Gene therapy for arthritis: progress towards a clinical trial
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Citation for published version (APA):

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Empty capsids and macrophage inhibition/depletion increase rAAV-transgene expression in joints of both healthy and arthritic mice.
ABSTRACT

Gene therapy has potential to treat rheumatic diseases, however, the presence of macrophages in the joint might hamper AAV mediated gene delivery. We demonstrate that in arthritic, but also in healthy mice, administration of agents that influence macrophage activity/number and/or addition of empty decoy capsids substantially improve the efficacy of rAAV5-transgene expression in the joint. Pre-treatment with triamcinolone or clodronate liposomes improved luciferase expression over a period of 4 weeks. Both the expression per joint, and the percentage of expressing knee joints improved. Similar results were seen when empty decoy capsids were added to full genome containing capsids in a 5:1 ratio. In a study to assess the duration of expression as well as to investigate the combination of these two approaches we observed a synergistic enhancement of gene expression, sustained for at least 6 months. The enhancement of gene expression was independent of the route of administration of triamcinolone (intra-articular or intramuscular). In healthy mice it was demonstrated that the combination improved expression of the transgene significantly, independently of the serotype used. These data have implications for future applications of gene therapy to the joint and for other tissues with an abundance of macrophages.

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Manuscript in preparation
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease affecting the synovial tissue in multiple joints. The synovial joint is characterized by a capsule surrounding the joint and the presence of lubricating synovial fluid within the synovial cavity. The intimal lining layer is composed of two cell types, intimal macrophages and fibroblast-like synoviocytes (FLS). The FLS are responsible for the lubrication of the joint (through production of hyaluronan), and the macrophages are responsible for the removal of debris and undesirable substances from the synovial fluid. During inflammation the presence of both cell types is increased by proliferation of the FLS and migration of macrophages to the joint and both cell types produce inflammatory mediators and bone- and cartilage-degrading enzymes.

Several biological therapies are now available for RA, significantly improving the outcome of the disease in many patients, but not all. Over 50% of patients will still have active disease and even the majority of clinical responders to biological treatment will have one or more actively inflamed joints. There remains an urgent need for the development of safe, efficacious, and cost-effective treatments to specifically target actively inflamed joints. Intra-articular gene therapy has the potential to treat arthritis, as the joint is an excellent target for the local delivery of gene therapy vectors.

There have been very few gene therapy clinical trials in RA, using either injection of cells that were genetically modified with a retrovirus expressing IL-1 receptor antagonist into the joint (ex vivo gene therapy), or injection of a recombinant adeno-associated viral vector (rAAV) encoding a TNF antagonist (in vivo gene therapy). While intra-articular administration of an rAAV2 vector encoding the TNF receptor IgG1 fusion gene (tgAAC94) was well tolerated, the treatment was not effective. It is likely that lack of efficacy was due to insufficient levels of gene expression. This notion is supported by the fact that local gene expression, as determined by a radioimmunoassay for TNFR:Fc protein in synovial fluid and an RT-PCR assay for mRNA specific to rAAV2-TNFR:Fc in synovial tissue, was not detected in the very limited number of samples that became available during the study. Moreover, we have shown that intra-articular injection of etanercept into the inflamed joint of RA patients does include clinical improvement, validating the therapeutic target when administered to the site of inflammation. Importantly, this trial provided encouraging data on safety and feasibility for further development of AAV-based intra-articular gene therapy in RA.

We are developing intra-articular gene therapy for RA using rAAV5. Comparing AAV serotype 1 to 5 we have shown that AAVS was the most efficient in transducing synovial tissue after intra-articular injection in two different animal models of RA. The main target cells for rAAV5 in the joint are FLS. AAVS is effective in delivering genes to both human and rodent FLS, in vitro as well as in vivo in rat and mouse models of RA. AAVS has the added benefit that the majority of the human population is seronegative for AAVS.

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 RA and in healthy animals. Here we present data that uncover an unexpected barrier to efficient gene transfer to the joint, namely, that synovial macrophages significantly inhibit AAV-mediated gene delivery, and we present strategies for overcoming this barrier. These data have implications for future applications of local gene therapy to the joint, or to other tissues that are abundantly infiltrated by macrophages.
METHODS

Vector and empty capsids production
An rAAV5 and rAAV2 vector was produced coding for Firefly Luciferase (Fluc) with a cytomegalovirus (CMV) promoter (rAAV5.CMV.Fluc and rAAV2.CMV.Fluc; Children’s Hospital of Philadelphia, Philadelphia, PA) as described previously. In brief, the plasmid encodes the Fluc gene under the control of the CMV promoter and a human growth hormone polyadenylation signal. The transgene cassette is flanked by AAV2 inverted terminal repeats and is packaged in capsid from AAV5 or AAV2. The genome containing vector and empty AAV capsid particles were purified by combined chromatography and cesium chloride density gradient centrifugation. Vector titers were determined by qPCR and expressed as viral genomes/ml (vg/ml).

Vector administration in mice
Intra-articular rAAV expression was investigated in male DBA mice (8-12 week old; Harlan Sprague Dawley, Horst, the Netherlands). Groups consisted of 5 to 18 animals per experiment (indicated per experiment in the legends of the figures). Mice were injected with rAAV5.CMV.Fluc or rAAV2.CMV.Fluc in both knee joints and monitored periodically for luciferase expression (as indicated in the legends of the figures, from 3 days up till a maximum of 6 months). For animals without arthritis the vector was administered on day 1, in arthritic animals the vector was injected on day 17 or 24 after immunization. Animals received 1.26e10 to 1.65e10 vg of rAAV per knee joint (in a volume of 5 ul) in healthy or CIA mice as indicated in the legends of the figures. Empty capsids were co-administered with the genome containing particles in several groups in a 1:5 or 1:20 full:empty ratio, as indicated.

Collagen-induced arthritis
Arthritis was induced and evaluated as previously described. Bovine type II collagen (2 mg/ml in 0.05 M acetic acid; Chondrex, Redmond, WA) was mixed in an equal volume of Freund’s complete adjuvant (2 mg/ml of Mycobacterium tuberculosis; Chondrex). The mice were immunized intradermally at the base of the tail with 100 µl of emulsion (100 µg collagen) on day 0. On day 20, mice received an intraperitoneal booster injection of 100 µg type II collagen in NaCl. The severity of arthritis was assessed using an established semiquantitative scoring system (0-4; 0 = normal, 1 = swelling in 1 joint, 2 = swelling in >1 joint, 3 = swelling in the entire paw, and 4 = deformity and/or ankylosis. The cumulative score for all 4 paws of each mouse was used to represent overall disease severity and progression. In the long-term follow-up study presented in figure 3, due to technical problems of the IVIS, data are not available for time point 8 weeks. In addition, a total of 15 animals (2-4 per group) were sacrificed prior to the end of the experiment due to reaching a humane endpoint (clinical score >12) before the end of the study.

Macrophage inhibition/depletion
Macrophage inhibition was initiated by two different compounds, clodronate containing liposomes (Ordered from www.clodronateliposomes.com; Dr N van Rooijen, Amsterdam, the Netherlands) and triamcinolone (B.-M.S., Utrecht, the Netherlands). Clodronate liposomes were injected intravenously at a dosage of 5ul/g bodyweight with a concentration of 5 mg liposomes per 1 ml suspension 2 days prior to intra-articular vector administration.
Triamcinolone (5 mg/kg) was administered intramuscularly in the femoral biceps muscle, in a volume of 50 µl also two days prior to vector injection. Taking into account the faster metabolic rate in mice (factor 12.5), a dose of 5 mg/kg bodyweight was used and injected intramuscularly, similar to the route of systemic administration in RA patients. Control groups received an intramuscular injection with 50 µl NaCl. In follow-up experiments, intramuscular injection of triamcinolone was compared to intra-articular administration of the glucocorticoid (same dose in a volume of 5 µl). A control group received an intra-articular injection with 5 µl NaCl.

**Imaging of luciferase expression**

Luciferase expression was measured at different time points after vector administration, from day 3 up till 6 months depending on the experiments. Luciferase expression was detected as described previously. In brief, D-luciferin potassium-salt substrate (Caliper Life Sciences, Hopkinton, MA, USA) was injected intraperitoneally (150 mg/kg of body weight, in a volume of approximately 200 µl). Photon counts were acquired 10 minutes after substrate administration for 5 minutes using a cooled charge-coupled device (CCD) camera system (Photon Imager, Biospace Lab, Paris, France) and image processing and signal intensity quantification and analysis were performed using M3 Vision (Biospace Lab). The number of photons emitted per second per square centimetre per steradian was calculated as a measure of luciferase activity.

**General animal conditions and ethics statement**

Immunization, intra-articular injections and in vivo imaging were performed under isoflurane anaesthesia (3% isoflurane and oxygen). At the end of the experiments, animals were sacrificed by cardiac puncture under isoflurane anaesthesia, followed by cervical dislocation. The studies were reviewed and approved by the animal care and use committee of the University of Amsterdam (Amsterdam, the Netherlands; Permit Numbers: ART 102881, ART 102656, ART 102793, ART 102948, ART 103021 and ART 111AB) and carried out in strict accordance with the recommendations in the Dutch Law on Animal Welfare (Dutch: “Wet op Dierproeven”). Animals were maintained under pathogen-free conditions in the animal facility of the University of Amsterdam.

**Statistical analysis**

Luminescence over time was investigated using generalized estimating equations (GEE) to allow for longitudinal analysis (including all available longitudinal data and allowing unequal numbers of repeated measurements). All other statistics were analyzed using where appropriate the T-test or Mann Whitney U in Graphpad Prism (Ja Jolla, CA, USA). For all tests, differences with a p-value of <0.05 were considered significant.

**RESULTS**

**Inflammation affects intra-articular rAAV5 gene expression**

A hallmark of synovial inflammation is the proliferation of FLS in the synovial tissue of RA patients. In a healthy joint the intimal lining layer is 1-2 cell layers thick, whereas in the inflamed joint this is increased to 8- to 10 cells. This is also true in mouse models of RA, including in
the collagen-induced arthritis (CIA) model. As FLS are the primary target cells for rAAV5 in the joint, we hypothesized that administration of rAAV5 after the onset of inflammation in the CIA model would lead to higher expression, due to a higher number FLS present in the joint at the time of inflammation. To test this hypothesis, we administered an rAAV5 vector encoding the Firefly luciferase gene (rAAV5.CMV.Fluc) intra-articularly in mice with CIA, before (day (d) 17) or after (d24) the onset of arthritis. Surprisingly, we found that administration of an rAAV5 vector after the onset of inflammation (d24) resulted in lower expression compared to vector administration before the onset of inflammation (d17) (Figure 1).

**Immunosuppressive agents improve rAAV5 transgene expression**

An explanation for decreased expression in animals with inflamed joints could be degradation or neutralization of the vector before it is able to transduce the target cells. During inflammation, there is not only an increase in the number of FLS, but there is also an increase in the number and activation of macrophages. We hypothesized that the decreased expression could be due to vector neutralization by macrophages (for example through phagocytosis or opsonization by soluble factors [complement]). To investigate this possibility we examined whether administration of agents that influence macrophage-activity and/or -number had an effect on rAAV5 expression.

Triamcinolone, a glucocorticosteroid, acts by inhibiting the activation and proliferation of immune cells, including macrophages. Alternatively, clodronate containing liposomes were used to deplete macrophages. The two agents were administered 48 hours before vector administration. Both triamcinolone and clodronate liposomes improved rAAV5.CMV.Fluc luciferase expression over a period of 4 weeks, showing that either depletion or inhibition of macrophages leads to an increase in gene expression (Figure 2a).

![Figure 1. Luciferase expression is influenced by vector administration before or after the onset of arthritis. (a) After induction of arthritis, mice (n = 5 per group) were injected with 1.65e10 vg/joint of a rAAV5-transgene vector encoding the gene for luciferase (rAAV.CMV.Fluc) intra-articularly in the knee joint either on day 17 before onset of arthritis or day 24 after onset of arthritis. Imaging was performed 3 days after vector injection and thereafter weekly up to 4 weeks (group day 24) or 5 weeks (group day 17). (b) The right panel shows a representative picture of the imaging results. Luminescence is shown per group as average; error bars, SEM.](image-url)
Figure 2. Effect of addition of clodronate, triamcinolone and empty capsids on rAAV5 luciferase expression. After induction of arthritis, mice (n = 5 per group) were i.a. injected with 1.65e10 vg/joint of rAAV5.CMV.Fluc vector. Imaging was performed 3 days after vector injection and thereafter weekly up to 4 weeks. (a) Addition of liposomal clodronate (5 μl/g i.v. in a conc of 5 mg/ml) and triamcinolone (5 mg/kg i.m.) resulted in higher levels of luminescence. (b) Addition of empty capsids in a 1:5 ratio (full to empty) with genome containing capsids improved luciferase expression. (c) The percentage of knee joints expressing a positive signal was improved 4 to 9 fold. (d) Luminescence 28 days after vector administration. Luminescence is shown per group as average; error bars, SEM. A positive signal was defined as a value 1.5 times above the upper limit of the value of control knee joints for at least one measurement in time. *p<0.05 compared to d24 (post-boost).
We hypothesized that an alternative approach to avoid macrophage vector neutralization was to add empty capsid particles upon vector administration. These empty capsids could act as a decoy and therefore increasing the chances that full virus particles will be able to reach the target cells. This strategy is similar to previously used to avoid neutralizing anti-AAV capsid antibodies. When empty capsids were added to full genome containing capsids in a 5:1 ratio, expression improved significantly (Figure 2b). These data supported our hypothesis that the vector is likely being neutralized by macrophages. In support of this hypothesis, we observed that all three treatment groups (triamcinolone, clodronate liposomes, empty capsid) also showed an increased percentage of positive joints (Figure 2c). In figure 2d the luminescence 28 days after vector administration is shown, demonstrating a significant difference between administration of the vector alone after onset of disease compared to the groups that received treatment. As triamcinolone is an anti-inflammatory agent, arthritis activity was closely monitored. As expected, mice treated with triamcinolone or clodronate liposomes showed a delayed onset of arthritis (results not shown).

**T**riamcinolone and decoy capsids have a synergistic effect on rAAV5 expression

As we had found that pharmacological inhibition of macrophage activity or addition of empty decoy capsids resulted in increased gene expression, we performed a long term follow up study to assess the duration of improved gene expression, as well as to investigate the combination of these two approaches. As triamcinolone is a therapeutic compound that is already approved for the treatment of RA, we selected this compound for the follow-up studies. The effect of addition of triamcinolone and/or empty capsids was analysed longitudinally using generalized estimating equations (GEE), allowing us to include all available longitudinal data and allowing unequal numbers of repeated measurements. Similar to the previous study, we observed increased expression using triamcinolone or empty capsid alone, however it was clear that the combination of macrophage inhibition and decoy capsids resulted in a synergistic enhancement of gene expression (Figure 3a and Table 1, 5.85 fold enhancement, p<0.001) and that this enhancement was sustained for up to 6 months post vector administration. As expected, due to its anti-inflammatory effect, arthritis activity was lower in groups treated with triamcinolone up till week 4 (Figure 3b), however long term arthritis activity was comparable between groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Luminescence coefficient</th>
<th>SE</th>
<th>P</th>
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<tr>
<td>rAAV.CMV.Fluc</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+ Triamcinolone</td>
<td>1.29</td>
<td>1.81</td>
<td>0.477</td>
</tr>
<tr>
<td>+ Empty capsid</td>
<td>0.86</td>
<td>1.81</td>
<td>0.635</td>
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<td>+ Triamcinolone and empty capsid</td>
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Local triamcinolone administration is as effective as systemic administration on increased gene expression of rAAV5

In the clinic, triamcinolone is usually administered locally by intra-articular injection into the inflamed joint. Therefore we compared the efficacy of local versus systemic administration of triamcinolone on enhancement of gene expression. To test this, triamcinolone was administered systemically (intra-muscular) or locally (intra-articular) two days prior to intra-articular administration of rAAV5.CMV.Fluc + empty capsid (empty:ful ratio of 5:1). As control, saline was injected intra-articularly. Luciferase expression was monitored over time by IVIS imaging up till 30 days. We observed similar increases in gene expression when triamcinolone was administered locally or systemically, indicating that local administration of triamcinolone was as effective as systemic administration (Figure 4).

AAV empty capsids improve transgene expression of rAAV5 in the absence of inflammation

All previous experiments were performed in CIA models, in which animals experienced significant inflammation in the joint at the time of vector administration. We then decided to investigate whether the enhancement in luciferase expression by adding empty capsids could also be seen in healthy joints. While the number of synovial macrophages increases during inflammation, even healthy synovium contains a significant number of macrophages. When empty capsids were added to genome containing particles in 2 different ratios we observed a dose dependent increase in luminescence (Figure 5). The increase was 4.8 fold on average in animals injected with empty capsids in a 5:1 ratio to full capsids (p < 0.01), and 20 fold when a 20:1 empty to full ratio was used (P<0.05).

Next, we investigated the effect of the combination of the two approaches on the expression of the transgene in healthy joints. As can be seen in Figure 6, both empty decoy capsid and triamcinolone treated animals showed increased gene expression compared with vector alone.
Figure 4. Improvement of intra-articular rAAV5-luciferase expression by addition of empty capsids and/or triamcinolone. Mice with CIA (n = 18 per group) were injected with triamcinolone either systemically (i.m.) or locally (i.a.) two days prior to i.a. administration of rAAV5.CMV.Fluc (1.65e10 vg/joint) + empty capsid (full:empty ratio of 1:5). Luciferase expression was monitored over time by IVIS imaging for a follow-up of 30 days after vector administration. Luminescence is shown per group as average; error bars, SEM.

Figure 5. Addition of empty capsids in 2 different ratios (1:5 and 1:20 full:empty) improves intra-articular rAAV5-luciferase expression in healthy mice. Healthy mice (n=7 per group) were injected i.a. with 1.65e10 vg/joint rAAV5-CMV-FLUC. In 2 groups empty capsids were added in different ratios. Luciferase expression was measured weekly until mice were sacrificed after 4 weeks. Luminescence at week 4 is shown per group as averages; error bars, SEM. * P < 0.05 and ** P < 0.01 in groups with empty capsid addition versus the control group that only received genome containing vector (one-tailed unpaired t test).
animals, but the combination of empty capsid and triamcinolone gave rise to the highest increase in gene expression levels, similar to what was observed in inflamed (CIA) joints. These results support the hypothesis that macrophages in the intimal lining layer inhibit AAV-mediated

**Figure 6.** Effect of empty capsid and triamcinolone on intra-articular rAAV5 gene expression in healthy mice. Healthy mice (n=17 per group, 34 total injected joints) were injected with 1.26e10 vg/joint rAAV5-CMV-Fluc intra-articularly +/- empty capsid (5:1 empty to full ratio) preceded 2 days prior with i.m. administration of either saline (NaCl) or triamcinolone. Luciferase expression was measured weekly by IVIS up to week 8. (a) Luciferase measurement over time for all groups. (b) Luciferase expression in all groups at week 8. Data shown are average per group ± SEM. * p<0.05, ** p<0.01, *** p<0.001 as determined by one-tailed Mann Whitney test.
expression, even in healthy joints, and that either adding decoy capsid particles and/or inhibiting macrophage activity can overcome this inhibition, leading to increased gene expression.

**Combination of empty capsid and triamcinolone is not specific for rAAV5, but enhances intra-articular gene expression using other serotypes as well**

Our studies thus far focused on AAV5 as this serotype is very effective in transducing FLS, however we hypothesized that the effect of macrophage neutralization on gene transfer using AAV is not serotype specific. AAV uptake by macrophages is a general phenomenon utilizing scavenger receptors, and thus 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. To test this hypothesis, we performed an experiment where we evaluated if triamcinolone and empty capsid could enhance gene expression from a serotype that is very different from AAV5, being AAV2. AAV5 and AAV2 share only 57% homology at the amino acid level, making them two of the most diverse serotypes of AAV known. To test this, we performed an experiment identical to that described above (Figure 6), but now with AAV2. Similar to results seen with rAAV5, both empty decoy capsid and triamcinolone treated animals showed increased gene expression compared with vector alone animals following rAAV2 administration (Figure 7). The combination of empty capsid and triamcinolone gave rise to the highest increase in gene expression levels, similar to what was observed in rAAV5 treated animals, providing further validation of this approach.

**DISCUSSION**

While there have been many advances in the treatment of RA, there remains an urgent need for safe and effective therapies targeting inflamed joints that are unresponsive to systemic therapies. To address this need, we and others have proposed local delivery of rAAV as a potential platform for the long term expression of therapeutic genes.\textsuperscript{14,25-27} rAAV vectors have been extensively used for in vivo gene therapy and have been shown to be safe and effective in pre-clinical models as well as in clinical trials.\textsuperscript{4,28}

In the course of developing an rAAV-based gene therapy product for the treatment of RA, we have used rAAV5 expressing firefly luciferase combined with in vivo imaging of mice to investigate factors affecting intra-articular gene expression. Of importance, we found that, following intra-articular administration of rAAV5 in arthritic animals, not all joints are effectively transduced (usually <50%), and expression in injected joints was quite variable. To ensure sustained local production of effective doses of therapeutic proteins in the joint, in particular in the rheumatoid synovium, an optimized gene delivery system is clearly needed.

Here we present data showing that synovial macrophages represent a previously unrecognized barrier for efficient intra-articular gene transfer. Our data suggests that agents that can deplete and/or inhibit activation of macrophages (clodronate liposomes or triamcinolone), as well as the addition of empty decoy capsids to the vector preparation, can improve luciferase expression after local injection in the joint. Using a combination of both approaches resulted in a synergetic effect. Interestingly, also in healthy joints the expression of luciferase was improved using a combination of triamcinolone and empty capsids, and this was not limited to the use of an rAAVS vector but also applies to other serotypes.
Triamcinolone, a glucocorticosteroid which is frequently used in the treatment of RA, had a positive effect on transgene expression in a similar order of magnitude as seen for clodronate liposomes. Systemic administration of glucocorticosteroids is known to exert a local effect in the joint by decreasing the number of macrophages in synovial tissue of RA patients\cite{29}, indicating that the effect of triamcinolone is at least partly related to the depletion of macrophages.\cite{30} Both systemic and intra-articular administration of glucocorticosteroids can be accompanied by side-effects.\cite{31} In case of mono- or oligoarthritis, the preferred route is intra-articular. We demonstrated that intra-articular injection of triamcinolone was equally effective in increasing gene expression compared with systemic administration, which is an important finding in view of the possible application of this approach for intra-articular gene therapy in RA.

The findings reported in this manuscript may also be relevant for the interpretation of the lack of efficacy in the phase I/II clinical trial with tgAAC94. In this study, adults with persistent moderate or severe inflammation in a clinically inflamed joint received a single intra-articular injection of rAAV2 expressing the human TNFR:Fc gene.\cite{7} Arthritis activity is strongly correlated with macrophage infiltration of the synovial tissue.\cite{32} Based on the findings presented here, we postulate that the presence of macrophages at the site of injection may have diminished the transduction efficacy of the vector, thereby preventing efficient transgene expression to induce a therapeutic effect. Other factors, including neutralizing antibodies, may also have played a role.

For transduction efficacy of rAAV vectors, the presence of macrophages is not only important in inflamed tissue, but as presented here, also in healthy tissues. Thus, the combination of triamcinolone and empty capsid is not only effective for increasing expression in diseased joints, but also in healthy joints. These results may be relevant for a wider range of applications using rAAV vectors delivered to tissue containing macrophages. This notion is supported by a previous study showing that blocking rAAV8 uptake by macrophages via scavenger receptors (using polynosinic acid (poly[i]) can reduce AAV endocytosis in vitro, and that poly(i) enhances liver targeted gene expression in vivo in a rat model of hyperbilirubinemia.\cite{33} As expected, we found that enhancement of gene expression by macrophage inhibition is applicable to AAV serotypes other than AAV5 as well.

Recently, it was reported that addition of a significant amount of empty capsids to the AAV transgene composition after intravenous administration is able to overcome the inhibitory effect of (pre-existing) neutralizing antibodies and has an ameliorating effect on transgene expression in the liver.\cite{24} It was shown that addition of empty decoy capsid to the final vector formulation can adsorb these antibodies and thus overcoming their inhibitory effect. Based on these findings, we hypothesized that empty capsids could act in a similar manner as decoy for macrophages, thereby preventing uptake and degradation of the transgene-containing rAAV vector. Indeed, we found that addition of empty decoy vectors to the full vector preparation in a ratio of 1:5 or 1:20 (empty to full) has a positive effect on transgene expression in the joint. The exact mechanism of action of these decoys is currently not known, and it is unclear if empty capsids have a higher affinity for macrophages compared to full capsids, or if the abundance of total capsid particles is overwhelming the macrophage scavenger pathway, resulting in more rAAV particles able to transduce the synovial FLS.

Empty capsids can be part of the vector preparation or be produced independent of the rAAV vector batch, and if desired, added to AAV vector preparations, or administered separately to an individual. In future studies, the ratio of empty to full particles needs to be
Figure 7. Effect of empty capsid and triamcinolone on intra-articular rAAV2 gene expression in healthy mice. Healthy mice (n=17 per group, 34 total injected joints) were injected with 1.26e10 vg/joint rAAV2-CMV-Fluc intra-articularly +/- empty capsid (5:1 empty to full ratio) preceded 2 days prior with i.m. administration of either saline (NaCl) or triamcinolone. Luciferase expression was measured weekly by IVIS up to week 4. a) Luciferase measurement over time for all groups. b) Luciferase expression in all groups at week 4. Data show is average per group + SEM. ** p<0.01, *** p<0.001 as determined by one-tailed Mann Whitney test.

investigated in more detail, also in the context of the level of inflammation and number of macrophages present. Reducing the number of capsids present in the preparation may also reduce immunotoxicity. An interesting approach to further enhance the safety of the use of empty capsids to bind neutralizing antibodies, is the mutation of the receptor binding site of AAV2 to generate an empty capsid mutant that can adsorb antibodies but cannot enter a
target cell, thereby also reducing the potential for targeted immunotoxicity. Whether such an approach is also feasible in reducing the detrimental effects of macrophages on transgene expression needs to be evaluated in future studies.

In conclusion, we provide evidence that intra-articular macrophages represent a barrier to efficient gene transfer, and that triamcinolone or empty decoy capsids alone result in improved gene expression in inflamed and healthy joints. Importantly, the combination of a glucocorticosteroid and empty capsids led to a synergistic increase in gene expression. These data have implications for future applications of gene therapy to the joint, but also to other tissues with an abundance of macrophages.

ACKNOWLEDGEMENTS

The authors want to thank L Bevaart and FA Koopman for their contribution in writing the application for the animal ethical committee and their assistance in getting the studies initiated.
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