Gene therapy for arthritis: progress towards a clinical trial
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General Discussion and Summary
During the past two decades significant progress has been made in the treatment of rheumatoid arthritis (RA). Several biological therapies are now available, improving the outcome of RA in many patients, but not all. Current therapies require systemic and repeated administration, through intravenous or subcutaneous injections in order to reach sufficiently high levels of the therapeutic protein in the affected joints. At any time >50% of patients will still have active disease in spite of the available treatments, and the majority of clinical responders to biological treatment will still have one or more actively inflamed joints. Intra-articular gene therapy could provide a potential solution by providing long lasting local treatment for arthritis, with prolonged expression of a therapeutic protein at the site of inflammation after a single injection. In addition, for patients with persistent or recurrent mono- or oligoarthritis not necessarily in need of systemic biological treatment or those not eligible to receive biological treatment, local gene therapy could provide benefit.

We developed a viral vector, ART-102, for intra-articular treatment of inflammatory arthritis. ART-102 is a recombinant adeno-associated virus (AAV) type 5 vector genetically designed to encode the gene for the immunomodulatory protein human Interferon-β (hIFN-β) under control of a nuclear factor κB (NF-κB) promoter. The inflammation-inducible NF-κB promoter ensures controlled expression of the transgene only in the presence of inflammation.

Chapter 2 and 3 in this thesis provide background information on the selection of this vector and therapeutic target. In Chapter 2 the concept of local gene therapy is described, including different delivery methods, viral and non-viral vectors, and potential therapeutic targets for the treatment of RA. Adeno-associated virus has become a frequently used vector for in vivo gene therapy in different diseases, due to its favorable characteristics, including its non-pathogenicity, its ability to induce long-term transgene expression, and its high safety profile. For administration in joints, AAV serotype 5 demonstrated to be most effective in transducing synovial tissue as compared to AAV1 to 4. The potential of IFN-β as a novel therapeutic target is concisely discussed in Chapter 3. After promising results in animal models of arthritis, the systemic administration of IFN-β protein three times weekly did not result in clinical improvement in patients with RA. Possibly, higher and more constant levels of the therapeutic protein at the site of inflammation (the joint) are required to attain a beneficial clinical effect, a goal which could be reached through the approach of local gene therapy. This concept was tested in different animal models of arthritis, showing promising results.

Over the last years further evidence for the use of IFN-β in the treatment of arthritis has accumulated. To deliver active IFN-β to the site of inflammation a fusion protein was generated by linking mouse IFN-β with the latency-associated protein (LAP) fused by an matrix metalloproteinase (MMP) cleavage site. In its inactive form, this protein remains inactive and has a very long half-life (approximately 55 hours). However upon expression at sites of inflammation, cleavage by MMPs releases active IFN-β. Systemic administration of plasmids coding for LAP-IFN-β or LAP- IFN-β fusion proteins improved arthritis activity in the collagen-induced arthritis (CIA) model of RA. Compared to IFN-β protein, the LAP fusion proteins showed increased efficacy, presumably because of the specificity of the fusion protein for inflamed joints. The anti-inflammatory role of IFN-β in arthritis is also supported by recent genetic studies. Protein tyrosine phosphatase nonreceptor type 22 (PTPN22) is a gene involved in immune function and a variant (PTPN22W) has been identified as one of the strongest genetic risk factors for autoimmune diseases, including RA. Innate immune cells (e.g. macrophages)
from patients with the PTPN22W variant produce less IFN-β after immune activation, leading to decreased immune suppression. In addition, the disease-associated PTPN22W variant failed to promote type 1 IFN upregulation and type 1 IFN-dependent suppression of experimental arthritis.\textsuperscript{11} Taken together these data strongly support the rationale for the use of IFN-β in the treatment of arthritis as discussed in chapter 3.

In part I and part II of this thesis both preclinical and clinical aspects of ART-I02 gene therapy were investigated, together supporting progress with ART-I02 towards a phase 1 clinical trial for the local treatment of joint inflammation in patients with RA.

**PRE-CLINICAL STUDIES FOR THE DEVELOPMENT OF ART-I02**

In Chapter 4 we demonstrated that transduction of RA fibroblast-like synoviocytes (FLS; the main target cells in joints for rAAVS) with ART-I02 resulted in high expression levels of bioactive human (h)IFN-β. Transduction of FLS of other species also achieved high levels of hIFN-β, which proved to be bioactive in FLS from rhesus monkeys but not in FLS from rodents. Transgene expression and bioactivity of hIFN-β in RA FLS were unaltered in the presence of methotrexate, a finding of importance for future clinical trials with ART-I02, as methotrexate is frequently used as the single concomitant antirheumatic medication in clinical trials. In vivo studies with ART-I02 in rats demonstrated that the majority of vector DNA remained in the joint after intra-articular injection. Local expression of a marker gene (Luciferase) expressed from a rAAVS vector was confirmed by in vivo imaging in mice, showing sustained expression up to 6 months (see also chapter 6) after vector injection.

In previous publications we have shown that the expression of the transgene was mainly in FLS of the joints of rats and mice.\textsuperscript{2,3,7,14} Transgene expression at the site of inflammation after intra-articular injection has been demonstrated for different transgenes and in different animal models, further validating the concept of local gene therapy for arthritis.\textsuperscript{7,14-18} We cannot exclude the possibility that other tissues surrounding the joint space are also transduced to a certain extent. Since we focus on the delivery of genes encoding therapeutic proteins in the joint, surrounding tissues could contribute to the therapeutic protein with a beneficial effect.

Biodistribution and germline transmission are important safety issues that need to be considered with regard to the clinical application of ART-I02. With the use of a very sensitive PCR (100 viral copies/100 ng DNA detection limit) distribution to organs other than the target was shown, however spread to other organs was magnitudes lower than levels in the injected joints and lymph nodes, especially compared to intravenous administration. The degree of biodistribution is related to the route of administration, for example, whereas intravenous administration results in diffuse distribution, intra-ocular (subretinal) injections result in minimal spread of vector genomes to peripheral tissues in non-human primates.\textsuperscript{19} While the joint is not a closed compartment, the majority of the injected vector particles remains in the joint followed by the draining lymph nodes. In our study, spread to the gonads was transient and vector copies were not detectable after 4 weeks. In the GLP biodistribution study with ART-I02 injection in healthy rats, low vector copies numbers were detected in ovaries and testis at the highest dose group 90 days after vector injection.\textsuperscript{20} A validated qPCR was used in this study and instead of
the ankle joint, the knee joint was injected. Since low ART-102 copy numbers were detected in the reproductive organs, a germline transmission study has been designed to detect possible paternal and maternal germline transmission of ART-102. The presence of low vector copy numbers in the reproductive organs are in line with a biodistribution study after intravascular AAV5 administration in rabbits, in which low vector DNA copies were present in the testes 30 weeks after injection; the presence of low copy numbers in semen was only transient and rapidly cleared from semen within 5 weeks. Another study that should be mentioned is a germline transmission study evaluating germline transmission of the AAV-5 vector, the same serotype as ART-102. Mice of either gender were injected intravenously with AAV5-PBGD at $5 \times 10^{14}$ genomic copies/kg, a 676x higher dose/kg as used for ART-102 in the rat studies. Vector DNA was detected in reproductive organs of the treated animals but not in the non-treated (mating) animals. There was no vector DNA in uterus or placenta of non-treated animals or in the fetuses. As data from these studies prove that there is no risk for germline transmission when using AAVS with these high doses, it is not expected that germline transmission will be observed with ART-102.

Next, we assessed biodistribution and safety as well as initial efficacy in a larger animal model (Chapter 5). The CIA model in rhesus monkeys allowed us to investigate the biodistribution, safety and initial efficacy of ART-102, as hIFN-β is also biologically active in non-human primates (NHP) (see also Chapter 4). Intra-articular injection of ART-102 was well-tolerated in this animal model and did not induce adverse events. The highest copy numbers of vector DNA were detected in synovial tissue of the injected joint as well as the draining lymph node. Importantly, there was no persistence of ART-102 sequences in gonads, brain, heart, bone marrow, kidney, lungs, muscle, or thymus. The maximal feasible dose resulted in stabilization or decrease of joint swelling, which was not observed in the placebo group. In addition, a reduction in histological inflammation and bone erosion scores was observed in these animals. This effect is most likely ART-102 treatment related and IFN-β expression was detected by immunohistochemical staining in the ART-102 injected PIP joints. High neutralizing antibody (Nab) responses to the vector were detected in all monkeys, and 5 monkeys developed an rAAV5-specific T cell response; the response was near the detection level. This could represent cross recognition of AAV5 epitopes by T cells primed during exposure of the animals to the wild-type virus. Additionally, it should be noted that similar capsid responses in NHPs were observed during preclinical administration of AAV vectors to the subretinal space. It appears unlikely that these responses have an impact on transduction efficacy or safety of the approach.

However, immune responses to the vector and the transgene could have a negative effect on transgene expression or re-administration of the vector. Exposure to wild-type AAV or to AAV vectors, and the consequent activation of innate and adaptive immunity to vector and transgene leads to both antibody and cell-mediated responses. Results from clinical trials and studies of gene transfer conducted in either small or large animal models showed that Nab titers as low as 1:5 can completely block AAV vector transduction, and that AAV vectors remain susceptible to antibody-mediated neutralization for several hours after intravascular delivery. The presence of pre-existing (neutralizing) antibodies to various AAV serotypes following natural exposure to wild-type AAV is a common phenomenon. In adults, anti-AAV2 antibodies are the most prevalent (up to 70% of healthy individuals are positive), followed by serotypes like AAV5, AAV9, and AAV8, which are much less prevalent. AAV5 has a very low seroprevalence in healthy subjects (about 30-40%); in
RA patients the prevalence of AAV5 neutralizing antibodies was only 3-5%.\textsuperscript{26} Vector administration may result in long-lasting high-titer anti-AAV neutralizing antibodies, which prevent vector readministration. The effect of neutralizing antibodies on transduction is dependent on the route of administration, dose of vector and type of vector.\textsuperscript{27} Studies defining the optimal strategy or combination of strategies, to successfully treat subjects with preexisting antibodies to AAV due to natural infection or to prior administration of AAV vectors, are ongoing. Such strategies have included selection of naïve subjects, serotype switching, capsid modifications, plasmapheresis, transient immunosuppression (e.g. B-cell depletion through rituximab), use of specific delivery techniques and modulation of the antibody response with empty capsids.\textsuperscript{27}

The capsid-specific T-cell response consists of recognition of vector capsid antigens by T cells and a response directed against transduced cells. Natural infection with wild-type AAV also triggers cell-mediated immune responses against the capsid, which results in a reservoir of memory CD8+ T cells that can be reactivated upon vector administration. This can cause the destruction of transduced cells harboring AAV capsid antigen in the context of MHC class I, as it has been observed in subjects enrolled in AAV vector-mediated liver gene transfer trials.\textsuperscript{28} However, in clinical studies with alipogene tiparvovec (an approved medicine for the treatment of lipoprotein lipase deficiency) and alpha-1-antitrypsin long term expression was observed in spite of a cellular immune response against the vector. In both studies AAV1 vectors were administered intra-muscularly.\textsuperscript{29,30} Notably, experience from the AAV8 gene therapy trials in hemophilia B subjects suggests that timely administration of immunosuppression can prevent detrimental effects of capsid-directed T cell immunity.\textsuperscript{31} Whether intervention strategies currently in use to block T cell-mediated clearance of transduced cells will be safe and effective for all gene therapy indications needs further investigation. Results from novel preclinical models and clinical studies will help to address these questions and to reach the goal of developing safe and effective gene therapy protocols.\textsuperscript{32} The effect on the formation of capsid-specific T-cells after rAAV5 vector injection in the joint is currently unknown and will need to be monitored in a future clinical trial.

In an effort to improve transduction efficiency, we have investigated factors that can influence in vivo transduction of rAAV vectors after intra-articular injection in animal models of arthritis and in healthy animals. The finding that synovial macrophages inhibit AAV mediated gene delivery, led to further studies investigating strategies for overcoming this barrier (Chapter 6). Both in arthritic but also in healthy mice pretreatment with agents that influence macrophage activity or number (triamcinolone and clodronate liposomes) resulted in increased rAAV5 transgene expression in the joint over a period of 4 weeks. Improvement was seen also on the percentage of expressing knee joints. Another strategy previously used, was to add empty decoy capsids to the full capsid formulation. This effect was contributed to absorption of pre-existing neutralizing antibodies.\textsuperscript{33} In our experiment, adding empty capids to the vector preparation resulted in improved transgene expression. However in our mouse model mice are negative for rAAV5 neutralizing antibodies, and we postulate that the effect observed was a result of empty capsids acting as a decoy for synovial macrophages, thereby increasing the chances that full virus particles could reach the target cells. When both approaches were combined, we observed a synergistic enhancement of gene expression, which was sustained for at least 6 months. Interestingly, the enhancement of gene expression was independent of the route of administration of triamcinolone, either intra-articular or intramuscular.
To put these findings in a broader perspective, these strategies were assessed in healthy mice since it is known that also healthy synovium contains macrophages. The addition of empty capsids to the vector preparation and injection of triamcinolone before intra-articular injection of the vector resulted in significant improvement in gene expression even in the absence of inflammation. Moreover, we tested the effect of these strategies using an AAV serotype very different from AAV5, being AAV2. As AAV uptake by macrophages is a general phenomenon utilizing scavenger receptors, this should not be limited to one specific serotype, or any specific virus as macrophages are known to take up a wide range of viruses and bacteria. It was demonstrated that the combination of triamcinolone and empty capsids improved expression of the transgene significantly, independently of the serotype used.

These data not only have implications for future applications of local gene therapy to the joint, but also to other tissues that have an abundance of macrophages. The importance of depletion or inhibition of macrophage activity was supported by an in vivo study with rAAV8 liver-directed gene therapy for inherited hyperbilirubinemia. Recombinant AAV8 uptake by macrophages and Kupffer cells in the liver was improved by blocking a macrophage scavenger receptor. Additional studies are warranted to further develop these strategies into clinical applications.

CLINICAL ASPECTS OF THE DEVELOPMENT OF INTRA-ARTICULAR GENE THERAPY

The concept of intra-articular protein therapy was first investigated in patients with inflammatory arthritis in a proof of mechanism study (Chapter 7). The effects of intra-articular TNF blockade were assessed using etanercept (TNF soluble receptor) as a tool compound, for the first time in a randomized placebo-controlled design. The study suggested that intra-articular etanercept administration was safe and well-tolerated. After a single intra-articular injection of etanercept there was immediate clinical improvement of the target joint, which lasted up to 2 weeks after injection. The duration of the clinical effect was expected based on the half-life of etanercept; serum levels were detectable up to 3 weeks after injection. This study supports the concept of intra-articular targeted therapy aimed at long-term inhibition of inflammatory cytokines, for instance by gene therapy.

We would envision that the initial clinical trial with intra-articular gene therapy would be an experimental medicine trial in RA patients. In addition to clinical parameters, we would include soluble biomarkers, synovial tissue analysis to evaluate a variety of effects ranging from transduction efficiency to measurement of CD68 positive macrophages, and MRI of the joints. These studies would provide a deeper understanding of the effects of intra-articular gene therapy in RA, and would allow an informed go/no go decision with regard to further clinical development. In the clinical trial with intra-articular etanercept (Chapter 7) we used a composite change index (CCI) to determine clinical improvement. The CCI combines both single-joint patient reported and physician assessed outcomes. The CCI has been used in previous clinical trials investigating local treatments, however, the scoring system had not been validated before, unlike other outcome measures for evaluation in patients with polyarthritis, like the disease activity score of 28 joints (DAS28). In Chapter 8, we investigated the responsiveness and discrimination of the CCI as a single-joint assessment by evaluation of different statistical indicators. Responsiveness (the ability to measure a clinically important
change) was assessed by means of the standardized response means (SRM) and the Guyatt’s effect size. Construct validity (the degree to which a test measures what it claims, or purports, to be measuring) was assessed by comparing the CCI and its components to the Health Assessment Questionnaire (HAQ) using Spearman’s rank correlation. Demonstrating a high SRM and high Guyatt’s effect size, the use of the CCI as a single-joint assessment after single-joint intervention was supported by this study. Also, the CCI correlated moderately well with the HAQ. In previous studies different versions of the CCI have been used, also including a 6-parameter variant with a CCI score ranging from 0-12. Other combined single-joint outcome measures include the Thompson Knee Index (THOMP) and the Knee Joint Articular Index (KJAI). Both indices are based on physician assessments and include assessments of knee tenderness and swelling. With the combination of several local parameters they provide severity indices, yet valuable information on patient-reported outcomes or assessment of treatment efficacy is left out. Alternatively, to measure a clinical effect, single components can be used, such as patient-reported outcomes, which have also been shown to have construct validity and to be responsive. An advantage of the CCI is that, by incorporation of both patient reported outcomes and physician assessments, it offers a more complete evaluation of a single-joint response. Moreover, the index does not include general parameters like acute phase reactants, thereby excluding the possible influence of persistent arthritis in other joints. With new local therapies evolving this index may be useful in future clinical trials.

Finally, in Chapter 9 we summarized the current status of developments in the field of viral gene therapy using adeno-associated virus as a vector, with a special focus on arthritis. As a relatively new therapeutic modality, it has been explored in a wide variety of diseases, including haemophilia, Leber’s congenital amaurosis and lipoprotein lipase deficiency. While for many diseases gene therapy functions as a means to replace a missing or defective gene, for the treatment of RA, where not one single gene is causative of the disease, gene therapy may be used to restore the cytokine balance by over-expressing immunomodulatory cytokines or inhibiting pro-inflammatory cytokines, targeting of signal transduction pathways, and the induction of apoptosis in synovial tissue. The few clinical trials performed in rheumatologic diseases have aimed at targeting proinflammatory cytokines (by overexpression of a TNF soluble-receptor and an interleukin 1-receptor antagonist), demonstrating safety and providing encouraging data on the feasibility of in vivo gene therapy. We discuss some of the key hurdles and potential solutions that may be faced during development of viral gene therapy in Chapter 9. Besides the development of immune responses (as discussed earlier), other hurdles include the packaging limit of AAV vectors, limited transduction of the target tissue and host genome integration.

**FUTURE PERSPECTIVE - TOWARDS A CLINICAL GENE THERAPY TRIAL**

Gene therapy has recently become a reality for patients in the Western world with the approval of alipogene tiparvovec for the treatment of lipoprotein lipase deficiency. There is significant progress and interest in the field of cell-and gene-therapy, as also illustrated by the recent submission of a marketing application to the European Medicines Agency (EMA) for a cell therapy (GSK2696273) to treat patients with adenosine deaminase severe combined immunodeficiency (SCID) in Europe.
immunodeficiency syndrome (ADA-SCID) for whom no suitable human leukocyte antigen (HLA)-matched related stem cell donor is available.

Bringing AAV gene therapy to the clinic requires a relatively long process. We describe in this thesis part of this process for ART-I02, including pre-clinical studies performed with ART-I02 as well as a proof of mechanism clinical trial with targeted intra-articular treatment. The finding of efficient transduction of FLS in vitro, local expression in joints, and limited biodistribution in 2 animal models of RA all support progress towards a phase 1 experimental medicine clinical trial evaluating intra-articular treatment of patients with RA. Other parts of this process include GLP rodent studies to further assess safety and biodistribution in healthy animals. These studies showed a favorable safety profile but there is a requirement for an additional germline transmission study. The clinical trial assessing intra-articular anti-TNF treatment provided further support for the concept of intra-articular therapy in patients with inflammatory arthritis.

There are remaining hurdles for the clinical application of gene therapy, as described earlier. Humoral and cellular immunity may influence efficacy and duration of transgene expression, and more research is needed on potential solutions to address this. Another challenge lies in obtaining sufficient eligible patients for inclusion in clinical trials as recruitment rates can be slow due to improved disease control in arthritis and competitive clinical trials. ART-I02 comprises an AAV5 vector and the IFN-β gene. Interferon-β is a natural protein, which is immunomodulating rather than immunosuppressive. Importantly, IFN-β protein treatment is already an approved therapy for patients with multiple sclerosis, and there is an extensive safety database available. In view of its high safety profile, lack of toxicity and its ability through different serotypes to efficiently transduce a wide variety of tissues and cells, AAV vectors have been identified as the current most promising gene therapy vector for nonlethal diseases. Clinical trials investigating AAV-based gene therapy for the treatment of different diseases, such as hemophilia B, have demonstrated initial safety and long term expression and efficacy. Progression with ART-I02 towards a clinical trial will represent the next step in the development of intra-articular treatment that could be used in the context of personalized health care for patients with persistent monoarticular or oligoarticular manifestations.
REFERENCES


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