Addressing immune tolerance issues in inflammatory bowel disease and adeno-associated virus based gene transfer

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Chapter 8

Mir-142-3p target sequences reduce transgene directed immunogenicity following intramuscular AAV vector-mediated gene delivery

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Abstract

Background

Muscle represents an important tissue target for adeno-associated virus (AAV) vector-mediated gene transfer in muscular, metabolic or blood related genetic disorders. However, several studies demonstrated the appearance of immune responses against the transgene product after intramuscular AAV vector delivery which resulted in a limited efficacy of the treatment. Use of microRNAs (miRNAs) that are specifically expressed in antigen-presenting cells (APCs) is a promising approach to avoid those immune responses. Cellular mir-142-3p, which is APC-specific, is able to repress translation of its target cellular transcripts by binding to a specific target sequences.

Methods

In this study, we explored the potential of mir-142-3p specific target sequences to reduce or abolish immune responses directed against ovalbumin (OVA), a highly immunogenic protein, expressed as transgene and delivered by AAV1 vector administered intramuscularly.

Results

The occurrence of immune responses against OVA transgene following intramuscular delivery by AAV have been previously described and resulted in loss of OVA protein expression. In the present study we demonstrate that OVA protein expression was maintained when mir-142-3pT sequences were incorporated into the expression cassette. The sustained expression of OVA protein over time correlated with a reduced increase in anti-OVA antibody level. Furthermore, no cellular infiltrates were observed in the muscle tissue when AAV1 vectors containing 4 or 8 repeats of mir-142-3p target sequences after OVA sequence were used.

Conclusions

The rising humoral and cellular immune responses against OVA protein after intramuscular delivery can be efficiently reduced by the use of mir-142-3p target sequences.

Introduction

Adeno-associated virus (AAV) vectors are one of the most promising systems for achieving therapeutic gene delivery for treatment of genetic and chronic diseases [1-3]. AAV vectors are able to transduce efficiently a wide variety of tissues and can provide long term expression of delivered gene after single administration [4-6]. Furthermore, AAV vectors are not associated with any pathology and are replication-defective [7]. They have been successfully employed in treatment of genetic disorders in preclinical studies [8-11] as well as in clinical trials [1, 3, 12, 13].

Muscle represents an important tissue target for AAV vector-mediated gene transfer in muscular [14], metabolic [1] or blood related genetic disorders [1, 15, 16] such as haemophilia B in which liver directed approach might be not possible due to high prevalence of hepatitis in the patient population [17-20]. However, several studies demonstrated the appearance of immune responses against the transgene product after intramuscular AAV vector delivery which resulted in a limited efficacy of the treatment [15, 21, 22]. These observations correlate with accumulating evidence that AAV serotypes are able to transduce antigen-presenting cells (APCs) [23-25] and, consequently, can mediate the appearance of immune responses against the transgene products in case of AAV-based delivery in the muscle [24].

The cellular and humoral immune responses that can occur against the delivered transgene product might result in a loss of transgene expression, as reported in animal [22, 26] and patient studies [27, 28]. A commonly used approach to avoid transgene product directed immunogenicity consists of immune suppression protocols. However, immune suppression protocols have not always proven to be effective [29], as observed in a clinical study where AAV-based gene therapy was used in patients with Duchenne's muscular dystrophy [27]. Furthermore, immune suppression involves strong, systemic drugs that can lead to serious side effects and complications [30, 31]. Therefore, it is of importance to find an alternative way of tolerance induction towards the transgene product.

Cellular microRNAs (miRNAs) are considered to be an important component of the gene expression regulatory network [32, 33]. According to miRBase (central online repository for miRNA) 21643 mature miRNA are identified in 168 species. Many miRNAs are expressed in a tissue specific manner and have an important role in maintaining tissue specific functions and differentiation [34, 35]. Haematopoietic mir-142-3p is a miRNA which is specifically expressed in antigen presenting cells (APCs) and is able to repress translation of its target transcripts by binding to specific target sequences. Fusion of mir-142 -3p target sequences to a transgene sequence has been shown to mediate inhibition of gene expression in haematopoietic lineage cells, including APCs *in vitro* and *in vivo* [36]. The use of mir-142-3p target sequences was shown to prevent immune responses towards the transgene product in mice when a lentiviral vector was used for gene delivery targeting the liver [37, 38].

In the present study we explored the potential of incorporating mir-142-3p-specific target sequences to reduce or abolish immune responses directed against ovalbumin (OVA), a highly immunogenic protein, expressed as transgene delivered by an AAV vector administered intramuscularly.

Materials and methods

Ethics statement

All animal experiments were approved by the local animal welfare committee (University of Amsterdam).

Plasmid constructs

Constructs containing the expression cassette CMV-ovalbumin (CMV-OVA-WPRE) with 2, 3, 4, 6, 8, 10, 11 and 14 mir-142-3p target sequences (CMV-OVA-WPRE-mir-142-3pT) were generated by insertion of multiple copies of synthesized and annealed oligonucleotides of mir-142-3p target sequences into pVD272 plasmid (CMV-OVA-WPRE expressing plasmid) at the 3'-untranslated region downstream to OVA cDNA and WPRE sequence. The presence and orientation of mir-142-3p target sequences was verified by sequencing (**Figure 1.A**).

The mir-142-3p expressing plasmid (**Figure 1.A**) was generated as followed. A 436 bps fragment of mouse genomic DNA containing the pri-mir-142 (MI0000167) precursor was cloned by PCR amplification with mmu-mir142f (5'GAAGAAGAGGCTCATCTGGC3') and mmu-mir142r (5'CAAGTATCAGGG GTCAGGAAG3') primers into pCR-TOPO Blunt plasmid vector (Invitrogen, Carlsbad, CA). Next, the pri-mir-142 expression cassette was subcloned into pcDNA6.2-GW/EmGFP-miR vector (Life Technologies, Grand Island, NY). The presence of pri-mir-142 precursor was verified by sequencing. Pri-mir-142 expressed two mature miRNAs from 5' and 3' arm, named mir-142-5p and mir-142-3p. Since we are interested in the mir-142-3p, we refer to it solely from now on.

As the negative control, which does not recognize the binding sites for mir-142-3p, pcDNA6.2-GW/EmGFP-miR-neg (Life Technologies, Grand Island, NY) was used and it was named miScr.

In vitro transfection experiments

Hek293T cells were co-transfected with the plasmid encoding mir-142-3p and with the constructs containing the expression cassette CMV-OVA alone, or associated with 2, 3, 4, 6, 8, 10, 11 or 14 mir-142-3p target sequences (CMV-OVA-mir-142-3pT). Co-transfections were performed with 50 ng of OVA expressing plasmids and different amounts of mir-142-3p expressing plasmid (5, 10 and 50 ng) with the use of lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 hours of incubation, the medium was collected and tested for OVA expression in an OVA specific enzyme-linked immunosorbent assay (ELISA).

AAV production

The AAV1 vectors batches (AAV1-CMV-OVA, AAV1-CMV-2xmir-142-3pT, AAV1-CMV-4xmir-142-3pT and AAV1-CMV-8xmir-142-3pT) were produced in insect cells according to a technology adapted from R. M. Kotin [39]. The AAV batches were purified with an AVB sepharose column using the ÄKTA explorer system (GE Healthcare). Diafiltration and concentration of the AAV elution in PBS-/-, 5% sucrose buffer was performed with the use of

hollow fiber membrane (Spectrum labs). The titer of AAV vector genomes copies (gc/ml) in the final product was determined by Taqman QPCR amplification. Infectivity of all AAV1 batches was demonstrated *in vitro* by OVA specific ELISA performed on medium from Hek293T that were transduced with AAV1 batches with different MOI's (Multiplicity Of Infection) (data not shown).

Mice experiments

Male C57BL/6 mice (8-10 weeks) were obtained from Harlan and maintained in specific pathogen-free conditions at animal facility.

In the first *in vivo* experiment, mice (n=6/group) were injected intramuscularly with PBS or 1 x 10^{14} gc/kg of AAV1-CMV-OVA, AAV1-CMV-OVA-2xmir-142-3pT, AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xTmir-142-3pT. In the second *in vivo* experiment, mice (n=3/group) were injected intramuscularly with PBS and two different doses (5 x 10^{13} gc/kg, 1 x 10^{14} gc/kg) of AAV1-CMV-OVA, AAV1-CMV-OVA-2xmir-142-3pT, AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xmir-142-3pT. Blood was collected weekly by submandibular vein puncture in tubes containing 2 µl of heparin/PBS. Plasma was isolated after centrifugation for 5 min at 5000 rpm and frozen at -80°C until further analysis. OVA protein level, anti-OVA antibody level and anti-AAV1 antibody level were determined in specific ELISA assays as described below.

Assessment of OVA protein expression, anti-OVA antibody level and anti-AAV1 antibody level

Expression of OVA protein in mouse plasma was measured by OVA specific ELISA. Nunc MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with 1:2500 rabbit anti-OVA (Fitzgerald) and OVA protein in samples was detected with 1:2000 rabbit anti-OVA-biotin (Fitzgerald) and 1:1000 Streptavidin-HRP (DAKO). Measured OD (450 nm) values between 0 and 1.5 were determined to correspond to a protein concentration range between 0 and 450 ng/ml.

The level of anti-OVA antibody in mouse plasma was measured by anti-OVA specific ELISA. Nunc MaxiSorp® flat bottom 96-well plates (ThermoScientific) were coated with 0.5 μg/ml OVA protein and anti-OVA antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO). Measured OD (450 nm) values between 0 and 3 were determined to correspond to an antibody concentration range between 0 and 400 ng/ml.

The level of anti-AAV1 antibody in mouse plasma was measured by anti-AAV1 specific ELISA. MaxiSorp® flat bottom 96-well plates (ThermoScientific) were coated with AAV1 and anti-AAV1 antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO).

Histological analysis

Mouse muscle tissue was obtained at sacrifice, 8 weeks after intramuscular injections (groups were injected with: PBS, AAV1-CMV-OVA, AAV1-CMV-OVA-4xmir-142-3pT, and AAV1-CMV-OVA-8xmir-142-3pT) and was frozen in isopentane cooled with liquid nitrogen. Frozen sections, 7μm thick were stained for the presence of $CD8^+$ lymphocytes (Rat anti-mouse CD8a, clone 53-6.7, BD Pharmigen™), $CD4^+$ lymphocytes (anti-mouse CD4, clone LT3T4, BD Pharmigen™), neutrophils (Rat anti-mouse mAb NIMP-R14, Abcam) and macrophages (Rat anti-mouse, clone F4/80, home-made) and OVA protein (anti-OVA antibody; Fitzgerald Inc.). A counter staining with haematoxylin was performed.

Results

Reduction of OVA expression from CMV-OVA constructs containing mir-142-3p targets upon *in vitro* co-transfection with mir-142-3p expressing construct

Constructs containing the expression cassette CMV-OVA alone or linked to 2, 3, 4, 6, 8, 10, 11 and 14 mir-142-3p target sequences (CMV-OVA-mir-142-3pT) were generated (**Figure 1.A**). The inhibitory effect of mir-142-3p on the

level of OVA expression in the CMV-OVA constructs containing mir-142-3p targets was evaluated *in vitro*. Increasing the number of mir-142-3p targets resulted in decreased level of OVA protein expression when mir-142-3p expression plasmid was added in amount of 5 or 10 ng. With the highest amount of mir-142-3p (50 ng) the correlation between increasing amount of mir-142-3p target sequences and decrease of OVA expression was lost. This result shows that mir-142-3p target sequences in the CMV-OVA constructs were recognized by mir-142-3p. However, at a high level of mir-142-3p expression, an increasing number of mir-142-3p target sequences did not result in higher inhibition of OVA protein expression (**Figure 1.B, C**).

Incorporation of mir-142-3p target sequences after OVA transgene sequence is correlated with a sustained expression of OVA protein *in vivo*

A mouse study was performed to explore the effect of mir-142-3p target sequences on OVA expression *in vivo*. For this study, constructs containing 2, 4 and 8 mir-142-3p target sequences were chosen. C57BL/6 mice were injected intramuscularly with PBS, AAV1-CMV-OVA, AAV1-CMV-OVA-2xmir-142-3pT, AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xmir-142-3pT. The expression level of OVA protein was monitored in the plasma of injected mice for 4 weeks.

In control mice that were injected with AAV1-CMV-OVA only, the expression of OVA protein in the plasma increased until 1 week before returning to basal level after 2 weeks. OVA expression remained at base line until sacrifice of the mice. In contrast, the plasma of mice injected with AAV1-CMV-OVA associated with mir-142-3pT sequences (AAV1-CMV-OVA-mir-142-3pT), OVA protein expression was detectable starting from 1 week. In mice that were injected with AAV1-CMV-OVA-2xmir-142-3pT, OVA expression was stable over the first three weeks following administration before it started to rise. In mice that were injected with AAV1-CMV-OVA-4xmir-142-3pT and AAV1-CMV-OVA-8xmir-142-3pT, OVA expression was higher compared to the level achieved with AAV1-CMV-OVA-2xmir-142-3pT. In addition, OVA levels were continuously increasing over the whole observation period of 4

weeks. In contrast to the *in vitro* experiments, OVA protein expression was correlated with the increase of mir-142-3pT sequences (**Figure 2**).

Based on the higher level of OVA expression observed, AAV1-CMV-OVA-4xmir-142-3pT and AAV1-CMV-OVA-8xmir-142-3pT were used in the subsequent *in vivo* study.

Mir-142-3p target sequences reduce OVA directed immunogenicity following AAV1 intramuscular delivery

C57/BL6 mice were injected intramuscularly with PBS, AAV1-CMV-OVA AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xmir-142-3pT. In order to determine a possible influence of the AAV vector dose on the immune response to OVA protein, two different AAV vector doses were used (5 x 10^{13} and 1 x 10^{14} gc/kg) in the present study. The dose-dependent effect of mir-142-3p target sequences on the development of humoral immune response against the OVA transgene was monitored for 7 weeks, while cellular immune responses were assessed by immunohistochemistry at the sacrifice, 8 weeks after intramuscular injection.

Elevated levels of anti-OVA antibodies were observed in mice injected with 5 x 10^{13} or 1 x 10^{14} gc/kg of AAV1 (CMV-OVA) (**Figure 3.B, D**), which correlated with the loss of OVA protein expression (**Figure 3.A, C**). In some animals, the anti-OVA antibody level was already reduced after week 4 however the OVA protein expression remained absent. In contrast, low anti-OVA antibody levels (**Figure 4.B, D** and **5.B, D**) and a sustained expression of OVA protein (**Figure 4.A, C** and **5.A, C**) were detected in mice that were injected with 5 x 10^{13} or 1 x 10^{14} gc/kg of AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xmir-142-3pT. To determine whether the decrease of humoral immune response was specific for the OVA protein, anti-AAV1 antibody levels were measured in parallel. Anti-AAV1 antibody levels were observed over time in all animals injected with the different AAV1 vectors but were absent in PBS injected mice (**Figure 6**). In all mice injected with AAV1 vectors anti-AAV1 antibody levels increased until week 3 and remained at this level until the end of experiment. The data obtained indicate that the humoral immune response was reduced specifically with respect to OVA protein.

In addition to the systemic humoral immune response, the local cellular immune response was assessed. Haematoxylin and eosin staining of muscle tissue sections showed the presence of numerous cellular infiltrates in mice injected with 1 x 10^{14} gc/kg of AAV1-CMV-OVA (**Figure 7.B**). Specific staining of these sections showed that the infiltrates were composed of CD8 positive cells (**Figure 8.B**), CD4 positive cells (**Figure 9.B**), macrophages (**Figure 10.B**) and neutrophils (**Figure 10.F**). The cellular infiltrates were shown to colocalize with cells expressing OVA (**Figure 11.B**). No substantial cellular infiltrates were observed in the muscle tissue of mice that were injected with 1 x 10^{14} gc/kg of AAV1-CMV-OVA-4xmir142-3pT (**Figure 7.C, 8.C, 9.C, 10.C, G**) or AAV1-CMV-OVA-8xmir142-3pT (**Figure 7.D, 8.D, 9.D, 10.D, H**), similar to the control mice injected with PBS (**Figure 7.A, 8.A, 9.A, 10.A, E**).

Inclusion of the mir-142-3pT sequences does not alter mRNA transcription of the OVA transgene

Total RNA from the muscle tissue of mice injected with PBS or 1 x 10^{14} gc/kg of AAV1-CMV-OVA, AAV1-CMVOVA-4xmir-142-3pT and AAV1-CMV-OVA-8xmir-142-3pT was isolated, reverse transcribed into cDNA, and analyzed by qPCR with primer sets specific for OVA and b-actin. No significant differences in the mRNA levels of OVA in the muscle were observed between mice injected with AAV1-CMV-OVA, AAV1-CMV-OVA-4xmir-142-3pT or AAV1-CMV-OVA-8xmir-142-3pT (*data not shown*). Additionally, the amount of genome copies of (CMV-OVA) DNA present in the muscle of the mice was not significantly different between groups (*data not shown*). Overall, those results indicate that the inclusion of mir-142-3pT sequences does not influence mRNA transcription of the transgene, nor does it influence the infectivity of the AAV itself.

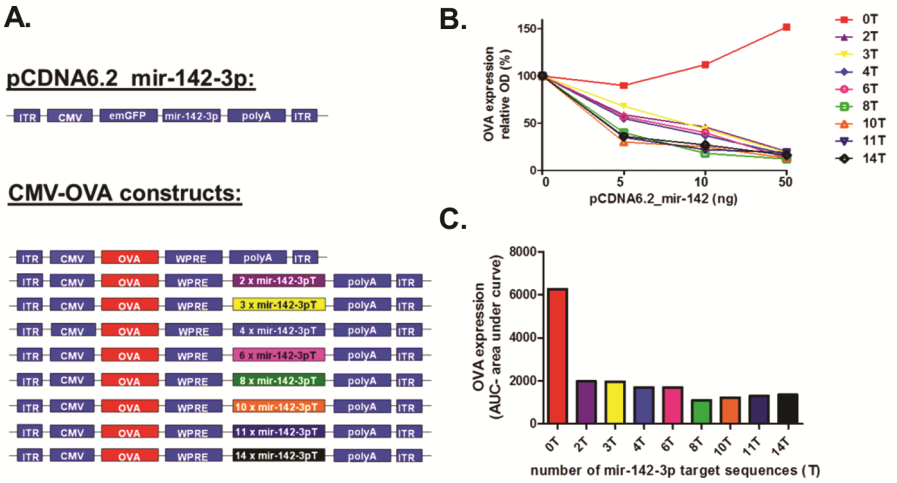

Figure 1. Inhibition of OVA protein expression from CMV-OVA-WPRE plasmid constructs containing mir-142-3p target sequences upon *in vitro* co-transfection with mir-142-3p expressing plasmid (pCDNA6.2_mir-142-3p). A: Mir-142-3p expressing construct, CMV-OVA-WPRE construct and CMV-OVA- WPRE constructs with mir-142-3p target sequences; **B, C:** Relative OVA protein expression after co-transfection. Inhibition by mir-142-3p was expressed as relative OD450, and was calculated by dividing the OD450 of mir142-3p by OD450 of miScr, multiplied by 100.

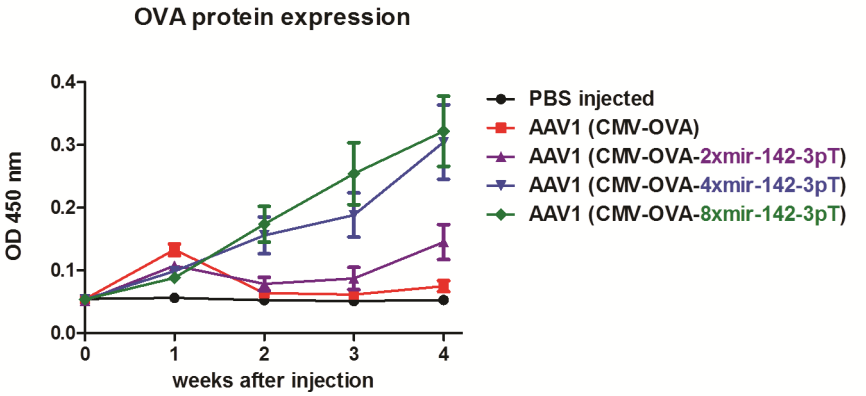

Figure 2. Sustained OVA protein expression level correlates proportionally to the number of mir-142-3p target sequences incorporated after OVA transgene sequence. Mice were injected with PBS, AAV1-CMV-OVA ($1x10^{14}$gc/kg), AAV1-CMV-OVA-2xmir-142-3p ($1x10^{14}$gc/kg), AAV1-CMV-OVA-4xmir-142-3p ($1x10^{14}$gc/kg) and AAV1-CMV-OVA-8xmir-142-3p ($1x10^{14}$gc/kg). Points represent mean ± SD (n=6).

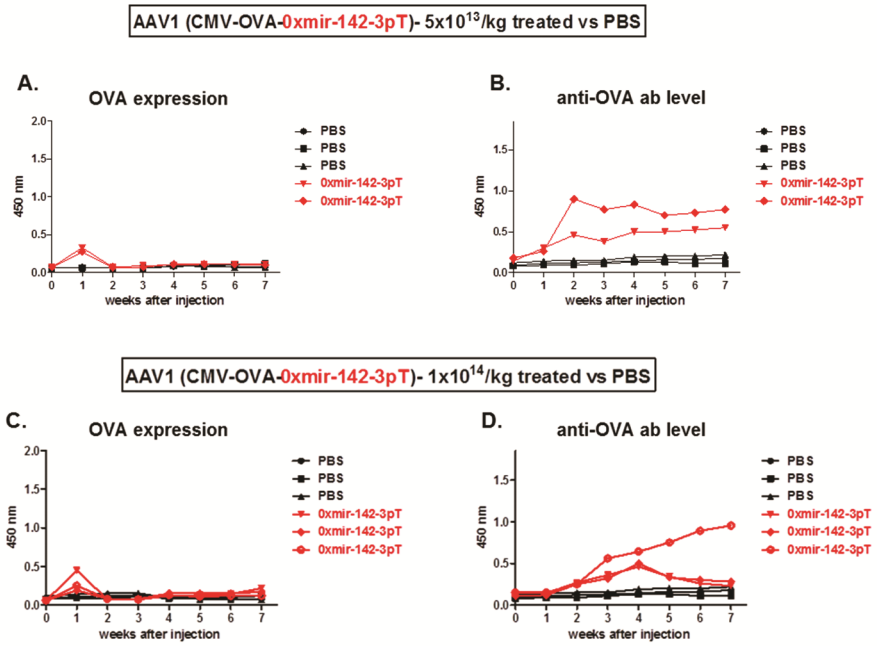

Figure 3. Loss of OVA protein expression that correlates with raising anti-OVA antibody levels following AAV1 intramuscular delivery. Expression of OVA protein after intramuscular delivery of $5x10^{13}$ and $1x10^{14}$ gc/kg of AAV1-CMV-OVA raises atweek1, although it is totally lost in subsequent weeks (**A, C**). It correlates with raising anti-OVA antibody levels (**B, D**). Black lines on the graphs represent PBS-injected control mice. OD between 0 and 1.5 equals the range of 0–450 ng/ml for OVA ELISA; OD between 0 and 3 equals 0–400 ng/ml for anti-OVA ELISA.

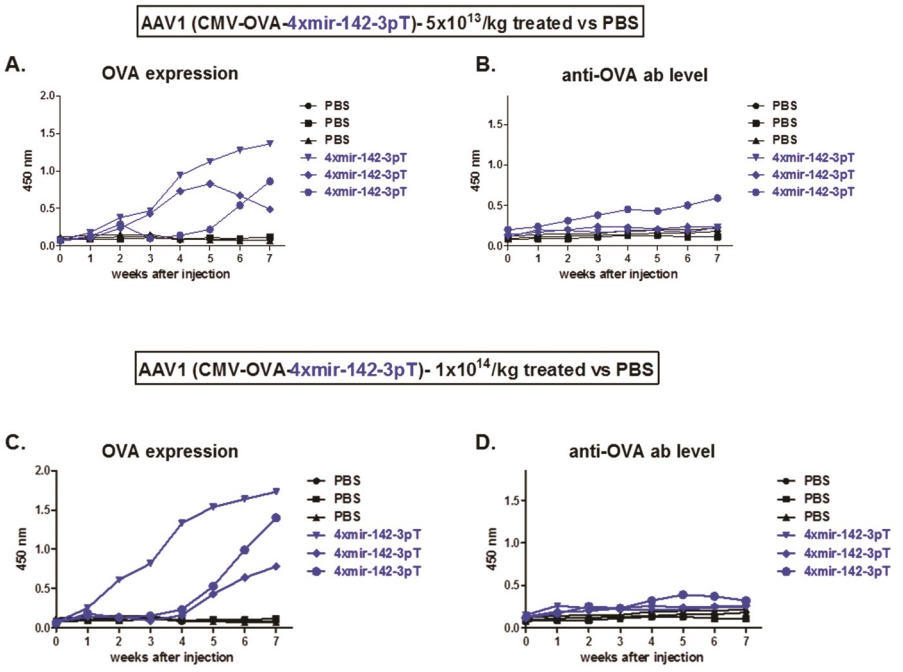

Figure 4. Mir-142-3p target sequences reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery. Intramuscular delivery of AAV1-CMV-OVA-4xmir-142-3p shows sustained expression of OVA protein (**A, C**) which correlates with low levels of anti-OVA antibody (**B, D**) after use of $5x10^{13}$ and $1x10^{14}$gc/kg dose. Black lines on the graphs represent PBS injected control mice. OD between 0 and 1.5 equals the range of 0–450 ng/ml for OVA ELISA; OD between 0 and 3 equals 0–400 ng/ml for anti-OVA ELISA.

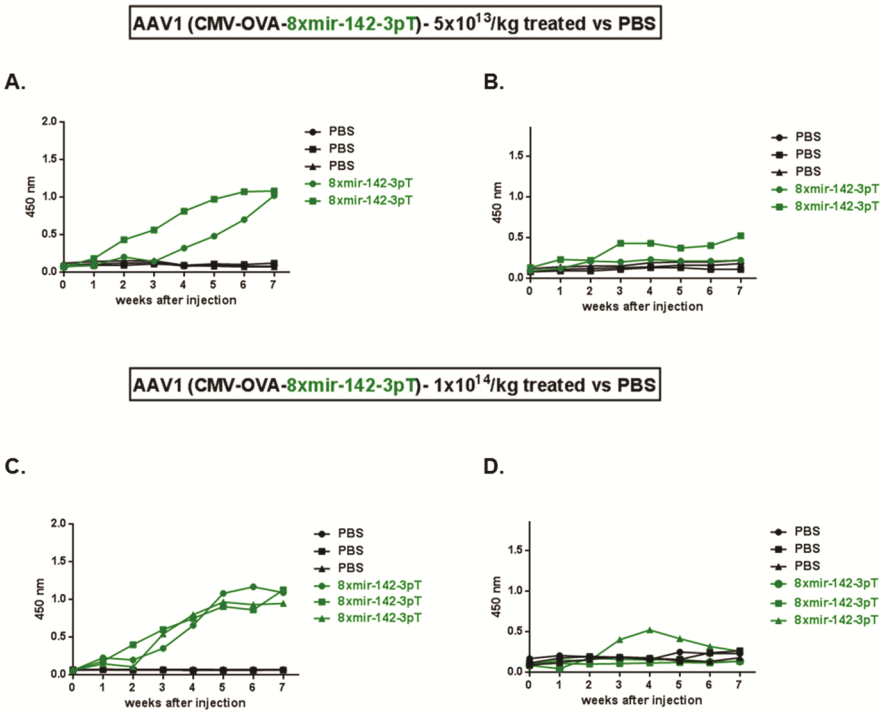

Figure 5. Mir-142-3p target sequences reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery. Intramuscular delivery of AAV1-CMV-OVA-8xmir-142-3p shows sustained expression of OVA protein (**A, C**) which correlates with low levels of anti-OVA antibody (**B, D**) after use of $5x10^{13}$ and $1x10^{14}$gc/kg dose. Black lines on the graphs represent PBS injected control mice. OD between 0 and 1.5 equals the range of 0–450 ng/ml for OVA ELISA; OD between 0 and 3 equals 0–400 ng/ml for anti-OVA ELISA.

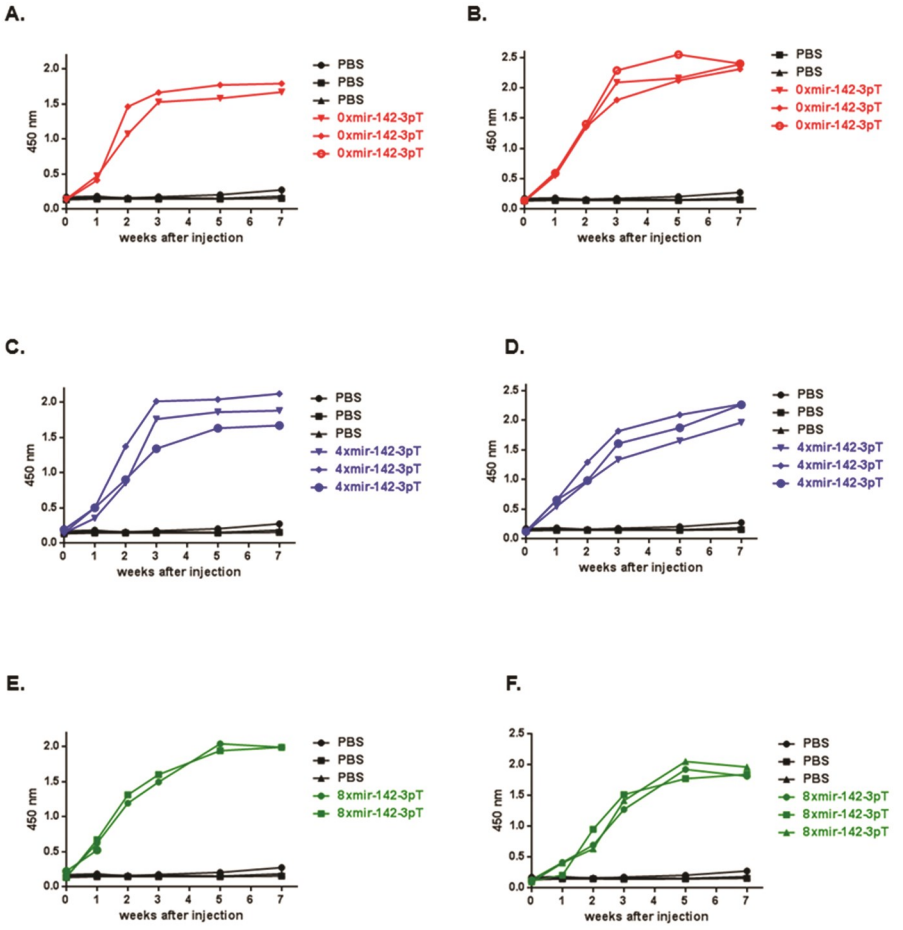

Figure 6. Total anti-AAV1 antibody levels after intramuscular delivery of different AAV1 batches at $5x10^{13}$gc/kg (A, C, E) and $1x10^{14}$gc/kg (B, D, F) dose. Anti-AAV1 antibody level remains the same in all AAV1 injected groups (**A-D**). Black lines on the graphs represent PBS injected control mice.

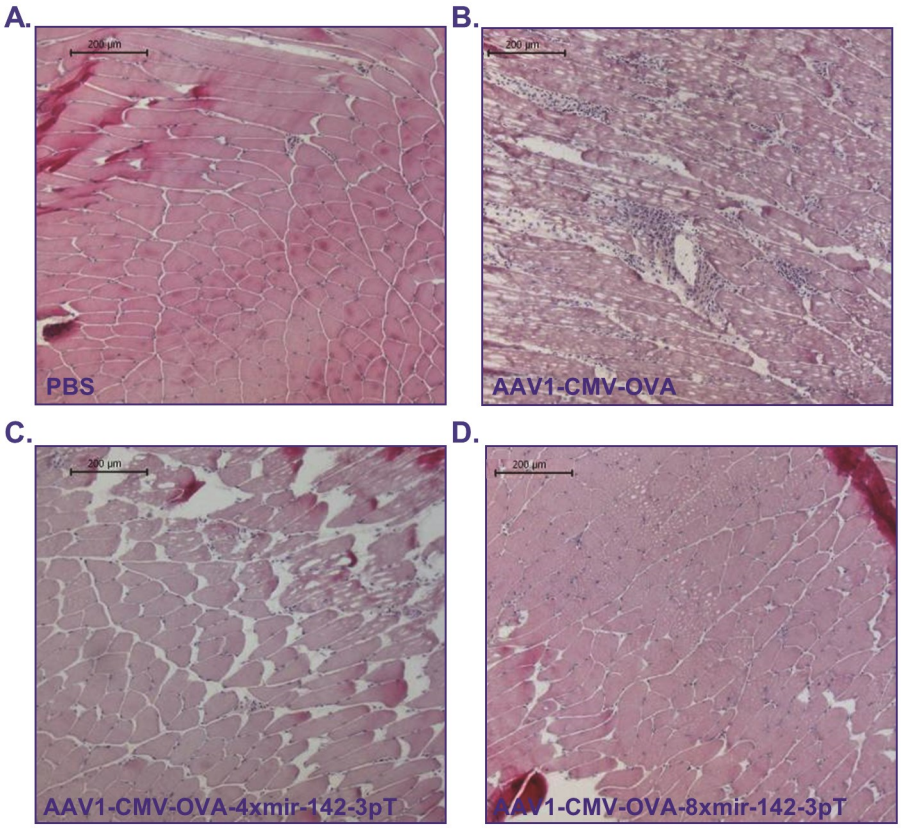

Figure 7. Haematoxylin and Eosin staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

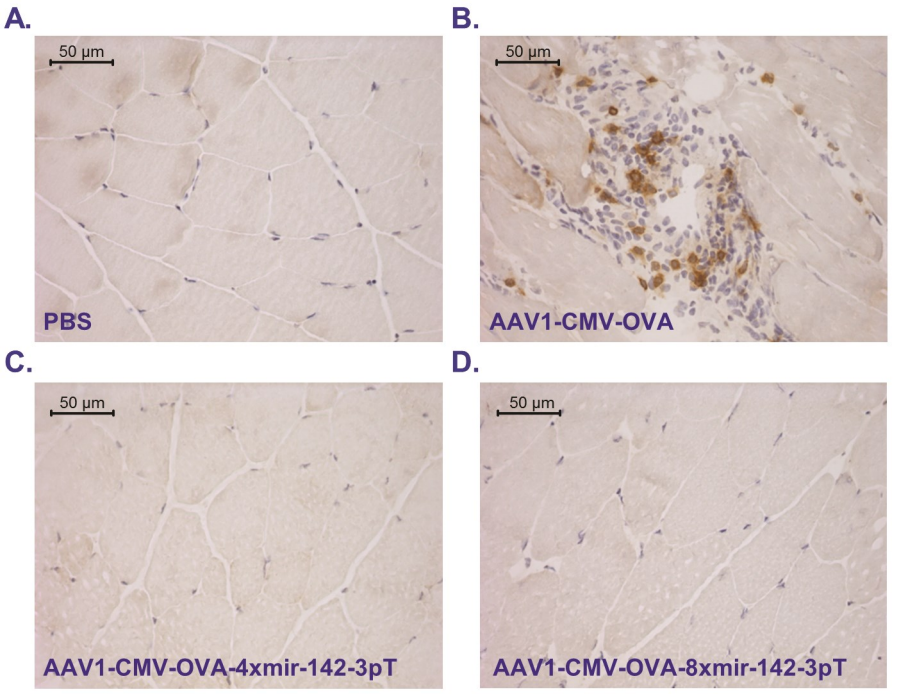

Figure 8. Anti-CD8 staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

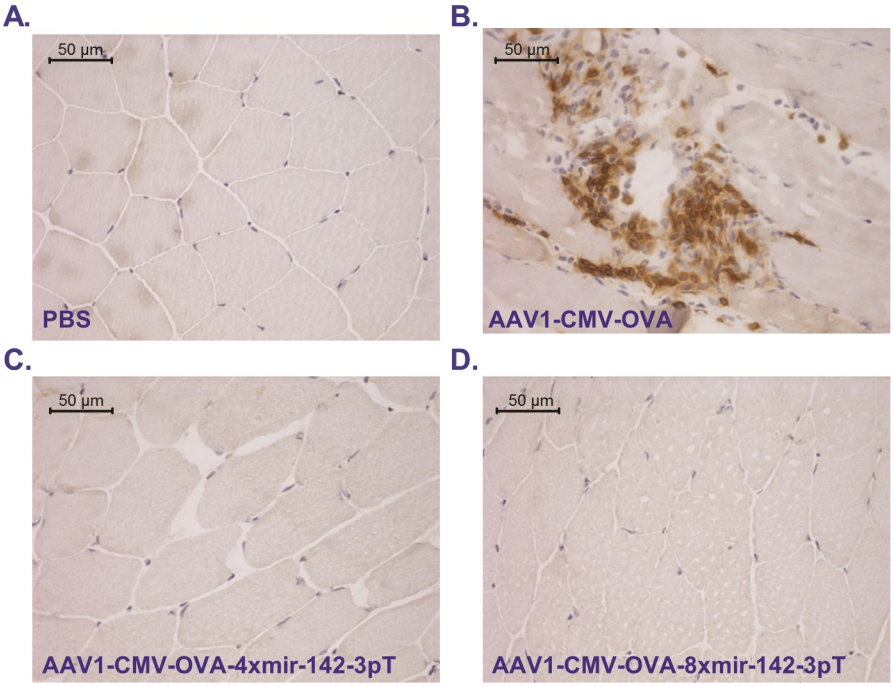

Figure 9. Anti-CD4 staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

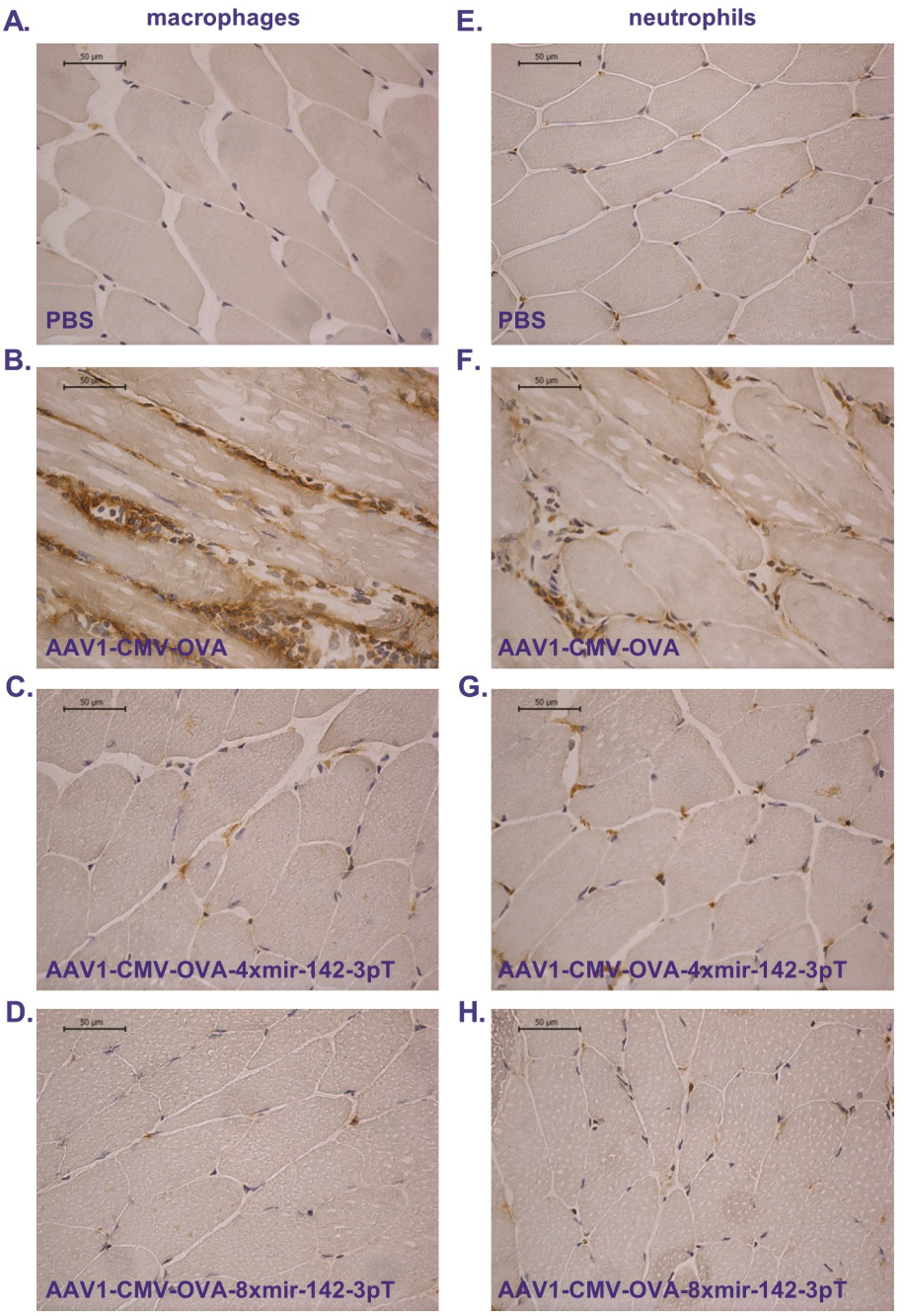

Figure 10. Anti-macrophage and anti-neutrophil stainings of mouse muscle tissue after intramuscular delivery of PBS (**A, E**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B, F**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C, G**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D, H**).

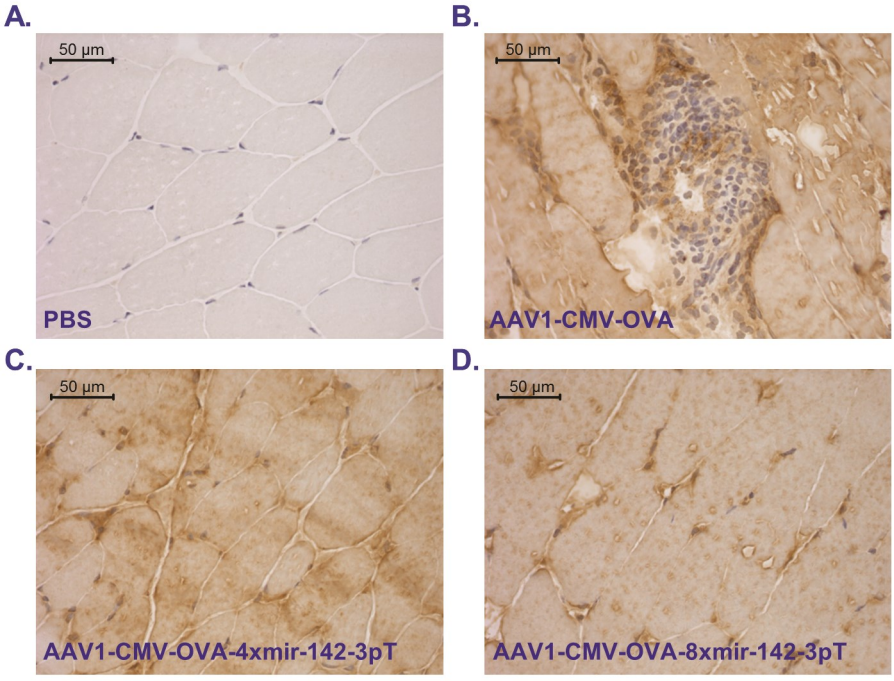

Figure 11. Anti-OVA staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

Discussion

In the present study we report an approach to prolong transgene expression after AAV vector-mediated intramuscular delivery by reducing immune responses directed against the transgene with the use of mir-142-3p target sequences.

AAV has been shown to be a promising vector for therapeutic gene delivery to a variety of tissues as a treatment for monogenic diseases [16, 40-43]. Both the safety and efficacy of AAV vectors was demonstrated in preclinical and clinical studies [1-3, 12, 13]. A very attractive target tissue for AAV vector-mediated gene therapy is muscle which is easily accessible and rich in vascular blood supply, providing an efficient transport system for the secreted proteins. Muscle has been a target tissue for gene therapy for neuromuscular diseases, metabolic disorders [1, 14, 15] and haemophilia, in case the liver cannot be considered as a target because of advanced liver diseases [17, 19]. However, immune responses against transgene products have been reported after AAV vector-mediated intramuscular delivery. They lead to destruction of transduced cells and consequently to loss of transgene expression [28]. Therefore, development of strategies to prevent immune responses against the transgene product is of great interest.

Current clinical protocols for avoiding those immune responses involve use of drug induced immune suppression. However, they are based on the use of a wide range of medications that have various side effects, can lead to many complications [16,19] and cannot guarantee the desired effect as demonstrated in Duchenne 's muscular dystrophy clinical trial where immune responses against mini dystrophin transgene after intramuscular delivery with AAV vector were observed despite use of immune suppression [27, 30, 31].

Many studies report immune responses against the transgene products after intramuscularly delivery by AAV vectors. However, when several groups describe sustained transgene expression without any signs of immune response [16, 43], others report cellular and humoral immune responses against neoantigens [26, 28]. The immunogenic potential of the transgene used seems to be an important factor in development of immune responses [44-46]. Further-

more, recent reports show that certain AAV serotypes, specifically AAV1, 2 and 5 vectors can transduce APCs and mediate potent immune response against transgene products [23, 25, 47].

Therefore, different strategies, such as the use of muscle specific promoters [28, 48, 49] are being developed in order to minimize the expression of transgene protein in APCs. An attractive alternative to modulate immune responses against proteins delivered by AAV vectors is miRNA-based regulation of transgene expression. miRNAs are small non-coding RNAs that are able to repress translation of target cellular transcripts and have a specific expression profiles in different tissues [35, 50]. Therefore, the incorporation of specific miRNA target sequences after the transgene sequence can repress transgene expression in particular cell types. Transgene expression from vectors incorporating target sequences for mir-142-3p, which is the haematopoetic-specific miRNA, was shown to be effectively suppressed in APCs [36-38, 51, 52]. Therefore, immune responses towards the transgene product could be prevented as it has been demonstrated in mice which were injected intravenously with lentiviral vectors [36-38]. Only a few studies have been reported in which the mir-142-3pT sequence was part of the expression cassette in AAV based vector [53, 54]. In each of those studies the liver was the target organ and no efficacy of mir-142-3pT sequences in preventing immune responses could be demonstrated. Qiao *, et al.* [54] were unable to draw conclusions from their study as the transgene expression was lost not as a result of immune clearance but due to promoter shut-off. While Contugno *, et al.* [53] did not observe reduction of the immune responses against the transgene nor its improved expression in the liver.

The present study was aimed to explore the potential of mir-142-3p target sequences to reduce the immune responses against a transgene product delivered intramuscularly by AAV. As a model, we used the OVA protein as it has been previously described in mice that AAV-mediated intramuscular delivery of OVA elicits systemic and local cellular and humoral immune responses against OVA [26]. In the time frame of the present study, we were able to demonstrate that the systemic OVA expression was maintained when mir-142 -3pT sequences were incorporated to the expression cassette. The sustained

expression of OVA over time was associated with a reduced increase in anti-OVA antibody levels.

The occurrence of cellular immune responses against OVA transgene following intramuscular delivery by AAV have been described previously [26]. In the present study, cellular infiltrates were also observed after intramuscular OVA delivery by AAV1 vector. However, no cellular immune responses were observed after addition of four or eight repeats of mir-142-3p target sequences to the OVA construct, which proves that incorporation of mir142-3p target sequences has the potential to reduce local, cellular immune responses as previously mentioned by Boisgerault*, et al.* [55]. The decrease of systemic OVA protein expression in the plasma of mice that were injected with AAV1-CMV-OVA correlates with the elevated level of anti-OVA antibodies and the local cellular immune responses that colocalize with OVA-expressing cells. However, no total clearance of the OVA protein from the muscle tissue was demonstrated at this time point. This result is different from that reported by Wang et al. [26], where local OVA expression in the muscle tissue was still present at day 10 but lost at day 30 after AAV delivery. However, it should be noted that the AAV serotypes used in those experiments were different as in the present study AAV1 (and not AAV2) was used. Furthermore, even though we are unable to directly compare the AAV doses used in the two studies, the dose used by Wang et al. [26] was reportedly lower. Consequently, the kinetics of total clearance of the OVA protein from the muscle tissue may be different.

In summary, the data obtained in the present study indicate that the rising humoral and cellular immune responses against OVA protein after intramuscular delivery can be efficiently reduced by use of mir-142-3p target sequences that prevent expression of OVA protein in APCs. Overall, this study identifies a promising approach for gene therapy applications because it could be applied as a "safety lock" for any intramuscular AAV vector based therapeutic gene delivery. Further investigations are currently pursued to evaluate the impact of mir-142-3pT regulated AAV gene delivery on the normal miRNA profile in the muscle tissue.

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