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Title Page

Title: Disease-regulated gene therapy with anti-inflammatory interleukin-10 under control of the CXCL10 promoter for the treatment of Rheumatoid Arthritis.

Short title: Disease-regulated IL-10 gene therapy

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Abstract

Objective. Disease-inducible promoters for the treatment of rheumatoid arthritis (RA) have the potential to provide regulated expression of therapeutic proteins in arthritic joints. In this study, we set out to identify promoters of human genes that are upregulated during RA and are suitable to drive the expression of relevant amounts of anti-inflammatory Interleukin-10 (IL-10).

Methods. Microarrays on RA joint tissues was compared to healthy controls. The CXCL10 promoter was obtained from human cDNA and cloned into a lentiviral vector containing firefly luciferase. The promoter inducibility was determined in primary synovial cells and in THP1 cells. The luciferase gene was replaced with IL-10 to determine the therapeutic properties of the CXCL10p-IL10 lentiviral vector.

Results. Microarray analysis yielded a list of 22 genes upregulated during RA. Of these genes, CXCL10 showed the highest induction in LPS-stimulated synovial cells. The promoter activation was strongest at 8-12h after stimulation with the pro-inflammatory cytokine TNFα and was re-inducible after 96h. The CXCL10 promoter showed a significant response to RA patient serum, compared to serum from healthy individuals. Primary synovial cells transduced with CXCL10p-IL10 showed a great increase in IL-10 production after stimulation, which reduced the release of pro-inflammatory cytokines TNFα and IL-1β.

Conclusion. The selected proximal promoter of the CXCL10 gene responds to inflammatory mediators present in serum of RA patients and transduction by the lentiviral CXCL10p-IL10 vector reduces inflammatory cytokine production by primary synovial cells from RA patients. CXCL10 promoter-regulated IL-10 overexpression can thus provide disease-inducible local gene therapy suitable for RA.
Gene therapy for rheumatoid arthritis (RA) enables transduced joint cells to express biological drugs. Because the therapy can be applied locally and requires fewer injections, the side effects of continuous systemic drug administration can be reduced. In addition, off-target effects can be even further reduced by using a disease-responsive promoter that is only active during disease flares and silent during remission.\textsuperscript{1}

Derivatives of currently applied biological drugs have been tested as a potential gene therapy in multiple clinical studies after successful application in animal models of RA, including the Interleukin-1 receptor antagonist (IL1-RA) and the soluble extracellular domain of the TNF receptor fused to an IgG tail.\textsuperscript{2,3} No severe adverse side effects were reported that could be contributed to the gene therapies.\textsuperscript{4} However, only minor clinical improvements were observed. These results solicit for the use of a more potent anti-inflammatory transgene, like Interleukin-10 (IL-10). IL-10 has pleiotropic effects and can inhibit the development of Th1 cells and the production of multiple pro-inflammatory cytokines as comprehensively reviewed by Bijjiga and Martino.\textsuperscript{5}

Clinical studies with recombinant IL-10 are impeded by the short half-life of IL-10 \textit{in vivo}.\textsuperscript{6} In addition, Crohn’s disease patients systemically treated with high doses of recombinant IL-10 showed a significant reduction in hemoglobin levels.\textsuperscript{7} These issues might be circumvented in RA by using local gene therapy. Recent studies have successfully attempted to provide disease-inducible expression of IL-10 in murine experimental arthritis.\textsuperscript{8,9} The promoters that were tested for therapeutic application with IL-10 in these studies were the IL1E-IL6P, a combination of the \textit{IL-1\beta} enhancer element and the \textit{IL-6} promoter, the \textit{Saa3} promoter and the \textit{Mmp13} promoter.
The translation of the use of inducible promoters from experimental arthritis to the human situation is hampered by both differences in the disease process and the subsequent activation of the associated genes, as well as the differences in the sequences of homologous promoters. For instance, the Saa3 promoter showed the highest inducibility in previous studies, but is only present as a pseudogene in humans.

In this study, we show the upregulation of the human CXCL10 promoter in joint tissue of RA patients and the inducibility of the isolated CXCL10 promoter in THP-1 monocytes and in primary synovial cells. When the CXCL10 promoter was used to drive the transcription of the IL-10 transgene in a lentiviral vector, IL-10 production was significantly increased in cells stimulated with pro-inflammatory cytokines. The increased production of IL-10 resulted in a decreased release of pro-inflammatory cytokines by synovial cells, indicating the potential of the CXCL10p-IL10 construct to provide a disease-regulated production of anti-inflammatory IL-10.

Materials and methods

Patient material

Synovial samples for microarrays were obtained during surgery or by fine-needle arthroscopy and stored in liquid nitrogen until further processing. In total, an additional 8 synovial biopsies for digestion were obtained during surgery from the Department of Orthopedics (Radboud University Medical Center, Nijmegen, The Netherlands) or the Sint Maartenskliniek, Nijmegen, The Netherlands. Synovial biopsies were transported in DMEM culture medium, supplemented with 1% penicillin/streptomycin at 4°C. First, the pieces were
confirmed to contain a synovial lining by histological analysis of cryosections. Examples of the sample cryosections are shown in Figure S1.

Subsequently, the pieces were digested with 50 µg/ml Liberase TM (Roche, Basel, Switzerland) for 1h at 37°C in plain RPMI culture medium. The digestion was quenched by adding 10% fetal calf serum (FCS). Subsequently, the synovial cells were passed through a 70 µm cell strainer (Corning, NY, USA) and centrifuged for 5 min at 1500 rpm. Red blood cells were lysed using RBC lysis buffer (155 mM NH₄Cl, 12 mM KHCO₃, 0.1 mM EDTA, pH 7.3) for 2 min at RT.

Sera from RA patients were obtained from early RA at the Department of Rheumatic Diseases, Radboud University Medical Center, Nijmegen, Netherlands. Sera from age- and sex matched healthy controls were obtained from the Sanquin Bloedbank, Nijmegen, The Netherlands. Serum samples were stored at -80°C. All patients included in this study gave their informed consent and the study protocol was approved by the Medical Ethics Committee. In total, material of 28 patients was included in this study.

**Microarray analysis**

Total RNA was isolated from the synovial samples using the RNeasy kit for fibrous tissues (Qiagen, Venlo, Netherlands). 100 ng of total RNA was used for the preparation of biotinylated cRNA as described earlier¹¹ and hybridized to a U133Plus 2.0 oligonucleotide array (Affymetrix, Santa Clara, CA, USA), according to the Affymetrix Expression Analysis Technical Manual for two-cycle amplification. The arrays were scanned using the GeneChip Scanner (Affymetrix) and analyzed using the GeneChip Operating Software version 1.4 (Affymetrix). Array normalization and expression value calculations were performed by the DNA-Chip analyzer version 1.3 (www.dchip.org) using the Invariant Set normalization and the Model-based method.¹² The RA synovium samples were obtained from the GEO database.
(13, accession number GSE9027). The control samples were deposited under accession number ...

Cell culture

THP-1 monocytes were seeded in a 24-well plate at 3x10E5 cells/well for RNA analysis and multiplex assay or in a µClear white 96-well plate (Greiner Bio-one, Alphen a/d Rijn, Netherlands) at 5x10E4 cells/well for luciferase measurements in RPMI culture medium, supplemented with 10% FCS, 1 mM pyruvate and 1% pen/strep. Synovial cells were centrifuged and resuspended after red blood cell lysis and cultured at similar concentrations and conditions. All cell cultures were kept in humidified atmosphere at 37°C and 5% CO2. The cells were stimulated with *E. Coli* lipopolysaccharide (LPS) (Invivogen, San Diego, CA, USA), Pam3CSK4 (Invivogen), rhIL-1β (R&D systems, Oxford, UK), rhTNFα (Abcam, Cambridge, UK) or rhIL-10 (Life Technologies Europe, Bleiswijk, Netherlands) at concentrations indicated in the text.

RNA isolation and qPCR

Total RNA from cells was isolated using TRI Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer’s recommendations and treated with DNase I (Invitrogen, Carlsbad, CA, USA) for 15 min. cDNA was synthesized by reverse transcription PCR using MLV Reverse transcriptase (Invitrogen) and oligo(dT) primers. The qPCR was performed using Power SYBR Green PCR master mix (Aplied Biosystems, Waltham, MA, USA) and the StepOnePlus Real-Time PCR system (Applied Biosystems) according to the manufacturer’s instructions. The reaction volume contained 5 µl SYBR master mix, 1 µl 2 µM forward primer, 1 µl 2 µM reverse primer and 3 µl cDNA template. Primer sequences are
listed in Table S1. The PCR protocol consisted of 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

**Plasmid cloning**

For the production of lentiviral vectors, we made use of the third generation self-inactivating lentiviral (sin) vector. Cloning of the pRLL-cPPT-PGK-luc-PRE-SIN vector has been described previously. The 535 bp proximal promoter of the *CXCL10* gene was isolated from purified human genomic DNA (Promega, Madison, WI, USA) using Phusion polymerase (New England Biolabs, Ipswich, MA, USA), the forward primer TTTTGTCACTCAAGGAGGACTGTCCAGGT and reverse primer TTTTGCCTAGCGGTGCTGAGACTGGAGGTTTC. The primers introduced restriction sites for SalI and NheI respectively. The promoter was first cloned into PCR-Script using the PCR-Script CAM cloning kit (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. SalI and NheI (New England Biolabs) were used to clone the CXCL10 promoter in the pRRL-cPPT-mcs-luc-PRE-SIN vector. The human *IL-10* transgene was isolated from cDNA obtained from primary human macrophages by nested PCR using forward primer AAAGAAGGCATGCACAGCTCA and reverse primer TGGAAGCTTCTGTTGGCTCC to obtain a 903 bp PCR nest. The 537 bp gene was subsequently isolated from the nest with forward primer GCTAGCGCCACCATGCACAGCTCAGCAGCT and reverse primer TCTAGATCAGTTTCGTATCTTTATTATCTCAGCT, which introduced restriction sites for NheI and XbaI. NheI and XbaI (New England Biolabs) were used to replace the firefly luciferase gene in the pRLL-cPPT-CXCL10-luc-PRE-SIN by the therapeutic *IL-10* gene.

**Lentivirus production**
293T cells were seeded at density 1x10^5 cells/cm² in a T75 culture flask in DMEM medium, supplemented with 10% FCS, 1 mM pyruvate, 1% pen/strep and 0.01 mM cholesterol (Sigma) one day prior to the plasmid transfection. The cells were co-transfected with a calcium phosphate precipitate containing 5.3 µg sin vector, 4.0 µg MDL packaging plasmid, 3.5 µg VSV-G expression plasmid and 1.8 µg RSV-REV expression plasmid in medium without antibiotics. After 16h, the medium was replaced and new complete medium containing the produced virus was collected after 24h and 48h. The supernatant was passed through a 0.22 µm Stericup filter (Millipore, Bedford, MA, USA). Subsequently, 32 ml supernatant was layered on 4 ml 20% sucrose (Sigma) and centrifuged for 2h at 25k rpm in a Surespin 6x50ml swingout bucket rotor (Sorvall) in a Discovery 100WX ultra centrifuge (Sorvall). The viral vectors were resuspended in sterile PBS and stored at -80°C. Virus concentrations were determined using the INNOTEST HIV antigen mAb kit (Diasorin, Saluggia, Italy) and expressed as ng p24/µl. Viral transduction of cells was performed with 50 ng virus/5x10^4 cells in complete medium supplemented with 8 µg/ml polybrene (Sigma).

**Luciferase measurements**

The luciferase measurements were performed in 96-well white clear-bottom plates (Greiner Bio-one) in total volumes of 50-60 µl using the BrightGlo luciferase assay system (Promega). 30 µl BrightGlo substrate was added to cells and light production was measured on a Lumistar luminometer (BMG, Offenburg, Germany). The values were corrected for the background signal and depicted as relative light units (RLU).

**Multiplex Elisa assay**

Cytokine and chemokine concentrations were determined by luminex multianalyte technology on the Bio-Plex 200 (Bio-Rad, Hercules, CA, USA) in combination with Bio-Plex pro human
cytokine kits (Bio-Rad) according to the manufacturer’s protocol. For IL-10, MCP-1, IL-6 and IL-8 measurements, the culture supernatants were first diluted 25x. Samples below the detection limit were set at the lowest measureable quantity to perform statistical analysis.

Statistical analysis

Statistical analysis was performed using the Student’s t-test, 1-way ANOVA and 2-way ANOVA. Results are depicted as mean +/- SD and P-values < 0.05 were regarded as significant.

Results

Microarray analysis of RA synovium samples

Potential disease-regulated promoters can be obtained from endogenous promoters of genes upregulated during disease. Therefore, we analyzed 20 microarrays of RA patient synovium biopsies and compared the gene expression levels to 8 control synovium biopsies without arthritis. The criteria for candidate genes included at least 10-fold upregulation in RA with P<0.01 compared to control and a minimal average relative probe intensity of 500 (arbitrary units) in RA to find promoters of sufficient capacity. The genes that do not code for a transcribed mRNA were removed from the list. In addition, the immunoglobulin genes were removed, as their proximal promoters rely on B-cell-specific factors and are dependent on intronic enhancer elements.15 22 genes met these criteria and are summarized in Table 1.

The upregulation of the genes in the RA patient synovium was confirmed by qPCR on a subset of the biopsies (Supplemental Figure 2, Table 1).
We first determined if the gene upregulation in the joints was the result of pro-inflammatory signaling and if inflammation-sensitive cells can activate the gene promoters after stimulation in-vitro. Human synovial biopsies were digested and stimulated for 6h with bacterial lipopolysaccharide (LPS), which activates the pro-inflammatory TLR4 signaling. Several genes that were selected from the microarrays were upregulated after stimulation, of which CXCL10 showed the highest induction (Figure 1A). These results confirm that a pro-inflammatory stimulus can activate transcription of the genes and the promoters of these genes therefore might be applicable for disease-regulated gene therapy. Similar results were obtained in human THP-1 monocytes after stimulation with the synthetic lipopeptide Pam3CSK4, which triggers TLR2 signaling\(^\text{16}\) (Figure 1B). Based on the microarray and the high inducibility in both synovial cells and THP1 cells, the CXCL10 promoter was selected for the remainder of this study.

**Promoter inducibility**

The proximal promoter of the CXCL10 gene was obtained from human cDNA and cloned into a lentiviral vector expressing the luciferase reporter gene. THP1 cells were transduced with the inducible reporter construct and stimulated with pro-inflammatory cytokines IL-1β, TNFα and TLR triggers LPS and Pam3CSK4 for 6h. The promoter showed a robust response to all stimuli (Figure 2A). In primary RA synovial cell culture, the isolated promoter was inducible by IL-1β, TNFα and LPS, but not by Pam3CSK4 (Figure 2B).

An important characteristic of disease-inducible therapy for RA is the net response of the promoter to the balance of pro- and anti-inflammatory proteins present in the patient. Therefore, we compared the CXCL10 promoter response to serum from 63 RA patients to serum from 62 age- and sex matched healthy donors. The RA patient sera showed a stronger
induction of the \textit{CXCL10} promoter compared to healthy control sera (Figure 2C). This confirms that the inducible \textit{CXCL10} promoter is more active in RA patients.

For the most accurate treatment of an RA patient, the inducible promoter activity should return to basal levels if the disease activity decreases and should be activated again if a new stimulus appears. Therefore, we compared the kinetics of the \textit{CXCL10} promoter to the constitutive active PGK promoter at different time points after stimulation with TNF\(\alpha\). Indeed, the PGK-luciferase activity remained around basal levels for 96 hours (Figure 2D). The \textit{CXCL10} promoter showed highest activity at 8-12h after the stimulation (Figure 2E), although the peak activity was still below PGK-level. As the TNF\(\alpha\) was used and degraded by the cells,\(^{17}\) the promoter activity decreased. Re-stimulation of the cells with TNF\(\alpha\) 8h prior to the 96h time point resulted in a significant second induction of the promoter.

\textit{Recombinant human IL-10 effects on cytokine production}

The therapeutic potential for IL-10 was assessed using recombinant human IL-10 (rhIL-10). Primary RA synovial cell cultures of 3 patients were stimulated for 6h with LPS with or without the addition of rhIL-10. Despite differences in absolute quantity between patients, the production of TNF\(\alpha\) was increased after LPS stimulation and could be significantly reduced with rhIL-10 (Figure 3A). To determine the effects of the cytokines produced by the synovial cells on the \textit{CXCL10} promoter, THP1 cells transduced with \textit{CXCL10prom-luciferase} were stimulated with the synovial cell culture supernatant of one of the patients from Figure 4A (Figure 3B, closed bars). To test the effects of residual LPS and IL-10 that might be left in the supernatant from the synovial cells, LPS and rhIL-10 effects were first tested separately (open bars). No direct activation of the \textit{CXCL10} promoter by rhIL-10 was observed and LPS showed some activation of the \textit{CXCL10} promoter. However, the cell culture supernatant from
LPS-stimulated synovial cells showed a stronger induction compared to LPS alone. If the synovial cells were treated with rhIL-10, the capacity of its supernatant to activate the CXCL10 promoter was significantly reduced. This shows that activated synovial cells produce the required factors to activate the CXCL10 promoter and that IL-10 can diminish this production. All conditions were also tested in the presence of the specific TNFα-inhibitor Etanercept. In the presence of Etanercept, the CXCL10 promoter induction by synovial cell supernatant was significantly lower, indicating that TNFα produced by the stimulated synovial cell culture is the most important trigger for CXCL10 promoter activation in this setting.

Effects of CXCL10-IL10 lentivirus on cytokine production

The potential of the CXCL10 promoter to produce therapeutic quantities of IL-10 was tested by replacing the luciferase reporter transgene by the coding region of the human IL-10 gene (CXCL10p-IL10). THP-1 cells transduced with PGK-luciferase control lentiviral vector produced low quantities of IL-10 (Figure 4A). After transduction with CXCL10p-IL10, the basal activation of the of the CXCL10 promoter already resulted in increased IL-10 levels. Stimulation of the transduced cells with Pam3CSK4 caused a significant increase in production of IL-10. Pam3CSK4-stimulated THP-1 cells with control virus showed a marked increase in production of inflammatory cytokines MCP-1, IL-6 and IL-8 (Figure 4B-D). The release of these cytokines was inhibited by the CXCL10p-IL10 lentivirus, which shows the anti-inflammatory potential of the CXCL10p-IL10 treatment.

Subsequently, the CXCL10p-IL10 lentivirus was tested in synovial cell cultures of three patient donors. After transduction with control virus or CXCL10p-IL10, the cells were stimulated with 5 or 100 ng/ml LPS. Only minor quantities of IL-10 were produced by cells with the control virus (Figure 5A). After transduction with the CXCL10p-IL10 lentivirus,
basal production levels of IL-10 were observed, which significantly increased after LPS stimulation in all patients, although the magnitude varied between patients. LPS triggered the release of TNFα, which was reduced by the CXCL10p-IL10 treatment of the cells (Figure 5B). With the exception of the third donor, the same effects were also observed for IL-1β. In the third donor, IL-1β levels remained low after stimulation with LPS (Figure 5C). The production of MCP-1, IL-8 and IL-6 were also decreased in cells transduced with CXL10p-IL10, although the decrease was not significant for most samples (Supplemental Figure 3).

These results show that in the synovial cells of the patient, the CXCL10p-IL10 gene therapy can provide inducible expression of therapeutic levels of IL-10 that can suppress the production of pro-inflammatory cytokines.

**Discussion**

In this study we showed that the promoter from the CXCL10 gene is inducible by pro-inflammatory TLR triggers and cytokines. In human THP-1 monocytes, the promoter showed a significant induction by RA patient serum, compared to healthy control serum. Using the CXCL10p-IL10 lentivirus in LPS-stimulated cells from RA patient biopsies, therapeutic levels of anti-inflammatory IL-10 were produced that could reduce the release of pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8 and MCP-1.

The promoters of genes that were upregulated in RA synovium microarrays are the possible candidates for disease-regulated therapy. We first determined if the gene upregulation was the result of pro-inflammatory signaling rather than an altered cell composition in RA synovium by using broad TLR triggers which activate the major inflammatory transcription factors. TLRs are important players in RA and can be triggered by damage-associated molecular patterns (DAMPs) present in the RA joint. A TLR2 trigger was used for THP1 cells, because TLR2 shows the highest expression in THP1 cell. In contrast, TLR2 is not
uniformly expressed on synovial fibroblasts and is upregulated after stimulation.\textsuperscript{21,22} Therefore, the synovial cell cultures were stimulated with a TLR4 ligand. Based on the obtained data, the promoter of the \textit{CXCL10} gene was selected. In accordance with our results, previous studies have shown increased levels of \textit{CXCL10} in synovial fluid of RA patients.\textsuperscript{23} \textit{CXCL10} is a C-X-C motif ligand chemokine, responsible for the recruitment of several immune cell types into the inflamed joint.\textsuperscript{24} The isolated region of the \textit{CXCL10} promoter contains predicted binding sites for immunoregulatory transcription factors C/EBP\textbeta, NF-\kappaB, AP-1 and an interferon-stimulated response element (ISRE).\textsuperscript{25} Point mutations in the NF-\kappaB and the ISRE sites resulted in reduced promoter activity after stimulation with TNF\alpha and IFN\gamma or by TLR triggers in hepatocytes.\textsuperscript{26} Interestingly, promoter constructs with mutations in the AP-1 or C/EBP\textbeta binding sites showed increased activity upon stimulation, indicating orchestration of the promoter activity by multiple factors.

The \textit{CXCL13} gene, also known as B-lymphocyte chemoattractant, showed the highest upregulation in the RA microarrays and in stimulated THP-1 cells. In mice, the promoter of \textit{CXCL13} contains a TATA-box and transcription factor binding sites.\textsuperscript{27} In humans this region is conserved, but located in a large intron in the 5’UTR. We isolated both the proximal promoter region from the human transcription start site and the first intron, but we could not observe an upregulation of either promoter activity after stimulation (data not shown). In addition, we did not find an upregulation of endogenous \textit{CXCL13} in Pam3CSK4-stimulated synovial cells.

During RA pathogenesis, cells in the joint produce chemokines and cytokines to induce pannus formation and attract different immune cells to the joint, including neutrophils, monocytes, T-cells and B-cells.\textsuperscript{28,29} As a consequence, the synovial cell cultures after digestion of the synovium are of heterogeneous composition with patient-to-patient variation. This results in differences in cytokine production levels. For example, IL-1\beta has been
suggested to be predominantly produced by activated monocytes and macrophages.\textsuperscript{30} The third donor in Figure 5 showed the highest TNFα production upon stimulation, but only a very low production of IL-1β, which might reflect a low abundance of monocytes.

In general, the cells in the synovium of RA patients have an inflammatory phenotype, which has also been observed in microarrays. Compared to healthy controls, the signaling networks associated with upregulated genes in RA include inflammatory response, T-cell activation and apoptosis.\textsuperscript{31} As a result, the cytokine concentrations are increased in the synovial fluid of RA patients.\textsuperscript{32} The inflammatory phenotype of the synovial cells in this study is reflected by the basal production of IL-1β (Figure 5C) and IL-10 after CXCL10p-IL10 transduction (Figure 6A) and by a relatively high basal activity of the \textit{CXCL10} promoter in RA synovial cells (Figure 2B).

To avoid effects of the basal activation state on the \textit{CXCL10} promoter, THP-1 cells were used to study promoter activation. THP-1 cells are human monocytes which are responsive to multiple TLR ligands and cytokines and can subsequently activate different inflammatory pathways.\textsuperscript{33} In addition, gene expression analysis of peripheral blood in RA patients showed an enrichment in monocyte-specific transcripts.\textsuperscript{34} indicating that THP-1 is a sensitive cell model, relevant to RA. Indeed, the \textit{CXCL10} promoter in THP-1 cells showed a strong induction by the cytokines produced by stimulated synovial cells. In addition, the responsiveness of the \textit{CXCL10} promoter in a monocyte cell line to RA serum factors might enable the use of the promoter to treat systemic components of the disease, like atherosclerosis and autoimmunity.

Patients with inherited deficiencies in IL-10 signaling are rare, but suffer from early-onset inflammatory bowel disease (IBD).\textsuperscript{35} In addition, some studies found that the frequency of a polymorphism associated with low serum levels of IL-10 was increased in RA patients.\textsuperscript{36,37} Currently, research is focusing on obtaining long-term active IL-10 targeted to the inflamed
joint both by disease-responsive gene therapy and by fusing IL-10 to homing antibodies to provide regulated suppression of the inflammation.

In conclusion, we have shown the temporal and spatial activation of the CXCL10 promoter, which was used to provide inducible expression of the anti-inflammatory IL-10. The vector was capable of reducing the release of pro-inflammatory cytokines by RA synovial cells and might therefore be suited to provide local and disease-responsive gene therapy in RA patients.

Acknowledgements

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Author disclosure statement

No competing financial interests exist.

References


Figure 1: Upregulation of candidate genes in synovial cells and THP1 cells after TLR stimulation. A) Gene expression levels in primary cell cultures of digested synovial biopsies from patient 21 after 6h stimulation with 100 ng/ml LPS. Genes are sorted from left to right according to fold induction over unstimulated cells. B) Gene expression levels in THP-1 cells stimulation with 100 ng/ml Pam3CSK4. Expression levels are depicted as threshold cycle (CT) +/- SD, corrected for GAPDH expression. Statistical analysis was performed by Student’s t-test. * = P<0.05, ** = P<0.01, *** = P<0.001.
Figure 2: Inducibility of the CXCL10 promoter in synovial cells and THP1 cells. A) Relative light signal of CXCL10 promoter-luciferase construct after stimulation with pro-inflammatory factors. THP1 cells were transduced with CXCL10 promoter-luciferase lentiviral vector and stimulated for 6h with 10 ng/ml IL-1β, 10 ng/ml TNFα, 100 ng/ml LPS or 100 ng/ml Pam3CSK4. Light values are shown as relative light units (RLU) +/- SD. B) CXCL10 promoter-luciferase construct activation in primary RA synovial cell culture from patient 22. C) CXCL10 promoter-luciferase activation by RA patient serum. A stably transduced THP1 clone was stimulated with 10% RA patient serum or age- and sex-matched healthy control serum (HC). The light signal after 6h is shown as RLU, corrected for the average signal from the control group +/- SD. D,E) THP-1 cells were transduced with lentiviral PGK-luciferase vector (D) or CXCL10-luciferase (E) and stimulated with 10 ng/ml TNFα. At multiple time points, the luciferase activity was determined. Light values are shown as relative light units (RLU) +/- SD. A second 10 ng/ml TNFα stimulation was provided at 88h, 8h prior to the last measurement. For statistical analysis, the re-stimulated sample was compared to the 96h time point.
point without re-stimulation. Statistical analysis was performed by Student’s t-test compared to the medium control. * = P<0.05, ** = P<0.01, *** = P<0.001.
Figure 3: TNFα levels in the supernatant of stimulated synovial cells and the activation of the CXL10p-luciferase by the supernatant. A) Synovial biopsies from patient 23, 24 and 25 were digested using Liberase and passed through a cell strainer. After red blood cell lysis, cells were seeded and allowed to attach and recover. Subsequently, the synovial cell suspension was stimulated for 6h with 5 ng/ml LPS and 20 ng/ml recombinant human IL-10. The cell
culture medium was removed and the produced TNFα was measured using multiplex assay.

**B** THP1 cells stably transduced with the CXCL10p-luciferase reporter lentivirus were stimulated for 6h with medium containing 20 ng/ml recombinant IL-10 (+I10), 5 ng/ml LPS (+L5), 100 ng/ml LPS (+L100) or a combination (open bars). In addition, the cells were stimulated with the cell culture supernatant obtained from Figure 4A (closed bars) that might contain residual IL-10 (I10) or LPS (L(5) and L(100)). All samples were tested in the presence and absence of 50 ng/ml TNFα-inhibitor Etanercept. Light values are shown as relative light units (RLU) +/- SD. Results were similar with supernatants from all three patients (data not shown). Statistical analysis between subgroups of different stimulations was performed by 2-way ANOVA and other comparisons were performed by Student’s t-test. ** =P<0.01, *** =P<0.001.
Figure 4: Production and effects of IL-10 in CXCL10p-IL10 transduced THP1-cells. A) THP-1 cells were transduced with lentiviral vectors containing PGK-luciferase or CXCL10p-IL10. After 48h, the cells were stimulated with medium control or with 100 ng/ml Pam3CSK4 for 24h. IL-10 production was measured in the culture supernatant by multiplex assay. Statistical analysis was performed by 1-way ANOVA. B-D) THP-1 cell culture supernatant concentrations of MCP-1 (B), IL-6 (C) and IL-8 (D). Statistical analysis was performed by Student’s t-test, compared to the stimulated control cells. * =P<0.05, *** =P<0.001.
Figure 5: Production and effects of IL-10 on TNFα and IL-1β in CXCL10p-IL10 transduced synovial cells. Synovial tissue biopsies from patient 26, 27 and 28 were digested and cells were transduced with lentiviral PGK-luciferase (control) or lentiviral CXCL10p-IL10. After a minimum of 48h after transduction, the cells were stimulated for 24h with 5ng/ml LPS or 100 ng/ml LPS. The supernatant was collected and the concentrations of IL-10 (A), TNFα (B) and
IL-1β (C) were measured by multiplex assay. The cytokine concentration of samples left of the dotted line are shown on the left y-axis and the samples on the right of the dotted line are depicted on the right y-axis. Statistical analysis was performed by 1-way ANOVA. * \( =P<0.05 \), ** \( =P<0.01 \), *** \( =P<0.001 \).
Supplemental Figure 1: Examples of histological sections of the synovium samples used in this study from patients 23, 24, 26 and 27. 7 µm cryosections were stained with haematoxylin and eosin.

Supplemental Figure 2: Gene expression levels of genes upregulated in RA in synovium biopsies. n=4 and n=5 samples per group were measured and the expression levels are
depicted as threshold cycle (CT) +/-SD, corrected for GAPDH expression. Statistical analysis was performed by Student’s t-test. * = P<0.05, ** = P<0.01.
Supplemental Figure 3: Production and effects of IL-10 on MCP-1, IL-8 and IL-6 in CXCL10p-IL10 transduced synovial cells. Synovial tissue biopsies from patient 26, 27 and 28 were digested and cells were transduced with lentiviral PGK-luciferase (control) or lentiviral CXCL10p-IL10. After a minimum of 48h after transduction, the cells were stimulated for 24h with 5ng/ml LPS or 100 ng/ml LPS. The supernatant was collected and the concentrations of
MCP-1 (A), IL-8 (B) and IL-6 (C) were measured by multiplex assay. Statistical analysis was performed by 1-way ANOVA. * =P<0.05, ** =P<0.01, *** =P<0.001.
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<td>889.9 (741.1)</td>
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<td>regulator of G-protein signalling 1</td>
<td>49.6 (63.0)</td>
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<td>CXCL13</td>
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<td>SLAM family member 7</td>
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Table 1: List of genes upregulated in RA synovium, compared to control synovium.
20 microarrays on RA synovium and 8 microarrays on control synovium. The average probe intensity was calculated with dchip using the invariant set normalization method and the model-based method. Depicted are the average intensity (+/- standard deviation) of the microarray sets, the index RA/HC, the P-value calculated by Student’s t-test and the ΔΔCt values of the control PCR of RA biopsies compared to healthy controls. HC = healthy control; RA = rheumatoid arthritis.
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<tr>
<th>Gene symbol</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
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<td>TCCACTGTGGAAATACCGA</td>
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<td>CCCGAGAAGGATATTCAAGAG</td>
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<td>CTCAAGCTGTAGATGAGTGGG</td>
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<td>AGAAGTTTGGAGAGGGCTGAGA</td>
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<td>CXCL9</td>
<td>GGAGTGCAAGGAAACCCAGTA</td>
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Table S1: List of primer sequences for qPCR analysis
Disease-regulated gene therapy with anti-inflammatory interleukin-10 under control of the CXCL10 promoter for the treatment of Rheumatoid Arthritis. (doi: 10.1089/hum.2015.127)

This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.
Supplemental Figure 2: Gene expression levels of genes upregulated in RA in synovium biopsies. n=4 and n=5 samples per group were measured and the expression levels are depicted as threshold cycle (CT) +/- SD, corrected for GAPDH expression. Statistical analysis was performed by Student’s t-test. * =P<0.05, ** =P<0.01.