctionele cytokines zijn, kan men er zijn of haar creativiteit in ieder geval goed op botvieren.

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8. Takahashi N, Ostendorf B, van Kilsdonk JWJ, Boonefaes T, **de Hooge ASK**, Bruggeman SWM, Rotties P, Alonso A, Smeets R, Joosten LAB, Lubberts, Schneider M, Swart GWM, van den Berg WB: Nucleoporin 88kDa in rheumatoid arthritis: a novel regulator of NF-kappa B nucleocytoplasmic trafficking and chronic inflammation. Submitted.
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Curriculum Vitae

Alfons de Hooge werd geboren op 3 april 1971 te Rotterdam. Van 1983-1989 doorliep hij het vwo aan het Revius Lyceum te Doorn. Vervolgens heeft hij van 1989-1990 de jaarcursussen natuur- en scheikunde gevolgd van de Stichting Aanvullend Onderwijs te Utrecht. Van september 1990-augustus 1996 heeft hij de studie planteziektenkunde aan de Landbouwuniversiteit Wageningen gevolgd. Binnen deze studie is gekozen voor de oriëntatie biotechnologie en zijn drie projecten uitgevoerd. Van januari 1994-september 1994 heeft hij een afstudeervak gedaan bij de vakgroep virologie van de Landbouwuniversiteit Wageningen (thans Wageningen University) onder begeleiding van dr. Douwe Zuidema en mevr. Magda Usmany. Titel verslag: Produktie van lege deeltjes van het Mond- en Klauwzeer Virus met behulp van het Baculovirus/ T7 systeem.

Ter voorbereiding op de buitenlandse stage is van september 1994-december 1994 een cursus Spaans gevolgd in Salamanca. Van januari 1995-mei 1995 is een stageproject uitgevoerd in het laboratorium van prof. dr. Eladio Viñuela binnen het Centro de Biología Molecular van de Universidad Autónoma de Madrid. Titel verslag: The DP71L gene of African Swine Fever Virus and its role in inhibiting apoptosis of infected cells.

Ter afronding van de studie is van juni 1995-juli 1996 een extra stageproject uitgevoerd op de afdeling virologie van de faculteit diergeneeskunde (Universiteit Utrecht). Dit project stond onder begeleiding van drs. Martin Raamsman en prof. dr. Peter J.M. Rottier. Titel verslag: The Mouse Hepatitis Virus envelope protein: membrane topology and localization in the cell.

Na het afronden van zijn studie is van augustus 1996- juni 1998 gewerkt aan een project getiteld “intra-oculaire anti-angiogene gentherapie” van de vakgroep Interne Geneeskunde (prof. dr. Emile Voest en dr. Frits Fallaux) en het laboratorium voor fysiologische chemie (prof. dr. Peter van der Vliet) van het Academisch Ziekenhuis Utrecht (thans UMC Utrecht).

Van juli 1998-april 2003 is het in dit boek beschreven promotie onderzoek uitgevoerd op het onderzoekslaboratorium reumatische ziekten van het Academisch Ziekenhuis Nijmegen (thans UMC St.Radboud). Dit onderzoek stond onder begeleiding van dr. Fons van de Loo en prof. dr. Wim van den Berg.

Sinds juni 2003 is hij werkzaam als post-doc op het immunologisch laboratorium kindergeneeskunde van het Leids Universitair Medisch Centrum te Leiden. Hier werkt hij aan immunotherapie tegen Ewing’s sarcoma en de rol van het tumor antigen Prame in de biologie van deze tumor.


GLENMORANGIE
SINGLE HIGHLAND
MALT SCOTCH WHISKY
10 YEARS OLD
GLENMORANGIE
Connemara
PEATED SINGLE MALT
IRISH WHISKEY
PRODUCT OF IRELAND
BOWMORE
LEGEND
ISLAY
SINGLE MALT
SCOTCH WHISKY
MILLENNIUM


SIEMENS
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