The doctor within

Disease-regulated local gene therapy for rheumatoid arthritis

Jeroen Geurts

Een uitgave over ziekte-gereguleerde lokale gentherapie voor reumatoïde artritis.

De dokter van binnenuit

De grote nadelen van de nieuwste generatie geneesmiddelen tegen reumatoïde artritis, zogenaamde biologicals, zijn een verhoogde kans op infectieziekten en hoge kosten. Een langdurige en afgestemde lokale productie van biologicals in het ontstoken gewricht lijkt een goed alternatief om deze nadelen te ondervangen.

Gentherapie, het inbrengen van therapeutisch genetisch materiaal in cellen van de patiënt, heeft zich ontwikkeld tot een werkzame methode om dit doel te bereiken. In het ideale geval wordt de biological bovendien geproduceerd naar gelang de behoefte van het ontstoken gewricht en functioneert de virale vector zo als de *"dokter van binnenuit"* door te diagnoseren en daarop gebaseerd de gentherapeutische behandeling te doseren.

In dit proefschrift beschrijven we de ontwikkeling en toepassing van biologische sensoren bestaande uit gen promoters, welke gevoelig zijn voor gewrichtsontsteking. Ontstekingsgevoelige promoters werden geselecteerd met behulp van een muismodel van reuma. Deze bleken vervolgens, wanneer ingebracht in cellen van een muizenknie, aangezet te worden tijdens ontsteking en de sterkte bleek zelfs afhankelijk van de mate van ontsteking. Toepassing van deze ziekte-gereguleerde gentherapie in muizen bleek uiteindelijk net zo effectief, echter veel veiliger dan onafgestemde, continue productie.

De *"dokter van binnenuit"* zou in de nabije toekomst toegepast kunnen worden voor gentherapeutische diagnostiek en behandeling van reuma.

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Contents

1. General introduction

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints and mainly targets the joint capsule, articular cartilage and bone. Key features of the disease include autoimmunity, chronic inflammation and connective tissue destruction. RA affects approximately 1% of the adult population worldwide and is associated with an increased mortality [1]. While the exact etiology remains to be elucidated, several genetic loci and environmental factors have been associated with disease susceptibility and severity. Genome-wide association studies have identified at least 31 RA risk alleles of which many, i.e. *HLA-DR1, PTPN22, CTLA-4* and *STAT4*, are related to immune system function [2]. Environmental susceptibility factors linked to RA include smoking and several infectious microorganisms such as bacteria and viruses [3]. Current treatments for RA aim at alleviating disease symptoms and halting its progression. Standard therapies include non-steroidal anti-inflammatory drugs, disease-modifying anti-rheumatic drugs, glucocorticoids and biologicals.

RA pathogenesis involves a complex interplay of various cell types of the immune system and resident cells of the joint [4-10] **(Figure 1).** After the clinical onset of disease the synovial membrane, consisting of synovial fibroblasts and macrophages, becomes hyperplastic and is infiltrated by immune cells such as monocytes, T- and B-cells, dendritic cells and neutrophils. Joint inflammation induces the release of pro-inflammatory mediators which promote cartilage destruction and bone erosion mediated by the activities of synovial fibroblasts, osteoclasts, and chondrocytes. The immune and destructive processes that take place in the rheumatic joint are governed by a complex and hierarchical network of cytokines, chemokines and proteolytic enzymes. In this vast network, interleukin (IL)-1 and tumor necrosis factor (TNF) have originally been identified as crucial mediators of RA pathogenesis in humans [11,12]. Extensive studies in experimental arthritis using TNF-/IL-1-deficient mice or blocking agents, such as antibodies and soluble receptors, have confirmed the pivotal roles of TNF and IL-1 in joint inflammation and destruction, respectively [13-19]. More importantly, these studies have revealed the therapeutic potential of TNF and IL-1 targeting for treatment of RA. Currently, TNF, IL-1 or IL-6 blockers are being applied as biologicals, while targeting alternative cytokines (e.g. IL-17) continues to be investigated in animal models and clinical trials [20]. Blocking TNFα using antibodies or soluble receptors represents the standard biological therapy for RA and shows efficacy in approximately seventy percent of the patients.

However, despite its efficacy systemic anti-TNF treatment suffers from several disadvantages. First and most prominently, the percentage of patients that fulfills more than seventy percent improvement of disease parameters (ACR70 criteria) is limited to only twenty percent [21]. Second, the role of TNFα in anti-infectious pathways leads to a significantly increased

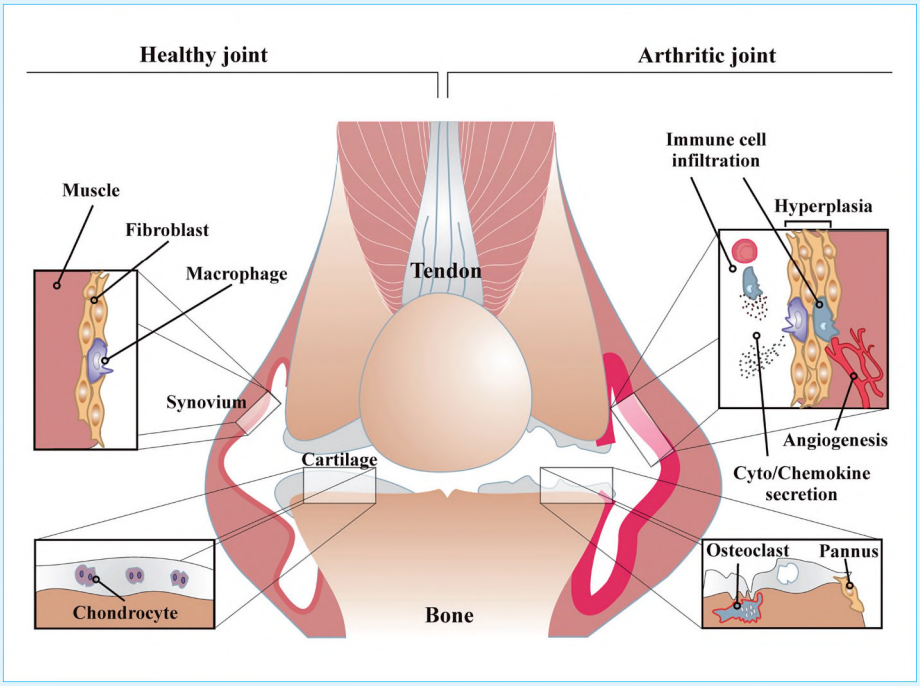

Figure 1.1

Key players in joint pathology of rheumatoid arthritis

The healthy joint is lined by an thin inner membrane called synovium, which consists of synovial fibroblasts and macrophages. Its main function is secretion of synovial fluid, which serves to lubricate the joint and reduces friction between joints and bones. Chondrocytes, which reside in the cartilage, control the synthesis and turnover of the extracellular matrix. The hallmarks of RA include inflammation and irreversible joint destruction. The latter is mediated via several mechanisms including enhanced osteoclast activities, cytokine-induced chondrocyte catabolism, chondrocyte death and invasion of bone and cartilage by the inflamed synovium (pannus). Joint inflammation is characterized by infiltration of both joint cavity and synovial tissue by immune cells, such as B-/T-cells, dendritic cells, neutrophils, and monocytes. In addition, hyperproliferation of synovial fibroblasts induces synovial hyperplasia and pannus formation and is accompanied by induction of angiogenesis. These pathological processes are largely mediated via the actions of cytokines and chemokines.

susceptibility to opportunistic infections upon systemic anti-TNF treatment [22-24]. Third, thirty percent of RA
patients fail to respond to TNF blockers suggesting alternative molecular pathways underlying pathogenesis in these patients. Fourth and last, the required continuous mode of treatment makes anti-TNF therapies relatively costly. To tackle these issues, local and sustained delivery of biologicals to arthritic joints appears an ideal treatment alternative. Over the last decade, gene therapy has emerged as a promising approach for achieving these goals.

Gene Therapy for RA

Gene therapy comprises the transfer of a gene product into a patient's cell in order to treat a disease. Monogenic diseases caused by single gene disorders were the first to be considered to be treated by insertion of a functional copy of the affected gene into target tissues [25]. Consecutively, transfer of therapeutic genes has been evaluated for a large number of lethal and non-lethal diseases including cancer, neurodegenerative diseases, diabetes and RA.

In terms of both safety and efficacy, the relatively confined joint space appears ideally suited for a local treatment based on gene therapeutic delivery of intracellular or secreted gene products such as short hairpin RNAs and proteins. Synovial fibroblasts make up the largest population of resident cells in the synovium (**Figure 1**) and are considered as the primary target cells for *ex vivo* and *in vivo RA* gene therapy. These are readily transducible by several viral vectors including retrovirus, adenovirus, adeno-associated virus, lentivirus and potentially sustain long-term transgene expression [26-31]. Initial proof of principle has been established by successful treatment of experimental arthritis based on local gene transfer of interleukin-1 receptor antagonist (IL1Ra) [26,32]. These encouraging studies were followed up by local delivery of IL-4 and IL-10 as cartilage-protective and anti-inflammatory cytokines, respectively [31,33-35]. Currently, a substantial number of gene therapy approaches has convincingly shown therapeutic efficacy in experimental arthritis, based on cytokine blocking, inhibition of angiogenesis, induction of synovial apoptosis and targeting of signal transduction pathways [36].

Recently, a Phase 1/2 clinical study of intra-articular adeno-associated virus serotype 2-mediated delivery of a human TNFα receptor-immunoglobulin Fc fusion gene has been completed [37]. The study demonstrated safety of local viral vector delivery and showed an improvement of clinical outcome measurements in treated patients. An alternative approach developed by Evans and coworkers [28,38], based on engraftment of autologous synovial fibroblasts retrovirally-transduced *ex vivo* with the IL-1 receptor antagonist gene, resulted in reduction of joint pain and swelling and prevented flares of joint inflammation [39]. Despite the optimistic results arising from these clinical studies, gene therapy for RA remains in its infancy and could still profit greatly from innovations in vector design and definition of novel targets.

Regulated Transgene Expression in RA Gene Therapy

A major advantage of a local gene therapeutic treatment for RA is that it allows precisely regulated transgene expression using a transcriptional targeting strategy. This strategy is based on the application of promoters that display preferential activity in specific cell types or under specific conditions, such as inflammation. The rationale for using transcriptional targeting for RA gene therapy is twofold. First, the course of RA is fluctuating rather than continuous resulting in periods of increased disease activity or flares alternating with periods of relative quiescence. Ideally, the induction of transgene expression parallels these flares in an offer-meets-demand fashion. Second, with an emphasis on safety transgene expression should be turned off after having effectively suppressed arthritis. Munford and Varley were the first to develop a transcriptional targeting strategy for inflammation [40]. The strategy was based on a two-component expression construct in which the murine complement 3 promoter regulates production of HIV-Tat protein, which in turn stimulates expression of a transgene downstream of a HIV-LTR promoter (C3-Tat/HIV). Our group and Miagkov *et al.* simultaneously demonstrated therapeutic efficacy in experimental arthritis of intra-articular C3-Tat/HIV-driven expression of human IL1Ra or IL-10, respectively [41,42]. However, the immunogenicity of HIV-Tat protein and its transactivating potential of host genes [43,44] hampered its application for gene therapy. This has led to the development of alternative transcriptional targeting strategies using both synthetic and endogenous promoter constructs, such as the hybrid IL-1 enhancer/IL-6 promoter [45,46].

Promoters and Signal Transduction

A promoter is defined as the *cis*-regulatory DNA region upstream of a gene that regulates timing, spacing and the level of its transcription under given environmental conditions. Roughly, a promoter can be divided into a core-, proximal-, and distal-promoter region. The core-promoter encompasses the 80–100 base pair (bp) region surrounding the transcription start site (TSS) and is required for assembly of the pre-initiation complex that contains RNA polymerase II [47]. The proximal-promoter (up to 500 bp upstream) contains pivotal *cis*-regulatory elements that interact with *trans*-acting transcription factors (TFs) and largely determine cell- or context-specific gene expression [48,49]. The distal-promoter together with distal up/downstream enhancers/silencers (up to 100 kB) further regulate the complex expression patterns of genes *in vivo.*

In RA, numerous stimuli trigger intracellular signaling cascades leading to activation of a specific set of TFs that bind the promoter and induce expression of pro-inflammatory genes (**Figure 2**). Synovial fibroblasts are potently activated by cytokines, such as IL-1 and TNF , and exogenous and endogenous toll-like receptor (TLR) 2, 3 and 4 ligands [8,11,12,50-53]. After ligation of their respective receptors, expression and secretion of pro-inflammatory mediators including cytokines, chemokines and matrix metalloproteinases (MMPs) is induced through multiple signal transduction pathways including nuclear factor-κB (NF-κB) and the mitogen-activated

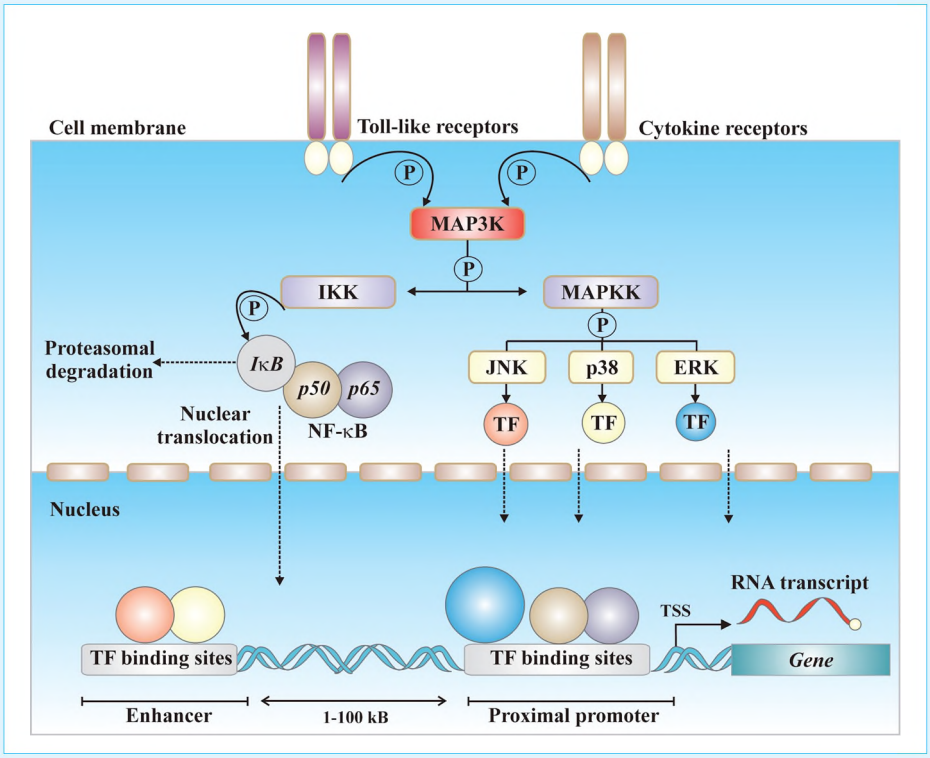

Figure 1.2

Inflammatory signal transduction leading to induction of gene transcription

Upon ligand binding receptor-associated kinases induce phosphorylation of MAP3Ks. Consecutively, these phosphorylate their respective targets including IKK and MAPKKs. IKK induces degradation of the inhibitor IκB, which enables nuclear translocation of transcription factor NF-κB. MAPKKs phosphorylate their downstream targets JNK, p38 and ERK ultimately leading to activation and nuclear translocation of several transcription factors. In the nucleus, these factors bind their cognate sequences in enhancer and promoter regions thereby inducing transcription of downstream genes.

P = phosphorylation, **TF** = transcription factor, **TSS** = transcription start site.

protein kinase (MAPK) families: p38, *c*-Jun-N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK) [54]. The latter kinases are regulated through phosphorylation by their upstream MAPK kinases (MAPKK). NF-κB signaling is regulated through phosphorylation of the inhibitor of kappaB (IκB) by IκB kinase (IKK), which leads to proteasomal degradation of the prior. In turn, MAPKK kinases (MAP3Ks) control the activation of IKK and MAPKK and are activated through interactions with receptor-associated proteins, such as IL-1R-associated kinases (IRAKs) and TNFR-associated factors (TRAFs). The MAP3K family comprises numerous members of which MEK kinase-1, -2 (MEKK1,-2) and transforming growth factor-β activated kinase 1 (TAK1) are most abundantly expressed in RA synovial fibroblasts [55]. Ultimately, signal transduction pathways lead to activation of TFs that translocate to the nucleus and cooperatively induce transcription of their target genes. Pivotal TFs in pathogenesis of human and experimental arthritis include activator protein-1 (AP-1), CAAT/enhancer-binding proteins (C/EBPs), NF-κB and signal transducers and activators (STAT)-1 and -3 [56-58].

Outline of the thesis

Despite its potential for addressing safety and efficacy of gene therapy approaches for RA, transcriptional targeting strategies are only scarcely explored. Therefore, the aim of the present thesis was to rigorously design a transcriptional targeting strategy for RA and evaluate its applications in gene therapeutic treatment and diagnostics.

In *Chapter 2* we have reviewed the status of vectors in RA gene therapy with an emphasis on targeting resident cells of the joint. Among the viral vectors reviewed were adenovirus and lentivirus, which we used as gene transfer vectors in *Chapters 3-7*.

In *Chapter 3* we designed proximal-promoters for transcriptional targeting using a computational approach on a microarray study of murine collagen-induced arthritis. We found an enrichment of NF-κB, AP-1 and C/EBPβ TF binding sites in the promoters of genes upregulated during arthritis. Nine identified promoters were responsive to pro-inflammatory stimuli *in vitro* and based on its excellent transcriptional properties the serum amyloid A3 *(Saa3)* promoter was selected as a promising candidate for transcriptional targeting.

In *Chapter 4* we explored the application of the computed promoters as a diagnostic tool for assessing joint inflammation in experimental and human arthritis. Analyzing promoter responses in flares of streptococcal cell wall-induced arthritis and synovial fibroblasts of rheumatoid arthritis patients, we found that specifically a *Saa3*-promoter could distinguish between inflammatory subtypes.

In *Chapter 5* we investigated the impact of transcriptional targeting on safety of RA gene therapy. To this end, we evaluated the effects of constitutive and transcriptionally-targeted IL-4 gene therapy in healthy and arthritic joints. Transcriptional targeting showed therapeutic

efficacy in arthritis while preventing transgene-induced side effects under healthy conditions, thereby providing proof-of-principle that it is a safer mode of gene therapy.

Chapters 6 and *7*, describe two novel gene therapy approaches based on RNA interference-mediated knockdown of TNF receptor-1 and targeting multiple signaling cascades though inhibition of transforming growth factor-β-activated kinase 1 (TAK1).

Chapter 8 reviews recent developments and future perspectives of transcriptional targeting in gene therapy approaches for autoimmune and inflammatory disease, in particular RA.

In the final chapter, the results described in this thesis are summarized and discussed.

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Gene therapy works in animal models of rheumatoid arthritis … *so what!*

2. Gene therapy works in animal models of rheumatoid arthritis … *so what!*

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Abstract

Rheumatoid arthritis (RA) is a systemic disease with polyarticular manifestation of chronic inflammation in the knees and small joints of hand and feet. The current systemic anti-tumor necrosis factor (TNF)-α therapies with biologicals ameliorate disease in 60% to 70% of RA patients. However, biologicals must be given systemically in relatively high dosages to achieve constant therapeutic levels in the joints, and side effects have been reported. To this end, local gene delivery can provide an alternative approach to achieve high, long-term expression of biologicals, optimizing the therapeutic efficacy and minimizing systemic exposure. Evidence from animal models convincingly supports the application of local gene therapy in rheumatoid arthritis, but preclinical studies remain necessary to evaluate the merge of cell-specific targeting, viral vector development, and disease-regulated transgene expression to optimize efficacy and safety.

Introduction

For the title of this review, we have chosen a provocative remark sometimes raised by preclinical gene therapy (GT). We hope this shows the eagerness to proceed into humans. The implementation of GT in the clinic will be delayed until researchers can determine whether results from GT trials in experimental arthritis models is sufficient to turn this technology into the long-promised magic bullet.

In 1990, the first GT clinical trial was approved, and the technology rapidly emerged as an innovative form of treatment for inherited and acquired genetic disorders such as cancer [1]. The principles of GT as designed in the 1990s became the foundation of GT in preclinical practice of today. Originally, GT was used to transfer genes in order to supplement a gene product for several purposes: 1) to restore a deficiency; 2) to overcome, counteract, or correct a defect; or 3) to restore the normal physiology and function of cells and organs. The first phase I clinical trial in RA, initiated in Pittsburgh in 1995 by Evans *et al.* [2], used the *ex vivo* method. Autologous synovial fibroblasts from the metacarpophalangeal (MCP) joints were transduced *in vitro* with a derivative of the Moloney murine leukemia virus (MLV) encoding human IL-1Ra, and then they were transplanted back into the MCP joints. Nine patients underwent this treatment at the time of joint replacement. No adverse effects were reported, and 11 of 12 joints expressed *IL-1Ra*

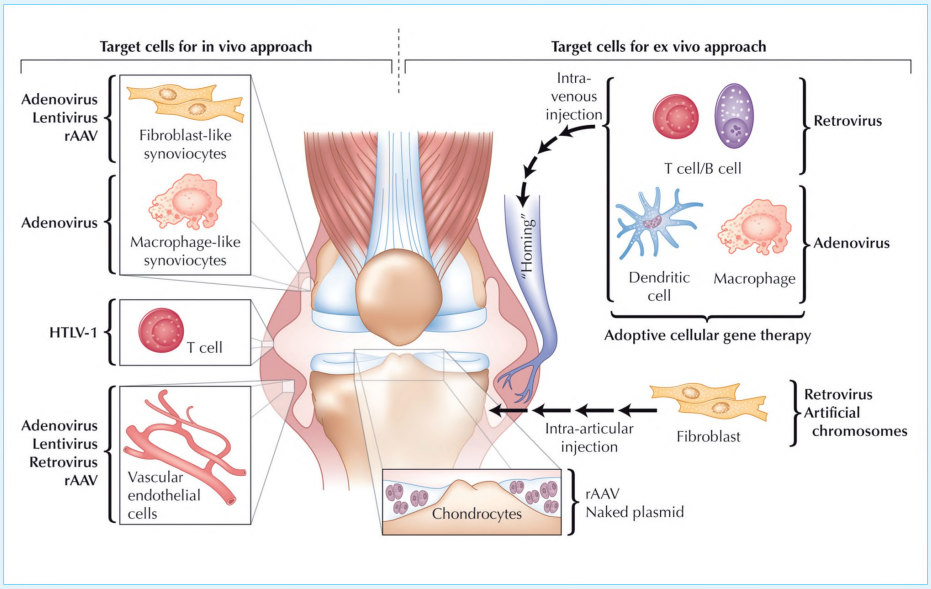

Figure 2.1

Gene transfer methods and targeted cells in experimental arthritis

Schematic presentation of the various gene transfer methods and their targeted cells that are used in experimental models of rheumatoid arthritis. In the in vivo gene transfer approach (left panel), vectors are injected either locally (intra-articular, peri-articular) or systemically (intramuscular, intravenous). Fibroblast-like synoviocytes are most efficiently transduced in vivo by a large variety of viral vectors while other resident cells (e.g. macrophages or T-cells) require specific viruses or adaptor molecules for transduction. In the *ex vivo* gene transfer approach (right panel), autologous fibroblasts, antigen-presenting cells (i.e. dendritic cells, macrophages, or B cells) or T-cells (primary or hybridoma cells) are transduced virally and consecutively transferred in the animal intra-articularly (fibroblasts) or intravenously (adoptive cellular gene therapy). HTLV-1—human T-cell lymphotropic virus type 1; rAAV—recombinant adeno-associated virus.

one week after transplantation. However, this *ex vivo* method requires a laborious and costly procedure to obtain and culture autologous cells.

On the other hand, direct injection of genes into the inflamed joint is simple, and several viral and nonviral vehicles are applicable and under review. Using this direct approach, a phase I dose escalation study in RA patients started in 2003 examining the intra-articular administration of tgAAC94, a recombinant adeno-associated virus (AAV) containing the tumor necrosis factor receptor-Fc *(TNFR:Fc)* immunoglobulin fusion gene [3]. No significant safety issues were noted in the 14 RA patients treated in the first two cohorts, and a second phase I/II was approved and

initiated in 2005. The completion of two phase I clinical trials of GT in RA in the past 16 years is not a success story.

The field of GT suffered a major setback in 1999, when 18-year-old Jesse Gelsinger died during a clinical trial to treat a genetic liver disorder. Then, approximately 2 years later, two of 10 boys enrolled in a French GT trial to cure their X-linked severe combined immunodeficiency (SCID) syndrome developed leukemia after initial encouraging results [4]. (A third patient later developed the disease.) These events set the entire GT field back, especially in non–life-threatening diseases such as RA. Meanwhile, the development of this sophisticated approach continued to show that GT in animal models of RA works.

Gene Therapy Approaches for Arthritis

In RA, the whole spectrum of inflammatory cells is present in the inflamed joint. These cells, together with the resident cells of the synovial membrane, are all potential targets for GT (**Figure 1**). For this application, local gene transfer into the inflamed joint is the most likely approach. Successes have been reported using different vectors (viral and nonviral), route (local and systemic), and mode of administration (injection, gene-gun, electroporation), and using a large arsenal of therapeutic transgenes. For cell-killing (also called suicide GT, gene scalpel, or genetic synovectomy), the emphasis is on cell-targeting depending on the receptor-binding characteristics of the vector, and only short-term expression is required. Whereas for modulation studies (e.g. restoring the cytokine balance), the emphasis lies on long-term transgene expression rather than cell-specificity. Since joint inflammation in RA patients often shows a relapsing course, transcriptional regulated transgene expression will be more efficacious.

Targeting Inflammatory Cells

Cells of the adaptive immune system

The *ex vivo* method of transplanting genetically modified immune cells into recipient mice is called adoptive cellular GT (ACGT). In this approach, isolated primary spleen cells, T-cells (T-cell hybridomas), and antigen presenting cells (i.e. dendritic cells [DCs], B-cells, macrophages) are virally transduced and transferred in the arthritic animal. Two adoptive transfer strategies have been shown to cause significant immune changes and ameliorate disease: 1) collagen-specific T-cell hybridoma transduced to express anti-TNF single chain antibodies or IL-12 p40, and 2) splenocytes from arthritic animals expressing soluble TNFR or transforming growth factor (TGF) ß [5–8]. Adoptive transfer of antigen-specific T-cell hybridomas, antigen-pulsed B-cells, or macrophages transduced to express *IL-4* home to the joint and exert a prophylactic effect that relies on the anti-inflammatory property of *IL-4* [9,10].

An effective approach in the treatment of collagen-induced arthritis (CIA) is the eradication of antigen-specific T cells by transfer of antigen-pulsed DCs that overexpress *TRAIL* or *FasL* [11,12]. Since DCs do not migrate to the joint, they probably exert their effect in the spleen and lymph nodes. The most sophisticated approach came from Zhang *et al.* [13]. They developed a dual ACGT strategy, in which they transferred collagen type II pulsed antigen-presenting

cells adenovirally transduced to express either 1) *FasL* to eradicate antigen-specific T-cells, or 2) transmembrane activator and calcium modulator and cyclophilin ligand interactor (*TACI*) to block antigen-specific B-cell activation. The dual ACGT completely prevented murine CIA, but a therapeutic regimen failed to affect established disease. Overall, the implementation of ACGT to treat early RA needs further preclinical investigation and more importantly requires knowledge of the putative autoantigen.

T-cells

In vivo targeting of T lymphocytes in the inflamed joint will be difficult, since they reside in the synovial sublining layer, often as lymphoid aggregates forming ectopic germinal centers that are less accessible to (viral) vectors. *In vitro*, vectors derived from oncoretroviruses such as the MLVs are frequently used as tools to transduce T cells. However, these viruses cannot transduce nonproliferating cells, probably due to post entry-block [14]. Activation of the T-cell receptor (TCR) by anti-CD3 and the CD28 coreceptor stimulates proliferation and renders these resting T cells permissive for retroviral transduction [15]. Lentiviral vectors (LV) can transduce both resting and dividing cells. However, costimulation is still needed, because T cells must enter the G1b phase of the cell cycle to facilitate transduction [15]. Maurice *et al.* [16] genetically engineered HIV-based lentiviruses that display a single chain of the anti-CD3 OKT monoclonal antibody on the surface, and this strongly increased transduction of T-lymphocytes.

Direct delivery of oncoretroviruses into the joint resulted in poor transduction of the synovium, which was greatly improved by lentiviruses; however, transduction of the infiltrating T cells was not demonstrated in these studies [17]. Even excessive amounts of adenoviruses or AAVs only achieve moderate transduction of T lymphocytes [18,19]. Human T-lymphotropic virus type 1 (HTLV-1) is related to deltaretroviruses and has a natural tropism for CD4+ T cells [20]. Furthermore, HTLV-1 antigens and immunoglobulin (Ig) M antibodies are found in chronic inflammatory arthropathies, and transgenic mice carrying the env-Px region of HTLV-1 spontaneously developed inflammatory arthropathies [21]. For this, HTLV-1 can be a valuable vector tool for T-cell–specific gene delivery in the inflamed joint of RA.

Cells of the innate immune system: Macrophages and neutrophils

Macrophages are abundantly present in inflamed joints, and the type A synovial lining cells are tissue-specific macrophages. Macrophages are prime target cells of GT in arthritis, as they produce proinflammatory cytokines and cartilage destructive enzymes. Macrophages express constitutively high levels of the high-affinity Fcγ receptor I. This receptor can be targeted selectively using a bispecific hybrid adapter protein consisting of the amino-terminal extracellular domain of the human CAR protein and the Fc region of the human IgG1 protein. Up to a 250-fold increase of transgene expression in CAR-negative human monocytic cell lines expressing the high-affinity Fcγ receptor I (CD64) was achieved with adenoviruses in the presence of this adaptor molecule [22]. Therefore, adaptor molecule mediated targeting should be explored in experimental arthritis. Macrophages also express CD44, and the introduction of heparan sulfate binding moieties on

fiber coat proteins facilitates adenoviral uptake via CD44. Intra-articular delivery of these modified adenoviruses into the rat ankle joint with adjuvant induced arthritis leads to enhanced transgene expression due to inflammation-induced upregulation of CD44 [23].

Large numbers of neutrophils are present in the RA synovial fluid, and they release large amounts of matrix-degrading enzymes and proinflammatory mediators. However, the lifespan of these cells is short due to apoptosis, making these cells poor targets for GT approaches. More importantly, these cells can scavenge viral vectors very efficiently due to their opsonization with antibodies and complement, thereby reducing the transduction of the synovium [24].

Targeting Resident Articular Cells

Synovial fibroblast-like cells

The RA fibroblast-like synoviocytes (FLS) are resident cells that also produce proinflammatory cytokines and matrix-degrading enzymes, as exemplified by the invasive pannus tissue that destroys cartilage and bone. RA-FLS do not express CAR and are poorly permissive to adenoviruses for the most commonly used serotype 5 (Ad5). Adenoviruses from subgroup B (serotypes 11, 16, and 35) do not use CAR for infection of cells. Chimeric adenoviruses of serotype 5 but carrying fibers from subgroup B are superior in transducing human synoviocytes [25]. Interestingly, 70% to 90% of RA patients have neutralizing antibodies against serotype 5 in their synovial fluid, whereas this is less frequent for serotype 35 [26].

CAR-independent cell entry of adenoviruses can be realized by modification of the fiberknob through introducing the arginine-glycine-aspartic (RGD) acid motif in the H1 loop. RGD-binding integrins such as αVβ3 and αVβ5 are expressed on FLS and other CAR-deficient cells as lymphocytes and macrophages [27]. We demonstrated that RGD-modified Ad5 were more efficient in transducing the murine synovial tissue, and that the higher *IL-1Ra* transgene expression results in a more efficacious treatment of CIA [28]. It is known that the members of adenovirus subgroup B have naturally short fibers. Hence, shortening the shaft of the adenoviral fiber from 22 to 7 fiber shaft repeats or ablating the fiberknob markedly enhanced (40- to 50-fold) the transduction of CAR-negative rheumatoid synovial fibroblasts, CD14+ macrophages, and CD3+ lymphocytes *in vitro* and murine synovial tissue *in vivo* [29]. Pro-inflammatory cytokines and joint inflammation further enhanced the transduction with these modified adenoviruses, due to upregulation of the integrins by inflammation. Furthermore, these shaft-modified adenoviruses partially escaped viral neutralization by human synovial fluid [29].

The adenoviral genome resides as episomal DNA in the nucleus and is lost during cell division, but under naïve conditions it remains in the murine knee joint for over 12 weeks. However, with the conventional constitutive promoters the transgene expression was short-lasting. As a result, adenoviruses are more suitable for the delivery of pro-apoptotic ("killer") genes when genetic synovectomy is required and for proof-of-principle studies in animal models of RA.

In contrast to retroviruses, LV and AAV do not need cell proliferation for efficient transduction of the synovium. Most commonly used self-inactivating (SIN)-LV are derived from HIV type 1 pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G). The advantage of

VSV-G over other retroviral envelope proteins is its broad host range, enabling infection into a wide variety of cell types [30]. Direct injection of VSV-G pseudotyped SIN-LV into rat or mouse knee joint cavities results in transduction of the synovial lining cells, but transduction of all synovial cell layers was seen when injected into inflamed joints [31]. Transgene expression peaked at day five after injection and declined thereafter to approximately 30% at day 21 [17]. Human primary synoviocytes, rabbit HIG-82 synovial fibroblasts, rat skin fibroblasts, rat synovial fibroblasts, and mouse NIH-3T3 fibroblasts can efficiently be transduced by SIN-LV, whereas RAW 264.7 macrophages and EL4.NOB1 thymoma were poorly transfectable (unpublished data). This suggests some selectivity of VSV-G pseudotyped LV for fibroblasts-like cells.

Currently, AAV is the first choice for gene transfer in arthritis, since no AAV-based pathology is found in humans. AAV is a non-enveloped, single stranded (ss) DNA virus of the human parvovirus family. The 4.7 kilobase (kb) AAV genome contains two open reading frames, rep and cap, which are flanked by 145 base pair (bp) inverted terminal repeats (ITRs). These ITRs comprise the origins of replication and the packaging signal, and they serve as cis elements for chromosomal integration. The recombinant AAV vector is devoid of viral genes and only possesses the ITRs, thereby becoming replication incompetent and non-immunogenetic, an advantage over other viral vectors used in GT. Twelve human and nonhuman primate AAV serotypes are known to exist, and their heterogeneity in capsid proteins determines their different host range and tissue tropism: muscle (AAV1/2/10), liver (AAV7/8), and retina (AAV4) [32]. Most studies in experimental arthritis are done with AAV2, and the main cell-type transduced by local application is the synovial lining cells with conflicting reports on chondrocyte transduction. AAV vectors provide long-term expression after local application in the inflamed joint.

Interestingly, the AAV-*IL-1Ra* transgene expression in naïve rat knees was undetectable, but even 100 days later, it could be upregulated by lipopolysaccharide-induced joint inflammation [33]. The transduction ability of AAV is species specific. AAV can enter mouse fibroblasts but is retained in the early endosomes due to impaired viral trafficking to the nucleus [34]. Goater *et al.* [35] found that the low AAV transduction of the synovium was significantly enhanced in the *TNF*-transgenic mice and correlated with joint damage. Direct injection of the AAV virus into the arthritic joint showed considerably higher expression of the *IL-4* transgene than injection into a naïve joint [36]. Thus, recombinant AAV vectors are even more attractive for GT, because they give rise to low basal levels of transgene that increase with inflammation. The clinical efficacy of local GT with rAAV-*sTNFR* has been demonstrated in both spontaneous arthritis in TNF-transgenic mice and in streptococcal cell wall (SCW) arthritis in rats [37,38].

Eighty percent of humans have antibodies directed against AAV2, and 30% express neutralizing antibodies, which may hamper its therapeutic application in RA patients. The capsid protein of AAV5 shares the lowest homology (60%) with all the other AAV serotypes, and antibodies against AAV5 are rarely found in humans.

Apparailly *et al.* [39] compared the efficiency of AAV2 with a chimeric AAV2/5 (i.e. an AAV2 pseudotyped with capsids of AAV5) for transduction of the murine arthritic joint. In knees with CIA, both AAV2 and AAV2/5 infected the synovial lining tissue and adjacent bone marrow but not

the cartilage, bone, or muscle. Quantitative analysis of transgene expression showed markedly enhanced expression with the cross-packaged AAV2/5. The same investigators also examined this apparent difference in transduction efficiency between AAV2 and AAV2/5 in a rat adjuvant arthritis model after local delivery in the inflamed ankle joints [40]. Again, the synovial tissue was the dominant transduction site using AAV2/2 and AAV2/5, in particular the fibroblasts. In addition, AAV2 pseudotyped for capsids of AAV1, 3, and 4 failed to transduce the joint.

Strikingly, both studies showed similar transduction efficiencies *in vitro* between AAV2 and AAV2/5 for different human cell-types: chondrocytes, osteocytes, myoblasts, and primary synoviocytes [39,40]. This finding was unexpected, since AAV2 uses heparan sulfate proteoglycans, integrins, and fibroblast growth factor receptor-1, whereas AAV5 uses N-linked sialic acid and the platelet growth factor receptor for cell binding and internalization. Furthermore, it does not explain the profound improved transduction properties of AAV2/5 in mice. Species differences could be the underlying reason, and Gao *et al.* [32] found that AAV2/5 raised higher titers of neutralizing antibodies than AAV2/2 in the rabbit. This finding warrants further investigation in humans.

Chondrocytes

Cartilage pathology is striking in RA, and chondrocytes play a pivotal role in the underlying processes. Therapeutic options include either reducing the synthesis of cartilage catabolic cytokines (eg, *IL-1*) and destructive enzymes (eg, metalloproteinases) or enhancing the anabolic capacity of chondrocytes in an attempt to counteract the matrix loss. Therefore, targeting the chondrocytes with GT is a valid option.

In vitro, isolated articular chondrocytes are easy to transduce with all viral vectors known today. *In vivo*, these cells are sealed in a complex and dense pericellular matrix. Based on their physical size, it is highly unlikely that AAV, adenoviruses, and LV with respective diameters of 20 nm, 80 nm, and 100 nm can transduce chondrocytes. Controversially, it was reported that AAV can transduce chondrocytes, probably related to the extent of cartilage damage [35,41,42]. To overcome this barrier effect, Grossin *et al.* [43] injected naked plasmid DNA into the rat knee joint, and they were able to transduce 30% to 50% of chondrocytes in the patella at all depths of cartilage using electroporation.

Vascular endothelium

Angiogenesis is an ongoing process in the deeper layers of the inflamed synovium, and it plays a central role in the perpetuation of chronic synovitis. The vascular endothelial growth factor (*VEGF*) promotes angiogenesis. Intravenous injection of adenoviruses expressing human soluble VEGF receptor 1 at the onset of murine CIA significantly reduced paw swelling and disease severity [44]. TNF is a proangiogenic cytokine, and direct injection of a LV expressing endostatin, an antiangiogenic peptide derived from collagen XVIII, into the joints of huTNF-transgenic mice before arthritis onset reduced vascularization and overall arthritis index 8 weeks later [45]. Transplantation of retroviral transduced NIH3T3 cells expressing angiostatin gene, an internal

fragment of plasminogen, into the knee joint of DBA1/J mice before onset of CIA dramatically prevented angiogenesis. As a result, pannus formation and cartilage erosion are reduced [46].
The enzyme plasmin is essential in endothelial cell migration, and systemic adenoviral-mediated overexpression of urokinase plasminogen activator receptor molecule also prevents angiogenesis and as a result ameliorates CIA [47]. The vascular endothelium of new blood vessels expresses high levels of αVβ3 and αVβ5 integrins, which can be targeted by adenoviruses with RGD-modified fiberknobs [27]. Recombinant AAV1 and AAV5 efficiently transduce vascular endothelial cells via 2,3-linked sialic acid, which is their high affinity receptor on endothelial cells. White *et al.* [48] changed the AAV2 capsid by introducing phage display selected vascular endothelium binding peptides. The capsid-modified AAV showed a different tropism with reduced hepatic uptake and enhanced uptake by the vena cava after systemic delivery. This study showed proof of principle, but at present, no published data exists showing the targeting of newly formed blood vessels in arthritis.

Fine Tuning GT in Arthritis by Regulated Gene Expression

Clearly, different cell-types/organs involved in arthritis can be targeted, but the stringency of vector choice will depend on the therapeutic approach, the safety of the vector, and the route of application. The major challenge is a disease-regulated expression of a recombinant protein to meet the variable demands during RA (i.e. high expression during relapse and low expression during disease remission). Target cells must remain transduced for a long period of time to avoid frequent readministration.
Several vectors allow long-term expression because of either integration into the host genome or the formation of self-replication genomic particles [22]. The disadvantage of integration vectors, as with retroviruses, is the random integration into the host genome with the potential for activating oncogenes (called insertional mutagenesis). This risk became painfully apparent in the French GT trial for SCID-X1, in which three patients to date have developed cancer [49]. Wild-type AAV2 integrates at a specific locus on chromosome 19 in human cells through the activity of a specific replicase/integrase protein (Rep) of AAV. However, recombinant AAV2 is devoid of Rep and only integrates into the host genome at low frequencies. Recombinant AAV2 integration in mouse liver and cultured human cells was favored in actively transcribed genes, which can lead to small deletions of host chromosomal DNA [50]. This is worrisome and may have consequences for risk/benefit considerations, especially with the reported enhanced liver metastasis in 18-month-old mice that received a single AAV injection early in their lives [51]. Stable transgene expression primarily results from extrachromosomal localization of the genome of either circular or linear concatemers. A recent advantage is the creation of self-complementary duplex strand structures of the AAV genomes. These self-complementary AAV genomes do not need second strand synthesis and support stable and long-term transgene expression *in vivo* [52].
Excessive production of the transgene and spill-over to the system may cause serious side effects, as seen in some patients receiving anti-TNF biologicals. Ideally, gene transfer vectors must auto-regulate transgene expression in relation to the disease activity, turned on by relapse and

turned off when in remission. Varley *et al.* [53] showed that the promoters of the acute phase proteins *C3* and serum amyloid A (*Saa*) are particularly attractive candidates for transcriptional regulation of cytokine inhibitors. A two-component expression system was created, in which the *C3*-promoter regulates the production of the HIV transactivator of transcription (*Tat*) protein, which in turn regulates the HIV long terminal repeat (LTR) promoter to express the transgene of interest [54]. Miagkov *et al.* [55] and we [56] independently demonstrated the feasibility of this two-component system in adenoviral vectors for auto-regulated expression of *IL-10* and *IL-1Ra* in two different experimental arthritis models. In a prophylactic regimen, we demonstrated a superior effect of this two-component *IL-1Ra* expression system in murine CIA. These two *in vivo* studies showed proof of principle of the efficacy of a disease-regulated expression system in arthritis.

However, this promoter may not be suitable for RA, because the Tat is a foreign immunogenic protein and may have undesirable effects by transactivating host genes [56]. For future clinical application, novel disease-regulated promoters will be developed based on the expression of endogenous genes at the site of injury/inflammation. We demonstrated that a hybrid promoter consisting of the human *IL-1* enhancer region in front of the human *IL-6* promoter fulfills the criteria of a disease-inducible promoter: low basal transcriptional activity that is highly upregulated during acute joint inflammation and the flare-up of SCW arthritis [57]. This system was used successfully to auto-regulate expression of cartilage protective levels of *IL-4* in mouse CIA (unpublished data). This showed that controlled transcriptional regulation is a feasible GT approach for auto-regulated protein drug treatment in experimental arthritis. In the future, a combined system of disease-inducible/drug-regulated transgene expression will provide auto-regulation with the possibility to intervene from the outside as an extra safety measure. Gould *et al.* (personal communication) are exploring so-called hybrid promoters (e.g. a minimal CMV promoter with seven upstream tetO elements combined with four hypoxia responsive elements) to obtain a dual responsiveness to both pharmacological and pathophysiological regulation.

The non-immunogenic recombinant AAV vectors also show an attractive transgene expression profile even with constitutively active promoters, as reviewed earlier. The autonomously regulated transgene expression in AAV-transduced human RA-synovial fibroblasts was studied in more detail by Traister *et al.* [58]. To bypass the second-strand synthesis, they used double-stranded AAV vectors and consistently obtained higher transgene expression as compared to single stranded AAV2. Nevertheless, they found that with RA-synovial fluid or cytokines, the expression of different transgenes (*sTNFR-Ig, IL-10*, or *GFP*) was upregulated stimulated even when constitutive viral and non-viral promoters (CMV or chicken β-actin) were used for driving the transgene expression. The transgene expression returned to basal levels upon withdrawal and could be reinduced by re-exposure to these cytokines in a phosphatidylinositol 3-kinase dependent manner. Evidence shows that this could be due to either viral uncoating and trafficking or increased mRNA stability. However, a more likely explanation for this inducible expression is the favored integration site of AAV into active and potentially regulated genes. The observed slow response to cytokines and

arthritis (3–7 days to turn on or off) and the unknown long-term effects of AAV integration into the host genome are features that must be investigated in more detail before its application in GT for arthritis.

Conclusions

The field of vector development is ongoing. Recent novelties include the hybrid vectors of LV genome in an adenoviral backbone and the integration cassette of AAV in the helper-dependent adenoviral vector [59,60]. These hybrid vectors combine the efficient gene transfer and large packaging capacity of an adenoviral vector with stable integration from LV or AAV. The random integration of LV into the host genome can be dangerous. To avoid insertional mutagenesis, researchers have developed novel integrase-defective LVs that remain episomal as circular double-stranded viral DNA [61]. In cells that are essentially post-mitotic (eg, muscle, liver, brain, and retina), the episomal DNA does not need to replicate for long-term expression. Artificial chromosomes are advantageous in that they are non-integrating, have mitotic stability and can host multiple genes. Until now, the *in vivo* transfer of these large DNA strands (1–2 microns) is inefficient but can be used in the *ex vivo* gene delivery approach, as has been demonstrated recently in experimental arthritis [62].

Drug- and/or disease-regulated transgene expression will increase the efficacy and safety of local GT. Many strategies have demonstrated efficacy in preclinical studies: several transgenes, constitutive or regulated promoters, viral or nonviral vectors, and local or systemic delivery routes. However, no standardized side-by-side comparisons of different delivery systems have been performed. No consensus exists on the models used in reviewed studies, but the transplantation of synovial tissue biopsies into SCID mice could be a relevant model [63].

Gene therapists are considerably optimistic on the clinical approval of two GT cancer drugs in China based on the adenoviral delivery of the tumor suppressor gene *p53* [64]. In contrast, rheumatoid arthritis is a more complex autoimmune disease, and the success of GT will not only depend on our understanding of the pathophysiological processes underlying this disease but also on extensive preclinical research to test suitable transgene delivery and expression systems. The recent mouse study revealing the development of T-cell lymphomas in 33% of animals treated with the *IL-2RG* (as used in the French SCID trial) 1.5 years post-transplantation underlines the necessity of proper preclinical GT studies [65].

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Computational design and application of endogenous promoters for transcriptionally targeted gene therapy for rheumatoid arthritis

3. Computational design and application of endogenous promoters for transcriptionally targeted gene therapy for rheumatoid arthritis

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Abstract

The promoter region of genes that are differentially regulated in the synovial membrane during the course of rheumatoid arthritis represent attractive candidates for application in transcriptionally targeted gene therapy. In this study, we applied an unbiased computational approach to define proximal-promoters from a gene expression profiling study of murine experimental arthritis. Synovium expression profiles from progressing stages of collagen-induced arthritis were classified into six distinct groups using *k-means* clustering. Using an algorithm based on local over-representation and comparative genomics, we identified putatively functional transcription factor binding sites in TATA-dependent proximal-promoters. Applying a filter based on spacing between TATA box and transcription start site combined with the presence of over-represented nuclear factor-κB (NF-κB), AP-1, or CCAAT/enhancer binding protein β (C/EBPβ) sites, 382 candidate murine and human promoters were reduced to 66, corresponding to 45 genes. *In vitro*, nine out of ten computationally defined promoter regions conferred cytokine-inducible expression in murine cells and human synovial fibroblasts. Under these conditions, the serum amyloid A3 (*Saa3*) promoter showed the strongest transcriptional induction and strength. We applied this promoter for driving therapeutically efficacious levels of the interleukin-1 receptor antagonist (*Il1rn*) in a disease-regulated fashion. These results demonstrate the value of bioinformatics for guiding the selection of endogenous promoters for transcriptional targeted gene therapy.

Introduction

Selective spatial and temporal expression of a therapeutic transgene has a major impact on the safety and efficacy of gene therapeutic treatments for human disease. This can be accomplished by transcriptional targeting using endogenous or synthetic *cis*-regulatory DNA regions, which facilitate tissue/cell-specific or physiologically-regulated expression. Predominantly in cancer

gene therapy, numerous tumour/tissue-specific or drug/radiation-inducible promoters have been developed and applied [1].

With emphasis on the discontinuous clinical course of rheumatoid arthritis (RA), which is characterized by spontaneous remissions and exacerbations of joint inflammation, transcriptionally targeted gene therapy appears ideally suited. The few endogenous promoters that have been tested in RA thus far were empirically derived. Efficacious inflammation-responsive expression in experimental arthritis has been accomplished using the promoter elements of the murine acute-phase response gene complement factor 3 *(C3)* [2,3] and the human cytokine gene interleukin-6 *(IL6)* [4]. While *IL6* and *C3* promoters showed high specificity towards inflammation, they only conferred weak transcriptional activity that had to be enhanced artificially using a interleukin-1 enhancer region [5] or a recombinant HIV-1-*tat* transcriptional activator [6], respectively. However, the application of the latter approach is hampered due to the immunogenic properties of HIV-1-*tat*.

The promoter of a gene is defined as the *cis*-regulatory DNA region that drives transcription in response to environmental signals. Roughly, a promoter can be divided into a core-, proximal- and distal-promoter region. The core-promoter encompasses the 80-100 bp region surrounding the transcription start site (TSS) and is required for assembly of the pre-initiation complex [7]. Bioinformatic analyses of mammalian core-promoters have revealed that the spacing between the TATA box and TSS affects the transcriptional specificity of the downstream transcript and the highest specificity has been observed for TATA-TSS distances between -32 and -29 [8]. In mouse, the most common TATA-TSS spacings are 30 and 31 bp [9] and most functional TATA boxes reside in a window ranging from positions -34 to -27 relative to the TSS [8]. The proximal-promoter (-500/+200 relative to the TSS) contains modules of transcription factor binding sites (TFBS) that confer tissue- and context-specific expression [10]. Several bioinformatics tools have been developed for computational identification of these TFBS in eukaryotic promoters (reviewed in [11]). The accuracy of prediction of TFBS that are functional *in vivo* has been significantly enhanced using the combination of phylogenetic footprinting with sets of co-regulated genes [12]. These methods have been used to successfully identify cartilage- [13] or muscle-specific [14] *cis*-regulatory elements in mammals.

In this study, we computationally designed and verified endogenous proximal promoters from a gene expression profiling study of murine collagen-induced arthritis (CIA) for transcriptionally targeted gene therapy for RA. The 201 genes that were significantly regulated during advancing CIA severity stages, were divided in clusters of distinct expression profiles using *k-means* clustering. Next, over-represented TFBS per cluster were calculated in proximal-promoter sequences of the genes and their human orthologs that contained a putative TATA box within the -44 to -17 bp region relative to the TSS. We investigated the transcriptional properties of ten computationally identified proximal-promoter regions *in vitro* using lentiviral luciferase reporter. Nine out of ten promoters drove gene expression in a cytokine-inducible fashion. The serum amyloid A3 (*Saa3*) proximal-promoter conferred high transcriptional specificity and

activity towards inflammation and we demonstrated that this promoter improved the efficacy of transcriptionally targeted adenoviral gene therapy using interleukin-1 receptor antagonist (*Il1rn*) as a transgene.

Results

K-means clustering of CIA gene expression profiles

In local gene therapy for RA the synovial tissue, a thin layer of connective tissue consisting of fibroblast- and macrophage-like cells, is transduced by viral vectors [15]. To elucidate candidate genes whose proximal-promoter might confer inflammation-inducible expression, we analyzed gene expression profiles in inflamed synovium of mice with CIA. Whole genome expression profiles were obtained from synovial tissue biopsies of knee joints from both naïve and DBA/1J mice at day 30 of CIA using the Mouse Genome 430 2.0 array (**Figure 1a**). Prior to isolation, we semi-quantitatively scored the degree of joint inflammation and subdivided samples in four advancing stages of disease severity. For each stage, hybridization experiments were performed in triplicate and data normalized using MAS 5.0 software. Using Expander 4.1 software [16] we obtained 234 Affymetrix probes that correspond to 201 unique Ensembl genes whose expression was at least tenfold upregulated in at least three severity stages compared to naive conditions. Next, the standardized (mean 0 and variance 1) expression profiles were partitioned into distinct clusters using the *k-means* algorithm. Partitioning into six clusters resulted in the most optimal average separation (0.913) and homogeneity (0.938) of clusters. The average standardized expression levels of the clusters are plotted in **Figure 1b**. The annotation of the probe identities per cluster is documented in **Table S1**.

AP-1, C/EBPβ and NF-κB binding sites are over-represented in TATA proximal-promoters

To filter for promoters with high transcriptional specificity towards inflammation, we analyzed the murine and human orthologous proximal-promoters (-500/+200 region) for the presence of a putative TATA box within a -44 to -17 bp window relative to the TSS. The promoter sequences of 191 murine and human orthologs (**Table S2**) were retrieved from the Cold Spring Harbor Promoter database [17] and putative TATA box binding sites were searched with the program PATSER [18] using TATA_01 (M00252) and TBP_01 (M00471) position weight matrices (PWMs) in the TRANSFAC 7.0 database [19]. For 37 genes a putative TATA box was predicted in both murine and human promoter sequences. Additionally, a single TATA TFBS was predicted in promoter sequences 21 murine and 12 human genes (**Table 1**). The TATA proximal-promoters were used for subsequent identification of over-represented *cis*-regulatory elements. For this, we used Transcription Factor Matrix-Explorer software since it combines motif over-representation with comparative genomics and takes spatial conservation of *cis*-regulatory elements into account. Per cluster, over-represented TFBS were searched using PWMs corresponding to human or mouse binding factors in the public TRANSFAC and JASPAR databases [20]. Proximal-promoters from clusters 1 and 2, were significantly ($P<10^{-6}$) enriched for PWMs corresponding to the transcription factor Rel/nuclear factor-κB (NF-κB). In clusters 2,4,5 and 6 several PWMs for the transcription factor activator protein-1 (AP-1) were over-represented. In contrast, promoters of

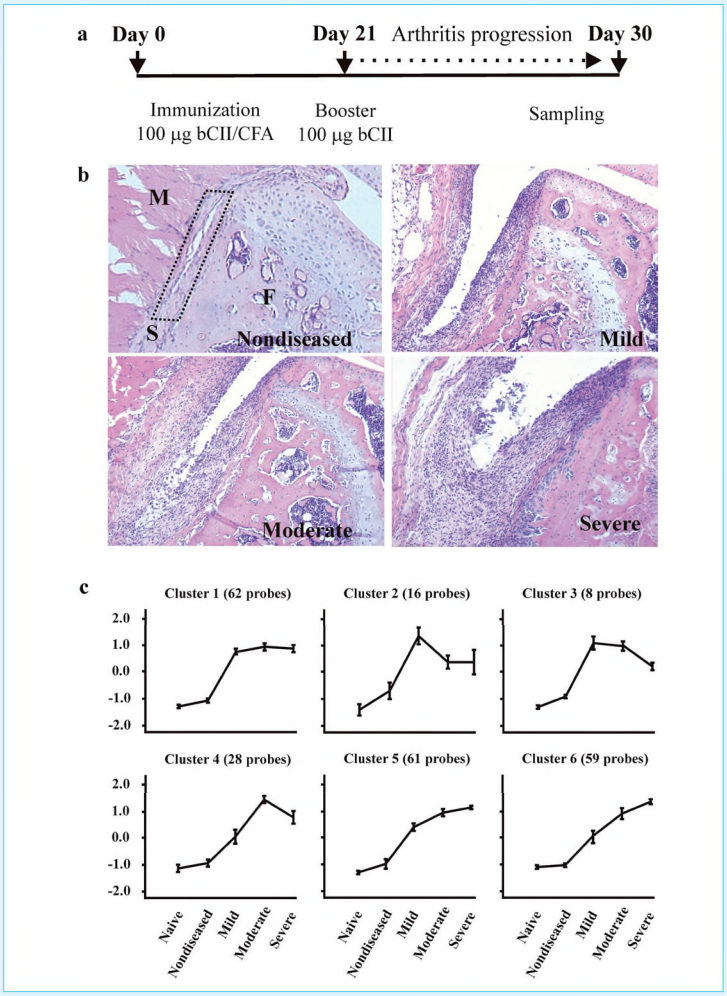

Figure 3.1

Clusteranalysis of gene expression profiles from collagen-induced arthritis

(a) Schematic overview of the collagen-induced arthritis model. DBA/1J mice were immunized at day 0 by intradermal injection of bovine collagen type II (bCII). Immunized mice received an intraperitoneal booster injection of bCII at day 21 which triggers arthritis onset. After 10 days of arthritis progression knee joints were macroscopically scored and divided in the four indicated severity stages. Representative hematoxylin & eosin stained tissue sections of knee joints from the four advancing severity stages are shown. **M** = muscle, **S** = synovium (dotted rectangle), **F** = femur. **(b)** Mean expression profiles of the eight clusters obtained with *k-means* clustering in EXPANDER 4.1. The Y-axis represents standardized expression levels. Error bars represent the ± 1 standard deviation of the members of each cluster about the mean of the particular severity stage.

Table 1. Putative TATA-dependent murine and human orthologous genes

Gene	Pos[a]	Score[b]	Ortholog	Pos	Score
Cluster 1					
			ADAMTS4	-30	5.29
Arg1	-30	4.58	*ARG1*	-32	6.36
Ccl20	-44	7.58	*CCL20*	-30	9.21
Clca1	-32	5.32*	*CLCA1*	-30	5.01*
Cxcl1	-36	8.70	*CXCL1*	-32	7.64
Cxcl2	-32	7.86	*CXCL2*	-44	7.64
			CXCL14	-35	5.28
Il4ra	-31	6.22			
Msr1	-29	5.38*			
			NCAPG	-23	5.41*
			OLFM4	-32	7.39
Rrm2	-30	8.12	*RRM2*	-38	5.58*
			SULF1	-40	8.61
Tnfaip6	-36	5.98*	*TNFAIP6*	-30	5.98*
Cluster 2					
Ccl2	-33	6.88	*CCL2*	-31	4.33
Ccl7	-30	9.34	*CCL7*	-17	9.75
Cxcl1	-36	8.70	*CXCL1*	-32	7.64
Gpr84	-38	4.48	*GPR84*	-34	3.95*
Il6	-30	5.28*	*IL6*	-34	3.79*
Ptgs2	-30	3.67	*PTGS2*	-30	7.52
Rrad	-33	7.12	*RRAD*	-31	6.47
Serpbin1a	-31	7.38	*SERPINB1*	-40	8.08
Socs3	-39	5.41			
Cluster 3					
Ankrd1	-32	8.41	*ANKRD1*	-35	7.43
Uhrf1	-38	7.67	*UHRF1*	-21	5.96
Cluster 4					
C1qtnf3	-32	7.29	*C1QTNF3*	-30	7.67
Clec4n	-31	4.76*			
Cxcl3	-33	9.13			
Ibsp	-30	5.28*	*IBSP*	-30	5.38*
			LRRC15	-31	7.33
Mmp13	-31	5.53	*MMP13*	-31	7.50
Pbk	-25	4.27*			
Pmaip1	-30	5.48			
Rspo2	-37	5.67*			
Tnfsf11	-33	4.32*			

Gene	Pos	Score	Ortholog	Pos	Score
Cluster 5					
			CA13	-24	8.70
Ccl12	-30	5.19			
Clec4e	-30	5.69			
Cxcl5	-32	6.41	*CXCL6*	-33	4.16
			EVI2B	-29	3.46*
Has1	-28	4.93			
Il1b	-33	7.08	*IL1B*	-33	7.64
Il1rn	-34	7.68	*IL1RN*	-40	4.32*
Il4ra	-31	6.22			
Lcn2	-30	9.39	*LCN2*	-31	7.71
Lcp1	-44	6.20*	*LCP1*	-42	6.20*
Lox	-30	3.84*			
			MS4A7	-29	5.93*
			NCAPG	-23	5.41*
			PAPPA	-30	4.98
Rassf5	-34	7.82			
Timp1	-31	4.06*	*TIMP1*	-30	5.49*
Tnc	-36	8.93	*TNC*	-34	9.38
Wwox	-35	6.71			
Cluster 6					
Chi3l1	-23	5.68	*CHI3L1*	-31	7.63
Crabp2	-27	8.50			
Hdc	-30	4.38*	*HDC*	-30	5.70
Il1rl1	-29	5.98*	*IL1RL1*	-29	3.46*
Ltb	-29	6.73	*LTB*	-30	6.41
Ltbp2	-29	6.73			
Mmp3	-34	7.89	*MMP3*	-33	6.95
Mmp9	-31	4.65*	*MMP9*	-35	4.82
Mxd1	-31	4.72*			
			PLEK	-31	6.44
S100a8	-30	9.83	*S100A8*	-29	8.97
S100a9	-30	8.31	*S100A9*	-31	9.81
Saa3	-33	5.47			
Slpi	-30	3.78*	*SLPI*	-31	4.99
Snai2	-36	4.61			
Sox4	-30	5.38*	*SOX4*	-21	5.38*
Spp1	-30	4.76*	*SPP1*	-32	5.83*

Putative TATA-box binding sites were identified by searching promoter sequences with TRANSFAC PWMs for TBP_01 (M00471) and TATA_01 (M00052) using the PATSER program. Sequences with scores higher than the cutoff calculated by PATSER were considered as putative binding site. Promoters with binding sites within the -44/-17 window relative to the TSS are displayed.

[a] Start position of the core TATA-motif

[b] Putative TFBS based on scores above the numerically calculated cutoff scores for TATA_01 (range: 4.36-11.76) and TBP_01 (range: 3.45-7.53)

* Hits for TBP_01 PWM

Table 2. Over-represented transcription factor binding sites in *k-means* clusters

PWM	Location	*P*-value	nh[a]	ns[b]	PWM	Location	*P*-value	nh	ns
	Cluster 1					**Cluster 4**			
NFKAPPAB65_01	-0095 / -0049	$8{,}53\times10^{-15}$	14	47%	FREAC-7	-0273 / -0029	$2{,}62\times10^{-10}$	32	76%
p65	-0095 / -0050	$4{,}06\times10^{-12}$	12	42%	FOXD3_01	-0428 / -0026	$1{,}08\times10^{-08}$	42	84%
MEF2	-0048 / -0019	$5{,}67\times10^{-12}$	15	66%	MEF2	-0445 / -0028	$1{,}53\times10^{-08}$	34	92%
CREL_01	-0095 / -0049	$6{,}55\times10^{-12}$	12	38%	AP1_C	-0276 / +0185	$4{,}83\times10^{-08}$	24	76%
c-REL	-0095 / -0049	$1{,}10\times10^{-11}$	12	38%	FREAC7_01 #	-0438 / -0020	$1{,}01\times10^{-07}$	40	84%
NFKB_C	-0156 / -0048	$7{,}25\times10^{-11}$	15	52%	OCT1_02	-0247 / -0098	$1{,}30\times10^{-07}$	16	61%
NRSF_01 *	+0008/ +0171	$6{,}55\times10^{-09}$	23	61%	AP1_Q6	-0050 / -0004	$9{,}81\times10^{-07}$	8	53%
IK_01 *	-0119 / -0046	$3{,}82\times10^{-08}$	12	38%					
FREAC-7	-0060 / -0029	$4{,}24\times10^{-08}$	13	47%		**Cluster 5**			
NF-kappaB	-0096 / +0061	$1{,}02\times10^{-07}$	13	52%	CEBPB_01	-0238 / -0044	$1{,}31\times10^{-07}$	25	50%
					FREAC7_01 #	-0051 / -0020	$3{,}30\times10^{-07}$	14	50%
	Cluster 2				GR_Q6	-0377 / -0035	$4{,}15\times10^{-07}$	36	57%
NFKAPPAB65_01	-0168 / -0053	$6{,}21\times10^{-11}$	14	58%	AP1_Q6	-0239 / -0047	$4{,}41\times10^{-07}$	22	57%
p65	-0186 / -0053	$6{,}22\times10^{-10}$	14	64%	HLF	-0098 / +0050	$5{,}73\times10^{-07}$	19	53%
CREL_01	-0165 / -0053	$1{,}06\times10^{-08}$	12	52%	SPI-B	-0138 / -0036	$8{,}70\times10^{-07}$	15	42%
c-REL	-0165 / -0053	$1{,}79\times10^{-08}$	12	52%					
NF-kappaB	-0168 / -0067	$9{,}46\times10^{-08}$	10	52%		**Cluster 6**			
NFKB_Q6	-0170 / -0057	$1{,}29\times10^{-07}$	11	58%	cEBP	-0454 / -0052	$3{,}32\times10^{-11}$	39	64%
NRSF_01 *	+0026 / +0102	$1{,}48\times10^{-07}$	13	52%	CEBPB_02	-0493 / -0041	$1{,}75\times10^{-10}$	43	71%
c-Fos	-0284 / -0037	$6{,}41\times10^{-07}$	19	76%	NF1_Q6	-0491 / -0047	$1{,}60\times10^{-09}$	30	60%
SRF	-0145 / -0018	$7{,}75\times10^{-07}$	10	47%	SPI-1	-0273 / -0066	$4{,}91\times10^{-09}$	47	78%
NFKAPPAB_01	-0168 / -0070	$8{,}10\times10^{-07}$	9	52%	CEBPB_01	-0183 / -0078	$2{,}12\times10^{-08}$	20	50%
					AP1_C	-0198 / -0072	$1{,}14\times10^{-07}$	18	35%
					PAX4_04 #	+0120 / +0167	$3{,}50\times10^{-07}$	17	50%
					Gfi	-0228 / -0051	$3{,}56\times10^{-07}$	22	53%
					CEBPA_01	-0282 / -0078	$3{,}78\times10^{-07}$	27	50%
					S8_01 #	-0236 / -0162	$4{,}13\times10^{-07}$	16	39%

Abbreviations: PWM, position weight matrix; TFBS, transcription factor binding sites.
Over-representation of hits for TRANSFAC and JASPAR PWMs in promoters sequences with a putative TATA-box between positions -44/-17 (Table 1), was calculated using TFM-Explorer. Hits with a *P*-value < 10^{-6} are regarded as significant.

[a] Total number of hits for PWM within promoter sequences

[b] Percentage of promoter sequences containing at least one hit for PWM

[#] TFBS for which only murine binding factors have been described.

[*] TFBS for which only human binding factors have been described.

Table 3. Significant transcription factor binding sites for NF-κB, AP-1 and C/EBPβ in TATA-dependent genes

NFkappaB65_01[a]			AP1_01[b]			CEBPB_01[c]		
Gene	**Pos.**	**ln(*P*)**	**Gene**	**Pos.**	**ln(*P*)**	**Gene**	**Pos.**	**ln(*P*)**
Cxcl2	-70	-14.07	*CXCL14*	-96	-13.23	*IL6*	-158	-15.90
Cxcl1	-71	-14.07	*Rrad*	-143	-12.74	*Has1*	-198	-12.83
CXCL1	-79	-14.07	*Mmp9*	-90	-12.63	*Il6*	-158	-12.35
Cxcl5	-89	-14.07	*MMP9*	-79	-12.51	*Cxcl1*	-131	-11.94
CXCL2	-90	-14.07	*Il1rn*	-137	-12.13	*Saa3*	-79	-11.86
CXCL6	-92	-14.07	*MMP13*	-52	-11.80	*Saa3*	-154	-11.28
LTB	-86	-12.96	*IL6*	-286	-11.62	*LCN2*	-146	-11.11
Ltb	-89	-12.96	*LRRC15*	-52	-11.54	*PLEK*	-52	-10.91
NCAPG	-65	-12.35	*Il6*	-279	-11.37	*MMP3*	-115	-10.81
Cxcl3	-74	-12.24	*MMP3*	-75	-11.35	*Arg1*	-233	-10.19
RRAD	-112	-12.17	*Mmp13*	-52	-11.31	*CXCL14*	-110	-10.02
Rrad	-163	-12.17	*OLFM4*	-140	-10.71	*MMP9*	-166	-9.91
Il6	-71	-11.75	*Wwox*	-200	-10.63	*Chi3l1*	-160	-9.53
IL6	-73	-11.75	*TNFAIP6*	-132	-10.52	*LCN2*	-85	-9.51
CCL20	-79	-11.68	*Tnfaip6*	-138	-10.52	*S100A8*	-136	-9.06
Ccl20	-96	-11.68	*Chi3l1*	-125	-10.37	*Ccl12*	-79	-9.02
Wwox	-100	-11.28	*Timp1*	-98	-9.90	*IL1B*	-95	-8.98
SOX4	-99	-10.59	*TIMP1*	-105	-9.90	*Lcn2*	-188	-8.86
Rspo2	-119	-10.34	*Ccl2*	-58	-9.73	*SLPI*	-96	-8.74
Il1rl1	-138	-10.13	*Socs3*	-201	-9.29	*ARG1*	-81	-8.68
Ccl2	-154	-9.83	*Lcn2*	-277	-8.83	*Il1b*	-95	-8.66
			Il1b	-230	-8.79	*Has1*	-144	-8.62
			Ptgs2	-279	-8.78			
LCP1	-449	-12.71	*EVI2B*	-401	-11.95	*ANKRD1*	-304	-13.92
Ptgs2	-403	-12.17	*Ccl2*	-456	-10.93	*Wwox*	-422	-13.79
PTGS2	-449	-12.17	*CHI3L1*	-367	-10.84	*Ccl7*	-460	-12.86
LCN2	-175	-10.79	*Mmp9*	-488	-10.37	*Wwox*	-400	-12.56
Lcn2	-229	-10.79	*Il4ra*	-416	-10.06	*Ankrd1*	-301	-11.79
IL1B	-296	-10.71	*Arg1*	-410	-10.04	*MMP9*	-269	-11.05
Il1b	-303	-10.64	*CXCL6*	-323	-9.81	*Slpi*	-458	-10.97
TNC	-222	-10.57	*Slpi*	-490	-9.81	*Serpinb1a*	-408	-10.60
CCL7	-306	-10.38	*IL6*	-490	-9.58	*Slpi*	-281	-10.34
PLEK	-337	-10.27	*TIMP1*	-359	-9.56	*Chi3l1*	-356	-9.85
MMP9	-329	-10.20	*Cxcl5*	-417	-9.56	*MMP9*	-446	-9.65
MS4A7	-494	-9.83	*UHRF1*	-468	-9.49	*Tnfaip6*	-444	-9.41

Putative binding sites were identified by PATSER using TRANSFAC matrices for [a]NF-κB (M00052), [b]AP-1 (M00517) and [c]C/EBPβ (M00117). Hits are ranked according to their ln(*P*) values and separated into hits lying within (upper panel) or outside (lower panel) the conserved windows identified by TFM-Explorer (Table 2).

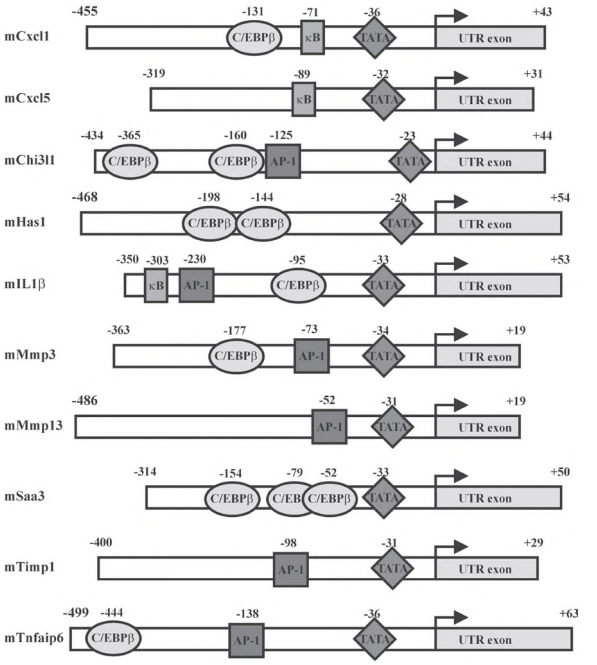

Figure 3.2

Schematic map of promoter regions with putative cis-regulatory elements

Indicated promoter regions were inserted upstream of the firefly luciferase cDNA in a SIN lentiviral backbone. The position of putative binding sites for TATA, NF-κB, AP-1 and C/EBPβ identified by PATSER (Table 1, 3) are indicated. Promoter regions were derived from the murine genes CXC ligand 1/5 (*Cxcl1/5*), chitinase 3-like 1 (*Chi3l1*), hyaluronan synthase 1 (*Has1*), interleukin-1 beta (*IL1β*), matrixmetalloproteinase 3/13 (*Mmp3/13*), serum amyloid A3 (*Saa3*), tissue inhibitor of metalloproteinase 1 (*Timp1*) and TNF alpha-induced protein 6 (*Tnfaip6*).

cluster 5/6 genes, whose transcriptional activity correlates most closely with disease severity, were enriched for binding sites CCAAT/enhancer binding protein β (C/EBPβ). The majority of over-represented *cis*-regulatory elements were spatially conserved in a narrow 200-100 bp window upstream (NF-κB -170/-50, AP-1 -290/-30, C/EBPβ –240/-45) of the putative TATA box binding site (**Table 2**).

Target genes of these transcription factors were identified using PATSER with high quality TRANSFAC PWMs, compiled from more than twenty experimentally verified binding sites,

NFkappaB65_01 (M00052) , AP1_01 (M00517) and CEBPB_01 (M00109). *Bona fide* hits ($P<10^{-8}$) were ranked according to the log ratio of their *P* values (ln *P*) (**Table 3**). From the 97 TATA-dependent promoters, 66 contained a spatially conserved NF-κB, AP-1 or C/EBPβ TFBS. Without taking spatial conservation into account, 81 promoters contained a putative binding site. This strategy, has narrowed down the number of candidate proximal-promoters for transcriptional targeting in arthritis and provides useful information for rational selection of the promoter region.

Construction and validation of transcriptionally targeted SIN lentiviral vectors

We developed ten transcriptionally targeted lentiviral vectors, in which the firefly (*Photinus Pyralis*) luciferase cDNA expression is under control of computationally identified proximal-promoters. All promoters comprised the upstream region containing the identified over-represented TFBS, the TSS and a part of the 5'UTR-exon (**Figure 2**). To evaluate the basal transcriptional activity of the cloned promoter regions, murine NIH-3T3 fibroblasts were co-transfected with promoter constructs and as internal control reporter pRL-TK encoding constitutively active *Renilla Reniformis* luciferase. Cells were transfected with a promoterless or constitutively active cytomegalovirus immediate early (CMV) promoter construct as negative and positive control, respectively. Assessment of the relative luciferase activity at two days after transfection revealed that the basal transcriptional activity of all generated constructs was approximately three- to tenfold lower than obtained with the CMV promoter (1141±48). The strongest and lowest basal activity in murine fibroblasts was observed for the promoter regions of *Cxcl5* (332±12), *Il1b* (331±10), *Mmp13* (398±26), *Tnfaip6* (342±8) and *Saa3* (96±3), *Mmp3* (112±10), respectively (**Figure 3a**). Next, we determined the responsiveness and kinetics of promoter activities to a pro-inflammatory stimulus. Murine RAW 264.7 macrophages (**Figure 3b**) or NIH-3T3 fibroblasts (**Figure 3c**) were transduced with lentiviral promoter-luciferase vectors and after two days challenged with lipopolysaccharide (LPS, 50 ng/ml). Luciferase activity was consecutively measured at two-hour intervals (0-6 hours) and twenty four hours. With exception of the *Tnfaip6* (-499/+63) promoter, all constructs showed induction of promoter activity in response to toll-like receptor 4 (TLR4) triggering by LPS in macrophages. In contrast, a clear promoter response in fibroblasts was only observed for *Saa3* (-314/+50), *Cxcl1* (-455/+43), *Cxcl5* (-319/+31), *Il1b* (-350/+53) promoters. Since our computational analyses included the promoter regions of human orthologs (**Table 1**) and the majority of over-represented TFBS were conserved between species (**Table 3**), we investigated whether the murine promoters would be responsive in synovial fibroblasts isolated from RA patients (RASF). Primary synovial fibroblasts (*n*=4) were transduced with lentiviral vectors containing the *Saa3*, *Cxcl1*, *Cxcl5* or *Mmp13* promoter. Two days after transduction RASF were stimulated for twenty four hours with either recombinant human IL-1β (0.25 ng/ml) or hTNFα (1 ng/ml) alone, or the combination of the two cytokines (**Figure 3d**). The *Saa3* promoter activity was most strongly induced by stimulation with hIL-1β (17±2) or hTNFα (15±2) and the induction was less than additive for the combination of stimuli (22±3). As in murine fibroblasts, the *Cxcl5* and *Mmp13* were modestly and nonresponsive,

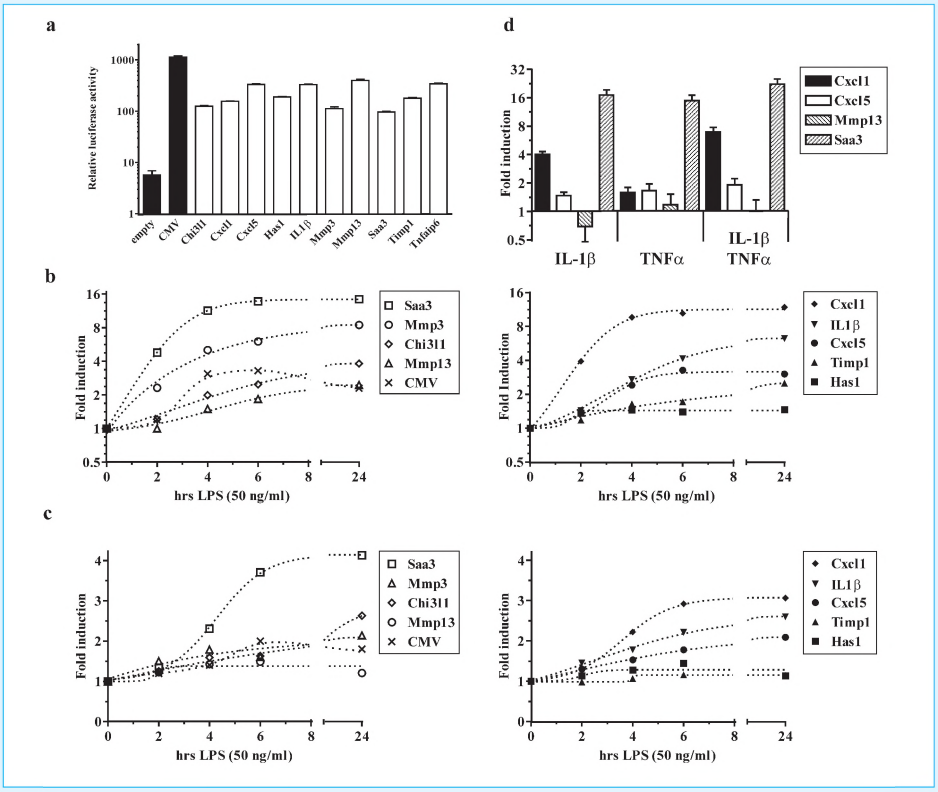

Figure 3. 3

Experimental verification of transcriptionally targeted vectors

(a) Dual luciferase assay of basal promoter activity in NIH-3T3 fibroblasts. Cells were co-transfected with 500 ng transcriptional targeted vector and 50 ng pRL-TK. Promoterless (empty) and CMV-promoter vectors (black bars) served as negative and positive controls, respectively. Promoter activity is expressed as relative (firefly/renilla) luciferase activity +SEM (n=3). Kinetics of promoter induction by TLR4 triggering in murine RAW 264.7 macrophages **(b)** and NIH-3T3 fibroblasts **(c)**. Two days after transduction with lentiviral vectors, cells were stimulated for indicated time points with lipopolysaccharide (50 ng/ml). Luciferase activities are represented as fold induction over unstimulated conditions. **(d)** Induction of promoter activity in rheumatoid arthritis synovial fibroblasts (n=4 donors). Fibroblasts were transduced with lentiviral vectors and stimulated for twenty four hours either recombinant human IL-1β (0.25 ng/ml) or hTNFα (1 ng/ml) alone, or the combination. Luciferase activities are expressed as fold induction +SEM.

respectively. Strikingly, the *Cxcl1* promoter activity was induced by hIL-1β (4.0±0.3) but only marginally by hTNFα (1.6±0.2), while the combination of cytokines synergistically activated the *Cxcl1* promoter (7.0±0.8).

Saa3 promoter drives therapeutically efficacious Il1rn in a disease-regulated fashion

Previously, we have demonstrated therapeutic efficacy of transcriptionally targeted IL-4 gene therapy in CIA using a hybrid promoter consisting of the enhancer region of *IL1B* (-3690/-2720) fused to the proximal promoter of IL6 (-163/+12) (IL1E/IL6P) [4]. However, when the IL-4 cDNA was replaced for the Il1rn cDNA we failed to prevent the development and progression of CIA (data not shown) as described for constitutive overexpression of this transgene [2,21]. We sought to address this efficacy issue by substituting the IL1E/IL6P promoter for a computationally identified promoter. Based on *in vitro* validations of promoter strength and responsiveness (**Figure 3**), the *Saa3* promoter was selected as the most promising candidate for such a substitution. Firstly, we compared the promoter strength under arthritic conditions *in vivo*. Knee joints of C57Bl/6 mice were transduced by lentiviral vectors and after seven days an acute arthritis was induced by intra-articular injection of zymosan A (180 µg). Twenty four hours after challenge we assessed luciferase activity *ex vivo* (**Figure 4a**). To control for amplification of transgene expression by inflammation-induced proliferation of lentivirally transduced synovium [22], knee joints were transduced with a vector encoding phosphoglyceratekinase-1 (PGK) promoter-driven, constitutively expressed luciferase. Under naive conditions transcriptionally targeted vectors exhibited equal and low luciferase activities, approximately a log-fold lower compared to PGK-driven expression. One day after induction of arthritis, we measured a two-fold upregulation of PGK-driven luciferase expression. In contrast, under arthritic conditions *Saa3* and *IL1E/IL6P* promoter activities were twenty five- and eight-fold upregulated, respectively, and *Saa3* promoter strength was approximately six fold higher compared to *IL1E/IL6P*. Next, we generated adenoviral vectors encoding *Saa3* or *IL1E/IL6P*-driven *Il1rn* and measured the Il1rn protein levels under basal and stimulated conditions. HeLa cells were transduced (MOI 10) at day one and the day thereafter left untreated or stimulated for twenty four hours with hTNFα (10 ng/ml). Basal and induced levels were 152.9±34.2 vs. 325.0±50.8 ng/ml (CMV) and 2.8±0.2 vs. 49.6±8.1 ng/ml (*Saa3*) (n=4). Protein levels for *IL1E/IL6P*-driven *Il1rn* were around the detection limit of the ELISA (1 ng/ml). Next, we tested therapeutic efficacy of these vectors in an *in vitro* assay system (**Figure 4b**). NIH-3T3 fibroblasts stably transfected with the luciferase gene downstream of 5 tandem repeats of NF-κB binding sites were transduced (MOI 10) at day one with adenovirus encoding transcriptionally targeted *Il1rn* and at day two with constitutively expressed *Il1b*. As a positive and negative control, cells were transduced at day one with adenovirus encoding constitutively expressed *Il1rn* (CMV) or non-encoding adenovirus (del). At day three, we assessed Il1rn protein levels by ELISA and IL-1β-induced NF-κB activation by luciferase assay at day three. As expected, CMV-driven *Il1rn* expression (56.8±11.8 ng/ml) completely suppressed IL-1β-induced NF-κB activation ($P<0.01$). Empty control virus and IL1E/*IL6P*-driven *Il1rn* expression (undetectable) failed to significantly inhibit NF-κB activation. In

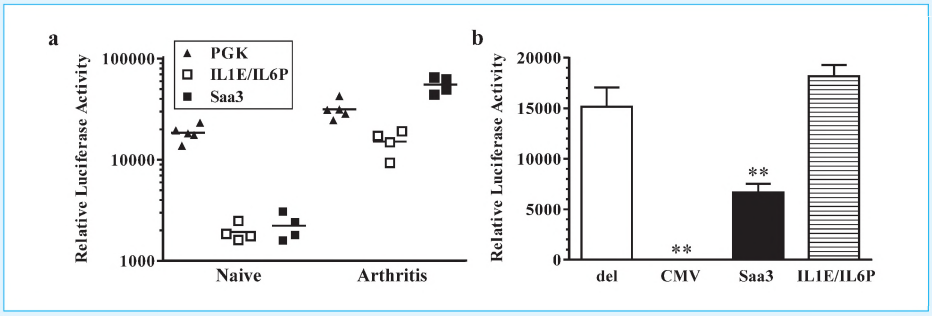

Figure 3.4

Saa3*-promoter drives therapeutically efficacious *Il1rn

Comparison of therapeutic efficacy using *Saa3* versus IL1E/IL6P promoter for disease-regulated *Il1rn* expression. **(a)** Promoter activity in transduced synovium of naïve and arthritic C57Bl/6 mice. Knee joints were injected with 300 ng $p24^{gag}$ equivalents lentivirus encoding PGK, *Saa3* or IL1E/IL6P-luciferase. Seven days after transduction, arthritis was induced by intra-articular injection of 180 µg zymosan A. After twenty fours luciferase activity was assessed ex vivo. Data are represented as individual relative luciferase activities; horizontal bars indicate the means per group. **(b)** Efficacy of transcriptionally targeted adenoviral vectors expressing Il1rn. NIH-3T3-5xNF-κB-Luc were transduced at MOI 10 with control vector (del) or adenovirus encoding CMV, *Saa3*, IL1E/IL6P-driven Il1rn. After twenty four hours cells were transduced at MOI 10 with control vector (del) or Ad5.CMV-*Il1b*. The day thereafter, IL-1β-induced NF-κB activation was assessed by luciferase assay. Data are represented as relative luciferase activities +SEM (n=4) and differences were determined using ANOVA with Dunnett's post-test. ** P<0.01.

contrast, *Saa3*-driven *Il1rn* expression (11.1±1.7 ng/ml) led to a significant (P<0.01) reduction (~56%) of IL-1β-induced NF-κB activation. These results indicate that the promoter strength and responsiveness of *Saa3* might be sufficient to provide therapeutically efficacious protein levels in a transcriptionally targeted *Il1rn* gene therapy approach for experimental arthritis.

Discussion

Endogenous proximal-promoters that confer a range of transcriptional activities in an inflammation-specific fashion are of great value towards tailor-made transcriptionally targeted gene therapy for RA. To this end, we used a gene expression profiling study of CIA for elucidating disease-regulated genes and performed computational analyses on the proximal-promoter regions to define DNA regulatory elements that can be applied for transcriptional targeting. Using this approach we narrowed down the number of candidate murine and human promoters from 382 to 66, corresponding to 45 unique genes.

In our approach we took the spacing between TATA box and TSS as a filtering parameter for transcriptional specificity. The rationale for this was derived from recent studies [8,9] showing the association of TATA-dependent transcription with tissue/context-specific gene expression. Moreover these studies demonstrate that the TATA-TSS spacing affects the transcriptional specificity of the downstream transcript. We predicted putative *bona fide* TATA boxes in using matching of PWM models. Generally, the score of a predictive PWM model is highly correlated with the strength of the protein-DNA interaction [23]. However, a genome-wide characterization of the interaction between the TATA box and the pre-initiation complex has revealed the absence of a global correlation between PWM score and tissue specificity [8]. These findings are reflected in our computations in which we could not establish any correlation between PWM score and spacing. Accordingly, in the experimental verifications using promoter-luciferase constructs we did neither find an apparent correlation between TATA box scores and basal promoter activity in a murine fibroblast cell line.

Apart from the TATA-TSS spacing, the identification of putative TFs that govern a particular expression profile was the second key determinant for selection of promoter regions. As demonstrated in similar approaches [13,14], the combination of co-regulated genes with phylogenetic footprinting proved fruitful for identification of over-represented TFBS. Supporting evidence for the functional relevance of the predicted over-represented TFBSs in RA is substantial. The over-representation of NF-κB and AP-1 binding sites corresponds to the pivotal role that has been implied for these factors in human RA, CIA and immunity [24-27]. Enhanced expression and DNA binding activity of C/EBPβ in synovial tissue of RA patients has been implicated in the pathology [28] and chronicity [29] of disease. The latter coincides with the enrichment of C/EBPβ binding sites in the CIA clusters in which the gene expression profile was closely correlated with disease severity. The additional enrichment of Spi-1 (Pu.1) and C/EBPα binding sites in cluster 6, is expected to arise from the increasing infiltration of the inflamed joint in CIA by myeloid (monocytes, dendritic cells, neutrophils) and lymphoid (T and B cells) cells. Pu.1 and C/EBPα are key factors in development of myeloid and B cells [30]. Interestingly, promoters of myeloid and B-cell-specific genes often contain a regulatory module consisting of a C/EBPα motif in close proximity (<60 bp) of the Pu.1 motif [31]. Indeed, the conserved locations of Pu.1 (-273/-66) and C/EBPα (-282/-78) in our analyses indicate the presence of such myeloid-specific modules. Since synovial fibroblasts represent the target cells for local gene therapy [15,32], the identification of myeloid-specific modules might be exploited to further refine the number of candidate promoters. Besides aforementioned TFs, we expect an contribution of the TFs signal transducers and activators of transcription-1/3 (STAT-1/3) [33] and interferon regulatory factor-1 (IRF-1) [34] in synovial inflammation. Indeed, scanning TATA-dependent promoters with corresponding PWMs (STAT_01 and IRF1_01) we found 15 and 36 promoters with a putative binding site, respectively (data not shown). However, these hits were evenly dispersed over the clusters on a wide range (-500/-100) of positions, which explains the absence of over-representation for these TFBS in our analyses. The accuracy of the approach for inferring functional *cis*-regulatory elements is not only demonstrated by our

experimental verifications but also by literature confirming the functionality of computationally-identified top-ranking TFBS in several promoters such as *Il1b* [35], *Cxcl1* [36], *Saa3* [37], *Timp1* and *Mmp3/13* [38].

The strength of our approach lies in the combination of gene expression profiling for identification of candidate genes with computational prediction of functional endogenous proximal promoters. These are expected to contain evolutionarily conserved and by natural selection optimal combinations of and spacings between TFBSs. This represents a major advantage over recent efforts that aim at tailoring gene expression using genetic engineering of synthetic promoters [25,39,40]. While these studies have demonstrated encouraging results for modulating transgene expression in prokaryotes, the extrapolation to eukaryotic transcriptional regulation by modules proves far more complex and the screening of hundreds of randomly assembled synthetic promoters is laborious and time-consuming. Still, the identified over-represented TFBS and their distance constraints might be useful for guiding the rational design of a synthetic promoter. For example, it was demonstrated for RA gene therapy that a synthetic promoter consisting of six tandem repeats of NF-κB consensus sites drives efficacious [41] expression of anti-TNFα. The functionality of this promoter might be explained by the fact that the construct consists of a TFBS that is over-represented in inflammation-induced genes and positioned within the evolutionary conserved -170/-50 region upstream of the TSS.

The pitfall of using *Il1rn* as a transgene lies in the fact that it needs to be present in at least 100-fold molar excess in order to block the effects of IL-1β on synoviocytes and chondrocytes [42]. However, using *Saa3*-driven *Il1rn* expression we obtained a more than 50% inhibition of an CMV-driven, exaggerated and non-physiological amount of IL-1β. The relatively high transcriptional strength of the *Saa3* proximal promoter has been demonstrated by Varley and coworkers [43]. In their study, *Saa3*-driven luciferase expression exceeded that of the CMV promoter in response to cytokine-rich conditioned medium prepared from the culture supernatant of LPS stimulated human peripheral blood monocytes. In combination with our computational analyses and verifications, the *Saa3* promoter is the most appropriate endogenous promoter for transcriptionally targeted *Il1rn* gene therapy for arthritis.

For the panel of inflammation-responsive promoters we envisage two major applications. First, these promoters can be applied in non-invasive imaging approaches of joint inflammation or activation of particular transcription factors, e.g. AP-1 (*Mmp13*) versus NF-κB (*Cxcl1*), in human and experimental arthritis. Whether these proximal promoters confer differential expression patterns during experimental arthritis will be investigated using an *in vivo* imaging approach. Second, differential transcriptional activities can be exploited for gene therapy tailored to transgene properties, e.g. a promoter with strong transcriptional activity for generating the required excess of Il1rn expression and a promoter with low basal activity for minimizing *Il4*-induced side effects.

Materials and methods

Animals

Male 10-12 week old DBA/1J mice were obtained from Janvier (Le Genest Saint Isle, France). C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany). During viral experiments, mice were housed in low-pressure isolator cages. The animals were fed a standard diet with food and water *ad libitum*. All *in vivo* studies complied with national legislation and were approved by the local authorities of the Care and Use of Animals.

Induction of collagen-induced arthritis

Bovine collagen type II (bCII) was dissolved in 0.05 M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* strain H37Ra, Difco Laboratories, Detroit, MI, USA). DBA-1/J mice were immunized intradermally at the base of the tail with 100 µl of emulsion (100 µg of bCII). On day 21, the mice were given an intraperitoneal booster injection of 100 µg bCII dissolved in PBS. Mice were sacrificed on day 30 by cervical dislocation. Prior to biopsy, knee joints were scored visually using four grades: nondiseased, mild (minor swelling, color change due to infiltration), moderate (marked swelling) and severe (severe swelling, patella tendon structure not visible). Synovial tissue samples from the lateral and medial sites (n=24, 12 knee joints per severity stage) were isolated in a standardized manner using a 3 mm biopsy punch (Stiefel, Wächtersbach, Germany), as described previously [44]. Samples were pooled in a randomized fashion to generate triplicates consisting of eight biopsies.

RNA isolation

Total RNA from biopsy punches was prepared by TRIzol extraction (Invitrogen Life Technologies), and purified on an affinity resin (RNeasy Kit, Qiagen) according to the manufacturer's instructions. Quantity and purity were assessed by the absorbance at λ = 260 nm (A_{260n}m) and the ratio A_{260nm}/A_{280nm}. Integrity of the RNA was confirmed by non-denaturing agarose gel electrophoresis. Total RNA was stored at -80 °C until further processing.

Oligonucleotide array

1 µg of total RNA was used as starting material for cDNA preparation. Generation of biotinylated cRNA and subsequent hybridization, washing and staining of MOE 430_2 oligonucleotide arrays (Affymetrix, Santa Clara, CA) were performed according to the Affymetrix Expression Analysis Technical Manual for one-cycle amplification. The arrays were then scanned using a laser scanner GeneChip®Scanner (Affymetrix) and data were analyzed and normalized using Affymetrix Microarray Suite (MAS) 5.0 software according to the manufacturer's instruction. The gene expression data were deposited in the GEO database under accession number GSE13071.

Clusteranalysis

Filtering, standardization and cluster analysis were performed using Expression Analyzer and Displayer (EXPANDER) software version 4.1 [16]. The mean (n=3) expression values calculated from the normalized microarray data using Excel served as input. After filtering for tenfold regulated probes, the expression values were standardized using the mean 0 and variance 1 algorithm. Cluster analyses were performed using *k-means* clustering with the number of clusters set to 6.

Promoter sequence retrieval

Affymetrix probe identifiers were converted into ENSEMBL gene identifiers (NCBI m37 mouse assembly) and RefSeq DNA identifiers (mm8) using BioMart (http://www.biomart.org). Human orthologs were retrieved from the Mouse Genome Database (MGB) [45]. We extracted the proximal promoter regions of these genes (-500/+200) from the Cold Spring Harbor Laboratory mammalian promoter database [17] using RefSeq identifiers. When multiple promoters existed for a gene, we selected either a promoter from the Eukaryotic Promoter Database (EPD) or Database of Transcription Start Sites (DBTSS). When the prior two were unavailable, we selected the promoter with the shortest distance to the gene.

Identification of transcription factor binding sites

The PATSER program [18] at the Regulatory Sequence Analysis Tools server (http://rsat.ulb.ac.be/rsat) [46] was used to search position weight matrix models collected from the public TRANSFAC 7.0 database [19]. Both strands of the promoter sequences were scanned in our analyses. Sequences with scores higher than the cutoff calculated by PATSER were considered as putative TFBS [47].

Identification of over-represented TFBS

Promoter sequences with a putative TATA binding site were scanned, per cluster, for 322 vertebrate matrices from the TRANSFAC 7.0 and JASPAR [20] databases using TFM Explorer (http://bioinfo/lifl.fr/TFM-Explorer) [12]. The ratio parameter, indicating the minimal average density of hits in the cluster relative to the reference model, was set to 4.0. PWMs with a P-value $< 10^{-6}$ were regarded as significantly over-represented.

Cell culture

Mouse embryonic fibroblasts (NIH-3T3), macrophages (RAW 264.7) and human HeLa cells were cultivated in DMEM with 1 mM pyruvate, 40 µg/ml gentamycin, and 5% or 10% fetal calf serum (FCS), respectively. Early-passage rheumatoid arthritis synovial fibroblasts (kind gift from R.W. Kinne, Universital Hospital Jena, Eisenberg, Germany) were maintained in DMEM supplemented with 1 mM pyruvate, 80 µg/ml gentamycin, and 10% FCS. Cells were kept at 37 °C in a humid atmosphere containing 5% CO_2.

Plasmids

Renilla luciferase vector pRL-TK was obtained from Promega (Madison, WI, USA). For generation of recombinant lentiviral vectors we made use of the third-generation self-inactivating transfer vectors pRLL-cPPT-PGK-mcs-PRE-SIN containing the human phosphoglyceratekinase (PGK) promoter and the promoterless pRLL-cPPT-mcs-PRE-SIN (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). For cloning we used *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA). All generated constructs were verified by sequencing. The firefly luciferase cDNA from pGL3b (Promega, Madison, WI, USA) was transferred as an *NheI/XbaI* fragment into the multiple cloning site generating pRLL-cPPT-PGK-Luc-PRE-SIN and pRLL-cPPT-Luc-PRE-SIN. The plasmid pRLL-cPPT-CMV-Luc-Pre-SIN was generated by PCR cloning the CMV promoter from pShuttle-CMV (Stratagene) into *SalI/HpaI* sites using primers FW 5' GTCGACTAGTAATCAATTACGGGG-3' and RV 5'GTTAACGGATCTGACGGTTCAC-3'. The murine promoter sequences were PCR cloned from liver genomic DNA into *SalI/NheI* sites of pRLL-cPPT-mcs-Luc-PRE-SIN using primers in **Table S3**. The IL-1E/IL-6P promoter was transferred as a *SalI/NheI* fragment from pGL3-IL-1E/IL-6P [5] into pRLL-cPPT-mcs-Luc-PRE-SIN. The cDNA of the mouse *Il1rn* gene was transferred as a *SalI/XbaI* fragment from pShuttle-CMV-Il1rn [48] into pRLL-cPPT-PGK-mcs-PRE-SIN. For constructing pRLL-cPPT-*Saa3*-Il1rn-PRE-SIN, Il1rn was amplified from pShuttle-CMV-Il1rn using primers FW 5'-GCTAGCGCCACCATGGAAATCTGCTGGGGAC-3' and RV 5'-TCTAGACTATTGGTCTTCCT-GGAAG-3' introducing a 5' *NheI* and 3' *XbaI* site and blunt ligated into the SrfI site of pCR-Script Amp SK(+) (Stratagene, La Jolla, CA, USA). The luciferase cDNA was removed from pRLL-cPPT-*Saa3*-Luc-PRE-SIN by restriction with *NheI/NsiI* and religated with a *NheI/PstI* fragment from pPCR-Script-Il1rn (-). A SalI/XbaI fragment from pRLL-cPPT-*Saa3*-Il1rn-PRE-SIN containing the *Saa3* promoter and Il1rn cDNA was transferred to pShuttle-polyA to give pShuttle-*Saa3*-Il1rn-polyA. The cDNA of mouse interleukin-1 beta (*Il1b*) was PCR cloned from reverse transcribed C57Bl/6 synovial tissue RNA into the *KpnI/XhoI* sites of pShutlle-CMV using primers FW 5'-AAAGGTACCGCTATGGCAACTGTTCC-3' and RV 5'-TTTCTCGAGTTAGGAAGACAC-3'.

Lentiviral vector production

Packaging of VSV-G pseudotyped recombinant lentiviruses was performed by transient transfection of 293T cells. The day prior to transfection, 293T cells were seeded in a T75 flask at $1x10^5$ cells/cm^2 in DMEM supplemented with 10% FCS, 1 mM pyruvate, 40 µg/ml gentamycin and 0.01 mM water-soluble cholesterol (Sigma). Cells were co-transfected with 19 µg transfer vector, 14 µg *gag/pol* packaging plasmid (pMDL-g/p-RRE), 4.7 µg *rev* expression plasmid (RSV-REV) and 6.7 µg VSV-G expression plasmid (pHIT-G) by calcium phosphate precipitation. Transfections were performed in 6 ml DMEM without antibiotics and cholesterol and proceeded for 16 hours. Thereafter medium was replaced with fully supplemented DMEM and supernatant harvested after 24 and 48 hours. Cell debris was removed by centrifugation at 1500 rpm for 5 minutes at 4 °C, followed by passage through a 0.45 µm pore PVDF Durapore filter (Millipore, Bedford, MA, USA). For concentration by ultracentrifugation 28 ml supernatant was layered on 4 ml 20%

sucrose solution and centrifuged at 25.000 rpm in a Surespin 630 rotor (Sorvall). Pelleted viruses were resuspended in sterile PBS and stored at -80 °C. Viral titers were determined by assaying $p24^{gag}$ values with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abbott, Il, USA) and expressed as ng $p24^{gag}$/µl.

Adenoviral vectors

Replication-deficient adenoviral vectors (E1/E3 deleted) Ad5.*Saa3-Il1rn*, Ad5.IL-1E/IL-6P-*Il1rn*, Ad5.CMV-*Il1rn*, Ad5.CMV-*Il1b* and Ad5.del were prepared according to the AdEasy system (Stratagene), with the exception that replication-competent recombinant free viral particles were produced in E1 transformed N52E6 amniocyte cells [49]. Viruses were purified by two consecutive CsCl2 gradient purifications and stored in small aliquots at -80 °C in buffer containing 25 mM Tris, pH 8.0, 5 mM KCl, 0.2 mM $MgCl_2$, 137 mM NaCl, 730 µM Na_2HPO_4, 0.1% (w/v) ovalbumin and 10% (v/v) glycerol. The infectious particle titer (ffu) was determined by titrating vector stocks on 911 indicator cells and measuring viral capsid protein immunohistochemically 20 hours after transduction.

Luciferase measurements

For *in vitro* reporter studies, cells were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium). The day after, cells were transduced with 50 ng $p24^{gag}$ equivalents lentivirus in 50 µl medium supplemented with 8 µg/ml polybrene (Sigma) for 4 hours at 37 °C. Cells were serum starved (1% FCS) for two days and subsequently stimulated with recombinant human IL-1β (R&D Systems Europe, Oxford, UK), TNFα (Abcam, Cambridge,UK) or *E. Coli* LPS (Sigma) for indicated hours and subsequently lysed in ice-cold lysis buffer (0.5% NP-40, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl, 10 mM Tris-HCl pH 7.5). Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to total protein content of the cell/tissue extracts. For transient transfection experiments, cells were seeded at 70% confluency in a 24-wells plate and co-transfected with 500 ng firefly luciferase reporter and 50 ng renilla luciferase reporter (pRL-TK) using Arrest-In (Open Biosystems, Huntsville, AL, USA) according to the manufacturer's instructions. Cells were serum starved (1% FCS) for two days and firefly and renilla luciferase activities were quantified using the Dual Luciferase Reporter Assay System (Promega). For *in vivo* studies, knee joints were injected with 300 ng $p24^{gag}$ equivalents lentivirus in a total volume of 6 µl. Seven days after transduction, knee joints were injected with 180 µg zymosan A/6 µl PBS (Sigma). After one day patellae with surrounding tissue were dissected, put in 250 µl cell culture lysis buffer (Promega) and snap frozen in liquid nitrogen. Supernatant was centrifuged at 13.000 rpm for 5 minutes and luciferase activity assayed as described above.

In vitro Il1rn-efficacy assay system

NIH-3T3 fibroblasts stably transfected with a 5xNF-κB luciferase reporter were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium). The day thereafter, cells were transduced at a multiplicity of infection (MOI) of 10 with *Il1rn*-encoding or control non-encoding adenovirus (Ad5.del) in DMEM for 4 hours at 37 °C. After twenty four hours medium was aspirated, cells were rinsed and transduced at MOI 10 with *Il1b*-encoding or Ad5.del virus in DMEM for 4 hours at 37 °C. The day after the second transduction, IL-1β-induced NF-κB activation was measured by assessing the luciferase activity as described above.

Il1rn ELISA

White high-binding flat bottom 96-well plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) were coated with the capture antibody rat anti-murine Il1rn (MAB480, R&D Systems, Minneapolis, Minnesota, USA) at 3 µg/ml in 0.1 M carbonate buffer pH 9.6 and incubated overnight at 4 °C. Non-specific binding sites were blocked with 1% BSA in PBS for 1 hour at room temperature. Between subsequent incubations, wells were rinsed three times with 0.1% Tween-20 in PBS. Twenty four hour culture supernatants (100 µl) from 3 x 10^5 HeLa cells transduced with adenoviral *Il1rn*-expressing vectors at a MOI 10 were added to coated wells and incubated for 3 hours at room temperature. The plates were then incubated with the biotinylated goat anti-murine Il1rn (BAF 480, R&D Systems) at 0.2 µg/ml in 0.1 % BSA/PBS for 2 hours, followed by a 30 minute incubation with streptavidin-conjugated horseradish peroxidase (Dako, Glostrup, Denmark) at 0.25 µg/ml in PBS. Antibody complexes were detected by incubation with the Supersignal ELISA Pico Chemiluminescent Substrate (Pierce, Rockford, Illinois, USA) and luminescence quantified in a luminometer.

Statistics

Data are represented as means+s.e.m. and significant differences were calculated using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison test (GraphPad Prism 5.02, San Diego, CA, USA). *P*-values less than 0.05 were regarded significant.

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Supplementary material online

Table S1. Annotation of the probe identities per *k-means* cluster.

Table S2. Proximal (-500/+200) promoter sequences used in this study.

Table S3. Supplementary Methods: Primers

(http://www.nature.com/mt/journal/v17/n11/suppinfo/mt2009182s1.html)

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A novel *Saa3*-promoter reporter distinguishes inflammatory subtypes in experimental arthritis and human synovial fibroblasts

4. A novel *Saa3*-promoter reporter distinguishes inflammatory subtypes in experimental arthritis and human synovial fibroblasts

Submitted for publication

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Abstract

Objective: To evaluate the applicability of a lentiviral (LV) *Saa3*-promoter luciferase (Luc) reporter for assessing inflammation subtypes in experimental arthritis and synovial fibroblasts (SF) from osteoarthritis (OA) and rheumatoid arthritis (RA) patients.

Methods: Lentiviral transduction of murine synovium and human SF was optimized using a cholesterol-additive during LV production. In mice, synovium was transduced *in vivo* and two flares of acute joint inflammation were induced by intra-articular injection of streptococcal cell wall (SCW) material into the knee joint cavity. The time course of synovial inflammation was assessed using *ex vivo* luciferase assays, ^{99m}technetium (Tc) uptake and histological analysis. SF (n=12) of RA and OA patients were stratified by hierarchical clustering of whole genome expression profiles. Relative *Saa3*-promoter responses were determined in cytokine- or toll-like receptor-stimulated SF subgroups.

Results: *Saa3*-promoter activity was transiently and strongly upregulated at one and two days after first and second SCW challenge, respectively. Promoter activities during acute inflammation correlated with Tc uptake measurements and histological assessments, whereas only the *Saa3*-promoter activity correlated with histological changes during the relapse stage of the disease. *Saa3*-promoter reporter, but not Tc uptake, responded to SCW challenge in a dose-dependent fashion. Molecular stratification defined two inflammatory SF subtypes, unrelated to disease classification. Relative *Saa3*-promoter responses to IL-1β, TNFα and TLR4 agonist were significantly increased in OA/RA SF with a high (n=3) compared to a low inflammatory subtype (n=3).

Conclusion: A transcriptional *Saa3*-promoter reporter demonstrates a robust and feasible method for assessing the course and severity of experimental arthritis and to distinguish molecularly distinct inflammatory SF subtypes from a heterogeneous patient population.

Introduction

Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease that mainly affects the synovial joints that ultimately leads to joint destruction. Prior to and during joint inflammation the synovial lining tissue, consisting of synovial fibroblasts (SF) and macrophages, becomes activated. In both experimental and human arthritis, SF have been identified as cells that actively drive inflammation and joint destruction [1-3]. Moreover, transmigration of activated RASFs has been implicated in mediating the spreading of destructive arthritis to unaffected joints [4]. Due to their key role in RA pathogenesis, SF are regarded not only as major target cells for treatment, but also for diagnostic assessment of disease activity or phenotype.

Non-invasive assessment of disease activity is frequently performed using radiographic agents such as ^{99m}technetium pertechnetate (^{99m}Tc) [5]. Histological and immunohistochemical analyses of synovial tissue comprise the classic ex vivo methods for assessing the grade of inflammation and molecular pathology in human and experimental arthritis and have evidently pointed out that RA is a heterogeneous disease [6,7]. These findings have been corroborated by various gene expressing profiling studies of synovial membranes and isolated SF using microarrays [8-13]. Moreover, these have shown that disease heterogeneity is stably imprinted in SF and molecularly distinct inflammatory subtypes can be discerned.

Gene expression is regulated at multiple levels including epigenetic, transcriptional and translational control. Transcriptional control is exerted by the differential activation of transcription factors (TF) and pivotal TFs implied in RA pathogenesis include nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and CCAAT/Enhancer binding protein β (C/EBPβ) [14-16]. Activity of TFs can be dynamically assessed using transcriptional assays based on expression of luminescent, fluorescent or enzymatic reporter genes driven by a promoter containing TF-response elements. Until now, the diagnostic potential of transcriptional reporters in RA has not been extensively investigated.

Recently, we have designed a number of promoter-luciferase reporters, using gene expression profiling data of inflamed synovium in murine collagen-induced arthritis (CIA), that demonstrated responsive to experimental arthritis and inflammatory stimuli in human SF [17]. These reporters contained composite elements of AP-1, NF-κB and C/EBPβ binding sites. Notably, we found an enrichment of C/EBPβ-binding sites in the promoters of genes whose expression correlated with disease severity in CIA, in particular the promoter of serum amyloid A3 (*Saa3*). This led to the hypothesis that a *Saa3*-promoter reporter might serve as a novel diagnostic tool for assessing inflammatory subtypes in arthritis.

Therefore, we performed kinetic analyses of *Saa3*-promoter activity in SCW arthritis and compared this novel method with ^{99m}Tc uptake and histological assessment of joint inflammation. In addition, we evaluated whether *Saa3*-promoter responses in SF would be able to discriminate between SF of low and high inflammatory expression profiles, as deduced by gene expression profiling.

Results

Optimal transduction efficiency of synovial fibroblasts using cholesterol-optimized lentivirus.

HIV-1 recombinant lentiviruses (LV) have been demonstrated to efficiently transduce murine SF *in vivo* [17,18]. To establish a novel LV vector-based method for assessing synovial inflammation, we determined optimal transduction efficiency of murine synovium using cholesterol-optimized LV particles (**Figure 1a**) [19]. Cholesterol addition during LV production resulted in maximally six-fold increased functional titers ($P<0.001$) at a cholesterol concentration of 0.01 mM. Using conventionally-produced LV, maximal transgene expression *in vivo* was reached at 300 ng of LV p24^{gag} equivalents per joint (**Figure 1b**). In contrast, cholesterol-optimized LV showed a four-fold increase in transgene expression at the same particle titer (**Figure 1c**). Fluorescence microscopy of tissue sections demonstrated that increased transgene expression resulted from an enhanced transduction efficiency of the synovial lining layer (**Figure 1d**).

Next, we determined optimal transduction efficiency of human SF isolated from tissue biopsies. The fibroblast phenotype of isolated cells was confirmed by the abundant surface expression of Thy-1 (**Figure 2a**). Transduction efficiency reached approximately 98% of the starting from 100 ng of p24^{gag} LV per 1.6x10^5 cells and the optimum was reached at 200 ng, respectively (**Figure 2b,c**)

Saa3-promoter activity correlates with histological assessment of joint inflammation in SCW arthritis

Using optimal transduction parameters described in the previous section, we investigated the kinetics of *Saa3*-promoter activity during two consecutive flares of SCW-induced arthritis. Histological analysis revealed a large inflammatory infiltrate at one day post-challenge, which was most prominently located in the joint cavity (exudate, score: 2.05±0.37) and marginally in the synovium (infiltrate, score 0.30±0.11) (**Figure 3a**). At six days after SCW challenge exudate was resolved (0.06±0.13), whereas the infiltrate remained and showed an increase compared to day one (1.19±0.37) (**Figure 3b**). In line with these results, the induction of *Saa3*-promoter activity peaked at day one after challenge (35.3±6.8-fold), declined significantly at 2,4 and 6 days (~12-fold), but did not return to basal levels (**Figure 3c**). A second SCW injection strongly reactivated *Saa3*-promoter activity at one (23.3±6.8-fold) and three days (40.3±8.7-fold) after challenge. To assess whether these results are specific for the *Saa3*-promoter, we performed the same experiment using a *Cxcl1*-promoter reporter. Our previous computational analyses demonstrated that, unlike *Saa3*, expression of *Cxcl1* does not correlate with disease activity in CIA and its promoter contains two strong NF-κB motifs. Cxcl1-promoter activity showed peak

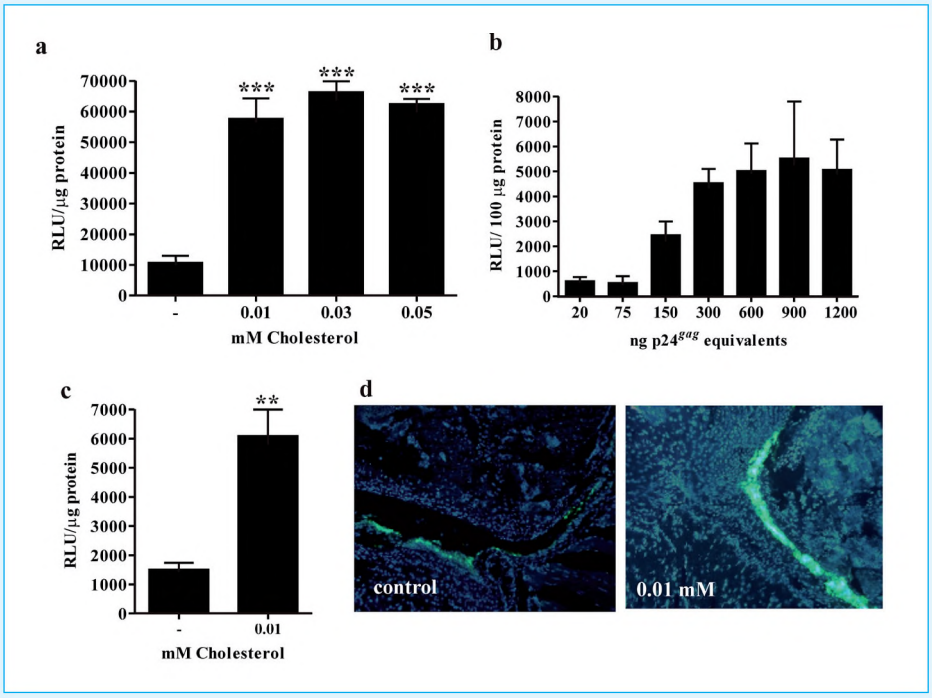

Figure 4.1

Enhanced transduction efficiency of murine synovium using cholesterol-optimized LV

Enhanced transduction efficiency of murine synovium using cholesterol-optimized LV. **(a)**, 5x10^4 NIH-3T3 fibroblast were transduced with 50 ng p24^{gag} equivalents LV-PGK-Luc. Viral particles were produced in the presence of indicated amounts water-soluble cholesterol. Two days post-transduction luciferase expression was quantified using a luciferase assay. Data are expressed as RLU/µg protein +SEM (n=3). **(b-d)**, Analysis of transduction efficiency of murine synovial tissue in vivo. Knee joints of naïve C57Bl/6 mice were transduced with 300 ng p24^{gag} equivalents LV-PGK-Luc **(b,c)** or LV-PGK-GFP (d) produced in absence **(b)** or presence **(c,d)** of cholesterol. Five days post-transduction joints were dissected and transgene expression was quantified using a luciferase assay **(b,c)** or fluorescence microscopy **(d)**. Luciferase expression is expressed as RLU corrected for protein content of samples +SEM (n=3). **(d)**, Analysis of GFP expression in synovium using immunofluorescence microscopy of tissue sections. Statistical differences were determined using ANOVA with Bonferroni's post-test. ** P<0.01, *** P<0.001.

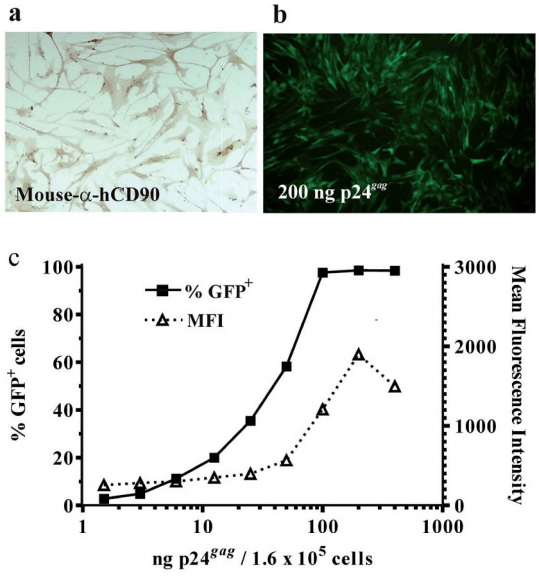

Figure 4.2

Transduction efficiency of human SF by cholesterol-optimized LV

Fibroblasts were isolated from the synovial tissue by consecutive digestion of the tissue with trypsin and collagenase p, followed by negative CD14 MACS isolation [48]. **(a)** Strongly positive immunohistochemical staining of CD14 MACS negatively isolated RA SF for the surface marker Thy-1 (CD90) confirms a fibroblast phenotype. **(b,c)**, $1.6x10^5$ SF were transduced with the indicated $p24^{gag}$ equivalents LV-PGK-GFP. After two days, GFP expression was evaluated using fluorescence microscopy, a representative picture is shown **(b)**. The optimal transduction efficiency was deduced using FACS analysis of the percentage GFP-positive cells (% GFP^+) and the protein expression level (MFI) **(c)**.

induction (6.9±2.5-fold) at day one and returned to nearly basal levels (~2-fold) from day two through six. However, a second SCW challenge failed to induce reactivation of *Cxcl1*-promoter reporter. These results suggest a unique correlation between *Saa3*-promoter activity in synovium and joint inflammation.

Saa3-promoter reporter provides increased sensitivity for detecting joint inflammation compared to ^{99m}Tc uptake

Bioluminescent imaging has shown potential for non-invasive assessment of inflammation in arthritis [20]. Therefore, we compared the performance of *Saa3*-promoter reporter with ^{99m}Tc uptake measurement, which is an well-established method for non-invasive quantification

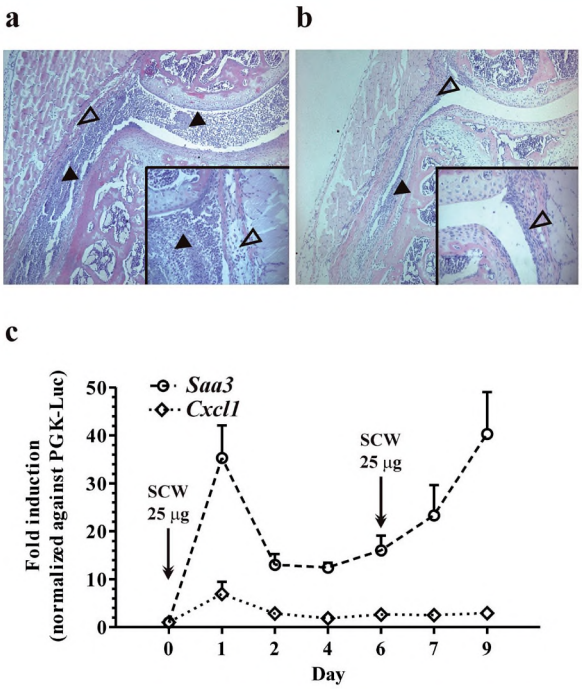

Figure 4.3

Saa3-promoter activity correlates with histological assessment of joint inflammation

(a,b) Knee joints of naïve C57Bl/6 mice were challenged with 25 µg SCW and mice were sacrificed at one **(a)** or six **(b)** days thereafter. Joint inflammation was assessed by evaluation of hematoxylin and eosin-stained tissue sections, representative pictures are shown. Filled and open arrowheads mark inflammatory infiltrate in the synovial cavity and synovial lining, respectively. Inlays show the synovial lining at a higher magnification. **(c)** Knee joints of naïve C57Bl/6 mice were transduced with 300 ng $p24^{gag}$ LV-*Saa3*-Luc, LV-Cxcl1-Luc or constitutively-active LV-PGK-Luc. Four and ten days post-transduction (days 0 and 6) acute arthritis was induced by intra-articular injection of 25 µg SCW (arrows). Luciferase activities were evaluated *ex vivo* at indicated time points, normalized versus PGK-driven luciferase, and represented as fold-induction+SEM (n=5) over naïve conditions.

of joint inflammation [5]. Both *Saa3*-promoter activation (15.0±1.5-fold) and ^{99m}Tc uptake measurement (1.84 ±0.02), revealed strong joint inflammation at day one after SCW challenge (**Figure 4a**). In addition, we observed a strong and significant correlation between both methods at this time point (P<0.0005, Pearson r 0.82) (**Figure 4b**). At day seven, in line with the observations from the previous section, *Saa3*-promoter reporter remained significantly

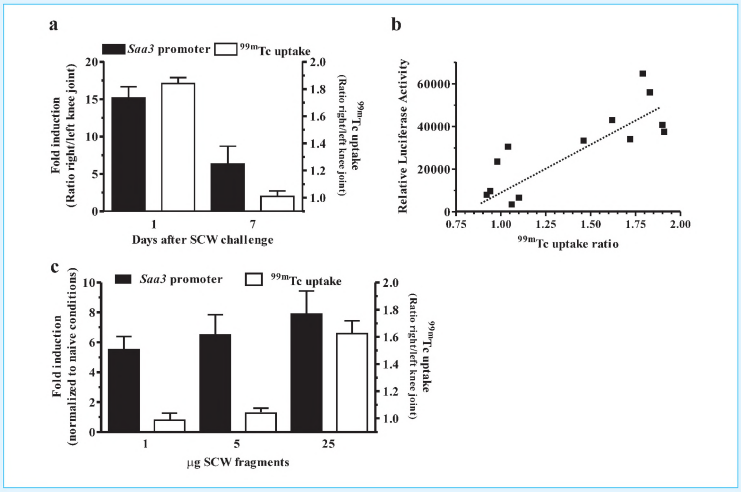

Figure 4.4

Saa3-promoter reporter vs. ^{99m}Tc uptake for assessing joint inflammation

Comparison of *Saa3*-promoter reporter versus ^{99m}Tc uptake for assessment of joint inflammation. **(a)** Both knee joints of naïve C57Bl/6 mice were transduced with 300 ng $p24^{gag}$ equivalents LV-*Saa3*-Luc. Seven days post-transduction, unilateral arthritis was induced by intra-articular injection of 25 µg SCW fragments (right joint). ^{99m}Tc uptake was assessed at indicated days and immediately thereafter joints were dissected and luciferase activities assessed. Bars represent means+SEM (n=4) of luciferase (black) and ^{99m}Tc uptake (white) ratios (right/left joint). **(b)** Correlation between *Saa3*-promoter reporter and ^{99m}Tc uptake ratios from non-inflamed and SCW-challenged knee joints. Data are collected from two independent experiments. **(c)** Sensitivity of *Saa3*-promoter reporter versus ^{99m}Tc uptake. Right knee joints were transduced with LV-*Saa3*-Luc and after 6 days challenge with indicated amounts SCW fragments. Bars represent means+SEM (n=3) of luciferase activity (black, fold induction over naïve conditions) and ^{99m}Tc ratios (white).

activated (6.3±2.4-fold) during relapse of joint inflammation. However, joint inflammation was not detectable at this time point using ^{99m}Tc uptake measurement (1.01±0.02).

These results prompted us to investigate the sensitivity of both methods by measuring the dose-response to SCW fragments (1, 5 and 25 µg) (**Figure 4c**). Whereas *Saa3*-promoter reporter showed a clear response for all doses and a trend towards stronger responses at higher dose (5.5±0.8, 6.5±1.3, 7.9±1.6-fold), joint inflammation as measured by ^{99m}Tc uptake was only evident (1.62±0.17) at highest SCW-dose. These data suggest that *Saa3*-promoter reporter is a more sensitive read-out for joint inflammation than ^{99m}Tc uptake.

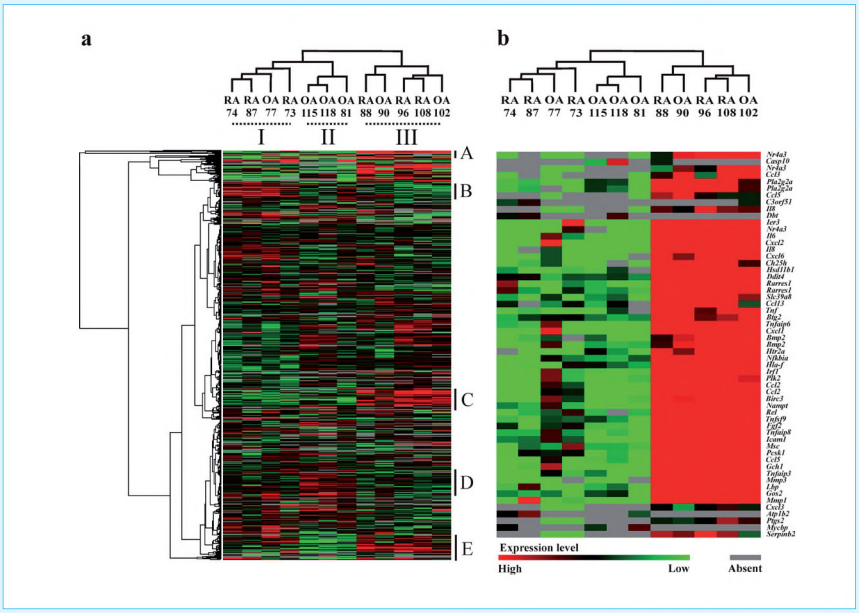

Figure 4.5

Hierarchical clustering of RA and OA SF gene expression profiles

(a,b) Hierarchical clustering of the expression of 7705 cDNAs in SF from patients with RA (*n*=6) or OA (*n*=6). Each row represents a single gene; each column represents an individual SF sample. Black, red and green bands indicate relative expression equal to, greater or lower than the median of all samples. Gray bands indicate the absence of expression of the corresponding cDNA. Color intensities indicate the magnitude of the deviation from the median (bottom, b). Subgroups are labeled I, II and III. Gene clusters with a related expression profile are labeled A through E **(a)**. **(b)** Detailed cluster diagram of subcluster A. Gene symbols are indicated.

Molecular stratification of inflammatory subtypes in OA/RA SF using gene expression profiling

Since *Saa3*-promoter reporter in murine synovium could discriminate the grade of inflammation in experimental arthritis, we hypothesized that this method might discriminate between high and low inflammatory synovial tissues from arthritis patients. Molecular stratification of inflammatory subtypes was performed using complete-linkage hierarchical clustering of whole-genome expression profiles of RA and OA SF (*n*=6 for each) (**Figure 5a**). Based on expression profiles, RA and OA SF were completely intermingled and three subgroups could be discerned. To define low and high inflammatory subtypes, we analyzed gene identities in the

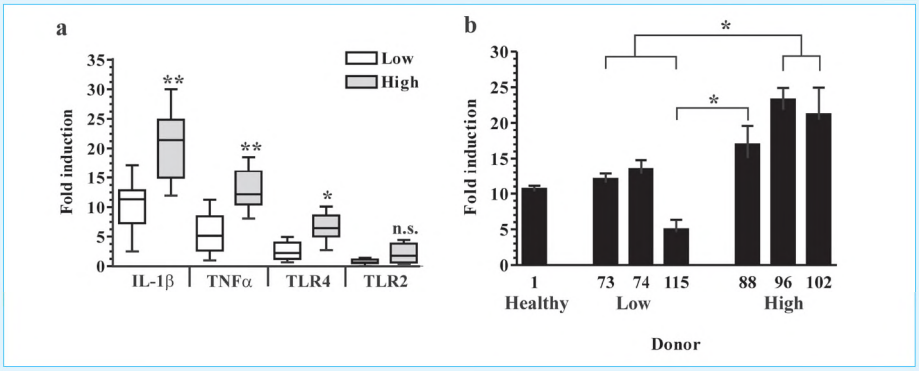

Figure 4.6

Saa3-promoter reporter distinguishes low and high inflammatory SF

(a) Low (*n*=3) and high (*n*=3) inflammatory SF were transduced in quadruplo with LV-*Saa3*-Luc and serum-starved for two days. Cells were stimulated for 6 hours with human IL-1β (1 ng/ml), TNFα (10 ng/ml), LPS or Pam_3Cys (both 1 µg/ml). Luciferase activities are given as fold-induction and represented as box plots indicating the sample minimum, lower quartile, median, upper quartile and sample maximum of low and high subgroups. **(b)** IL-1β-induced *Saa3*-promoter activation in individual SF of a healthy control, or SFs from low and high inflammatory subtypes. Data are represents as means+SEM. Statistical differences were determined using ANOVA with Bonferroni's post-test. * $P<0.05$,

subclusters (A through E) displaying clearly differential gene expression between subgroups (**Figure 5b**). Consistent with prior studies, group III could be classified as a high inflammatory subtype based on significantly increased expression of genes involved in chemotaxis, angiogenesis and TGFβ/activin A pathways [10,21,22].

Increased Saa3-promoter response in stimulated SF with high inflammation profile

Next, we evaluated *Saa3*-promoter responses in SF from low and high inflammation synovial tissue. Irrespective of the subtype, *Saa3*-promoter activity could be significantly induced by TNFα, IL-1β, LPS, but not Pam_3Cys (**Figure 6a**). Whereas basal promoter activity was equal between groups (data not shown), we observed significant higher induction of *Saa3*-promoter reporter in high inflammatory SF stimulated with IL-1β (10.1±1.4 vs. 18.3±1.4, $P<0.01$), TNFα (5.5±1.0 vs. 11.8±1.1, $P<0.01$), or LPS (2.6±0.4 vs. 7.1±0.7, $P<0.05$). Since IL-1β-induced *Saa3*-promoter response provided the largest readout window, we evaluated whether this response could discriminate inflammatory subtype in individual samples (**Figure 6b**). Within subgroups, individual *Saa3*-promoter responses did not differ significantly. Two out of three high inflammatory SF showed significantly increased ($P<0.05$) responses over all low

inflammatory SF, while the third revealed a clear trend towards a higher response that failed to reach significance. These results suggest that *Saa3*-promoter reporter might have a diagnostic value for classification of molecularly distinct low and high inflammatory synovial tissues.

Discussion

Regarding the significant role of activated SF in the local inflammatory processes within the arthritic joint, methods that allow quantitative measurement of inflammation through assessment of the degree of activation of these cells are of great value toward diagnostics of disease activity and treatment efficacy. In experimental arthritis, a number of methods has been developed for *ex/in vivo* assessment of synovial inflammation including histological analysis, ^{99m}Tc uptake, magnetic resonance imaging and ultrasound biomicroscopy [5,23,24]. In our study, we have introduced a lentiviral bioluminescent *Saa3*-promoter reporter as a novel, sensitive and feasible tool for discriminating between inflammatory subtypes in experimental arthritis and human SF. A bioluminescent promoter-reporter has several prominent advantages over the aforementioned methods.

First, the versatility of luciferase as a reporter gene enables quantitative analysis at several levels of resolution. We have assayed transgene expression in synovium *ex vivo* by performing a luciferase assay on a lysate from homogenized tissue. Alternatively, luciferase expression can be revealed and semi-quantitatively scored in histological tissue sections using standard immunohistochemical analysis. Most importantly, luciferase activity can readily be assessed non-invasively and in real-time using *in vivo* imaging systems. These offer high sensitivity and ease of use and have been successfully been applied for monitoring zymosan-induced arthritis [25,26], angiogenesis [27], and stem cell transplantations [28]. In a pilot experiment we were able to monitor inflammation knee and ankle joints of collagen-induced arthritis (CIA) mice using real-time imaging (unpublished observations).

Second, using standard molecular cloning it is possible to generate a diverse range of promoter-luciferase reporters that allow imaging of the activity of individual transcription factors, genes-of-interest or the specific processes with which they correlate, e.g. chemotaxis, cartilage destruction, inflammatory infiltration. Zhang and co-workers utilized this approach to create a number of transgenic luciferase-reporter mice for monitoring diverse cellular processes including DNA damage (*Gadd45* promoter), angiogenesis (*Vegfr2* promoter), and NF-κB signaling (*Ikba* promoter) [27,29,30].

Gouze and co-workers have demonstrated that intra-articular injection of lentivirus leads to predominant transduction of synovial fibroblasts, but not macrophages [31]. This means that our findings with *Saa3*-promoter reporter have to be interpreted within a synovial fibroblast-specific context. Acute SCW arthritis is initiated via a TLR2- and MyD88-dependent macrophage-driven mechanism [32,33], which suggests that activation of the promoter reporter most likely does not arise directly from SCW-induced TLR2 activation in synovial fibroblasts. In support of this hypothesis, we found only marginal induction of *Saa3*-promoter in human synovial fibroblasts upon TLR2 triggering.

Histological analyses of joint inflammation revealed a predominant exudate, consisting of polymorphonuclear neutrophils [34,35], at the peak of promoter induction. An outstanding question remains therefore whether correlation of *Saa3*-promoter in synovium with joint inflammation arises from a response to neutrophil-secreted molecules, synovial-derived cytokines, or both. In favor of the latter, the cytokine profile of IL-1β in SCW arthritis appears to coincide with *Saa3*-promoter activity, showing a peak of induction within 24 hours and declining thereafter [36]. Notably, upon repeated SCW challenge IL-1β protein was present at an increased level compared to the first flare, which corresponds to our findings that *Saa3*-promoter reporter was stronger induced after SCW restimulation. In addition, we have shown that IL-1β is a potent inducer of *Saa3*-promoter reporter in human SF. Recent studies have shown that a *Saa3*-promoter can be induced by S100A8 in macrophages, mononuclear cells and a tumor cell line [37]. S100A8 is highly expressed and secreted by activated neutrophils [38] and has been shown regulate joint inflammation in antigen-induced arthritis [39]. This might represent a mechanism that specifically links neutrophil infiltration with *Saa3*-promoter activation, although it remains to be verified if S100A8 can induce pro-inflammatory signaling in SF.

The observation that *Saa3*-promoter reporter in stimulated human SF could distinguish low and high inflammatory phenotypes, was our most clinically relevant finding. This phenomenon was observed for IL-1β, TNFα and TLR4 stimulation. Based on these findings it can be hypothesized that increased *Saa3*-promoter activation in SF results from differential receptor expression or a downstream protein that is common between pathways. However, in our gene expression profiling the respective receptors IL1R1, TNFR1 or TLR4 were absent from subclusters showing differential expression between low and high inflammatory groups. In contrast we did detect an increased expression of transcription factor CEBPB, which is a strong inducer of *Saa3*-promoter activity [40,41]. Previous studies have demonstrated enhanced expression and DNA binding of C/EBPβ in RA SF [42]. Additionally, Pope and co-workers have established a correlation between nuclear staining of C/EBPβ in synovial lining and the grade of inflammation [16]. Moreover, the correlation was not a disease-specific since it could be observed both in RA and OA. This corresponds to our finding that RA and OA expression profiles were not distinct but intermingled.

A recent study by van Baarsen *et al.* has shown that high inflammatory synovial gene expression profile correlates with higher disease activity, C-reactive protein (CRP) levels, erythrocyte sedimentation rates (ESRs), increased platelet numbers and a shorter disease duration in RA patients [12]. This indicates that there is a diagnostic value of determining low and high inflammatory subtypes of synovial tissue. We failed to observe a correlation with these clinical parameters in our study (data not shown), which is most likely caused by the intermingling of RA with OA samples, since the latter showed an overall shorter disease duration, lower CRP levels and ESRs. On the other hand, intermingling of RA and OA tissues corresponds with recent to recent findings of the involvement of synovial inflammation in the pathogenesis of OA and a change in the dogma that OA is not an inflammatory disease [43-45].

Our findings have demonstrated that lentiviral *Saa3*-promoter reporter is a novel and feasible tool with a potential diagnostic value in experimental and human arthritis. In the future, it seems valuable to explore the potential of real-time bioluminescent imaging of *Saa3*-promoter activity *in vivo* as a non-invasive molecular imaging approach in animal models. Apart from its potential in identifying inflammatory subtypes, elucidation of the molecular mechanism causing differential induction of *Saa3*-promoter activity in SF could provide valuable insight into pathogenesis of arthritis.

Materials and methods

Animals

C57Bl/6 mice were obtained from Janvier (le Genest-Saint-Isle, France). During viral experiments, mice were housed in HEPA filtered individually ventilated cages. The animals were fed a standard diet with food and water *ad libitum*. All *in vivo* studies complied with national legislation and were approved by the local authorities of the Care and Use of Animals.

SCW preparation and induction of SCW arthritis

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously [46]. Uni- or bilateral arthritis was induced by intra-articular injection of 25 µg of streptococcal cell wall (SCW) fragments (rhamnose content) in 6 µl of phosphate buffered saline (PBS), respectively, into both or only the right knee joints of naive mice.

Assessment of joint inflammation by ^{99m}Tc-uptake

Joint inflammation was quantified by ^{99m}Tc uptake in knee joints as described previously [5]. Briefly, animals were sedated by intraperitoneal administration of chloral hydrate. Approximately 20 µCi ^{99m}Tc in 200 µl saline was injected subcutaneously and after 15 minutes the accumulation of the isotope was determined by external gamma counting. The severity of inflammation is expressed as the ratio gamma counts of the inflamed (right) over the contralateral joint. Ratios larger than 1.1 are considered as inflammation.

Histology

Whole knee joints were isolated at day one or six after SCW challenge and fixed in 4% paraformaldehyde for 4 days. After decalcification in 5% formic acid, specimens were processed for paraffin embedding. Tissue sections (7 µm) were stained with haematoxylin and eosin. Histological changes were scored in the patella/femur region on five semi-serial sections of the knee joint, spaced 70 µm apart. Scoring was performed by two observers without knowledge of the group. Infiltration of cells was scored on a scale of 0-3, depending on the amount of inflammatory cells in the joint cavity (exudate) or synovial tissue (infiltrate).

Patients and samples

Synovial tissue was obtained from open joint replacement surgery or arthroscopic synovectomy at the Clinic of Orthopedics, Waldkrankenhaus "Rudolf Elle" (Eisenberg, Germany). Patients with RA or OA were classified according to the ACR criteria, as described previously [47]. SF were purified from synovial tissue as previously published [48]. Briefly, the tissue samples were minced, digested with trypsin/collagenase *p* (Sigma, St. Louis, MO), and the resulting single cell suspension cultured for seven days. Non-adherent cells were removed by medium exchange. SF were negatively purified using Dynabeads M-450 CD14 (Invitrogen, Carlsbad, CA).

Oligonucleotide arrays and clusteranalyis.

Second passage SF were used for oligonucleotide arrays as described in detail previously. Briefly, SF were serum starved in DMEM/1% FCS for 72 hours and RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Gene expression analysis was performed using the U95A oligonucleotide arrays (Affymetrix, Santa Clara, CA) and data were analyzed and normalized using MAS 5.0 software according to the manufacturer's instruction. The gene expression data were deposited in the GEO database under accession numbers GSE7669 and GDS2931. Complete-linkage hierarchical clustering was performed using the algorithm available at the Gene Expression Omnibus server [49].

Plasmids

For generation of recombinant lentiviral vectors we made use of the third-generation self-inactivating transfer vectors pRLL-cPPT-PGK-mcs-PRE-SIN containing the human phosphoglyceratekinase (PGK) promoter, pRLL-cPPT-PGK-GFP-PRE-SIN, and the promoterless pRLL-cPPT-mcs-PRE-SIN (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). Construction of promoter-luciferase constructs is described in detail in **Chapter 3**.

Lentiviral vector production

VSV-G pseudotyped recombinant LV particles were produced as described in **Chapter 3** with following modifications: Preceding calcium phosphate-based co-transfection of packaging plasmids and during virus production, HEK293T cells were cultured in medium supplemented with 0.01, 0.03 or 0.1 mM water-soluble cholesterol (Sigma). Viral particles were purified from supernatant by ultracentrifugation. Viral particle titers were determined by assaying $p24^{gag}$ values with an enzyme-linked immunosorbent assay (ELISA) kit (Abbott, IL, USA) and expressed as ng $p24^{gag}$/µl.

Luciferase measurements

For *in vitro* reporter studies, cells were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium). The day after, cells were transduced with 50 ng $p24^{gag}$ equivalents of lentivirus in 50 µl medium supplemented with 8 µg/ml polybrene (Sigma) for 4 hours at 37 °C. Cells were serum-starved (1% FCS) for two days and subsequently

stimulated with recombinant human IL-1β (R&D Systems Europe, Oxford, UK), TNFα (Abcam, Cambridge,UK), *E. Coli* lipopolysaccharide (LPS, Sigma) or Pam_3Cys (EMC Microcollections, Tübingen, Germany) for the indicated hours and subsequently lysed in ice-cold lysis buffer. Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to the total protein content of the cell/tissue extracts. For *in vivo* studies, knee joints were injected with 300 ng $p24^{gag}$ equivalents LV in a total volume of 6 µl. Four or seven days after transduction, knee joints were challenged with SCW fragments. At indicated time points, patellae with surrounding tissue were dissected, put in 250 µl cell culture lysis buffer (Promega) and snap frozen in liquid nitrogen. Supernatant was centrifuged at 13.000 rpm for 5 minutes and luciferase activity assayed as described above.

Statistics

Data are represented as means+SEM. and significant differences were calculated using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison test (GraphPad Prism 5.02, San Diego, CA, USA). *P*-values of less than 0.05 are regarded as significant.

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Application of a disease-regulated promoter is a safer mode of local IL-4 gene therapy for arthritis

5. Application of a disease-regulated promoter is a safer mode of local IL-4 gene therapy for arthritis

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Abstract

The application of disease-regulated promoters in local gene therapy for rheumatoid arthritis potentiates the development of a sophisticated treatment that relies on a restricted and fine-tuned supply of biologicals. Although several studies have investigated regulated promoters for achieving effective transgene expression during arthritis, none have explored their potential for minimizing deleterious effects arising from constitutive overexpression of transgenes under naïve conditions. Using naïve and collagen-induced arthritic mice, we examined the applicability of a hybrid interleukin-1 enhancer/interleukin-6 proximal promoter for achieving efficacious murine interleukin-4 gene therapy under arthritic conditions, while minimizing interleukin-4-induced inflammation under naïve conditions. We found strong upregulation of luciferase expression in virally transduced knee joints under arthritic conditions compared to levels in naïve animals. Besides its responsiveness, the promoter strength proved sufficient for generating therapeutically efficacious levels interleukin-4, as demonstrated by the successful protection against cartilage erosion in collagen-induced arthritis. Most importantly, promoter-mediated restriction of the potent chemotactic interleukin-4 in naïve animals strongly reduced the amounts of inflammatory cell influx. This study suggests the suitability of the interleukin-1 enhancer/interleukin-6 promoter hybrid for the development of a local gene therapy strategy for rheumatoid arthritis that requires fine-tuned and restricted expression of transgenes with a pleiotrophic nature.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that mainly affects synovial joints and which is characterized by inflammatory synovitis, ultimately leading to invasion and destruction of articular cartilage and bone [1,2]. Rather than a continuous clinical course, RA displays variable disease activity in a considerable fraction of patients resulting in spontaneous remissions and exacerbations of joint inflammation [3]. Although the etiology of RA remains to be elucidated, it is recognized that the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) are central mediators of inflammation and cartilage destruction, respectively [4]. Conventional treatments for RA are predominantly based on

systemic delivery of biologicals that target these proinflammatory cytokines, e.g. anti-TNF-α [5]. Although these treatments have shown to be therapeutically efficacious, the safety of the systemic mode of treatment is under dispute [6,7]. Local intra-articular gene therapy represents a promising alternative for coping with side effects and improving therapeutic efficacy. The joint resident cells, primarily synovial fibroblasts, are readily transduced by several viral vectors and potentially sustain long-term gene expression from both integrating and non-integrating vectors [8-10]. As demonstrated in animal models, the relatively confined joint space appears ideally suited for treatments relying on secretion of anti-inflammatory proteins such as IL-4, IL-10 or IL-1 receptor antagonist [11]. A strong advantage of gene therapy is the possibility to transcriptionally control transgene expression and effectuating protein supply that is restricted to arthritic conditions [12-15]. Moreover, by minimizing transgene expression under naïve conditions, a safer mode of gene therapy could be generated that allows the application of biologicals which have deleterious effects when expressed constitutively, such as IL-4.
Intra-articular IL-4 gene therapy has been demonstrated to prevent cartilage and bone erosion in experimental arthritis [16-19]. The anti-inflammatory capacity of IL-4 arises from its capacity to downregulate the production of proinflammatory and Th1 cytokines by inducing mRNA degradation and upregulating expression of inhibitors of proinflammatory cytokines. Reduction of Th1 cytokines results in suppression of macrophage activation and production of IL-1, IL-6, IL-8 and TNF-α [20]. In contrast to the annotated anti-inflammatory properties, IL-4 is also potent proinflammatory and chemotactic factor for fibroblasts and macrophages [20,21]. This is illustrated by the fact that overexpression of IL-4 in a naïve knee joints results in a severe joint inflammation that is characterized by synovial cell influx [18]. In this study we investigate whether controlled and temporal expression of IL-4 minimizes these side effects.

Results

IL-1E/IL-6P promoter is responsive to collagen-induced arthritis

Previously, we have shown that the hybrid IL-1E/IL-6P promoter, composed of the human interleukin-1 enhancer region (-3690/-2720) upstream of the human interleukin-6 proximal promoter (-163/+12), responds to a broad range of proinflammatory cytokines *in vitro* as well as to an acute joint inflammation induced by zymosan or streptococcal cell wall material [13,14]. Alternatively, we investigated the responsiveness of this hybrid promoter to collagen-induced arthritis, which is an animal model of arthritis that is characterized by immune-mediated polyarticular joint inflammation and irreversible cartilage damage. To this end, adenovirus (10^7 *ffu*) expressing IL-1E/IL-6P-driven luciferase was injected into either nonaffected or arthritic knee joints of collagen type II immunized DBA/1J mice. Luciferase activities in the synovium were measured at two or seven days after virus injection. Under naïve conditions, the basal promoter activities were low ($1.0x10^3$ RLU, not shown), in line with previous observations [13,14]. When arthritis developed at two and seven days after virus injection, luciferase activity was strongly increased ($3.0x10^4$ RLU) (**Figure 1**). As we described previously, the transduction efficiency of the synovial tissue in arthritic joints was hampered [22]. Still, luciferase activities were significantly

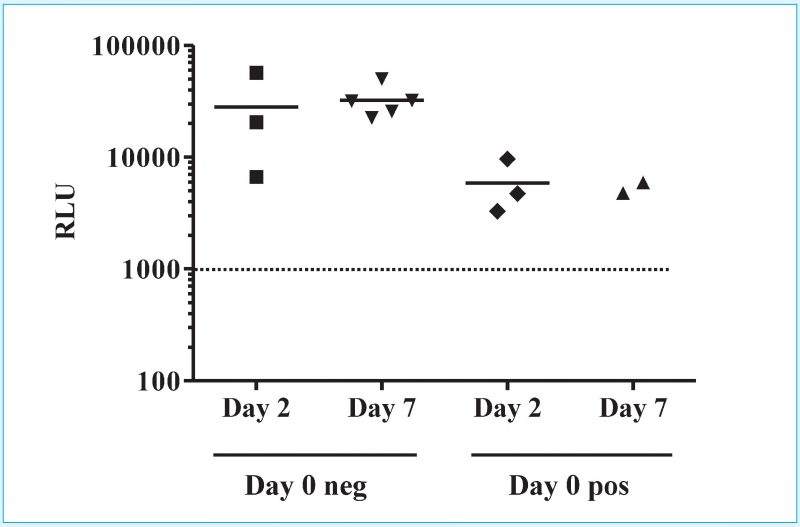

Figure 5.1
IL-1E/IL-6P promoter activity in collagen-induced arthritis

Collagen type II-immunized DBA/1J mice were injected intraarticularly with 10^7 *ffu* Ad5.IL-1E/IL-6P-Luc. Knee joints were scored macroscopically for inflammation at the time point of viral injection: negative (Day 0 Neg) or positive (Day 0 Pos). At day 2 and 7 post injection, arthritic joints were isolated. Luciferase activity in the synovium was assessed and expressed as relative light units (RLU). Squares and triangles represent individual luciferase measurements; horizontal bars indicate the mean per group; the dotted line indicates the mean expression level under naïve conditions.

increased over basal levels at day two and seven, although five- to six fold lower (5-6x10^3 RLU) than activities observed in joints that were non-inflamed at transduction. This shows a strong response of the IL-1E/IL-6P promoter to an immune-mediated inflammation. Furthermore, the IL-1E/IL-6P promoter activity was to the same extent at day two and seven, which illustrates that the promoter is not shut down when disease persists. This marks a strong advantage over the conventionally applied constitutively active viral promoters, such as the cytomegalovirus (CMV) immediate early promoter, which gives rise to a short expression peak at two days after transduction that is strongly declined after seven days [13,14].

Murine IL-4 does not influence IL-1E/IL-6P promoter activity in vitro

It has been reported that IL-4 synergizes with IL-1 in the release of several factors, e.g. IL-6, from fibroblasts,[23-25] but there is also evidence pointing to a suppressive effect of IL-4 on

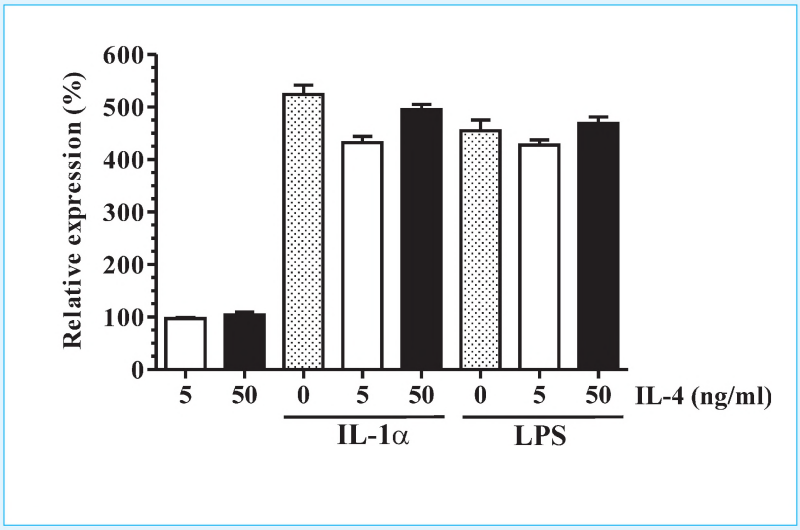

Figure 5.2

IL-4 does not influence IL-1E/IL-6P promoter activity *in vitro*

IL-4 effects on basal or cytokine-induced IL-1E/IL-6P promoter activity *in vitro*. NIH-3T3 fibroblasts were transduced at MOI 50 with Ad5.IL-1E/IL-6P-Luc and after one day stimulated for 24 hours with two concentrations IL-4 (5 or 50 ng/ml) alone, or in the presence of IL-1α (10 ng/ml) or LPS (2 µg/ml). Luciferase activity was assessed and given as relative expression (ratio x 100) as compared to unstimulated cells. Data are presented as means +SEM (*n*=4).

IL-1 induced secretion of matrix metalloproteinases [26,27]. From previous studies it was determined that IL-1 and lipopolysaccharide (LPS) strongly upregulate IL-1E/IL-6P promoter activity [13], therefore we sought to investigate whether expression of murine IL-4 might interfere with the inducibility of IL-1E/IL-6P promoter activity. To this end, murine 3T3 fibroblasts were infected with Ad5.IL-1E/IL-6P-Luc at a multiplicity of infection of 50. The day thereafter, cells were stimulated for 24 hours with recombinant murine IL-4 (5 or 50 ng/ml) in the absence or presence of IL-1α (10 ng/ml) or LPS (2 µg/ml) (**Figure 2**). IL-4 alone did not affect promoter activity; relative expression levels were 97±3 and 104±5 for 5 and 50 ng/ml IL-4, respectively. The proinflammatory stimuli IL-1α or LPS alone increased promoter activity approximately four to five fold, expression levels were 524±16 and 455±21, respectively. Addition of IL-4 resulted in neither additive nor suppressive effects on LPS-induced promoter activity. IL-4 showed a slightly suppressive, but non-significant, effect on IL-1α-induced promoter activity.

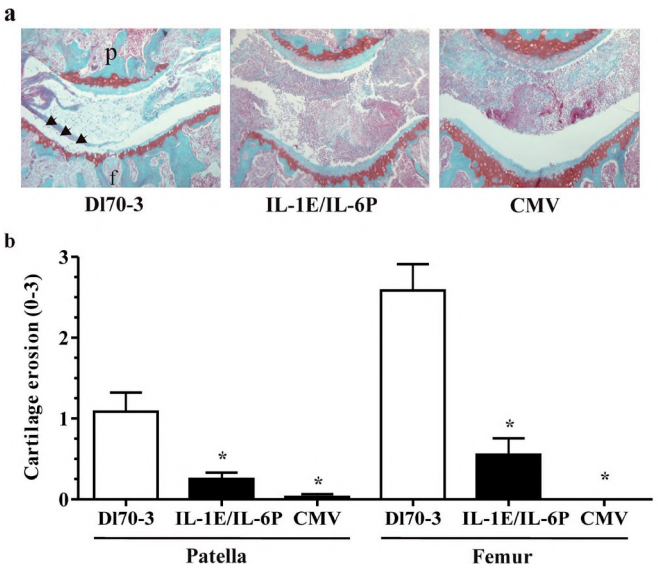

Figure 5.3

Disease-regulated IL-4 expression prevents cartilage erosion in CIA

Disease-regulated IL-4 expression prevents cartilage erosion in collagen-induced arthritis. **(a)** Safranin-*O*-stained tissue sections of arthritic knee joints, 7 days after injection with 10^7 *ffu* control vector (Dl70-3) or adenovirus encoding the murine IL-4 gene (IL-1E/IL-6P, and CMV). Arrowheads mark the sites of erosion. **(b)** Cartilage erosion was scored separately for the patella and femur on a 0-3 scale. Data are represented as means + SEM (n=10, IL-1E/IL-6P and CMV, n=6, Dl70-3) and differences determined using the Mann-Whitney U test. * P<0.05. **P** = patella, **F** = femur.

These results indicate that IL-4 does not influence basal or induced IL-1E/IL-6P promoter activity. It is expected that IL-1E/IL-6P-driven IL-4 expression *in vivo* will depend primarily on the presence of proinflammatory mediators rather than IL-4 itself.

Disease-regulated IL-4 expression prevents cartilage erosion in collagen-induced arthritis

Previously, we have shown that intraarticular overexpression of murine IL-4 provides protection against progression to major cartilage erosion in collagen-induced arthritis, presumably by suppression of IL-1β expression and nitric oxide synthesis [18]. To demonstrate that IL-1E/IL-6P-driven mIL-4 expression levels are therapeutically efficacious, 10^7 *ffu* Ad5.CMV-mIL-4 (positive control, constitutive IL-4 expression), Ad5.dl70-3 (negative control, no IL-4 production),

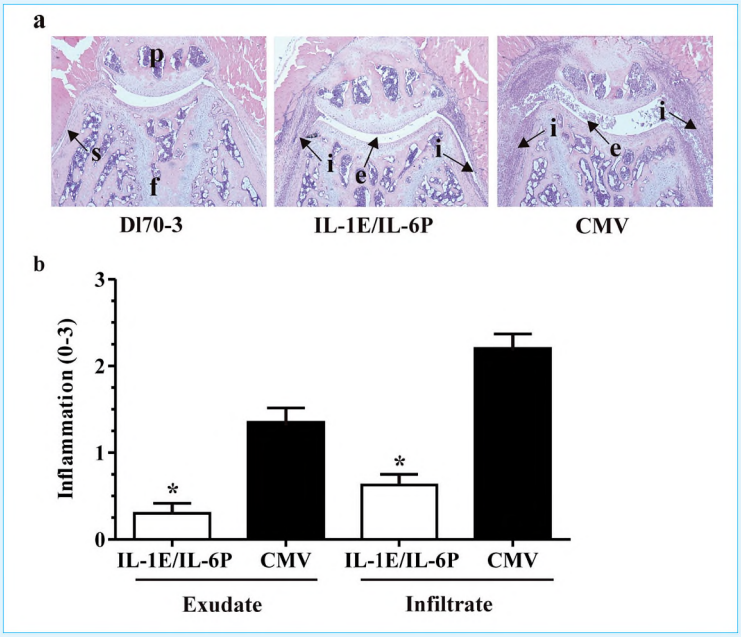

Figure 5.4

A disease-regulated promoter strongly reduces IL-4-induced inflammation in naive mice

(a) Haematoxylin and eosin-stained tissue sections of inflamed knee joints, 7 days after injection with 10^7 *ffu* control vector (Dl70-3) or adenovirus encoding the murine IL-4 gene (IL-1E/IL-6P and CMV). **(b)** Inflammatory infiltrate and exudate in the patella/femur region were scored on a 0-3 scale. Data are represented as means + SEM (n=10) and differences determined using the Mann-Whitney U-test. * P<0.05. **P** = patella, **F** = femur, **S** = synovium, **I** = infiltrate, **E** = exudate.

or Ad5.IL-1E/IL-6P-mIL-4 were injected in the knee joints of collagen type II immunized DBA-1/J mice, two days after they received a collagen booster injection. At day 30, incidence of arthritis was 100% in both IL-4 groups and 80% in the control group. As described previously for this vector dose [18], there were no significant differences between IL-4 treated and control groups in macroscopic clinical score of arthritis and histological score of inflammation (data not shown). Consecutively, histological knee joint sections were scored for cartilage depletion and cartilage surface erosions in the patella and femur region (**Figure 3a**). Profound proteoglycan depletion was observed in all groups, sustaining evidence that mIL-4 has no protective effect on cartilage proteoglycan depletion (data not shown). However, mIL-4 expression led to a significant decrease in erosion of patellar and femoral cartilage as compared to the control

group (**Figure 3b**). Whereas femoral cartilage was severely eroded (mean 2.6±0.3) in the control group, constitutive or disease-regulated expression of mIL-4 in arthritic mice revealed non- (CMV) or mildly eroded femoral cartilage (IL-1E/IL-6P: 0.6±0.2). Patellar cartilage was less severely eroded than femoral cartilage in the control group (1.1±0.2 out of 3.0), however the same trend of cartilage protection was observed in both IL-4 treated groups (CMV: 0.03±0.03, IL-1E/IL-6P: 0.3±0.1). This experiment proved that the IL-1E/IL-6P promoter activity in arthritic animals is sufficient to provide therapeutically efficacious mIL-4 levels.

IL-1E/IL-6P-driven IL-4 expression results in marginal inflammation in naive mice

Previously, it was demonstrated that constitutive intra-articular overexpression of mIL-4 for 7 days leads to substantial joint inflammation, characterized by massive infiltration of cells into the synovial cavity (exudate) and synovial tissue (infiltrate).[18] We hypothesize that disease-regulated mIL-4 expression will lead to low levels of mIL-4 under naïve conditions, thereby substantially reducing cell influx in naive mice. To verify this hypothesis, Ad5.IL-1E/IL-6P-mIL-4, Ad5.CMV-mIL-4 or Ad5.dl70-3 (10^7 *ffu*) was injected in the knee joints of naïve C57Bl/6 mice. Eight days after viral transduction mIL-4 levels were measured in patella washouts. Production of mIL-4 in mice treated with control adenovirus was below the detection limit (<5 pg/ml), which demonstrates that endogenous IL-4 levels in the joint are low. Driving IL-4 expression by the IL-1E/IL-6P promoter resulted in low basal levels (8.4±3.0 pg/ml) as compared to those obtained with the CMV promoter (>1000 pg/ml). Next, histological knee joints sections at day 7 were inspected for the presence infiltrate and exudate (**Figure 4a**). As expected, constitutive expression of IL-4 for seven days generated massive joint inflammation and treatment with control virus showed no signs of inflammation seven days after transduction. The IL-1E/IL-6P group showed only marginal amounts of inflammatory exudate. In line with the markedly reduced IL-4 levels, the extent of inflammatory infiltrate was profoundly reduced by putting IL-4 expression under control of a disease-regulated promoter (**Figure 4b**). Both infiltrate (0.63±0.13) and exudate (0.30±0.12) were strongly reduced in the IL-1E/IL-6P group compared to the CMV group (2.20±0.17 and 1.35±0.17 for infiltrate and exudate, respectively). These results show that the IL-1E/IL-6P promoter activity under naïve conditions is sufficient to maintain IL-4 expression at a minimal level.

Discussion

The concept of applying disease-regulated promoters in gene therapy for arthritis is a scarcely explored field. Nevertheless, this strategy contains a large potential since it might provide a fine-tuned supply of therapeutic proteins in an offer meets demand fashion. Perhaps even more importantly, tight control of transgene expression potentially minimizes the risk of deleterious effects under non-diseased conditions, such as toxicity or immune responses. This however, has not been investigated so far; similar studies focused rather on effective transgene expression under arthritic conditions [12,28-30]. In this study we demonstrate for the first time that disease-regulated mIL-4 expression by the IL-1E/IL-6P promoter provides not

only therapeutically efficacious protein levels under arthritic conditions, but also sufficiently low activity under naïve conditions. Minimizing the undesired effects of biologicals, in this case the potent proinflammatory and chemotactic properties of IL-4, requires promoters with low basal activity under naïve conditions. Promoter regions from acute phase proteins (APP) [28-33], inducible nitric oxide synthase (iNOS) [34], proinflammatory cytokines [13,14], and a nuclear factor-κB (NF-κB) responsive promoter [12] have been predominantly explored for their potential to effectuate arthritis-induced transgene expression. Although several of these appeared applicable for disease-regulated gene therapy, intrinsic properties limit their suitability for minimizing transgene expression under naïve conditions. Promoters from APP are in general well-suited for inflammation-induced gene expression, however absolute amounts of transgene expression are far lower than obtained with strong constitutive promoters [30,32]. To overcome this disadvantage, APP promoters were incorporated in a two-component enhancer system. This system relied on APP-regulated expression of the human immunodeficiency (HIV) transactivator of transcription (Tat) that consecutively stimulated protein expression from genes inserted downstream of a HIV long terminal repeat (LTR) promoter. Indeed, this resulted in a gain of transgene expression. However, APP promoter demonstrated leakiness under naïve conditions, which led to relatively high background luciferase expression that was equal to [13], or even surpassed CMV-driven expression [28]. An additional downside of this approach, is the use of the Tat protein, which possesses a large immunogenic potential due to its capacity to regulate numerous host genes involved in cell signaling and translation [35]. An NF-κB-responsive promoter showed strong upregulation by proinflammatory cytokines *in vitro* and in adjuvant arthritis in rat [12]. Strikingly, absolute expression levels exceeded those provided by a constitutive CMV promoter by far, both *in vitro* and *in vivo*. Moreover, the same phenomenon was observed for basal levels, indicating that there are substantial amounts of active nuclear NF-κB present under naïve conditions. Thus, turning off transgene expression under naïve conditions by transcriptional control via an NF-κB-responsive promoter appears not very feasible. Our results show that the hybrid human IL-1 enhancer/IL-6 promoter emerges as the most likely candidate thus far to achieve low expression under naïve conditions in combination with efficacious disease-regulated expression in arthritis [13,14]. The promoter regions of these interleukin genes were selected based on their crucial role in early onset and development of experimental arthritis [36,37]. Additionally, microarray analysis of collagen-induced and streptococcal cell wall arthritis revealed a strong correlation of both genes with disease severity and very low expression in naïve knee joints (unpublished data). In contrast, APP genes e.g. complement 3, were originally selected based on the acute phase response in the liver rather than arthritis models. Although these proteins also show induced production by inflamed RA synovial tissue [38], they are constitutively expressed under naïve conditions in synovial fibroblasts [39]. The latter also holds for NF-κB [40], which explains the relatively high basal levels obtained with these promoters under naïve conditions.

An important feature for the development of technologies for arthritis gene therapy would be long-term stable expression in the joint. Recently, it was reported that synovial

fibroblasts transduced with lentiviral or adenoviral vectors lost transgene expression in a promoter-independent fashion within 21 days [10], suggesting that transcriptional control of transgenes would not be beneficial. In our hands, however, we frequently experience rapid loss of transgene expression from constitutively active viral promoters, probably due to *in vivo* silencing caused by methylation of CpG motifs [41], histone modifications [42], cytokine-mediated promoter silencing [43] and vector clearance [44,45]. Strikingly, knee joints transduced with an IL-1E/IL-6P-Luc vector showed a robust response to inflammatory stimuli up to 90 days after viral transduction (unpublished data). Previously, we demonstrated that there is no significant difference in reduction of viral DNA from transduced synovium using the viral CMV or cellular IL-1E/IL-6P promoters [13]. It is our hypothesis that cellular promoters are less susceptible to promoter silencing and in fact support long-term expression in synovium as described by others [8,9].

Our data suggest that disease-regulated transcriptional control of transgene expression is a safer mode of local gene therapy for RA. The therapeutic potential of transgenes under arthritic conditions is retained, while undesirable effects under healthy conditions are effectively minimized. This enables the possible application otherwise disputable biologicals, such as IL-4. Currently, the IL-1E/IL-6P promoter seems very suitable for the development of gene therapy for RA that is based on fine-tuned and restricted expression of therapeutic genes of pleiotrophic nature.

Materials and methods

Animals

Male 10- to 12-weeks old DBA-1/J mice were obtained from Janvier (Le Genest Saint Isle, France). C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany). During adenoviral experiments, mice were housed in low-pressure isolator cages. The animals were fed a standard diet with food and water *ad libitum*. All *in vivo* studies complied with national legislation and were approved by local authorities of the Care and Use of Animals with related codes of practice.

Plasmids

Generation of the plasmid pShuttle-IL-1E/IL-6P-Luc, containing the hybrid IL-1 enhancer/IL-6 promoter sequence (IL-1E/IL-6P) was described previously [13]. For cloning, *Pfu* DNA polymerase (Stratagene, La Jolla, CA) was used. All generated plasmids were verified by sequencing. The plasmid pShuttle-IL-1E/IL-6P-mIL-4-polyA was prepared as follows: the IL-1E/IL-6P promoter was PCR-cloned from pUC18-IL-1E/IL-6P in the *Xba*I/*Eco*RV restriction sites of pShuttle (Stratagene, La Jolla, CA). The SV40 polyadenylation signal was PCR-cloned from pShuttle-CMV (Stratagene) in the *Bgl*II restriction site of pShuttle-IL-1E/IL-6P. Subsequently, the cDNA of mIL-4 was amplified from the recombinant adenoviral vector Ad5E1mIL-4 (Kind gift from C.D. Richards, McMaster University, Hamilton, Ontario, Canada) by using the primers (sense 5'-GATATC GCCACC ATGGGTCTCAACCCCC-3'; antisense 5'-GTCGAC CTACGAGTAATCCATTTGC-3')

introducing a Kozak sequence for enhanced translation [46] and inserted in the *Eco*RV / *Sal*I sites of pShutlle-IL-1E/IL-6P-polyA generating pShuttle-IL-1E/IL-6P-mIL-4-polyA.

Adenoviral vectors

Replication-deficient adenoviral vectors (E1/E3 deleted) Ad5.IL-1E/IL-6P-Luc, Ad5.IL-1E/IL-6P-mIL-4, and Ad5.dl70-3 were prepared according to the AdEasy system (Stratagene), with the exception that recombinant viral particles were produced in E1 transformed N52E6 amniocyte cells [47]. Replication deficient (E1 deleted) Ad5.CMV-mIL-4 was produced by infection of N52E6 cells with Ad5E1mIL-4. Viruses were purified by two consecutive $CsCl_2$ gradient purifications and stored in small aliquots at –80 °C in buffer containing 25 mM Tris, pH 8.0, 5 mM KCl, 0.2 mM $MgCl_2$, 137 mM NaCl, 730 µM Na_2HPO_4, 0.1% (w/v) ovalbumin and 10% (v/v) glycerol. The infectious particle titer (*ffu*) was determined by titrating vector stocks on 911 indicator cells and measuring viral capsid protein immunohistochemically 20 hours after transduction.

Luciferase measurements

For *in vitro* evaluation of the effect of mIL-4 on IL-1E/IL-6P promoter activity, NIH-3T3 cells were seeded at $3x10^4$ cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium) and maintained in Dulbecco's modified Eagle medium containing 5% fetal calf serum, 1% pyruvate and 40 µg/ml gentamycin at 5% CO_2/37 °C. The day after, cells were transduced with Ad5.IL-1E/IL-6P-Luc in serum-free medium at multiplicity of infection (MOI) 50 in a total volume of 50 µl for 3 hours at 37 °C. Thereafter, medium was replaced for serum-containing medium. After 24 hours, cells were stimulated with recombinant mouse IL-1α (10 ng/ml, R&D Systems, Minneapolis, MN) or lipopolysaccharide (2 µg/ml, Sigma, St. Louis, MO) in the presence or absence of recombinant murine IL-4 (5 or 50 ng/ml, kind gift of S. Gilles, Immunex, Seattle, WA) for 24 hours. Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI) by adding an equal volume of Bright-Glo to cells lysed in aquadest. Luminescence was measured on the Polarstar Galaxy (BMG, Offenburg, Germany) and expressed as relative light units (RLU) and normalized to total protein content of the cell/tissue extracts.

Induction of collagen induced arthritis (CIA)

Bovine collagen type II (CII) was dissolved in 0.05 M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freud's Complete Adjuvant (2 mg/ml of *M. Tuberculosis* strain H37Ra, Difco laboratories, Detroit, MI). The mice were immunized intradermally at the base of the tail with 100 µl of emulsion (100 µg of bovine CII). On day 21, mice were given an intraperitoneal booster injection of 100 µg bovine CII dissolved in PBS.

Study protocol and histology

To determine the response of the IL-1E/IL-6P promoter to an immune-mediated inflammation, 10^7 *ffu*/6 µl PBS luciferase-encoding adenoviruses were encoding were injected into the knee joints of immunized DBA-1/J mice, one day after they received a CII booster injection. Knee joints

were qualitatively scored for inflammation (negative or positive) at the time point of injection. At day 2 and 7 after virus injection, knee joints were qualitatively scored for inflammation and patellae with surrounding tissue were isolated in a standardized manner [48]. For luciferase measurements, patellae were put in 250 µl cell lysis buffer (Promega) and snap frozen in liquid nitrogen. Samples underwent four freeze-thaw cycles and were subsequently centrifuged for 10 minutes at 13000 rpm. Luciferase activity in the supernatant was determined as described above.

To evaluate effects of mIL-4 expression in naïve animals, 10^7 *ffu*/6 µl PBS adenovirus expressing mIL-4 (Ad5.IL-1E/IL-6P-mIL4 and Ad5.CMV-mIL-4) or an empty control (Ad5.dl70-3) was injected into the knee joints of C57Bl/6 mice. At day 8, mice were sacrificed by cervical dislocation. From four knee joints, patellae washouts were prepared for assessment of mIL-4 levels. Remaining whole knee joints were removed and fixed in 4% paraformaldehyde for 4 days. After decalcification in 5% formic acid, specimens were processed for paraffin embedding. Tissue sections (7 µm) were stained with haematoxylin and eosin (cell influx) or safranin-*O* (cartilage proteoglycan depletion). Histological changes were scored in the patella/femur region on five semi-serial sections of the knee joint, spaced 70 µm apart. Scoring was performed by two observers without knowledge of the group as described before [49]. Histopathological changes were scored using the following parameters. Cartilage depletion, defined as the loss of proteoglycan content, was scored on a scale ranging from 0-3 per region, depending on the intensity of staining in the cartilage. Infiltration of cells was scored on a scale of 0-3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, 3 = maximal cellularity) , depending on the amount of inflammatory cells in the synovial cavity (exudate) or synovial tissue (infiltrate) [50]. Cartilage erosion was graded on a scale of 0-3, ranging from no damage to compete loss of articular cartilage.

To assess the therapeutic efficacy of disease-regulated mIL-4 expression, 10^7 *ffu*/6 µl PBS adenovirus encoding IL-1E/IL-6P-mIL-4, CMV-mIL4 (positive control), or empty control (viral control) was injected at day 23 (onset arthritis) in knee joints of immunized DBA-1/J mice without macroscopic signs of arthritis in the paws. Development of arthritis in front and hind paws was macroscopically monitored (scores between 0-2) until day 30 (day 7, after intraarticular injection) Consecutively, whole knee joints were removed for histological analysis and cartilage erosion and depletion evaluated as described above.

Murine IL-4 ELISA

Patellae were incubated in RPMI 1640 (Gibco, 200 µl/patella), supplemented with 0.1% bovine serum albumin (Sigma), gentamycin (40 µg/ml) and 1% pyruvate for 1 hour at room temperature. Subsequently, supernatant was harvested and centrifuged for 5 minutes at 1000g. Murine IL-4 levels were determined using the Luminex multianalyte technology, using the BioPlex system in combination with BioPlex Mouse IL-4 Assay (Bio-Rad, Hercules, CA). Cytokines were measured in 50 µl washout medium. The sensitivity of the IL-4 assay was 5 pg/ml.

Statistical analysis

Data values are given as means+SEM and significant differences were determined using Student's *t*-test or Mann-Whitney *U*-test, unless stated otherwise. *P* values of less than 0.05 were regarded as significant.

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A crucial role for the TNF Receptor 1 (TNFR1) in synovial lining cells and the reticuloendothelial system in mediating experimental arthritis

6. A crucial role for the TNF Receptor 1 (TNFR1) in synovial lining cells and the reticuloendothelial system in mediating experimental arthritis

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Abstract

Introduction

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease that mainly affects synovial joints. Biologicals directed against tumor-necrosis-factor (TNF)-α are efficacious in the treatment of RA. However, the role of TNF receptor-1 (TNFR1) in mediating the TNFα effects in RA has not been elucidated and conflicting data exist in experimental arthritis models. The objective is to investigate the role of TNFR1 in the synovial lining cells (SLC) and the reticuloendothelial system (RES) during experimental arthritis.

Methods

Third generation of adenovirus serotype 5 were either injected locally in the knee joint cavity or systemically by intravenous injection into the retro-orbital venous sinus to specifically target SLC and RES, respectively. Transduction of organs was detected by immunohistochemistry of the eGFP transgene. An adenoviral vector containing a short hairpin (sh) RNA directed against TNFR1 (HpTNFR1) was constructed and functionally evaluated *in vitro* using a nuclear factor-κB (NF-κB) reporter assay and in vivo in streptococcal cell wall-induced arthritis (SCW) and collagen-induced arthritis (CIA). Adenoviruses were administered before onset of CIA, and the effect of TNFR1 targeting on the clinical development of arthritis, histology, quantitative polymerase chain reaction (qPCR), cytokine analyses and T-cell assays was evaluated.

Results

Systemic delivery of Ad5.CMV-eGFP predominantly transduced the RES in liver and spleen. Local delivery transduced the synovium and not the RES in liver, spleen and draining lymph nodes. *In vitro*, HpTNFR1 reduced the TNFR1 mRNA expression by three-fold resulting in a 70% reduction of TNFα-induced NF-κB activation. Local treatment with HpTNFR1 markedly reduced mRNA and protein levels of interleukin (IL)-1β and IL-6 in SLC during SCW arthritis and ameliorated CIA. Systemic targeting of TNFR1 in RES of liver and spleen by systemic delivery of Ad5 virus encoding for a small hairpin RNA against TNFR1 markedly ameliorated CIA and simultaneously reduced the mRNA expression of IL-1beta, IL-6 and Saa1 (75%), in the liver and that of Th1/2/17-specific transcription factors T-bet, GATA-3 and RORγT in the spleen. Flow cytometry confirmed that HpTNFR1 reduced the numbers of interferon (IFN)γ (Th1)-, IL-4 (Th2)- and IL-17 (Th17)-producing cells in spleen.

Conclusion
TNFR1-mediated signaling in both synovial lining cells and the reticuloendothelial system independently played a major pro-inflammatory and immunoregulatory role in the development of experimental arthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that mainly affects synovial joints and is characterized by inflammatory synovitis, ultimately leading to the destruction of cartilage and bone. The central role for tumor necrosis factor α (TNFα) in RA pathogenesis has been extensively demonstrated in experimental arthritis, such as successful treatment of murine collagen-induced arthritis (CIA) with TNFα-antibodies [1,2] and development of arthritis in transgenic mice overexpressing human TNF [3]. Most importantly, TNFα has been identified as a key cytokine in human RA [4], which has led to the development of effective treatment of disease by administration of neutralizing TNF-antibodies [5,6].

TNFα signaling is mediated via two distinct receptors encoded by the genes *Tnfrsf1a* (TNFR1) and *Tnfrsf1b* (TNFR2). The TNF receptors are single transmembrane glycoproteins and share only 28% homology, predominantly between their extracellular domains. Both TNFR1 and TNFR2 activate a wide range of pro-inflammatory signal pathways, leading to activation of nuclear factor-κB (NF-κB) and *c*-Jun N-terminal kinase, via recruitment of TNF receptor-associating factors (reviewed in [7]). Attenuation of CIA in TNFR1-deficient mice has demonstrated a dominant role of this receptor in disease [8,9]. Recent investigations on the cell-specific contribution of TNFR1-mediated signaling in RA pathogenesis, have revealed remarkably different functions of TNFR1 in mesenchymal or hematopoietic compartments. Cells from the prior compartment, in particular synovial fibroblasts (SF), have been identified as the primary targets for TNFα in the development of arthritis [10]. In contrast, TNFR1-mediated signaling in cells from the latter compartment, such as leukocytes, exerts an anti-inflammatory function [11,12].

This cell-specificity of TNFR1 function is highly relevant to the safety and efficacy of treatments that target TNFα signaling. Scintigraphic imaging of the biodistribution of radiolabeled anti-TNF after systemic administration in RA patients has shown that, apart from inflamed joints, antibodies accumulate in liver and spleen [13]. However, the function of TNFR1 expression in these secondary lymphoid organs and its contribution to RA pathogenesis remains to be elucidated.
In this study, we investigated the effects of TNFR1-mediated signaling in synovial lining cells (SLC) and the reticuloendothelial system (RES) during experimental arthritis. To this end, we used cell-specific RNA interference-mediated (RNAi) silencing of TNFR1 based on adenoviral delivery of a short hairpin RNA (shRNA)-expressing construct.

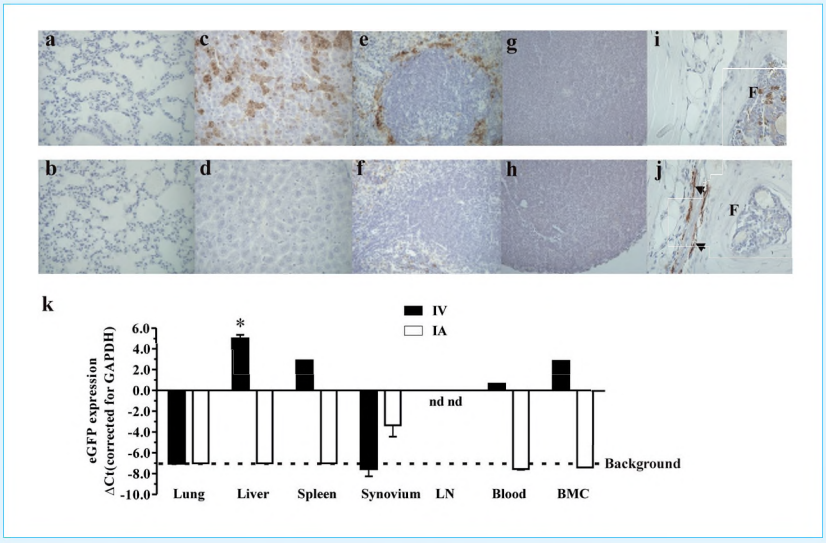

Figure 6.1

Biodistribution after local and systemic administration of adenovirus in mice

One day after collagen booster, non-arthritic DBA/1J mice were injected intra-articularly with 10^7 *ffu* or intravenously with $3x10^8$ *ffu* Ad-eGFP. After administration, eGFP was assessed by immunohistochemistry in lung **(a-b)**, liver **(c-d)**, spleen **(e-f)**, lymph nodes **(g-h)**, synovium **(i-j)** after local **(b-d-f-h-j)** or systemic **(a-c-e-g-i)** treatment. Sites of eGFP positive cells are indicated by arrows. The expression of eGFP mRNA levels **(k)** in each organ, blood and BMC. Draining lymph nodes were negative on IHC and qPCR (not detected). Data are represented as the difference in ΔCt values compared to the housekeeping gene GAPdH. mRNA levels that could not be detect are noted by 'nd' (not detectable). Background mean mRNA levels of eGFP are as low as the negative control. Bars represent means±SEM and statistical differences were determined using Student's *t*-test. * $P<0.01$.

Results

Biodistribution after local and systemic administration of adenoviruses in mice.

Ad5.CMV-eGFP was injected intravenously or intraarticularly one day after the bCII booster-immunization in mice that had no clinical signs of arthritis. One day later, liver, spleen, lung, blood, BMCs and synovium of the knee joints was isolated and prepared for immunohistochemistry (IHC) or processed for mRNA isolation. As expected, the systemically administered adenoviruses were scavenged by the reticuloendothelial system primarily in liver and spleen. IHC detection of eGFP transgene expression showed that in liver the Kupffer cells were predominantly transduced [14] and in the spleen the marginal metallophilic macrophages around the white pulpa [15] (**Figure 1a,c,e,g,i**). The synovium, draining lymph nodes and lung

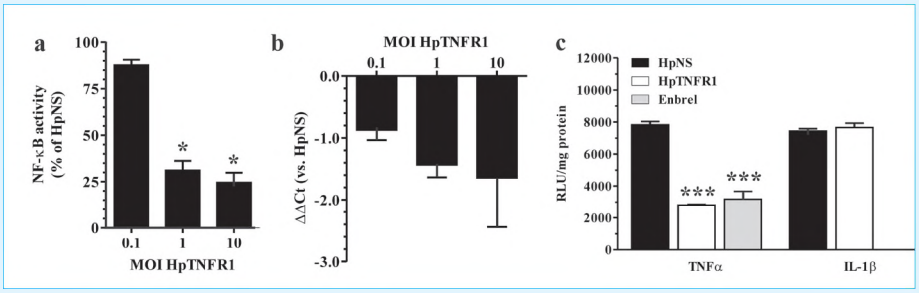

Figure 6.2

***In vitro* validation of HpTNFR1**

Validation of hairpin construct targeting TNFR1 in vitro. **(a)** NIH-3T3-5xNF-κB-luciferase cells were transduced at indicated MOI HpTNFR1 or HpNS and, after two days, stimulated with 10 ng/ml mTNFα for 6 hours. NF-κB-driven luciferase activity is represented as means+SEM (n=4) of percentages compared to the HpNS group. The production of HpNS transduced cells (doses MOI 10) with or without TNFα stimulation was 164.232±864 and 21.555±864 relative light units (RLU)/mg protein, respectively. **(b)** Expression of TNFR1 in NIH-3T3-5xNF-κB-luciferase cells transduced at indicated MOI with HpTNFR1. Data are represented as the mean (n=10) of the difference in TNFR1 ΔCt values compared to HpNS-treated group (ΔΔCt). **(c)** NIH-3T3-5xNF-κB-luciferase cells were transduced at MOI 10 with HpTNFR1 or HpNS or left untreated. After two days, untreated cells were pre-incubated for 1 hour with 10 µg/ml Enbrel and thereafter all groups were stimulated with 10 ng/ml mTNFα or mIL-β for 6 hours. Luciferase activity is represented as means+SEM (n=4). Statistical differences were determined using ANOVA with Bonferroni's post-test. *** P<0.001.

remained negative on IHC. A more sensitive detection method is RT-qPCR and at the mRNA level the spleen, liver, but also blood and BMCs were positive for eGFP, whereas the synovium remained negative (**Figure 1k**). One day after i.a. injection only synovial lining cells, probably type B cells (based upon their morphology), were transduced as shown by RT-qPCR and IHC, while lung, liver, spleen, draining lymph-nodes, blood and BMCs were negative on IHC (**Figure 1b,d,f,h,j**).

HpTNFR1 expression decreased TNFR1 mRNA expression and TNFα signaling in vitro

RNAi-mediated downregulation of gene expression involves both translational repression and accelerated mRNA turnover [16]. To investigate the efficiency of TNFR1 gene silencing by shRNA-expression, we transduced murine NF-κB luciferase reporter fibroblasts with adenoviral vector encoding a hairpin construct targeting TNFR1 (HpTNFR1) or a scrambled control sequence (HpNS). After two days, cells were stimulated with TNFα for 6 hours and TNFα-induced NF-κB activation and TNFR1 expression were quantified using a luciferase assay or qPCR, respectively (**Figure 2a,b**). At MOI 1 and 10, we observed a strong reduction of NF-κB activation (70%) in

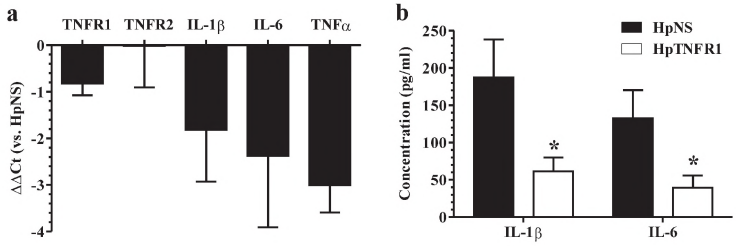

Figure 6.3

Effects of silencing TNFR1 in synovial lining cells during SCW arthritis

Knee joints of naïve C57BL/6 mice were injected with 10^7 ffu HpTNFR1 or HpNS and, two days post-transduction, joints were challenged with 5 µg SCW fragments. **(a)** Expression of indicated genes in synovial tissue at 24 hours after SCW challenge. Data are represented as means+SEM (n=3-6) of the difference in ΔCt values compared to the HpNS-group (ΔΔCt). **(b)** Cytokine protein levels in one hour cultures of synovial tissue explants isolated at 24 hours after challenge. Bars represent means+SEM (n=7) and statistical differences were determined using Student's t-test. * P<0.05.

the HpTNFR1-treated group as compared to the HpNS group. This was accompanied by a two- and three-fold reduction ($2^{\Delta\Delta Ct}$) of TNFR1 mRNA levels at MOI 1 and 10, respectively. Next, we investigated the specificity of the TNFR1-targeting construct (**Figure 2c**). NF-κB luciferase reporter fibroblasts were either transduced with HpTNFR1 or pre-incubated with a specific TNF antagonist (Enbrel) and then stimulated with TNFα or IL-1β. Both HpTNFR1 and Enbrel showed a strong reduction (90%) of TNFα-induced NF-κB activation. In contrast, HpTNFR1 treatment did not affect IL-1β-induced NF-κB activation indicating the specific targeting of TNFR1-mediated signal transduction.

TNFR1 silencing in synovial lining cells ameliorated arthritis

Previously, it was demonstrated that TNFR1 in SFs is essential to development of strictly TNF-driven arthritis [10]. Therefore, we sought to investigate whether this mechanism also holds for arthritis models that are known to be partly TNF-dependent including streptococcal cell wall (SCW) [17] and CIA [1]. Synovial lining cells from knee joints of naïve C57BL/6 were transduced by i.a. injection with adenoviral vectors encoding HpTNFR1 or HpNS. One day thereafter, joints were challenged with 5 µg SCW fragments and after twenty four hours synovial cytokine mRNA expression and protein levels were measured by qPCR and Luminex, respectively (**Figure 3a,b**). TNFR1, but not TNFR2, mRNA level was decreased (two-fold) in synovial tissue explants from the HpTNFR1-treated group. In addition, we observed a strong reduction (more than threefold) in mRNA levels of IL-1β, IL-6 and TNFα. Corresponding with these results, protein levels of IL-1β and IL-6 were significantly reduced

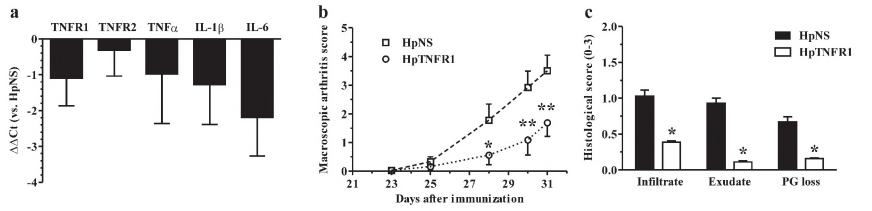

Figure 6.4

Silencing of TNFR1 in synovial lining cells ameliorates CIA

One day after collagen booster, knee joints of CIA negative mice were injected with 10^7 *ffu* HpTNFR1 or HpNS. **(a)** Expression of indicated genes in synovial tissue at day 26 of CIA. Data are represented as means+SEM (*n*=6) of the difference in ΔCt values compared to the HpNS-group (ΔΔCt). **(b)** Appearance of arthritis in fore and hind paws was monitored at indicated time points and scored for severity. **(c)** Histological analysis of inflammation (infiltrate, exudate) and proteoglycan depletion in patellar and femoral cartilage (PG loss) from knee joints isolated at day 31. Data are represented as means±SEM (*n*=9 mice) and statistical differences were calculated using Mann-Whitney *U*-test. * $P<0.05$, ** $P=0.01$.

in the HpTNFR1 compared to HpNS group. Next, knee joints of CIA negative mice were transduced with HpTNFR1 or HpNS at day one after booster (day 22). RT-qPCR analysis at day 26 showed a strong (more than fourfold) reduction in synovial mRNA levels of IL-1β, IL-6 and TNFα (**Figure 4a**). Arthritis development was monitored until day 31 (**Figure 4b**). While CIA incidence was equal between treatments, TNFR1 silencing clearly reduced macroscopic arthritis severity. Histology taken at day 31, revealed protection against cartilage destruction and a significant reduction in the amount of synovial inflammatory cell infiltrate and joint space inflammatory cell exudate (**Figure 4c**).

TNFR1 silencing in the reticuloendothelial system prevented collagen-induced arthritis development

Recently, it was shown that TNFR1 silencing in the radiosensitive hematopoietic compartment aggravates disease in CIA [12]. Secondary lymphoid organs, such as liver and spleen, are rich in mature and functional cells of hematopoietic origin, such as lymphocytes, monocytes and APCs. To delineate the function of TNFR1 in hepatic and splenic cells during arthritis, CIA negative mice (collagen type II immunized mice without macroscopic signs of arthritis) were injected i.v. with HpTNFR1 or HpNS at day one after booster injection (day 22). We monitored arthritis development until day 31 (**Figure 5a**). Up to day 30, incidence of arthritis in the paws of mice treated with HpTNFR1 (40%) was considerably reduced compared to HpNS treatment (83%) (data not shown). In addition, macroscopic arthritis scores were significantly reduced in the TNFR1 group. Histology

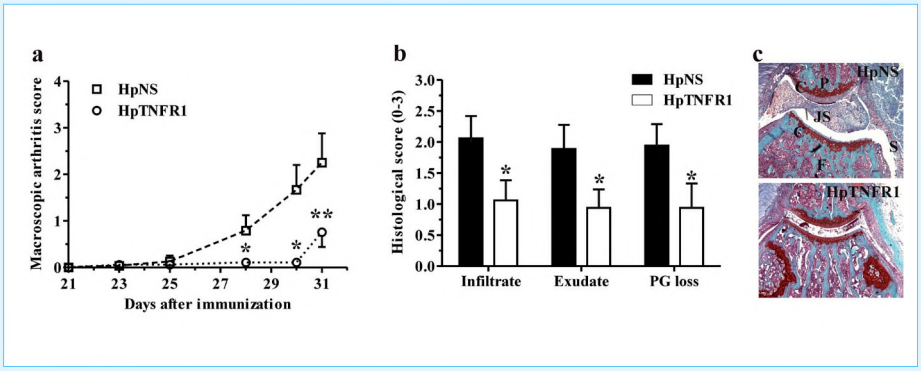

Figure 6.5

TNFR1 silencing in hepatic and splenic APCs ameliorates CIA

One day after collagen booster, CIA negative mice were injected intravenously with $3x10^8$ *ffu* HpTNFR1 or HpNS. **(a)** Appearance of arthritis in fore and hind paws was monitored at indicated time points and scored for severity. **(b)** Histological analysis of inflammation (infiltrate, exudate) and proteoglycan depletion in patellar and femoral cartilage (PG loss) from knee joints isolated at day 31. Data are represented as means±SEM (n=6 mice) and statistical differences were calculated using Mann-Whitney U-test. * $P<0.05$, ** $P<0.005$. **(c)** Representative picture of Safranin-O-stained tissue sections of knee joints from mice treated systemically with HpNS or HpTNFR1. Original magnification 40x. **C**=cartilage, **P**=patella, **JS**=joint space, **S**=synovium, **F**=femur.

of knee joints taken on day 31, confirmed a significant reduction in joint inflammation and revealed a strong suppression of cartilage proteoglycan depletion (**Figure 5 b,c**).

TNFR1 silencing in antigen-presenting cells reduced the number of T helper cells in spleen and dampened the acute-phase response in liver

To elaborate on the mechanisms behind HpTNFR1-mediated prevention of CIA, we analyzed pro-inflammatory gene expression in liver at disease-endpoint by qPCR (**Figure 6a**). This qPCR analyses on the splenocytes (**Figure 6b,c**) and cytokine measurements on the APC fraction (**Figure 6d**), respectively. FACS analysis showed a significant reduction in the number of $CD4^+$/$TCR\beta^+$T-cells, stained intra-cellularly for Th1 (IFNγ), Th2 (IL-4) or Th17 (IL-17) cytokine expression. This was accompanied by a strong decrease (more than fourfold) in mRNA expression of their respective transcription factors (T-bet, GATA-3 and RoRγT). Since both IL-1 and IL-6 have been described as crucial cytokines T-cell expansion and differentiation [18,19], we measured their production by TNFα-stimulated APCs in HpTNFR1 and HpNS-treated groups. Indeed, secreted IL-6 protein levels were significantly reduced in HpTNFR1 as compared to HpNS treated groups. Together, these data demonstrate a clear pro-inflammatory role of TNFR1 in SFs and splenic APCs.

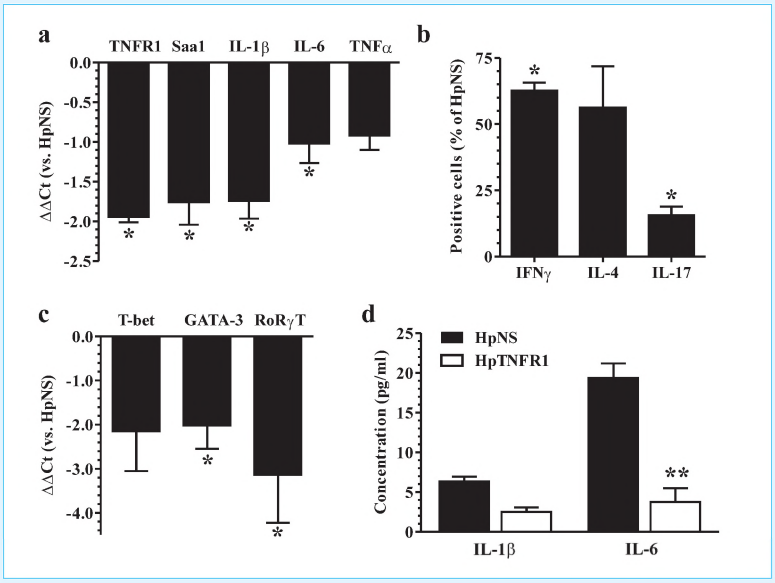

Figure 6.6

Effects of TNFR1 silencing in hepatic and splenic reticuloendothelial system

One day after collagen booster, CIA negative mice were injected intravenously with $3x10^8$ *ffu* HpTNFR1 or HpNS. **(a)** Expression of indicated genes in liver isolated at day 26. Data are represented as means (n=5) of the difference in ΔCt values compared to the HpNS-group (ΔΔCt). **(b)** Analysis of intra-cellular cytokine expression in T-cells isolated from spleen at day 26. Data are represented as mean+SEM (n=4) of the percentage positive cells compared to the HpNS-group. **(c)** Expression of indicated genes in isolated splenic T-cells. Data are represented as means (n=5) of the difference in ΔCt values compared to the HpNS-group (ΔΔCt). **(d)** Secreted cytokine levels from splenic APCs stimulated for 24 hours with 10 ng/ml mTNFα. Data are represented as means+SEM (n=5). Statistical differences were calculated using ANOVA with Bonferroni's post-test. * P<0.05, ** P<0.01.

Discussion

The pleiotropic biological and immunological activities of TNFa are determined by its cellular localization (transmembrane or soluble) [20-23] and the cell-specific relative abundance of its respective receptors, TNFR1 and TNFR2 [9,12,24,25]. The role of TNF as pivotal mediator of the cytokine cascade in inflammation and RA pathogenesis has been unequivocally established, but the relative contribution of specific cell types and TNF receptors has not been fully elucidated. Delineating the role of TNF and its receptors in different tissues and cell types relevant to disease may contribute to a better and safer TNF-targeting strategy in RA patients. While a number of studies using TNFR1-deficient mice have established the global contribution of signal transduction through this receptor in CIA [8,9,11,26], cell-specific functions of TNFR1 have thus far only been studied in SFs, bone marrow derived

macrophages, and radiosensitive hematopoietic cells [10,12,27,28]. In this study we have demonstrated that after local treatment in the knee-joint only the synovial lining cells were transduced and that there was no spill over to other organs. Gouze *et al.* [29] have shown that after intra-articular adenovirus delivery 75-90% of the transduced cells are positive for fibroblast markers (CD90, CD29,VCAM-1) and no transduced cells were positive for the macrophage marker CD11b. Ten percent of the transduced cells are positive for the APC marker CD86. After systemic delivery predominantly liver and spleen were transduced, while synovium remained negative. It is well documented that systemic intravenous delivery of adenoviruses targets the Kupffer cells in the liver [30] and marginal zone macrophages in spleen [31]. Stone *et al.* demonstrated that adenoviruses in the circulation become opsonized by blood platelets and that these aggregates are sequestered in the reticuloendothelial system [32]. Interestingly, depletion of synovial tissue macrophages [33] or the macrophages in spleen and liver [34] after local or systemic administration of clodronate-encapsulated liposomes demonstrates the crucial role of both the local and systemic macrophages in mediating experimental arthritis. For this, we can conclude that TNFR1-mediated signaling in joint, liver and spleen RES compartments contributes to the local joint inflammation and the development of autoimmunity during experimental arthritis.

The contribution of TNFR1-mediated signaling in SLCs to joint inflammation was investigated after SCW challenge. In the acute phase, SCW arthritis represents an innate immune response against SCW fragments that is driven by direct activation of macrophages [35,36]. TNFR1 silencing in SLCs resulted in a significant reduction of secreted IL-6 and IL-1β levels in the joint, which indicates an inhibition of the local cytokine cascade. The reduction of IL-6 is most likely a direct effect of TNFR1 silencing in SLCs, since previous studies demonstrated that TNF-induced IL-6 secretion in human RASF is mediated exclusively through TNFR1 [37,38]. In contrast, hematopoietic (neutrophils, monocytes), but not mesenchymal cells (SFs) were identified as the main source of IL-1 in TNF-driven joint pathology [39]. The observed reduction of IL-1β suggests that TNF signaling in SLCs plays an important role in chemoattraction of inflammatory cells. Indeed, histological analysis of HpTNFR1-treated joints in CIA showed almost complete prevention of IL-1-induced cartilage proteoglycan loss, which was accompanied by an impressive reduction of inflammatory cell influx. In line with the study of Armaka *et al.* [10] we have revealed a dominant role of TNFR1-mediated signaling in SLCs in joint inflammation.

Remarkably, we found that TNFR1 silencing in knee joints also protected the ipsilateral ankle joints in CIA mice. While such distal effects have been described before in local gene therapy approaches [40-42], the underlying mechanism is still not fully understood. However, such an effect might point toward a role of local TNFR1-mediated signaling in development of autoimmunity. In support of this, previous investigations using periarticular delivery of secreted transgenes, vIL-10 and TNFR, in CIA showed that distant anti-arthritic effects coincided with a reduction of specific collagen antibody titers and modulation of T-cell responses, respectively [42-44]. Notably, the beneficial systemic effects of periarticular TNFR gene therapy correlated well with circulating levels of the transgene [42]. In absence of transgene spillover to the circulation, distal effects have been attributed to antigen-primed APCs exposed to the therapeutic transgene traveling from treated to untreated joints [44-46]. In our approach, TNFR1-silencing was restricted to SLCs which would exclude transgene spillover or direct modulation of APCs as a causative for systemic effects. However, local TNFR1-treatment reduced local

levels of IL-6 and IL-1β. Both cytokines are implicated in APC function, which is in turn a prerequisite for induction of auto-reactive CD4+ T-cells and autoimmunity. Eriksson *et al.* demonstrated that IL-1 receptor type I is required for efficient activation of dendritic cells (DCs) [47]. IL-6 switches the differentiation of monocyte-derived APCs from DCs to macrophages [48]. The observed reduction of both IL-1β and IL-6 synthesis in the inflamed joint may result in the development of immature DCs, a differentiation state associated with a tolerogenic function of these cells. Alternatively, tolerogenic DCs can be induced by IL-10, a cytokine which inhibits the synthesis of IL-1 and IL-6 in monocytes and other cell types [49]. Alternatively, TNFR1-treatment might have affected the APC-like function of SLCs. Although SF are not considered to be professional APCs, approximately 60-70 percent of SF in the rheumatic joint expresses major histocompatibility complex (MHC) class II molecules and have the capacity to serve as accessory cells for superantigen-mediated T-cell activation [50-52]. Importantly, the interaction between cytokine-activated T-cells and SF was found to be dependent on transmembrane TNFα on the surface of T-cells and resulted in increased production of IL-6 and chemokine IL-8 [53]. Indeed, we found strongly decreased IL-6 production in HpTNFR1-treated joints, which may abrogate the ability of SLCs to present auto-antigens found within joint tissues.

Strikingly, systemic treatment with HpTNFR1 ameliorated CIA almost to the same extent as local treatment. We have previously shown that suppressor of cytokine signaling-3 (SOCS3) overexpression in splenic APCs ameliorates CIA via a general suppression of Thelper subsets [15]. Similarly, we observed a reduction in the number of Th1 (IFNγ, T-bet), Th2 (IL-4, GATA-3), and Th17 (IL-17, RoRγT) cells upon TNFR1 in splenic APCs after antigen booster injection. In line with these similar findings, it has been demonstrated that TNFR1-deficient murine myocardiocytes show increased expression of SOCS3 and reduced IL-6 secretion upon TNF infusion [54]. We have confirmed that the splenic APCs from HpTNFR1-treated mice produce markedly less IL-6 and IL-1β after TNF stimulation. As these cytokines are crucially involved in Th17 differentiation [18,19], the observed large reduction of Th17 numbers in spleen is not unexpected. Thus, TNFR1 modulation in the reticuloendothelial system has a clear-cut effect on immunity in CIA.

In a side by side comparison, we have demonstrated equal efficacies of local and systemic RNAi-mediated TNFR1-targeting gene therapy in alleviating CIA. Importantly, cell-specific gene therapeutic targeting of TNFR1 clearly modulated pro-inflammatory effects of TNFα, without interfering with protective effects of TNF signaling that have been described in hematopoietic cells [11,12]. It will be interesting to investigate whether TNFR1 knockdown in a therapeutic rather than a prophylactic regimen will be as effective and similar for local and systemic treatment.

Conclusions

Specific silencing of TNFR1 in synovial lining cells, hepatic and splenic reticuloendothelial system using an adenoviral-based RNAi approach revealed a dominant and clear pro-inflammatory role of TNF signaling in these cells during CIA. Systemic treatment dampened the liver acute phase response and reduced proliferation of T-helper subsets in spleen. Local treatment inhibited the pro-inflammatory cytokine cascade in the joint. Gene therapeutic targeting of TNFR1 may be a promising and safer approach for TNFα-blockade in RA patients.

Materials and methods

Animals

Male 10-12 week old DBA/1J and C57BL/6 mice were obtained from Janvier (Le Genest Saint Isle, France). During viral experiments, mice were housed in HEPA-filtered individually-ventilated cages. The animals were fed a standard diet with food and water *ad libitum*. All in vivo studies complied with national legislation and were approved by the local authorities of the Care and Use of Animals.

Induction of collagen-induced arthritis

Bovine collagen type II (bCII) was dissolved in 0.05 M acetic acid to a concentration of 2 mg/ml and was emulsified in equal volumes of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* strain H37Ra, Difco Laboratories, Detroit, MI, USA). DBA1/J mice were immunized intradermally at the base of the tail with 100 µl of emulsion (100 µg of bCII). On day 21, the mice were given an intraperitoneal booster injection of 100 µg bCII dissolved in phosphate buffered saline (PBS). Mice were killed on day 31 by cervical dislocation.

SCW preparation and induction of SCW arthritis

Streptococcus pyogenes T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously [55]. The resulting supernatant obtained after centrifugation at 10,000 g contained 11% muramic acid. Unilateral arthritis was induced by intra-articular injection of 5 µg of streptococcal cell wall (SCW) fragments (rhamnose content) in 6 µl of PBS.

Cell culture

Mouse embryonic fibroblasts (NIH-3T3) stably transfected with a 5xNF-κB luciferase reporter were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM pyruvate, penicillin-streptomycin (Lonza, Basel, Switzerland) and 5% fetal calf serum (FCS). Cells were kept at 37 °C in a humid atmosphere containing 5% CO^2.

Plasmids

For cloning we used *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and T4 DNA Ligase (New England Biolabs, Ipswich, MA). All generated constructs were verified by sequencing. The U6 promoter was PCR cloned from mouse genomic DNA into *XbaI/SalI* sites of pShuttle (kind gift of Bert Vogelstein, Howard Hughes Medical Institute, Baltimore, MD) to give pShuttle-U6 using primers FW 5'-TCTAGAGATCCGACGCCGCCATCTCTA-3' and RV 5'-GTCGACGT-TAACAAGGCTTTTCTCCA-3'. The target sequence for silencing the *Tnfrsf1a* gene was ATCTTCGGTCCTAGTAACT (bp 1170-1188, NM_011609.3) and as scrambled control sequence we used ACTCATGTCTTGATCAGCT (no complementary sequence in murine genome). The silencing cassette was constructed using following oligonucleotides: FW 5'-TG-*target*-TTCAA-

GAGA-*target reverse complimentary*-**TTTT**-TGCA-3' and RV 5'-AAAA-*target*-TCTCTTGAA-*target reverse complimentary*-CA-3' where the loop and polyA sequences are underlined and bold, respectively. Oligonucleotides (4.5 nM) were mixed in annealing buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES pH 7.4), heated for 5 minutes at 95 °C, and gradually cooled to room temperatures. Annealed DNA fragments were ligated in *HpaI/SalI* sites of pShuttle-U6.

Adenoviral vectors

Replication-deficient adenoviral vectors (E1/E3 deleted) Ad5.U6-HpTNFR1, Ad5.U6-HpNS (scrambled control) were prepared according to the AdEasy system [56], with the exception that replication-competent recombinant free viral particles were produced in E1 transformed N52E6 amniocyte cells [57]. Ad5.CMV-eGFP was a kind gift of Jay K. Kolls (Department of Pediatrics, Children's Hospital of Pittsburgh, Pennsylvania).

Viruses were purified by two consecutive $CsCl_2$ gradient purifications and stored in small aliquots at -80 °C in buffer containing 25 mM Tris, pH 8.0, 5 mM KCl, 0.2 mM $MgCl_2$, 137 mM NaCl, 730 µM Na_2HPO_4, 0.1% ovalbumin and 10% glycerol. The infectious particle titer (*ffu*) was determined by titrating vector stocks on 911 indicator cells and measuring viral capsid protein immunohistochemically 20 hours after transduction.

Study design and histology

To study which organs are transduced after systemic or local treatment, Ad5.CMV-eGFP was injected into naïve DBA/1J mice intravenously (i.v.) or intra-articularly (i.a.) with $3x10^8$ or 10^7 *ffu* adenovirus, respectively. One day later liver, spleen, lung, knee joints and draining lymph nodes, blood and bone marrow cells (BMCs) were isolated. They were fixated in 4% paraformaldehyde for 4 days for immunohistochemistry. After decalcification in 5% formic acid, specimens were processed for paraffin embedding. Tissue sections (7 µm) were stained with anti-GFP antibody. For mRNA measurement with RT-qPCR all parts were isolated.

DBA/1J mice were injected intravenously (i.v.) or intra-articularly (i.a.) one day after bCII booster (day 22) with $3x10^8$ or 10^7 *ffu* adenovirus, respectively. For the siRNA hairpin treated mice, three days post-transduction mice were sacrificed and synovium (i.a.) spleen, and liver (i.v.) were isolated. Development of arthritis in front and hind paws was macroscopically monitored (scores between 0-2) until day 31. The macroscopic arthritis score is based on the clinical signs of inflammation in each paw and ankle with a maximum score of 8 (1 for each hind paw and 1 for the ankle). Mice were killed at day 26 or 31 by cervical dislocation. At day 26, synovial tissue explants (i.a.), spleen and liver (i.v.) were removed. At day 31, ankle and knee joints (all groups) were removed and fixed in 4% paraformaldehyde for 4 days. After decalcification in 5% formic acid, specimens were processed for paraffin embedding. Tissue sections (7 µm) were stained with haematoxylin and eosin (cell influx) or safranin-*O* (cartilage proteoglycan depletion). Histological changes were scored in the patella/femur region on five semi-serial sections of the knee joint, spaced 70 µm apart. Scoring was performed by two observers without knowledge of the group as described

before. Histopathological changes were scored using the following parameters. Cartilage depletion, defined as the loss of proteoglycan content, was scored on a scale ranging from 0-3 per region, depending on the intensity of staining in the cartilage. Infiltration of cells was scored on a scale of 0-3 (0 = no cells, 1 = mild cellularity, 2 = moderate cellularity, 3 = maximal cellularity) , depending on the amount of inflammatory cells in the synovial cavity (exudate) or synovial tissue (infiltrate). Cartilage erosion was graded on a scale of 0-3, ranging from no damage to compete loss of articular cartilage.

Immunohistochemistry

Paraffin sections were stained with rabbit-anti-GFP (1:800) (#2555, Cell Signaling Technology, Beverly, MA) o/n at 4°C. After washing sections were incubated for 1 hour with biotinylated secondary antibody goat-anti-Rabbit-BIOT (1:400) (Vector Laboratories, Burlingame, CA). After washing, sections were incubated for 30 minutes with Vectastain (1:400) (Vector Laboratories). Thereafter sections were stained with 3,3'-diaminobenzidine and counterstained with haematoxylin, embedded in Permount (Thermo Fisher, Rockford, Il).

Spleen cell isolation and APC stimulation

Spleens were mashed and filtered, and erythrocytes were removed by osmotic shock. After washing, the splenic cell fraction was incubated in RPMI 1640 (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2 for 1 hour in order to separate adherent cells from nonadherent cells. The adherent cell fraction mainly consisting of macrophages is termed APC. Splenic APCs were stimulated for 24 hours with 10 ng/ml TNFα (Abcam, Cambridge,UK). Cytokine production was analyzed by Luminex.

Flow cytometry analysis

Total spleen cells obtained as described above, were cultured (10^6/ml) for 2 hours in RPMI 1640 (Invitrogen) supplemented with 10% FCS, penicillin-streptomycin, 1 mM pyruvate, 1 µl/ml Golgiplug inhibitor (BD Biosciences, San José, CA), 10 ng/ml PMA, 1 µg/ml ionomycin. Thereafter cells were labeled for 30 minutes at 4 °C with antibody TCRβ-FITC (1:200) and CD4-APC (1:100) or their respective isotype control antibodies. Cells were washed and consecutively fixed and permeabilized using cytofix/cytoperm solution (BD Biosciences). Thereafter, cells were incubated with phycoerythrin-labeled (PE) antibodies IFNγ-PE (1:200), IL-4-PE (1:200), IL-17-PE (1:500) (Biolegend, San Diego, CA) or appropriate isotype controls for 30 minutes at 4°C in PBS containing 1% bovine serum albumin (BSA), 2% FCS, 0.1% saponin. Analyses were performed on a BD FACSCalibur (BD Biosciences).

Cytokine measurements

Synovial tissue explants were incubated for one hour at RT in 200 µl RPMI 1640 supplemented with 0.1% BSA, penicillin-streptomycin and 1% pyruvate. Subsequently, supernatant was harvested and centrifuged for 5 minutes at 1000g. Murine IL-1β, IL-6 and TNFα levels were determined using the Luminex multianalyte technology, using the BioPlex system in combination with BioPlex Mouse Cytokine Assays (Bio-Rad, Hercules, CA). Cytokines were measured in 50 µl washout medium. The sensitivities were 5, <3 and 5 pg/ml for IL-1β, TNFα and IL-6, respectively.

Luciferase measurements

NIH-3T3-5xNF-κB-luciferase cells were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium). The day after, cells were transduced with adenovirus at indicated multiplicity of infection (MOI) in 50 µl DMEM for 4 hours at 37 °C. Two days post-transduction cells were stimulated with 10 ng/ml recombinant murine TNFα or IL-1β (R&D Systems, Abingdon, UK) for 6 hours and subsequently lysed in ice-cold lysis buffer (0.5% NP-40, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl, 10 mM Tris-HCl pH 7.5). Alternatively, TNFα was antagonized by pre-incubating cells for one hour with 10 µg/ml Enbrel (Wyeth Pharmaceuticals, Hoofddorp, Netherlands). Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to total protein content of the cell/tissue extracts using BCA protein assay kit (Thermo Fisher, Rockford, Il).

RNA isolation

Synovial and liver tissue was snap-frozen in liquid nitrogen and homogenized using a MagNa Lyser (Roche). Total RNA was extracted using TRI reagent (Sigma, St. Louis, MO). Isolated RNA samples were treated with RNase-free DNase I (Qiagen, Venlo, the Netherlands) for 15 minutes. Synthesis of cDNA was accomplished by reverse transcription PCR using an oligo(dT) primer and Moloney murine leukemia virus Reverse Transcriptase (Invitrogen).

Quantitative PCR

QPCR was performed using SYBR Green PCR Master mix and the ABI 7000 Prism Sequence Detection system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. Primers were designed over exon-exon junctions in Primer Express (Applied Biosystems Inc.) and used at 300 nM in the PCR reaction. PCR conditions were as follows: 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C. Gene expression (cycle threshold, Ct) values were normalized using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a reference gene ($\Delta Ct = Ct_{gene} - Ct_{Gapdh}$). Primer sequences are available on request.

Statistical analysis

Data are represented as means+SEM. and significant differences were calculated using Student's *t*-test, one-way analysis of variance (ANOVA) or Mann-Whitney *U*-test, as indicated (GraphPad Prism 5.02, San Diego, CA). P-values of less than 0.05 were regarded as significant.

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Toll-like receptor 4 signalling is specifically TAK1-independent in synovial fibroblasts

7. Toll-like receptor 4 signalling is specifically TAK1-independent in synovial fibroblasts

Submitted for publication

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Abstract

Objective

Activated synovial fibroblasts are key players in the pathogenesis of rheumatoid arthritis (RA) by driving inflammation and joint destruction. Numerous molecules including cytokines and toll-like receptor (TLR) ligands induce pro-inflammatory signalling and gene expression through a hierarchical network of kinases. Upstream mitogen-activated protein kinase kinase kinases (MAP3Ks) represent an attractive target for RA treatment. In this study we sought to determine the role of the MAP3K transforming growth factor-β activated kinase 1 (TAK1) in cytokine and TLR-mediated signalling.

Methods

TAK1 activity was inhibited using either a small molecule inhibitor or lentivirally-overexpressed kinase-inactive TAK1-K63W mutant in murine embryonic and human dermal and synovial fibroblasts. Fibroblasts were stimulated with IL-1, TNF, TLR2 or TLR4 agonists and responses were evaluated using transcriptional reporters and analysis of gene expression of collagenases (MMP3,13), cytokines (IL-1β,-6) and chemokines (IL-8, MCP-1).

Results

TAK1 inhibition abrogated cytokine- and TLR-induced activation of NF-κB and *Saa3*-promoter reporters in murine and human dermal fibroblasts. In synovial fibroblasts, TAK1 crucially regulated IL-1 and TNF-mediated NF-κB, but not *Saa3*-promoter activation. Furthermore, TAK1 was required for inducible mRNA expression of IL-1β, IL-6, IL-8, MMP3 and MMP13, but not MCP-1, in response to IL-1, .TNF and TLR2 agonist. Unexpectedly, TLR4-induced NF-κB activation and gene expression was fully TAK1-independent.

Conclusion

In general, TAK1 plays a prominent role in regulation of IL-1- and TNF-mediated signalling in fibroblasts. Interestingly, TLR4 signalling is specifically TAK1-independent in synovial

fibroblasts. Consequently, therapeutic TAK1 inhibition in arthropaties may not dampen the damage-associated molecular pattern-mediated TLR4 activation of synovial fibroblasts.

Introduction

Rheumatoid arthritis (RA) is a systemic chronic autoimmune disease that mainly affects the synovial joints that ultimately leads to joint destruction. Prior to and during joint inflammation the synovial lining tissue, consisting of synovial fibroblasts and macrophages, becomes activated and hyperplastic resulting in invasion and degradation of adjacent cartilage and bone. In both experimental and human arthritis, synovial fibroblasts have been identified as cells that actively drive inflammation and joint destruction [1,2]. Moreover, transmigration of activated RA synovial fibroblasts has been implicated in mediating the spreading of destructive arthritis to unaffected joints [3]. Due to their key role in RA pathogenesis, synovial fibroblasts are major target cells for treatment of disease. Synovial fibroblasts are potently activated by cytokines, such as IL-1 and TNF [4-6], and toll-like receptor (TLR) ligands [7-9]. TLRs 2, 3 and 4 are predominantly expressed in synovial fibroblasts and their expression is increased in RA patients [8,10]. Although TLRs are primarily activated by exogenous pathogens, they also recognize endogenous damage-associated molecular patterns (DAMPs) that are abundantly present in arthritic joints [7,11]. In experimental arthritis, it has been demonstrated that TLR4 activation promotes the onset and severity of disease [12]. Moreover, DAMP-mediated activation of TLR4 specifically in synovium has been found to be crucially involved in joint destruction [13].

After ligation of their respective receptors, expression and secretion of pro-inflammatory mediators including cytokines, chemokines and matrix metalloproteinases (MMPs) is induced through multiple signalling cascades including nuclear factor-κB (NF-κB) and the mitogen-activate protein kinase (MAPK) families p38, *c*-Jun-N-terminal kinase (JNK), and extracellular-signal regulated kinase (ERK) (reviewed in [14]). These kinases are regulated through phosphorylation by their upstream kinases IκB kinase (IKK) and MAPK kinase (MAPKK), respectively. In turn, MAPKK kinases (MAP3Ks) control the activation of IKK and MAPKK and are activated through interactions with receptor-associated proteins, such as IL-1R-associated kinases (IRAKs) and TNFR-associated factors (TRAFs). The MAP3K family comprises numerous members of which MEK kinase-1, -2 (MEKK1,-2) and transforming growth factor-β activated kinase 1 (TAK1) are most abundantly expressed in RA synovial fibroblasts [15].

TAK1 has been identified as the key regulator of IL-1, TNF and TLR-induced activation of NF-κB and MAPK pathways in mice [16-18]. Inhibition of TAK1 catalytic activity prevented chemical-induced inflammation in mice [19]. However, studies using cell-type specific TAK1-deficient mice have also revealed that TAK1 is a crucial regulator of homeostasis in cartilage, skin, epithelium and liver [20-23]. Therefore, insight into the cell-specific

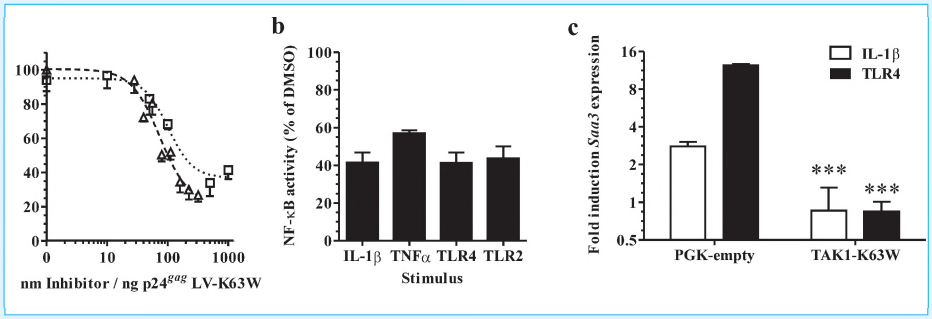

Figure 7.1

TAK1-dependent signalling in murine fibroblasts

TAK1-dependent NF-κB activation in murine fibroblasts. TAK1 inhibition by an inhibitor and LV-K63W (**a**). NIH-3T3-5xNF-κB-luciferase cells were either transduced with indicated $p24^{gag}$ equivalents LV-K63W (triangles) and control lentivirus (PGK-empty) or pre-treated with indicated concentrations inhibitor (squares) and vehicle. Two days post-transduction or one hour after pre-treatment, cells were stimulated with IL-1β (1 ng/ml) for 6 hours. (**b**) NIH-3T3-5xNF-κB-luciferase fibroblasts were pre-treated with 500 nM inhibitor or vehicle and, stimulated for 6 hours with murine IL-1β, TNFα, TLR2 and TLR4 agonists. NF-κB activation was measured by luciferase assay and data are expressed as relative activity compared to controls (mean±SEM, *n*=3). TAK1 inhibition significantly ($P<0.05$, ANOVA) reduced NF-κB activation though all stimuli. (**c**) Induction of *Saa3* mRNA expression by IL-1β or TLR4 agonist in NIH-3T3 stably transduced with LV-K63W or PGK-empty. Data are represented as fold induction (means+SEM, *n*=3)) over untreated cells. *** $P<0.001$ by ANOVA.

regulation of signalling pathways by TAK1 is indispensable for developing a treatment based on its inhibition.

Until now, it has been shown that TAK1 regulates the IL-1-induced JNK pathway and activator protein-1 (AP-1) transcription factor in synovial fibroblasts [24]. Here, we investigated the role of TAK1 in TNF-, TLR2-, and TLR4-mediated signal transduction and induction of pro-inflammatory gene expression in murine embryonic and primary human dermal and synovial fibroblasts. The present study reveals that TLR4 signalling is specifically TAK1-independent in synovial fibroblasts.

Results

TAK1 regulates cytokine- and TLR-induced NF-κB activation in murine fibroblasts

TAK1 has been identified as the crucial mediator of IL-1- and TNF-induced activation of NF-κB, JNK and p38 MAPK signal transduction pathways in NIH-3T3 fibroblasts [25]. Using a 3T3 NF-κB reporter cell line, we investigated TAK1-inhibitory efficiency of a small molecule inhibitor [19] and lentivirally-overexpressed kinase-inactive TAK1-K63W mutant (LV-K63W) (**Figure 1a**).

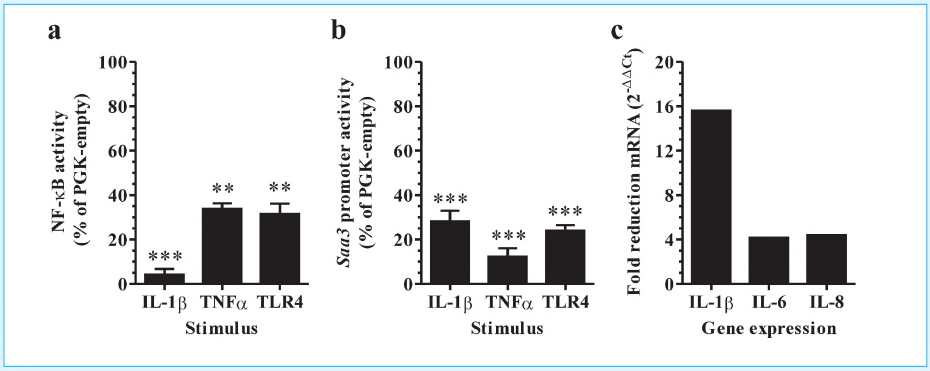

Figure 7.2

TAK1-dependent signalling in human dermal fibroblasts

TAK1-dependent NF-κB and *Saa3*-promoter activation in human dermal fibroblasts. Dermal fibroblasts were co-transduced with 150 ng LV-K63W / PGK-empty and 25 ng LV-*Saa3*/NF-κB-Luc, serum-starved for two days and thereafter stimulated with human IL-1β, TNFα and LPS (TLR4) for 6 hours. NF-κB **(a)** and Saa3-promoter **(b)** activation were determined using a luciferase assay. Data are expressed as relative activity compared to controls (mean+SEM, *n*=4). Induction of IL-1β, IL-6 and IL-8 mRNA expression by IL-1β was determined by RT-PCR **(c)**. Data are represented as fold reduction compared to PGK-empty ($2^{-\Delta\Delta Ct}$, ΔΔCt= ΔCt K63W - ΔCt control). Statistical differences were determined using ANOVA. ** $P<0.01$, *** $P<0.001$

Maximal inhibition of IL-1β-induced NF-κB activity was achieved at 500 nM inhibitor (66.2±8.6%) or 150 ng $p24^{gag}$ equivalents LV-K63W per $5x10^4$ cells (65.4±6.2%), respectively. There were no significant differences in maximal inhibition between inhibitor and LV-K63W, and aforementioned concentrations were applied throughout following experiments. Next, we analysed TAK1-mediated regulation of TLR-induced signal transduction (**Figure 1b**). Treatment of fibroblasts with inhibitor resulted in a significant reduction in NF-κB activation (~60%, $P < 0.05$) induced by TLR2 (Pam_3Cys) and TLR4 (LPS) agonists. As a control, IL-1- and TNF-induced NF-κB activation were also potently suppressed by inhibitor. Finally, we evaluated the effect of TAK1 inhibition on the inducible expression of serum amyloid A3 (*Saa3*), which has been identified as a strictly TAK1-dependent TNF target gene [25] (**Figure 1c**). Stable transduction with LV-K63W also completely prevented induction of *Saa3* expression by IL-1β and LPS. These results confirm the crucial role of TAK1 in mediating cytokine- and TLR-mediated signal transduction in murine fibroblasts.

TAK1-dependent signalling in primary human dermal fibroblasts

Since activated fibroblasts play an important role in the pathogenesis of chronic inflammatory diseases [1,26], inhibition of inflammatory signal transduction represents a straightforward treatment strategy. Therefore, we assessed the contribution of TAK1 to pro-inflammatory signal

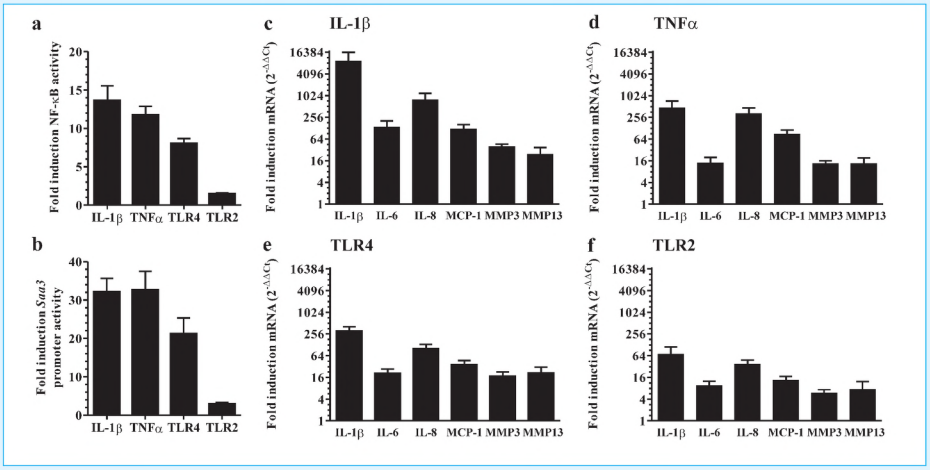

Figure 7.3

Inflammatory signalling in synovial fibroblasts

Induction of transcriptional reporters and pro-inflammatory gene expression in synovial fibroblasts. Fibroblasts were transduced with 25 ng LV-Saa3/NF-κB-Luc, serum-starved for two days and thereafter stimulated with indicated ligands for 6 hours. NF-κB (**a**) and Saa3-promoter (**b**) activation were determined using a luciferase assay. Data are represented as fold induction over basal levels (mean+SEM, *n*=4). Induction of pro-inflammatory gene expression was determined by RT-PCR (**c-f**). Synovial fibroblasts were serum-starved for two days, pre-incubated for one hour with vehicle, and either left untreated or stimulated for 6 hours with IL-1β (**c**), TNFα (**d**), LPS (TLR4, **e**), and Pam_3Cys (TLR2, **f**). Data are represented as fold induction ($2^{-\Delta\Delta Ct}$) compared to untreated cells (means+SEM, six donors).

transduction in primary human dermal and synovial fibroblasts. Overexpression of TAK1-K63W in dermal fibroblasts completely abrogated IL-1-induced NF-κB activation (**Figure 2a**). Also NF-κB activation through TNF- (66.2±2.5%) and TLR4- (68.5±4.6%) was strongly reduced. Since *SAA3* is a pseudogene in humans and TAK1 directly regulates the transcriptional activity of the *Saa3* promoter [25], we additionally used a *Saa3*-promoter luciferase reporter for studying the effect of TAK1 inhibition in human fibroblasts (**Figure 2b**). TAK1 crucially regulated IL-1, TNF- and TLR4-mediated activation of *Saa3*-promoter reporter as revealed by an approximately eighty percent reduction of luciferase activity. Reduction of NF-κB and *Saa3*-promoter activation through TAK1 inhibition correlated with a suppression of IL-1β, IL-6 and IL-8 gene expression (**Figure 2c**). These data identify TAK1 as a pivotal regulator of pro-inflammatory signalling in dermal fibroblasts.

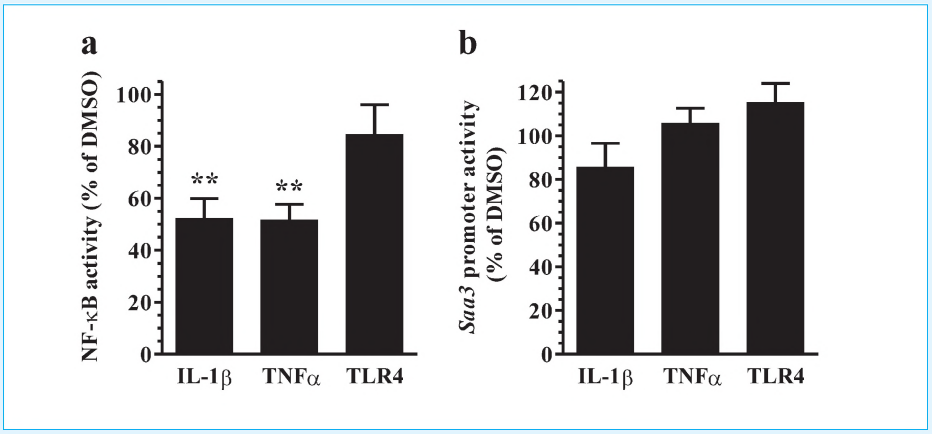

Figure 7.4

TAK1-independent TLR4 signalling in synovial fibroblasts

TAK1-independent TLR4 signalling and *Saa3*-promoter activation in synovial fibroblasts. Fibroblasts were transduced with 25 ng LV-*Saa3*/NF-κB-Luc and consecutively serum-starved for two days, pre-treated with 500 nM inhibitor or vehicle for one hour, and either left untreated or stimulated with human IL-1β, TNFα, and LPS (TLR4) for 6 hours. NF-κB (**a**) and *Saa3*-promoter (**b**) activation were measured using a luciferase assay and data are represented as relative activity (mean+SEM, four donors, quadruplicate measurements) compared to untreated cells. Statistical differences were determined using ANOVA. ** $P < 0.001$.

Cytokine- and TLR-mediated induction of transcriptional reporters and pro-inflammatory genes in synovial fibroblasts

In order to identify TAK1-dependent signalling pathways in synovial fibroblasts, we first validated whether cytokines and TLR ligands induced NF-κB and *Saa3*-promoter transcriptional reporters (**Figure 3a,b**) and pro-inflammatory gene expression (**Figure 3c-f**). We found strong upregulation of both reporter activities upon IL-1, TNF and TLR4 stimulation, whereas TLR2 stimulation only led to marginal induction. Accordingly, expression of NF-κB target genes IL-1β, IL-6 and IL-8 was strongly upregulated upon cytokine and TLR stimulation. Moreover, the magnitude of upregulation seemed to correlate with the extent of NF-κB and *Saa3*-promoter activation. Additionally, we detected significantly induced expression of AP-1 (MMP3/13) [27] and interferon regulatory factor-3 (MCP-1) [28] transcription factor target genes.

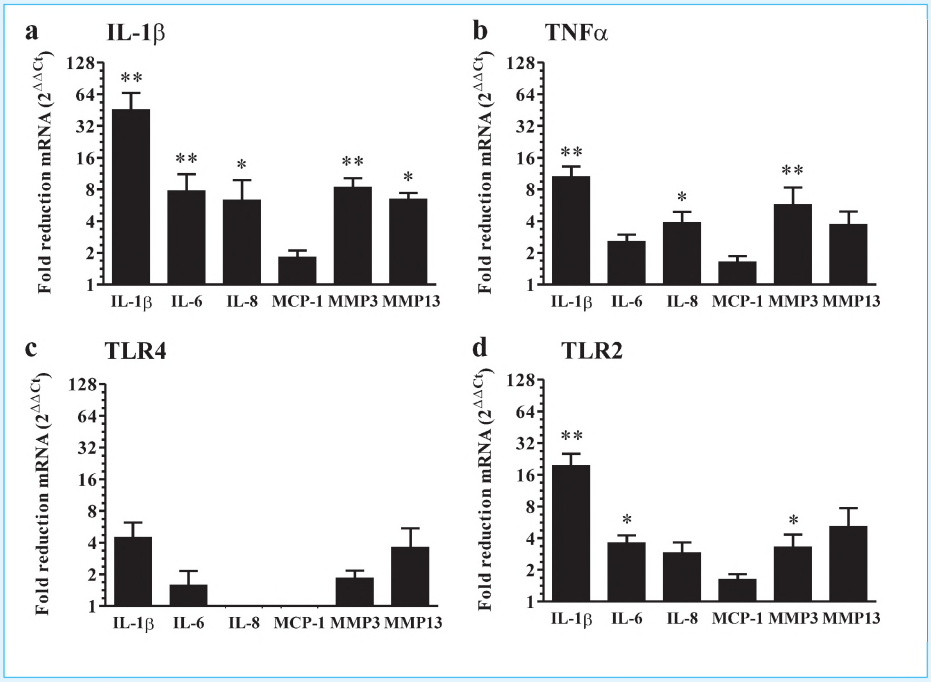

Figure 7.5

Effects of TAK1 inhibition on induction of pro-inflammatory gene expression in synovial fibroblasts

Effects of TAK1 inhibition on induction of pro-inflammatory gene expression in synovial fibroblasts. Fibroblasts were serum-starved for two days, pre-treated with 500 nM inhibitor or vehicle for one hour, and left untreated or stimulated with human IL-1β (**a**), TNFα (**b**), LPS (TLR4, **c**) and Pam_3Cys (TLR2, **d**) for 6 hours. Expression of indicated genes was measured using RT-PCR. Data are represented as fold reduction ($2^{-\Delta\Delta Ct}$) compared to DMSO-treated cells (mean+SEM, six donors). Statistical differences were determined using Repeated Measures ANOVA. * $P < 0.05$, ** $P < 0.01$.

TLR4-mediated signalling is TAK1-independent in synovial fibroblasts

Next, we repeated experiments as described above in the presence of TAK1 inhibitor. Consistent with our previous results in murine and human dermal fibroblasts, IL-1 (47.9±7.9%) and TNF-mediated (48.4±6.2) NF-κB activation was significantly reduced through TAK1 inhibition (**Figure 4a**). In contrast, induction of *Saa3*-promoter activation was unaffected by treatment with TAK1 inhibitor (**Figure 4b**). Surprisingly, both NF-κB and *Saa3*-promoter activation through TLR4 triggering demonstrated TAK1-independent, as indicated by a complete lack of inhibition of transcriptional reporters upon inhibitor treatment.

To corroborate these data, we analysed the effect of TAK1 inhibition on the induction of pro-inflammatory genes by cytokines (**Figure 5a,b**) and TLR agonists (**Figure 5c,d**). In line with promoter reporter measurements, induction of NF-κB target genes IL-1β, IL-6 and IL-8 through IL-1 and TNF was significantly reduced by TAK1 inhibitor treatment. Corresponding with the established regulation of JNK-AP-1 pathway by TAK1 [24], IL-1 and TNF-induced MMP3 expression was also clearly reduced. The IRF3-target gene MCP-1 demonstrated TAK1-independent for all applied stimuli. Whereas TAK1 inhibition resulted in suppression of TLR2-induced IL-1β, IL-6 and MMP3 expression, TLR4-induced gene expression was completely unaffected by inhibitor treatment. Together these data reveal TAK1-independent TLR4 signal transduction that is specific for synovial fibroblasts.

Discussion

Given the multitude of activating molecules and redundancy and complexity of MAPK signalling [29,30], upstream MAP3Ks are of particular interest as therapeutic targets as they potentially couple multiple receptors to downstream signalling pathways. In this study, we investigated the role of the MAP3K TAK1 in mediating pro-inflammatory signalling in fibroblasts and uncovered TAK1-independent TLR4 signalling specifically in synovial fibroblasts.

Although there is substantial evidence that IL-1 signals through both TAK1 and MEKK3 [31,32] information on a physiological role of these two kinases in synovial fibroblasts is limited. In two studies, analysis of MAP3K expression on mRNA and protein level in synovial fibroblasts revealed abundant expression of TAK1 and trace amounts of MEKK3 [15]. Knock down of MEKK3 did not affect IL-1β-induced MAPK activation and TAK1 was identified as the crucial mediator in JNK, but not ERK or p38 MAPK pathways. siRNA-mediated suppression of TAK1 did not significantly inhibit NF-κB nuclear translocation and DNA binding, but suppressed IL-6, a typical NF-κB target gene [24]. Hence, our results, using transcriptional reporter systems confirm that a significant portion of NF-κB activation through IL-1R and TNFR is mediated by TAK1, while p38 and ERK pathways maybe regulated independently from TAK1.

Unexpectedly, we discovered that TAK1 plays no role in TLR4-induced NF-κB activation and pro-inflammatory gene expression in synovial fibroblasts. Several studies have emphasized a central role for TAK1 in LPS/TLR4-mediated NF-κB activation in murine macrophages, embryonic fibroblasts, B-cells, and human HEK293 cells [16,17,33,34]. In addition, we have revealed that TAK1 regulates NF-κB in murine NIH-3T3 fibroblasts and primary human dermal fibroblasts. TLR4 activates signal transduction through TRIF- (TIR-domain-containing adapter-inducing interferon-β) and MyD88-dependent pathways. Upon ligand binding MyD88 is recruited, which subsequently leads to the recruitment and phosphorylation of IRAK proteins, which then interact with TRAF6. The activated complex activates downstream IKK and MAPKKs through an interaction with TAK1. The TRIF-dependent pathway activates the interferon regulatory factor-3 (IRF3) pathway through TANK binding kinase-1 (TBK1). NF-κB and MAPK pathways are activated through interaction with receptor interacting protein-1 (RIP1), which consecutively interacts with TRAF6 and TAK1 [35]. Maximal induction of inflammatory cytokines such as TNFα and IL-6

is dependent on the activation of both TRIF and MyD88 pathways [36]. Based on these studies, we expect TAK1-dependent regulation of MyD88- and TRIF-dependent NF-κB and MAPK activation, but not the IRF3 pathway. The latter was confirmed by our RT-PCR analysis results, which indicated indeed that regulation of the IRF3 target gene MCP-1 [28] was not inhibited by treatment of synovial fibroblasts with TAK1 inhibitor. Inhibition of IL-1β-induced NF-κB activation by inhibitor treatment suggested a MyD88-TAK1-IKK pathway in synovial fibroblasts. Based on the latter, and our observations that TAK1 does not mediate TLR4 signal transduction in synovial fibroblasts, at least two mechanism might underlie these remarkable results.

First, the TLR4-MyD88-IKK/MAPK pathway can be regulated by a MAP3K other than TAK1. Recent investigations have unravelled a TAK1-independent and MEKK3-dependent mechanism for TLR8-mediated IKK and JNK activation [37], and a similar pathway was discovered for IL-1 mediated NF-κB activation [38]. As in our study, NF-κB activity is not completely inhibited by the TAK1 mutant or the TAK1 inhibitor, and knowledge on regulation of MEKK3 in synovial fibroblasts is very limited. Therefore, MEKK3 is a likely candidate that accounts for TAK1-independent signalling in synovial fibroblasts. Thus, a divergence of IL-1R/TLR MyD88-dependent pathways into IL-1R-TAK1- and TLR4-MEKK3 routes could be a possibility.

Second, TLR4 signalling through the MyD88-dependent pathway might be less dominant or even ablated in synovial fibroblasts. A possible mechanism for such phenomenon has been described for LPS-tolerance induction in human monocytes and dendritic cells [39,40]. Upon restimulation with a TLR4 ligand tolerized cells show decreased TLR4-MyD88 complex formation, which results in impaired IRAK-1 phosphorylation [41]. Moreover, it has been shown IRAK-M was upregulated in RA synovial fibroblasts and which is a negative regulator of the MyD88-dependent pathway and is associated with tolerance induction [42,43]. If this were to take place in synovial fibroblasts, we would expect predominant signal transduction through TRIF. However, in the case of pre-dominant TRIF signalling we would still expect activation of the TRIF-TRAF6-TAK1 pathway, unless TAK1 can be bypassed for NF-κB and MAPK signalling in this route.

Circumventing TAK1 in TLR4 mediated NF-κB activation can be achieved by TANK, which was shown to slightly induce NF-κB transcription after overexpression [44]. Another pathway without involvement of TAK1 mediated NF-κB activation has been discovered in IL-1 signalling [45]. In this pathway, TRAF6 is associated with p62, which activates atypical protein kinase C and this subsequently leads to NF-κB activation.

Besides TLR4 signalling, *Saa3*-promoter activation demonstrated specifically TAK1-independent in synovial fibroblasts. This promoter is synergistically activated through cooperation of NF-κB and CAAT/enhancer-binding protein (C/EBP) transcription factors [46,47]. Therefore, TAK1-independent *Saa3*-promoter activity, despite TAK1-dependent NF-κB activity could point towards differential regulation of C/EBP transcription factors by TAK1 in synovial versus dermal fibroblasts.

In conclusion, using a previously characterized dominant-negative mutant or small molecule inhibitor of TAK1, we have found a more restricted role for this MAP3K in mediating

pro-inflammatory signalling in synovial fibroblasts. In the light of the recently established role of TLR4 in experimental arthritis [12,13,48], insight into TLR4 signalling in synovial fibroblasts is of particular interest for understanding pathogenesis and treatment. The underlying mechanism for TAK1-independent regulation of TLR4 signalling, potentially TLR4 tolerance or involvement of an alternative MAP3K, remains to be addressed in future research.

Materials and methods

Patients and samples

Human dermal fibroblasts were obtained from skin biopsies of healthy volunteers at the Radboud University Nijmegen Medical Centre (kind gift from J. Schalkwijk, Department of Dermatology). Synovial tissue was obtained from open joint replacement surgery or arthroscopic synovectomy at the Clinic of Orthopedics, Waldkrankenhaus "Rudolf Elle", Eisenberg, Germany (kindly provided by R.W. Kinne, University of Jena). Patients with RA or osteoarthritis were classified according to the ACR criteria. Synovial fibroblasts were purified from synovial tissue as previously published [49]. Briefly, the tissue samples were minced, digested with trypsin/ collagenase p (Sigma, St. Louis, MO), and the resulting single cell suspension was cultured for seven days. Non-adherent cells were removed by medium exchange. Fibroblasts were obtained by negative isolation using Dynabeads M-450 CD14 MACS purification.

Cell culture

Mouse embryonic fibroblasts (NIH-3T3) DMEM supplemented with 1 mM pyruvate, 40 µg/ml gentamicin, and 5 or 10% fetal calf serum (FCS), respectively. Stable transcriptional reporter cell line NIH-3T3-5xNF-κB-luciferase was described before [50]. Human dermal and synovial fibroblasts were cultivated in DMEM supplemented with 1mM pyruvate, 80 µg/ml gentamicin, and 10% FCS. Cells were kept at 37 °C in a humid atmosphere containing 5% CO_2.

Plasmids

For generation of recombinant lentiviral vectors we used of the third-generation self-inactivating transfer vector pRLL-cPPT-PGK-mcs-PRE-SIN (PGK-empty) containing the human phosphoglyceratekinase (PGK) promoter (kind gift from J. Seppen, AMC Liver Center, Amsterdam, The Netherlands). For cloning we used cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA). All generated constructs were verified by sequencing. A lentiviral transcriptional luciferase reporter containing four tandemly-arranged NF-κB binding sites (pTRH2-NF-κB-Luc) was purchased from System Biosciences (Mountain View, CA, USA). Construction of the *Saa3*-promoter luciferase reporter is described in **Chapter 3**. The cDNA sequences of a non- and EGFP-tagged kinase-inactive mutant of TAK1 (K63W) were PCR cloned from pEGFP-C1-TAK1-K63W (kind gift from M. Kracht, Rudolf-Buchheim-Institute for Pharmacology, Giessen, Germany) into

NheI/NsiI sites of PGK-SIN using the following primers: RV 5'-ATGCATTCATGAAGTGCCTTGT-CAG-3', FW 5'-GCTAGCGCCACCATGTCGACA GCCTCCGCCGCC-3' (non-tagged, Kozak sequence for enhanced translation introduced), and FW 5'-GCTGGTTTAGTGAACCGTCAG-3'(EGFP-tagged).

Lentiviral vector production

Packaging of VSV-G pseudotyped recombinant lentiviruses was performed by transient transfection of 293T cells. One day prior to transfection, 293T cells were seeded in a T75 flask at $1x10^5$ cells/cm^2 in DMEM supplemented with 10% FCS, 1 mM pyruvate, 40 µg/ml gentamicin and 0.01 mM water-soluble cholesterol (Sigma). Cells were co-transfected with 19 µg transfer vector, 14 µg *gag/pol* packaging plasmid (pMDL-g/p-RRE), 4.7 µg *rev* expression plasmid (RSV-REV) and 6.7 µg VSV-G expression plasmid (pHIT-G) by calcium phosphate precipitation. Transfections were performed in 6 ml DMEM without antibiotics and cholesterol and proceeded for 16 hours. Thereafter medium was replaced with fully supplemented DMEM and supernatant harvested after 24 and 48 hours. Cell debris was removed by centrifugation at 1500 rpm for 5 minutes at 4 °C, followed by passage through a 0.45 µm pore polyvinylidene fluoride Durapore filter (Millipore, Bedford, MA, USA). For concentration by ultracentrifugation 28 ml supernatant was overlaid on 4 ml 20% sucrose solution and centrifuged at 25.000 rpm for four hours in a Surespin 630 rotor (Thermo Fisher Scientific, Waltham, MA). Pelleted viruses were resuspended in sterile PBS and stored at -80 °C. Viral titers were determined by assaying $p24^{gag}$ values with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abbott Diagnostics, Hoofddorp, the Netherlands) and expressed as ng $p24^{gag}$/µl.

Luciferase measurements

For *in vitro* reporter studies, cells were seeded at 5 x 10^4 cells per well in a Krystal 2000 96-wells plate (Thermo Labsystems, Brussels, Belgium). The next day, cells were transduced with 25 ng $p24^{gag}$ equivalents lentivirus in 50 µl medium supplemented with 8 µg/ml polybrene (Sigma) for 4 hours at 37 °C and subsequently serum starved (1% FCS) for two days. Cells were pre-incubated for one hour with indicated concentrations TAK1 inhibitor (11,12-dihydro-5Z-7-oxozeaenol, AnalytiCon Discovery, Potsdam, Germany) or dimethyl sulfoxide (DMSO) as vehicle control. Followed by stimulation with recombinant murine or human IL-1β (1 ng/ml, R&D Systems Europe, Oxford, UK), TNFα (10 ng/ml, Abcam, Cambridge, UK), *E. Coli* LPS (1 µg/ml, Sigma), Pam_3Cys (1 µg/ml, EMC Microcollections, Tübingen, Germany) for indicated hours and subsequently lysed in ice-cold lysis buffer (0.5% NP-40, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl, 10 mM Tris-HCl pH 7.5, 1x protease inhibitor cocktail (Roche, Mannheim, Germany)). Luciferase activity was quantified using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) by adding an equal volume of Bright-Glo to the cell lysate. Luminescence was quantified in a luminometer (Lumistar, BMG, Offenburg, Germany), expressed as relative light units (RLU) and normalized to total protein content of the cell extracts.

RNA isolation

Cells were seeded at 90% confluency in 24-wells plates and serum starved (1% FCS) for forty eight hours. Consecutively, cells were pre-incubated with TAK1 inhibitor or DMSO for one hour and stimulated as mentioned above. Thereafter, cells were washed in ice-cold PBS and total RNA was extracted using TRI reagent (Sigma). Isolated RNA samples were treated with RNase-free DNase I (Qiagen, Venlo, the Netherlands) for 15 minutes. Synthesis of cDNA was accomplished by reverse transcription PCR using an oligo(dT) primer and Moloney murine leukemia virus Reverse Transcriptase (Invitrogen).

Quantitative PCR

QPCR was performed using SYBR Green PCR Master mix and the ABI 7000 Prism Sequence Detection system (Applied Biosystems Inc., Foster City, CA) according to the manufacturer's instructions. Primers were designed over exon-exon junctions in Primer Express (Applied Biosystems Inc.) and used at 300 nM in the PCR reaction. PCR conditions were as follows: 2 minutes at 50 °C and 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C. Gene expression (cycle threshold, Ct) values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene ($\Delta Ct = Ct_{gene} - Ct_{GAPDH}$). Primers sequences are available on request.

Statistics

Data are represented as means+SEM and significant differences were calculated using a one-way analysis of variance (ANOVA) or Repeated Measures ANOVA followed by Bonferroni's Multiple Comparison test (GraphPad Prism 5.02, San Diego, CA, USA), where appropriate. *P*-values of less than 0.05 were regarded significant.

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New developments in vectors: regulated promoters

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8. New developments in vectors: regulated promoters

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Introduction

Over the past decades gene therapy has emerged as a promising approach for treatment of a variety of diseases including monogenic diseases, cancer, neurodegenerative disorders, autoimmune and inflammatory diseases. However, efficacy and safety remain the major challenges for turning gene therapy into a clinical reality. Several advances in vectorology have provided opportunities to address these issues including transductional and transcriptional targeting of viral vectors. The prior involves the modification of the virus tropism in order to increase the efficiency and specificity of target cell transduction. The latter comprises the use of *cis*-regulatory elements, such as promoters and enhancers (Figure 1), to restrict transgene expression to specific tissues or patho-physiological conditions. Here we focus on recent developments and applications of endogenously-regulated promoter systems in gene therapy for autoimmune and inflammatory diseases, in particular rheumatoid arthritis (RA).

Disadvantages of constitutively active promoters

The vast majority of pre-clinical gene therapy studies rely on high levels of therapeutic proteins using viral promoters derived from cytomegalovirus (CMV), Rous sarcoma virus (RSV) or cellular promoters such as the human phosphoglycerate kinase-1 (PGK), elongation factor 1α (EF1α) or chicken β-actin promoter. Despite their short-term effectiveness in animal models, the applicability of these promoters for long-term therapy is hampered by a number of issues. First, transgene expression *in vivo* is often transient due to promoter attenuation. A comprehensive study of Chen and co-workers demonstrated a dramatic drop in CMV-driven transgene expression in liver and spleen starting from 3 days after intravenous delivery of adenovirus [1]. Upon intramuscular injection, expression levels decreased between 100- and 1000-fold within three weeks using both CMV, β-actin and EF1α promoters. A similar study, describing a rapid loss of CMV-driven transgene expression from muscle tissue, demonstrated rapid and extensive methylation of CpG sites in the promoter abolishing its transcriptional activity [2] (**Figure 1**). In synovial tissue, which represents the target site of local gene therapy for RA, rapid silencing of the CMV promoter is frequently observed [3-7]. Second, strong constitutive promoters might transactivate endogenous (onco)genes upon integrating in the genome. Weber *et al.* established

a cell-type specific correlation between transcriptional strength and transactivation potential of promoters, including PGK, EF1α and CMV [8]. A second study by Zychlinski and co-workers confirmed the transactivation potential of retroviral enhancer-promoters. Additionally, they demonstrated that the genotoxic risk of integrating vectors is considerably reduced using constitutive cellular promoters (PGK, EF1α) in combination with a self-inactivating vector design [9]. Third, systemic or local overexpression of biologicals regardless of the physiological demand can elicit serious transgene-induced side effects. Our group has demonstrated several undesired side effects in cytokine-based gene therapy approaches, arising from the pleiotropic nature of these proteins. Intra-articular gene therapy for experimental arthritis and osteoarthritis using respectively interleukin-4 (IL-4) [5,10] and transforming growth factor-β (TGFβ) [11], led to a protection against cartilage destruction. However, constitutive TGFβ expression also induced synovial fibrosis and osteophyte formation [12]. The chemotactic properties of IL-4 gave rise to a massive joint inflammation when this transgene was overexpressed in non-arthritic joints. Taken together, the development of long-term and safe gene therapeutic treatment requires a careful consideration of promoter selection. While strong ubiquitous promoters represent seemingly obvious candidates for gene therapy, tightly-regulated expression of transgene appears a key feature towards an effective and safe mode of gene therapy.

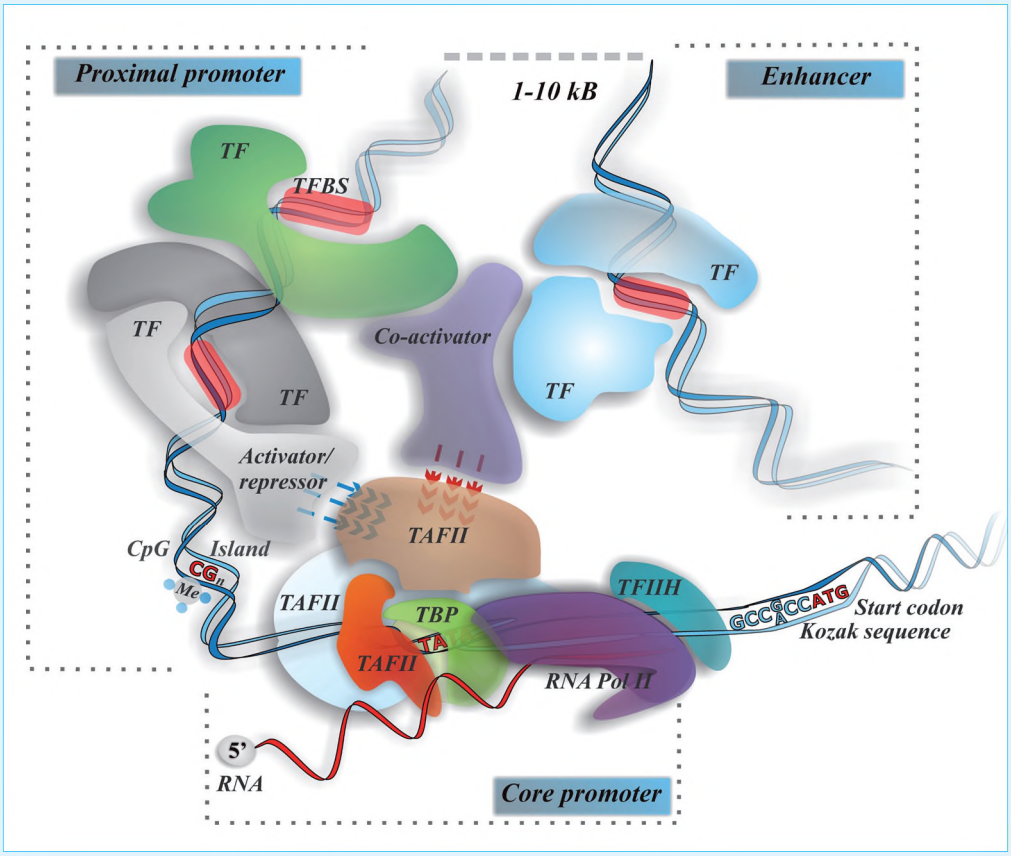
Transcriptional targeting

Transcriptional targeting strategies that are either based on cell-specificity or reactivity to physiological changes are explored extensively in cancer due to their unique expression of tumor specific (onco)genes and regional enriched expression of genes created by the hypoxic environment in tumors (reviewed by Robson and Hirst [13]). Although hypoxia and related processes such as neovascularisation also occur in the inflamed tissue, promoters of these genes or artificial promoters bearing consensus sequences of response elements for the hypoxia-inducible transcription factor (HIF-1α) have thus far not been explored in gene therapeutic approaches for chronic inflammatory of autoimmune diseases. Other strategies utilized in cancer gene therapy as radiation-, chemotherapy- and hyperthermia-inducible expression vectors make use of existing anti-cancer therapies [14] and are for this reason not used for transcriptional targeting of inflammation. Several chronic inflammatory diseases including RA, lupus and inflammatory bowel disease are characterized by a disease course which displays spontaneous periods of exacerbations and remission. Most ideally, expression of a therapeutic protein meets the variable demand during these diseases: high during a relapse and low during remission of the disease. For this, transcriptional targeting in these diseases is now based on the use inflammation-responsive rather than cell-specific promoters.

Acute phase gene promoters

The acute phase response (APR) is part of the innate immunity and comprises an early set of inflammatory reactions induced by infection or tissue injury [15]. The APR is accompanied by a rapid increase in acute phase protein (APP) levels in plasma including serum amyloid A

Figure 8.1



(*SAA*), complement factor 3 (*C3*) and C-reactive protein (*CRP*). The augmentation in serum SAA and CRP levels are sensitive biological laboratory markers in RA patients that correlate with disease activity [15]. The dramatic increase in APP levels are due to a strongly induced transcriptional rate arising from cytokine-mediated synergistic activation of APP promoters. Varley and co-workers pioneered the inflammation-inducible expression of recombinant proteins *in vivo* from APP promoters. Using intravenous administered adenoviral reporters harboring the firefly luciferase cDNA under transcriptional control of murine *Saa3* (-306/+33) or C3 (-397/+45) promoters they demonstrated a strong response upon systemic injection of lipopolysaccharide (LPS). Burke and co-workers applied the human CRP (-122/+672) promoter to generate transgenic-mice showing inflammation-inducible expression of granulocyte

Figure 8.1
Typical TATA-dependent promoter, its cis-regulatory sequences and interactions between regulatory proteins and the transcription initiation complex

Schematic overview of a typical TATA-dependent promoter, its cis-regulatory sequences and interactions between regulatory proteins and the transcription initiation complex. The lower part displays the core promoter region which is the essential sequence for transcription initiation. In eukaryotes, the most common type core promoter is the TATA box that is the binding site for the transcription factor TATA binding protein (TBP). After TBP binds the TATA box, a number of TBP-associated factors (TAFII) and RNA polymerase (Pol II) combine around the TATA box to form the preinitiation complex (PIC). The TAFII, TFIIH has helicase activity and is involved in opening the double helical DNA strands. The PIC only drives a low rate of transcription. The transcriptional rate is further enhanced or inhibited by regulatory factors along with any associated co-activators or co-repressors. The cis-regulatory elements that drive tissue- or context-specific expression are predominantly located in the proximal-promoter region, depicted in the left part of the diagram.
Regulatory factors consist of protein complexes, often hetero- or homodimers, that bind on their cognate binding sites (TFBS). Transcription factors (TF) can transactivate or repress the activity of the PIC and work cooperatively by stabilizing each other when forming DNA-protein complexes. Co-activators mediate the influence of regulatory factors through protein-protein interaction between a TF and the PIC. Additional fine-tuning of transcription is accomplished via more distal DNA elements as enhancers, displayed on the right part. These sequences exert their activating effects independently from position or orientation and are obtained by accomplishing a specific DNA formation. Extensive methylation of CG-rich regions in promoter sequences (CpG Islands), e.g. viral promoters, compromises the binding of regulatory factors and leads to inactivation of the promoter. The Kozak sequence (GCCRCC) is frequently placed directly upstream of the translation start codon (ATG) for enhanced translation of transgenes in gene therapy approaches.

macrophage colony-stimulating factor (GM-CSF) [16]. Upon systemic administration LPS, GM-CSF levels were increased approximately 150-fold within 6 hours and protein levels were 100-500 fold higher compared to endogenous GM-CSF. Transgenic *Saa1-Luc* mice harboring a 7.7 kB promoter of murine *Saa1* demonstrated strong induction of luciferase activity in multiple tissues after systemic LPS and tumor necrosis factor-α (TNF-α) treatment [17]. The strongest induction of luciferase activity, approximately 5000-fold, was observed in the liver whereas brain and spleen showed a less than two fold induction. During an acute arthritis induced by intra-articular injection of zymosan, luciferase levels were induced approximately twenty fold within four hours and declined to seven and six fold induction at one and five days after arthritis induction, respectively.

Pro-inflammatory cytokine and enzyme gene promoters

Apart from APR genes, the promoter regions of genes that are differentially regulated in chronic inflammatory processes such as cytokines, chemokines and matrix degrading enzymes, represent attractive candidates for development of disease-specific gene therapeutic vectors. Experimental arthritis models showed that the pro-inflammatory cytokines TNFα, IL-1, IL-6, IL-18 belong to early-responsive genes, which are upregulated at the primary onset and secondary flare-ups of experimental arthritis [18]. Especially IL-6 is identified as a marker of disease activity and the principal cytokine responsible for the acute phase response in RA patients. Serum IL-6 levels correlated with serum CRP levels and erythrocyte sedimentation rate (ESR) [19,20]. Also the synovial fluid levels of IL-6 correlate with serum CRP and ESR in patients with RA [21]. The synovial fluid concentration of IL-6 are higher than in serum with the synovial fibroblasts as the source of IL-6 with the highest production in the presence of lymphocytic follicles in the synovial tissue [19,20,22]. IL-6 is strictly regulated at the transcriptional level and several transcription factors, including NF-κB, activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) contribute to the complex regulation of this gene. For NF-κB and AP-1 factors a pivotal role has been implied in human RA, murine collagen-induced arthritis (CIA) and immunity [23-27]. Enhanced expression and DNA binding activity of C/EBPβ in synovial tissue of RA patients has been implicated in the pathology [28] and chronicity [29] of disease. Therefore, the IL-6 promoter appeared a prominent candidate to achieve disease-regulated gene therapy. While the minimal promoter of human *IL-6* (-163/+12) showed only little responsiveness, the upstream addition of the human *IL-1β* enhancer (-3690/-2720) mounted a robust response towards pro-inflammatory stimuli *in vitro* and *in vivo* [7]. A comparison of *in vivo* performance between the hybrid (IL-1E/IL-6) and C3-Tat/HIV promoters revealed a comparable responsiveness and transcriptional strength. Geurts and co-workers exploited the properties of this hybrid promoter to enable the use of an otherwise disputable biological, IL-4 [5]. They demonstrated effective protection against cartilage erosion in CIA by injection of knee joints with adenoviral vector containing IL-1E/IL-6P-driven murine IL-4. Perhaps even more importantly, restriction of *IL-4* expression to inflammatory conditions minimized the deleterious effects of this transgene under non-diseased conditions, effectuating a safer mode of IL-4 gene therapy for RA. Adriaansen, Khoury and co-workers constructed adeno-associated viral vectors encoding human soluble p55 TNF receptor coupled to the Fc part of murine IgG1 under control of a minimal CMV promoter containing six upstream NF-κB binding motifs derived from the HIV-LTR promoter. Using a local intra-articular gene therapy approach, this construct delayed onset and decreased the incidence of CIA in mice [30]. The NF-κB-responsive promoter demonstrated a transient responsiveness to repetitive LPS injections at one and eight weeks post-transduction, respectively. In addition, the same vector was used to for local gene therapeutic treatment of adjuvant arthritis in rats [31]. Adriaansen and co-workers found a superior therapeutic effect for disease-regulated expression of transgenes and suggested his phenomenon to arise from a more favorable expression profile during the disease course.

Nitric oxide (NO) is produced by many cell types and has been implicated in host defense and immunity, including modulation of inflammatory processes. The compound is synthesized via nitric oxide synthases (NOS) and the inducible isoform (*iNOS*) is predominantly expressed after exposure to pro-inflammatory stimuli. The induction of *iNOS* is not a disease-specific event and has been demonstrated in several autoimmune and inflammatory diseases such as sepsis, arthritides, systemic lupus erythematosus and type I diabetes [32]. Transgenic *iNOS*-luciferase mice showed a transient upregulation of luciferase activity in knee joints upon injection with zymosan. Luciferase expression peaked at four hours after the challenge, showing six fold upregulation, and returned to basal levels after 24 hours [33]. This group also created a transgenic luciferase-reporter mouse for imaging of angiogenesis using the murine vascular endothelial growth factor receptor 2 (*Vegfr2*) [34]. Since angiogenesis is a hallmark of a variety of inflammatory diseases, including RA, Vegf (receptor) promoters can serve as disease-regulated promoters for gene therapy. Considering the co-dependence of angiogenesis and chronic inflammation [35], for warranting a rapid response to acute inflammatory processes angiogenesis-specific promoters may not be most suitable. Prostaglandins (PGs), particularly PGE2 and prostacyclin (PGI2), are potent mediators of pain and inflammation. PGs are derived from arachidonic acid metabolism through constitutive and inducible cyclooxygenases: COX-1 and COX-2, respectively. An interesting development is the use of the *COX-2* promoter for the replication control of conditionally replicating adenoviruses (CRAds)[36]. CRAds are based on placement of essential early adenoviral E1 genes under specific promoters to mediate oncolytic potency on tumor cells. The COX-2-dependent CRAds might be applied for genetic synovectomy in a regulated fashion by killing the synovial cells when becoming active during inflammation. Rachakonda and co-workers used the canine *COX-2* (-1145/+93) promoter for cytokine-inducible expression of canine IL-4 in articular chondrocytes [37]. For the development of disease-specific gene therapy for canine osteoarthritis, Campbell and colleagues applied the matrix metalloproteinase-9 (*MMP9*) (-1984/-1) promoter [38]. In a human chondrosarcoma cell line this promoter responded strongly to TNFα but not IL-1β. Interestingly, the transcriptional strength of the promoter was significantly enhanced by addition of three or five tandem-arranged NFκB binding sites. However, the basal promoter activity increased accordingly indicating leakiness of the hybrid promoter *in vitro*.

Bioinformatics-driven promoter design

Undoubtedly, the availability of endogenous or artificial promoters that confer a range of transcriptional activities in an inflammation-regulated fashion would contribute substantially to tailor-made gene therapy. While the aforementioned studies have provided compelling evidence for the value of disease-regulated gene therapy, the number of experimentally-verified promoters is fairly low. In addition, the actual choice of promoter identity and region has been mostly an educated guess. The latter issues can be addressed by exploiting recent advances in bioinformatics that aid in inferring transcription regulatory networks [39-42] and understanding promoter architecture [43,44]. Computational analyses of promoter regions from genes

specifically expressed in human cartilage or nematode muscle successfully identified the relevant regulatory DNA elements [42,45]. Using gene expression profiling of target tissues for gene therapy, computational analyses can provide useful information for promoter design. Our laboratory has pursued this approach and performed a gene expression profiling study of synovial tissue from murine CIA to elucidate disease-regulated genes. The proximal (-500/+200) promoter regions of these genes were analyzed with motif scanning algorithms to identify relevant DNA regulatory elements. Transcription factor binding sites for NF-κB, AP-1 and C/EBPβ were significantly enriched and evolutionary conserved in the promoters of arthritis-induced genes. The corresponding promoter regions that contained these motifs conferred inflammation-inducible expression *in vitro* and *in vivo* (unpublished observations). The strength of these approaches have additionally been demonstrated by the Pleiades promoter project, which is aimed at designing well-defined human promoters for brain region or cell type-specific gene therapy [46-48]. These studies focused on profiling region-enriched gene expression within seventeen key areas of the adult mouse brain and used bioinformatics tools to elucidate the transcription factor combinations governing expression profiles. Li and co-workers managed to elucidate a regulatory network comprising fifteen transcription factors and 153 target genes within the mouse brain, whose promoters or DNA regulatory elements will be tested for promoter design in brain-specific gene therapy [49].

Transcriptional amplification strategy

In the above computational approach promoter selection is based on their expression profile and promoter strength as determined by the level of their inducibility to obtain an efficacious gene therapy strategy. However, to obtain a side effect-free physiological response the basal expression of candidate promoters must be low and show no leakiness. To reach all these prerequisites a transcriptional amplification strategy might be necessary to integrate in the inflammation-responsive system. In order to couple inflammation-inducibility with sufficiently high expression levels required to achieve biological effects, Varley and Munford developed a 2-component expression system in which the C3 promoter regulates the production of the human immunodeficiency virus-1 (HIV) transactivator of transcription (Tat) protein, which in turn regulates the HIV-1 long terminal repeat (LTR) promoter to express the gene of interest [50]. This system was highly responsive towards various inflammatory stimuli as TNFα, IL-1β, IL-6, and LPS/turpentine-induced peritonitis. The HIV-LTR is also directly responsive to nuclear factor-kappaB (NF-κB) activating cytokines (IL-1/TNF) and other factors such as LPS. More importantly it was demonstrated that this 2-component construct responded to an inflammatory reaction in a similar fashion in different organs as liver, spleen, kidney, lung and heart. We found that this system was also highly responsive in the joint towards a zymosan-induced joint inflammation in an IL-6-dependent fashion [51]. Miagkov and coworkers and our laboratory independently demonstrated the feasibility of this 2-component system for auto-regulated expression of therapeutics in experimental arthritis models [51,52]. Intra-articular injection of adenoviruses containing the *C3*-Tat/HIV-hIL-10 construct in arthritic

paws of rats completely prevented reactivation of arthritis by an intravenous challenge with group A streptococcal peptidoglycan-polysaccharide (PG-APS) [52]. We used this system for adenoviral overexpression of human interleukin-1 receptor antagonist (IL-1Ra) in knee joints of murine collagen-induced arthritis (CIA) and compared this with the effect of IL-1Ra expression under direct control of the conventional CMV promoter. In a prophylactic regimen we could demonstrate superior effectiveness of this 2-component IL-1Ra expression system in CIA. While the *in vivo* studies using the Tat/HIV based-approach provided proof of principle of the efficacy of local disease-regulated gene therapy for arthritis, side effects of Tat expression in host cells including dysregulation of cytokine expression [53,54] and promotion of chemotaxis [55] limit its applicability for a safe and long-term gene therapeutic treatment.

There are several new drug-inducible expression systems developed based on tetracycline, rapamycin and ecdysone that allows reversible and adjustable expression by an exogenous stimulus (extensively reviewed in [56]). These regulatable systems can be combined with cell-specific or physiologically responsive promoters for regulation of their respective transactivating proteins. Despite their robustness and specificity, long-term application of these systems might be hampered due to an immune-response against the artificial transactivators as has been described for tetracycline-regulated systems in larger animals [57,58]. Regulating the transactivator expression using inducible promoters with low basal activities may delay or even prevent this immune-response and prolong the *in vivo* life time of these systems. Furthermore, these drug-controlled systems provide an additional safety switch to shut-down the system in case of malfunction or deleterious effects.

Conditional RNA interference-based gene therapy

Until now we reviewed the transcriptional targeting strategy for tunable expression of proteins but we envisage the application of inflammation-controlled promoters for development of a gene therapeutic treatment relying on conditional RNA interference-mediated gene silencing. Gene knockdown in experimental models of arthritis has been shown to be therapeutically efficacious [59,60] but a systemic mode of treatment gives rise to the risk of impairing the normal physiological responses required to combat pathogens and injuries. Therefore, conditional knockdown of endogenous genes using inflammation-responsive promoters would be advantageous. However, viral systems for temporal and conditional knockdown are almost exclusively based on drug-controllable (tetracycline) expression of RNA polymerase (Pol) III promoter-driven short hairpin (sh) RNAs [61]. Due to tight restrictions for transcriptional start sites and the termination signal only a relative small number of RNA Pol II promoters are able to produce effective silencing (si) RNAs using the shRNA system. In contrast, microRNA-adapted shRNA systems, in which mature miRNA sequences are replaced with gene-specific duplexes, have no such limitations and therefore can accommodate Pol II-dependent cell- or conditions-specific promoters. Stegmeier and co-workers developed a sophisticated lentiviral platform for simultaneous production of a miR30-adapted shRNA and transgene [62], which could be adapted for developing a dual-approach gene therapy. However, attempts for

conditional expression of miRNAs using other than exogenously-controlled systems have been rarely undertaken. Yang and Paschen used the human heat shock protein 70 (*HSP70*) promoter for heat shock-induced silencing of genes [63]. Since the expression of HSP70 is up regulated in almost all inflammatory diseases [64], this represents a strong candidate promoter for inflammation-induced gene silencing. Alternatively, Yoshizaki and co-workers used the human E-selectin (-160/+1) promoter for conditional knock down of the *SELE* gene [65]. The promoter showed a two-fold increase in activity upon stimulation with TNFα, which proved sufficient for effectively reducing E-selectin expression.

Future perspectives

The efficacy of gene therapy for RA has been demonstrated extensively in animal models of disease using a variety of vectors and transgenes. These promising pre-clinical results have until now led up to the initiation of five human phase I clinical trials in RA [66]. Without exception, these trials relied on strong constitutive viral promoters e.g. the Moloney murine leukemia LTR [67,68] and CMV promoter [69] . This indicates that the selection of an appropriate promoter remains an underestimated aspect in the development of gene therapeutic treatment for arthritis. In contrast, transcriptional targeting has been recognized as an essential prerequisite towards safe human gene therapy in other diseases, predominantly cancer. Gene therapy for inflammatory diseases is, however, not based on gene correction as in monogenic diseases but on restoring the balance by up-regulating therapeutic - or down regulating disease-process implicated genes. In general, the latter genes often have important regulatory roles in normal physiology and immunity that needs to be maintained. For instance, current systemic anti-TNF-treatment in RA has been shown to cause side effects such as opportunistic infections, particularly tuberculosis, in a number of patients [70,71]. For this, local and transcriptionally targeted gene therapy may circumvent the current problems seen with systemic delivery of protein biologicals. We have reviewed that hijacking the local inflammatory gene regulation is a feasible approach for transcriptional targeting, which has demonstrated efficacy in animal models. These studies are summarized in **Table 1**. Together with transductional targeting via vector modification or detargeting transgene expression from non-target tissues using miRNA-regulatory elements [72,73], inflammation-responsive promoters will develop into a safer and efficient mode of gene therapy that is essential for treatment of non-lethal diseases.

Table 1. Inflammation-responsive promoters used in gene therapy approaches

Promoter	Vector	Inflammation model	Species	Ref.
Saa3	Adenovirus	LPS i.p. & turpentine s.c.	Mouse	[74]
C3	Adenovirus	LPS i.p. & turpentine s.c.	Mouse	[50-52,74]
CRP	Transgenic	LPS i.p.	Mouse	[16]
Saa1	Transgenic	LPS/TNFα i.p. & zymosan i.a.	Mouse	[17]
IL-1E/IL-6P	Adenovirus	SCW/zymosan i.a. & CIA	Mouse	[5,7]
6xNFkB/mCMV	Adeno-associated virus	Adjuvant arthritis	Rat	[31]
	Adeno-associated virus	CIA	Mice	[30]
iNOS	Transgenic	LPS/IFNγ i.p. & zymosan i.a.	Mice	[33]
Vegfr2	Transgenic	Oxazolone topical	Mice	[34]
cCox2	Plasmid	*In vitro*, TNFα/IL-1β	Dog	[37]
cMMP9	Plasmid	*In vitro*, TNFα/IL-1β	Human	[38]
HSP70	Plasmid	*In vitro*, heat shock	Mouse/Rat	[63]
SELE	Plasmid	*In vitro*, TNFα	Human	[65]

LPS, lipopolysaccharide; SCW, streptococcal cell wall; CIA, collagen-induced arthritis; i.a., intra-articular; i.p., intraperitoneal; s.c., subcutaneous

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Summary and Future Perspectives

9

9. Summary and Future Perspectives

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Summary

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints and mainly targets the joint capsule, articular cartilage and bone. Key features of the disease include autoimmunity, chronic inflammation and connective tissue destruction. Despite improved understanding of its pathology in the last decades, the etiology remains to be elucidated. Correspondingly, treatment strategies for RA aim at alleviating disease symptoms and halting its progression. The identification of pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) as crucial mediators of cartilage destruction and inflammation, has led to novel treatment strategies in the clinic based on neutralizing these cytokines using antibodies or soluble receptors. Despite therapeutic efficacy of these so-called biologicals, the required continuous and systemic mode of treatment has led to several issues, including serious side effects, limited clinical remission and high costs. To tackle these issues, local and sustained delivery of biologicals to arthritic joints appears an ideal treatment alternative. Gene therapy has emerged as a promising approach for achieving these goals. An additional advantage of gene therapeutic treatment of RA includes the opportunity to achieve fine-tuned regulation of therapeutic transgene expression using a transcriptional targeting strategy. Ideally, such a strategy results in gene therapeutic vector that serves as a "doctor within" by providing transgene expression in an offer-meets-demand fashion with respect to disease activity in the arthritic joint. The aim of the research presented here was to rigorously design a transcriptional targeting strategy for RA and to evaluate its application for gene therapeutic treatment and diagnostics. Additionally, we have sought to validate novel gene therapy approaches based on targeting of intracellular signal transduction pathways.

To this end, we first reviewed the current status of different viral vectors for in and *ex vivo* transduction of resident cells of the arthritic joint in **Chapter 2**. These studies revealed that synovial fibroblasts are considered as primary target cells for local RA gene therapy and they can be efficiently transduced using adenoviral (Ad) and lentiviral (LV) vectors. We corroborated these findings by investigating the transduction efficiency of murine synovium *in vivo* by LV and Ad vectors in **Chapters 4** and **6**, respectively. Immunohistochemistry analyses of joints injected intra-articularly with green fluorescent protein (GFP) expressing Ad or LV viruses, demonstrated predominant transduction of the lining layer of the synovium. Using cholesterol-optimized LV we managed to enhance transduction efficiency of synovium four-fold (**Chapter 4**). Moreover, we demonstrated that LV vectors can transduce human synovial fibroblasts *ex vivo* up to nearly

hundred percent. This feature was exploited in **Chapter 7**, which analyzed pro-inflammatory signal transduction in synovial fibroblasts from RA patients using LV-based transcriptional reporters.

In **Chapter 3** we focused on the development of a transcriptional targeting strategy for RA. We hypothesized that suitable promoters for this strategy could be deduced from gene expression analyses of inflamed synovial tissue. Using gene expression profiles from synovial tissues from joints with progressing stages of collagen-induced arthritis (CIA), we detected 201 genes whose expression was at least ten-fold upregulated from the onset of disease. Bioinformatic analyses of corresponding promoter regions indicated that transcription factor (TF) binding sites of nuclear factor-κB (NF-κB), activator protein-1 (AP-1) and CAAT/enhancer-binding protein β (C/EBPβ) were significantly over-represented. Using an in-house developed filtering algorithm, based on TATA-box position and presence of over-represented TFs, we reduced the number of candidate human and murine promoters from 382 to 66. Ten promoters were incorporated into LV luciferase reporter vectors and nine showed inducible expression by lipopolysaccharide (LPS) in murine macrophages and fibroblasts. The serum amyloid A3 (*Saa3*) promoter, which showed strong inducibility and transcriptional strength, was applied for disease-regulated expression of IL-1 receptor antagonist (IL1Ra) and demonstrated capable of neutralizing effects of IL-1 *in vitro*. These results highlighted the value of a bioinformatics approach for designing a transcriptional targeting strategy and suggested the *Saa3*-promoter as candidate promoter for IL1Ra gene therapy.

The diagnostic value of *Saa3*- and *Cxcl1*-promoters identified in **Chapter 3** was the research topic of **Chapter 4**. First, LV luciferase reporters were used to evaluate promoter activities during two flares of joint inflammation induced by streptococcal cell wall (SCW) fragments. We found that specifically *Saa3*-promoter activity correlated with histological and technetium uptake measurements of joint inflammation. A comparison of the conventional technetium uptake with assessment of *Saa3*-promoter activity revealed an increased sensitivity of the latter for detecting joint inflammation. These findings led to the hypothesis that this method also might discriminate between high and low inflammatory synovial tissues from arthritis patients. Indeed, the relative *Saa3*-promoter responses to IL-1, TNFα and LPS were significantly increased in synovial fibroblasts with a high inflammatory expression profile. Taken together the transcriptional *Saa3*-promoter reporter demonstrated to be a robust, sensitive and feasible method for distinguishing molecularly distinct inflammatory subtypes both in experimental arthritis and synovial fibroblasts from a heterogeneous patient population.

Having designed and evaluated transcriptional targeting strategies in the aforementioned chapter, we demonstrated the important proof of principle for enhanced safety of gene therapy using transcriptional targeting in **Chapter 5**. For this, we compared the effects of constitutively versus disease-regulated intra-articular expression of IL-4 under healthy and arthritic conditions.

Disease-regulated expression was achieved using a hybrid promoter consisting of the IL-1 enhancer fused to the IL-6 promoter (IL-1E/IL-6P). Regulated expression of IL-4 demonstrated successful protection against cartilage erosion in CIA. More importantly, restriction of IL-4 expression in healthy joints significantly decreased inflammation, which occurs as a side effect of sustained IL-4 expression. These results definitely showed that transcriptional targeting is safer mode of gene therapy by retaining therapeutic potential of transgenes under arthritic conditions while effectively minimizing transgene-induced side effects during disease remission.

In **Chapters 6** and **7**, we sought to validate novel gene therapy approaches based on targeting of signal transduction pathways in synovial fibroblasts. The central role of TNFα in RA pathogenesis, led to the development of an Ad vector that mediated knockdown of TNF receptor-1 (TNFR1) via RNA interference (**Chapter 6**). When applied for local gene therapy, this vector markedly reduced IL-1 and IL-6 mRNA and protein expression in SCW-induced arthritis. Local treatment of CIA led to a significant amelioration of disease as characterized by reduction of joint inflammation and cartilage proteoglycan depletion. Notably, systemic gene therapy, which targeted TNFR1 in the reticulo-endothelial system of liver and spleen, also effectively ameliorated CIA. The underlying mechanism for this appeared to be a reduction of activated T cells in spleen and a dampening of the acute phase response in liver. Since besides TNF signaling numerous signal transduction pathways contribute to RA pathology, we evaluated transforming growth factor-β-activated kinase 1 (TAK1) as a target for simultaneously inhibiting multiple signaling pathways (**Chapter 7**). TAK1 catalytic activity could be efficiently inhibited using LV-mediated overexpression of a TAK1 kinase-inactive mutant. Using LV transcriptional NF-κB and *Saa3*-promoter reporters we confirmed the central position of TAK1 in mediating IL-1, TNF and toll-like receptor 2/4 (TLR) signaling in murine fibroblasts and human dermal fibroblasts. However, we uncovered a more limited role for TAK1 in synovial fibroblasts since, contrastingly, TLR4 signaling and IL-1/TNF-induced *Saa3*-promoter activation were independent of this kinase. Nonetheless, cytokine-induced NF-κB activation could be dampened by TAK1 inhibition. The combined data from **Chapters 6** and **7** suggests that targeting of intra-cellular signal transduction holds promise for novel gene therapeutic treatment strategies for RA.

In **Chapter 8** we finally returned to transcriptional targeting strategies and reviewed its recent developments and future perspectives.

Future perspectives

The initial optimism surrounding the emergence of gene therapy as a cure for disease has been severely tempered by cases of vector-induced fatalities in clinical trials [1-3]. These serious adverse events have shifted the emphasis of vector design from efficacy to safety. Transcriptional targeting has been introduced as suitable strategy to increase both efficacy and safety of gene therapy approach by strictly controlling spatial and temporal expression of transgenes. The most common strategies include the use of endogenously (**Chapters 3-5**) or

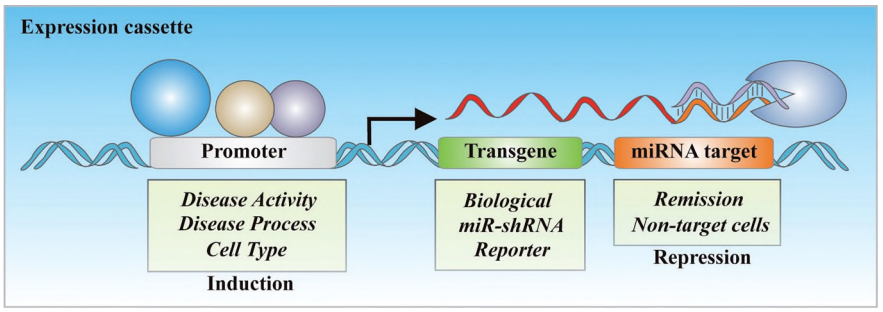

Figure 9.1
Future expression cassette for RA gene therapy

exogenously-controlled promoter systems [4]. Noteworthy, Brown and colleagues developed an intriguing novel approach that exploited endogenous microRNAs (miRNAs) for regulation of transgene expression [5]. Introduction of miRNA target sequences at 3' untranslated regions of transgene cDNAs resulted in strong suppression of its expression in cells expressing the corresponding miRNA. Adaptation of the miRNA-controlled system for RA gene therapy would require the identification of a miRNA whose expression inversely correlates with disease activity. Interestingly, comparison of miRNA profiles in synovial fibroblasts have revealed that miR-124a is significantly underexpressed in RA, which stimulates proliferation and chemokine secretion [6]. Based on these findings, miR-124a might be applicable for transgene regulation, provided that its expression level increases during RA remission. In terms of safety, regulation of transgene expression by a combination of endogenous inflammation-regulated promoters and miRNA-based suppression in remission appears an ideal combination. Another major advantage of using physiologically-regulated promoters is that their application leads to a strongly reduced risk of insertional mutagenesis [7]. Additionally, unpublised results from our laboratory suggested that the longevity of transgene expression might be elongated through transcriptional targeting. Whereas local adenoviral gene therapy using a constitutive promoter normally results in rapid loss of transgene expression within three weeks (**Chapter 5**), we were able to induce strong expression from such a vector at three months after transduction (unpublished observations).

Chapters 6 and **7** convincingly demonstrated that intracellular targeting of signal transduction pathways could be a valid strategy for gene therapy. Notably, selective knockdown of genes using short hairpin RNA interference (shRNA) appeared a powerful method for targeting signal transduction (Chapter 6). Ideally, shRNA-based gene therapy strategies would also incorporate

transcriptional targeting to ensure safety. However, due to tight restrictions for transcriptional start sites and the termination signal only a relatively small number of endogenous cellular RNA polymerase II-dependent promoters are able to effectively produce shRNA. Recently, Stegmeier and colleagues managed to address this issue by an miRNA-adapted shRNA design [8]. This design mimics a natural miRNA-30 primary transcript, which is naturally driven by a polymerase II-dependent promoter [9]. Yang and Paschen used this design in combination with a *Hsp70* promoter for heat shock-induced silencing of genes [10]. Importantly, these studies suggest that transient induction of miRNA-adapted shRNA is sufficient for temporal knockdown of genes. The development of inflammation-inducible RNAi gene therapy might therefore be feasible and has been pursued as a pilot experiment in our lab (unpublished results).

In **Chapter 3** we demonstrated that a computational approach on expression profiling data is a powerful strategy for developing transcriptional targeting for gene therapy. In principle, this approach can be applied to any expression profiling study as evidenced by D'Souza and coworkers, who followed an analog method to design brain region-specific gene therapy [11]. With respect to human gene therapy, computational analysis on expression profiles of synovial tissues could pave the way towards a personalized therapy. Supporting evidence for this hypothesis has been demonstrated in **Chapter 4**, in which we described that a *Saa3*-promoter shows a more potent response, and thus therapeutic transgene production, in synovial tissue from RA patients a high inflammatory profile. Therefore, in depth analyses of synovial tissue expression profiles of RA patients with different disease subtypes are warranted.

The differential transcriptional properties of the promoters identified in **Chapter 3** equip us with convenient tools for tailor-made gene therapy approaches. In addition, we demonstrated that transcriptional reporters could be of value for diagnostic imaging in arthritis (**Chapter 4**). Apart from feasibility, promoter-reporters could have several prominent advantages over present methods such as magnetic resonance imaging, ultrasound biomicroscopy and scintigraphy. First, several reporter genes are available that allow both *ex vivo* (**Chapter 4**) and non-invasive and real-time in vivo imaging [12]. Second, using standard molecular cloning techniques it would be possible to generate a diverse range of promoter-luciferase reporters for monitoring specific cell types, transcription factors or disease processes. Recently we have made promising advances towards real-time bioluminescence imaging of SCW-arthritis using promoter-reporters constructed in **Chapter 3** (unpublished results).

A future gene expression cassette for RA gene therapy, based on the perspectives sketched above, is illustrated in **Figure 1**.

Finally, recent glimpses of therapeutic efficacy of gene therapy in clinical trials have indicated that gene therapy still holds a great promise [13-17]. The search for the optimal vector for RA gene therapy is still ongoing [18]. This thesis shows that transcriptional targeting represents a valuable strategy towards a successful clinical application of gene therapy for RA.

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Nederlandse samenvatting

Reumatoïde artritis (RA) is een chronische ontstekingsziekte die voornamelijk de gewrichten aantast. RA wordt getypeerd door aanhoudende ontsteking van het synoviale membraan (synovitis), het weefsel dat de gewrichtsruimte van het gewrichtskapsel en ligamenten afsluit. Continue synovitis leidt tot onomkeerbare destructie van het bot en kraakbeen en uiteindelijk ernstige invaliditeit. Omdat deze ontstekingen voortkomen uit een immuunrespons tegen lichaamseigen factoren uit het gewricht, wordt RA tot de autoimmuunziekten gerekend. In de laatste decennia is onze kennis wat betreft de onderliggende mechanismen sterk toegenomen, echter de oorzaak blijft tot op de dag van vandaag onbekend.
Hierdoor richt de behandeling van RA zich niet op het bestrijden van de bron, maar op bestrijding van symptomen en remming van ziekteprogressie. De ontdekking dat de pro-inflammatoire cytokines interleukine-1 (IL-1) en tumor necrose factor (TNF) een cruciale rol spelen bij de inductie van kraakbeendestructie en gewrichtsonsteking, hebben geleid tot de ontwikkeling van een nieuwe generatie medicijnen welke gebaseerd zijn op het wegvangen van deze cytokines met behulp van antilichamen of vrije receptoren. Ondanks de therapeutische effectiviteit van deze zogenaamde biologicals heeft de vereiste continue en systemische behandelmethode enkele kritische beperkingen. Ten eerste wordt klinische remissie, ofwel meer dan zeventig procent afname van ziektesymptomen, slechts bereikt in twintig procent van de patiënten. Ten tweede spelen cytokines een belangrijke rol in dagelijkse afweer tegen bacteriën, waardoor patiënten een verhoogde kans op opportunistische infecties hebben. Ten derde zijn de jaarlijkse kosten voor biologicals hoog, tussen de tien en twintigduizend euro per patiënt, mede omdat deze continue toegediend moeten worden.
Om deze punten aan te pakken, lijkt een langdurige, lokale en afgestemde productie van biologicals in aangedane gewrichten een goed alternatief. Gentherapie heeft zich inmiddels ontwikkeld tot een veelbelovende methode om dit doel te bereiken. Een bijkomend voordeel van gentherapie is de mogelijkheid om de productie van het biological strikt te reguleren. In het ideale geval namelijk functioneert de gentherapie vector als een "dokter van binnenuit" en wordt de biological geproduceerd naar gelang de behoefte van het ontstoken gewricht. In dit proefschrift beschrijven we de ontwikkeling van een methode om tot ontstekingsafhankelijke productie van een biological te komen. De toepassing van deze methode in gentherapie en diagnostiek hebben we uitgebreid getest in RA diermodellen en patiëntenmateriaal. Tenslotte hebben we de toepasbaarheid onderzocht van nieuwe biologicals, welke gebaseerd zijn op het van binnenin blokkeren van pro-inflammatoire cytokine-geïnduceerde signaaltransductie in cellen van het synoviale membraan.

Allereerst hebben we een literatuuronderzoek uitgevoerd om inzicht te krijgen in de huidige mogelijkheden wat betreft het gebruik van verschillende virus vectoren voor het doelgericht inbrengen van genetisch materiaal in cellen van het ontstoken gewricht. Hieruit bleek dat fibroblasten uit het synoviale membraan gezien worden als primaire doelwitcel en deze

efficiënt genetische gemodificeerd kunnen worden door adenovirale en lentivirale vectoren. Deze bevindingen hebben we bevestigd in onze onderzoeken naar de toepasbaarheid van adenovirale (**Hoofdstuk 4**) en lentivirale (**Hoofdstuk 6**) vectoren voor genetische modificatie van het synoviale membraan in muizen. Hiertoe hebben we gebruik gemaakt van virussen die in plaats van een biological een groen fluorescent eiwit (GFP) produceren. Na injectie van deze virussen in het gewricht vonden we vrijwel uitsluitend GFP productie in de synoviale cellen aan de binnenkant van de gewrichtsholte. Daarnaast hebben we aan weten te tonen dat lentivirussen zeer efficiënt zijn in het inbrengen van genetisch materiaal in *in vitro* gekweekte human synoviale fibroblasten. Deze eigenschap hebben we benut in **hoofdstuk 7** waarin we de effecten van een nieuwe biological op signaaltransductie door cytokines bestudeerd hebben met behulp van lentivirale reporters .

Hoofdstukken 3-5 beschrijven, in chronologische volgorde, de ontwikkeling, diagnostische en therapeutische waarde van een gentherapie vector als "dokter van binnenuit" voor RA. Om te komen tot ontstekingsafhankelijke productie van een biological hebben we gebruik gemaakt van een zogenaamde induceerbare promoter, een DNA element welke genexpressie in respons op omgevingsfactoren reguleert. Geschikte promoters hebben we afgeleid uit een bioinformatische analyse van het genexpressieprofiel van ontstoken synovium van muizen met collageen-geïnduceerde artritis. Uit deze analyse bleek dat de promoters van ontstekingsafhankelijke genen een specifieke verrijking kennen van bepaalde transcriptie factor bindingsplaatsen. Transcriptie factoren (de)activeren genexpressie door binding aan de promoter. Bindingsplaatsen voor de activerende factoren nuclear factor-κB (NF-κB), activator protein-1 (AP-1) en CAAT/enhancer-binding protein β (C/EBPβ) waren verrijkt in promoters van ontstekingsafhankelijke genen. Mede op basis van aanwezigheid van bindingsplaatsen voor deze factoren konden wij het aantal kandidaatpromoters terugbrengen van 382 naar 66. Negen van tien uiteindelijk geteste promoters gaven een respons op een ontstekingsprikkel in gekweekte cellen, waarbij de promoter van het serum amyloid A3 gen (*Saa3*) het meest waardevolle profiel toonde: lage basale activiteit, welke tot wel twintigmaal toenam tijdens ontsteking. Hieruit blijkt dat bioinformatica analyses van toegevoegde waarde zijn voor het selecteren van promoters voor gereguleerde gentherapie.
Het diagnosticeren van gewrichtsontsteking is een essentieel onderdeel voor een afgestemde behandeling van RA. Daarnaast bestaat in preklinisch dierexperimenteel onderzoek naar nieuwe medicaties de vraag naar een accurate en minimaal-invasieve uitlees van synovitis. De hypothese in **hoofdstuk 4** luidde dat een ontstekingsafhankelijke promoter met een lichtgevend reporter eiwit in plaats van een biological mogelijk toepasbaar zou zijn in bovengenoemde toepassingen. Metingen van *Saa3*-promoter activiteit in het synoviale membraan van muizen tijdens twee opeenvolgende episodes van gewrichtsontsteking bleken specifiek en uitstekend te correleren met de mate van ontsteking gemeten aan de hand van histologie en technetium

opname methode. In vergelijking met technetium opname bleek de meting van *Saa3*-promoter activiteit zelfs gevoeliger met betrekking tot de detectie van ontsteking. In de humane situatie, bleek daarnaast de mate van cytokine-geïnduceerde *Saa3*-promoter activatie in gekweekte synoviale fibroblasten overeen te komen met het ontstekings-genexpressieprofiel. Tezamen suggereren deze data dat een *Saa3*-promoter reporter ontwikkeld zou kunnen worden tot een eenvoudige en gevoelige methode voor het diagnosticeren van synovitis.
Tenslotte hebben we de toepasbaarheid van ontstekingsafhankelijke promoters voor gentherapeutische behandeling van RA getest. In **hoofdstuk 3** toonden we aan dat de specifieke eigenschappen van de *Saa3*-promoter, met name snelle en sterke toename onder ontstekingscondities, ruim voldoende waren om *in vitro* de effecten van het cytokine IL-1 te neutraliseren met een receptor antagonist. In **hoofdstuk 5** bestudeerden we de effectiviteit en veiligheid van ziektegereguleerde gentherapie in een diermodel van RA. Als biological gebruikten we interleukine-4 (IL-4), een kraakbeenbeschermend cytokine dat bij langdurige expressie in het gewricht schadelijke bijwerkingen heeft. Zowel continue als ontstekingsafhankelijke productie van IL-4 tijdens artritis leidde tot succesvolle bescherming tegen kraakbeendestructie. Maar van groter belang was de bevinding dat in niet-ontstoken gewrichten de schadelijke bijwerkingen van IL-4 zeer sterk verminderd waren ten opzichte van continue expressie. Deze resultaten bewijzen namelijk onomstotelijk dat toepassing van ontstekingsafhankelijke promoters leidt tot een veiligere manier van gentherapie.

Hoofdstukken 6 en 7 beschrijven het onderzoek naar de toepasbaarheid van nieuwe biologicals, welke gebaseerd zijn op het van binnenin blokkeren van cytokine-geïnduceerde pro-inflammatoire signaaltransductie in cellen van het synoviale membraan. De eerste strategie is gebaseerd op vermindering van TNF receptor-1 (TNFR1) productie door specifieke afbraak van TNFR1 mRNA met behulp van RNA interferentie. Het verminderen van TNFR1 expressie in synoviale cellen leidde uiteindelijk tot een reductie van gewrichtsontsteking en kraakbeenschade in muizen met artritis. Noemenswaardig was het feit dat vermindering van TNFR1 productie in cellen van de milt en lever ook leidde tot een verbetering van artritis via een demping van immuunresponsen. Omdat naast TNF meerdere cytokines, waaronder IL-1, bijdragen aan de pathologie van RA hebben we blokkering van transforming growth factor-β-activated kinase 1 (TAK1) onderzocht als mogelijk doelwit. Dit eiwit speelt een centrale rol in pro-inflammatoire signaaltransductie door meerdere cytokines en toll-like receptor liganden in verschillende celtypen. In **hoofdstuk 7** hebben we onderzocht welke betrokkenheid TAK1 heeft bij pro-inflammatoire signaaltransductie in synoviale fibroblasten. In muis embryonale fibroblasten en humane huidfibroblasten bleek TAK1 een cruciale mediator van IL-1, TNF en TLR2/4 signaaltransductie. In synoviale fibroblasten daarentegen bleek de betrokkenheid van TAK1 meer gelimiteerd, met als meest opmerkelijke bevinding een complete TAK1-onafhankelijkheid van signaaltransductie door TLR4. Desondanks kon door het blokkeren van TAK1 de activatie van transcriptie factor NF-κB door IL-1 en TNF geremd worden. Deze gegevens suggereren dat het van binnenin blokkeren van cytokine-geïnduceerde

pro-inflammatoire signaaltransductie in cellen van het synoviale membraan een haalbare strategie zou kunnen zijn voor gentherapeutische behandeling van RA.

We eindigen met een overzicht van recente ontwikkelingen en toekomstperspectieven met betrekking tot het gebruik van reguleerbare promoters voor gentherapie in **hoofdstuk 8**.

Tot slot lijkt gentherapie haar grote belofte langzaam in te gaan lossen, gebaseerd op succesvolle behandeling van enkele humane ziekten in klinische trials. Hoewel de zoektocht naar de ideale virale vector voor RA gentherapie nog gaande is, hebben we in dit proefschrift aangetoond dat gereguleerde productie met behulp van ontstekingsafhankelijke promoters een waardevolle toevoeging kan zijn om te komen tot een veilige en effectieve gentherapeutische behandeling van rheumatoïde artritis.

Curriculum vitae

Nederlands

Jeroen Geurts werd geboren op 13 juni 1980 te Nijmegen. Na het behalen van zijn VWO diploma in 1998, begon hij aan de studie Scheikunde aan de Katholieke Universiteit Nijmegen (KUN). In het kader van de hoofdrichting Biochemie doorliep hij een stage bij de onderzoeksgroep Eiwitbiochemie van de KUN. Het onderzoek, onder leiding van Drs. John den Engelsman en Dr. Wilbert Boelens, resulteerde in een verslag getiteld: "αB-crystallin, a proteomics approach". Voor zijn nevenrichting klinische chemie werd een stageonderzoek uitgevoerd in het laboratorium Kindergeneeskunde en Neurologie van het UMC St. Radboud, onder begeleiding van Drs. Suzan Wopereis en Dr. Dirk Lefeber. Het resulterende stageverslag was getiteld: "Profiling of O-glycans of serum proteins". In mei 2005, werd het doctoraal examen Scheikunde met veel genoegen behaald.

Van mei 2005 tot september 2009 was hij werkzaam als junior onderzoeker in de onderzoeksgroep Rheumatology Research & Advanced Therapeutics van de afdeling Reumatologie van het UMC St. Radboud. Hier heeft hij onderzoek verricht naar de ontwikkeling en toepassing van ziekte-reguleerbare promoters in gentherapeutische behandeling van reumatoïde artritis. De resultaten van dit onderzoek, onder leiding van Dr. Fons van de Loo en Prof. Wim van den Berg, liggen ten grondslag aan dit proefschrift. Daarnaast was hij als lid van NCMLS PhD commissie verantwoordelijk voor de organisatie van de PhD retraite in 2007 en 2008.

Gedurende zijn promotietraject heeft hij meerdere malen zijn onderzoeksresultaten mogen presenteren op internationale congressen op het gebied van gen therapie en artritis. In 2008 ontving hij een "Young Investigator Award" voor zijn onderzoek gepresenteerd tijdens het Gene Therapy for Arthritis and Related Disorders congres in Seattle. In 2009 behaalde hij, namens UMC St. Radboud, de derde prijs in de "Beste PhD student onderzoek 2009" competitie tijdens de FIGON Nederlandse Geneesmiddelen Dagen in Lunteren.

Sinds november 2009 is hij werkzaam als post-doctoraal onderzoeker bij de groep Cel & Gentherapie van de afdeling Biomedizin van het Universitätsspital Basel te Zwitserland.

English

Jeroen Geurts was born on the 13th of June 1980 in Nijmegen. After obtaining his VWO diploma in 1998, he started his study Chemistry at the University of Nijmegen (KUN). For his major in Biochemistry he performed an internship at the Proteinbiochemistry group of the KUN. The research, under supervision of Drs. John den Engelsman and Dr. Wilbert Boelens, resulted in a report entitled: "αB-crystallin, a proteomics approach". For his minor in Clinical Chemistry he performed a student research project in the Laboratory Pediatrics and Neurology of the UMC St. Radboud. Supervised by Drs. Suzan Wopereis and Dr. Dirk Lefeber he wrote a report entitled: "Profiling of O-glycans of serum proteins". He graduated with distinction in May 2005.

From May 2005 until September 2009 he was a PhD student at Rheumatology Research & Advanced Therapeutics, department Rheumatology of the UMC St. Radboud. His research was focused on the development and application of disease-regulated promoters in gen therapy for rheumatoid arthritis. The results of the research, supervised by Dr. Fons van de Loo and Prof. Wim van den Berg, are described in this thesis. As a member of the NCMLS PhD committee he organized the PhD retreat in 2007 and 2008.

During his PhD training he presented his research at several international meetings of gene therapy and arthritis societies. In 2008 he received a "Young Investigator Award" for his research at the Gene Therapy for Arthritis and Related Disorders meeting in Seattle. As a candidate for the UMC St. Radboud, he won the third place in the "Best PhD student research 2009" competition at the FIGON Dutch Medicine Day in Lunteren.

Since September 2009 he is appointed as post-doctoral fellow at the Cell & Gene Therapy group, department Biomedicine of the University Hospital Basel, Switzerland.

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Dankwoord

„It only ends once. Everthing that happens before that, is just progress."

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