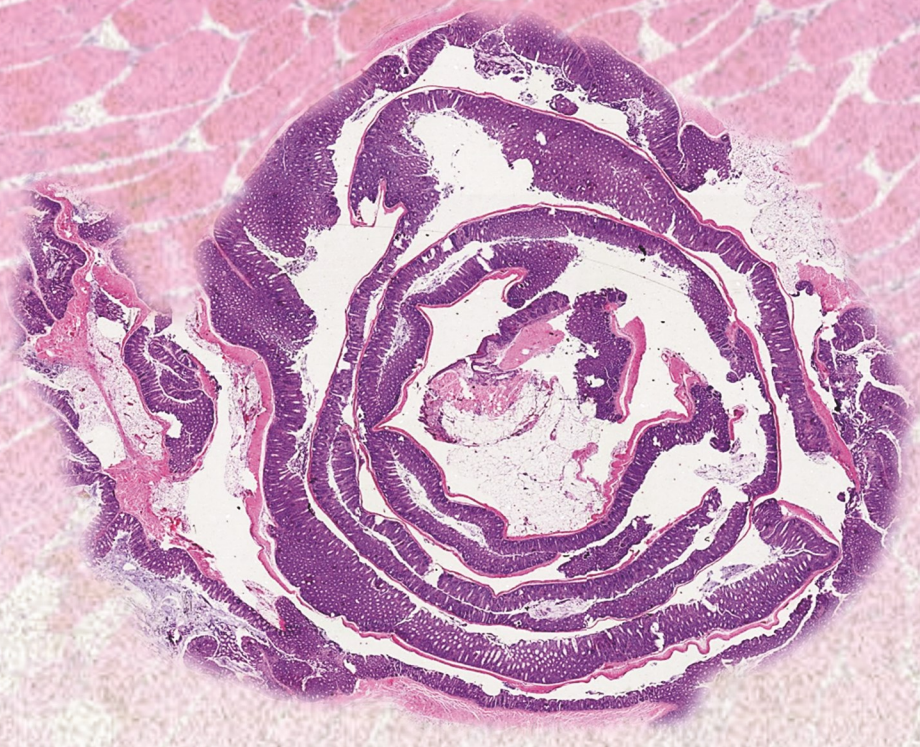
Addressing immune tolerance issues in inflammatory bowel disease and adeno-associated virus based gene transfer



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Contents

Part II

Scope & Outline

Breakdown of natural mechanisms of immune tolerance towards "self" antigens can result in autoimmune or allergic disease development. There is considerable experimental evidence that the response to antigen which might include full immunity or immune tolerance induction is dependent on the activation status of the antigen presenting dendritic cells. Current therapeutic options deplete immune cell populations, interfere with immune cell trafficking to the tissues, or inhibit inflammatory cytokine function and lymphocyte signaling. Although these treatments can be effective for certain diseases, they can also cause significant adverse effects and it is unlikely that they could induce stable, long-term immune tolerance. Cellular therapies using either tolerogenic dendritic cells or regulatory T cells may be able to achieve it. This thesis is focusing on cell-mediated induction of immune tolerance and consists of two parts. The studies described in **Part I** report the development of strategies for possible treatment of Inflammatory Bowel Diseases (IBD). Induction of immune tolerance, in IBD mouse model, with the use of regulatory T (Treg) cells generated *in vitro* by specific activation of naive T cells was achieved. Additionally Treg cells were also shown to be induced *in vivo* and restore intestinal immune tolerance with the use of adeno-associated virus (AAV) vector-based gene delivery. In relation to the use of AAV vector, **Part II** of this thesis is addressing the possibilities of tolerance induction to AAV capsid or transgene specific immune responses which can develop after AAV -based therapy.

Part I

Development of new treatment strategies for Inflammatory Bowel Diseases, which is a group of diseases considered to be autioimmune, is of great interest as currently there is no curative treatment. Cell and gene therapy approaches have been recently studied in relation to the inhibition of inflammation in the gastrointestinal tract. Treg cells have the ability to suppress immune responses and to sustain systemic immune balance. Therefore Treg cells have the potential to prevent inflammatory disorders by induction of immune tolerance. The major limitation to the use of natural Treg (nTreg) cells is their low availability

and unstable phenotype profile upon *ex vivo* expansion. Hence, use of *in vitro* generated induced Treg (iTreg) cells represents a good alternative. Currently, there are several techniques available to generate induced T regulatory (iTreg) cells, nevertheless all of them are to some extend impended with their own limitations. Therefore, establishing new, improved methods of iTreg generation is of great interest. The goal of the experiments presented in **Chapter 2** was to develop a new, straightforward method to generate *in vitro* functional and stable iTreg cells from CD4+CD25- human cells. Generated iTreg cells (TregPMA) proved to be functional *in vitro* in a mixed lymphocyte reactions (MLR's) as they suppressed proliferation of responder cells in a dose dependent manner. The protocol to generate TregPMA *in vitro* was also applied to murine cells. It has been described in **Chapter 3**. Functionality of the generated murine iTreg was demonstrated by amelioration of experimental colitis *in vivo* in a mouse model of IBD.

Also gene and cell therapy approaches have been recently studied in order to induce regulatory T (Treg) cells that would be able to inhibit inflammation in different tissues. In **Chapter 4** a gene delivery approach to promote Treg cells *in vivo* was explored. The delivery of regulatory T-cell epitope 167 (Tregitope 167) by adeno-associated virus (AAV) vector proved to induce Treg cells *in vivo* and ameliorated the experimental colitis. This study identifies AAV-based Tregitope 167 delivery as a new anti-inflammatory approach for induction of immune tolerance by Treg cells and in consequence possible treatment of autoimmune and inflammatory disorders on an example of IBD model.

Part II

The main obstacle in AAV-based gene delivery is the humoral immune response against AAV vector capsid that appears after primary AAV delivery. The neutralizing antibody (NAB) level that rises against AAV capsid, inhibit the AAV vector transduction upon re-administration of the AAV vector of the same serotype. In **Chapter 6** we demonstrate in a murine model that cross administration of AAV serotypes 5 and 1 can be an alternative for re-administration due to the lack of cross-reactivity of the NAB. Additionally, in **Chapter 7**, we explore different immune suppressive regimens for their ca-

pacity to decrease circulating anti-AAV NAB level that rises after primary AAV vector gene delivery. The aim of this study was to define the immune suppression strategy and time frame in which the decrease of the anti-AAV NAB level would allow the AAV re-administration.

Another concern in AAV vector-based gene therapy is the potential development of immune responses against the transgene product which might lead to loss of expression of the therapeutic transgene. Therefore, strategies to induce tolerance towards the transgene product are needed. In **Chapter 8**, we demonstrate the feasibility to use mir-142-3p target sequences to prevent immune responses against the transgene product after intramuscular AAV vector delivery.

Part I

Chapter 1

Introduction Part I

Gene and cell therapy based treatment strategies for inflammatory bowel diseases

Sander van der Marel, Anna Majowicz, Sander J. H. van Deventer, Daniel W. Hommes and Valerie Ferreira

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Abstract

Inflammatory bowel diseases (IBD) are a group of chronic inflammatory disorders most commonly affecting young adults. Currently available therapies can result in induction and maintenance of remission, but are not curative and have sometimes important side effects. Advances in basic research in IBD have provided new therapeutic opportunities to target the inflammatory process involved. Gene and cell therapy approaches are suitable to prevent inflammation in the GI tract and show therefore potential in the treatment of IBD. In this review, we are presenting the current progress in the field of both gene and cell therapy and future prospects in the context of IBD. Regarding gene therapy, we focus on viral vectors and their applications in preclinical models. The focus for cell therapy is on regulatory T lymphocytes and mesenchymal stromal cells, their potential for the treatment of IBD and the progress made in both preclinical models and clinical trials.

1

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory diseases most commonly affecting young adults [1-3]. The exact pathogenesis is unknown, but it is widely accepted that IBD result from an inappropriate response of a defective mucosal immune system to the intestinal flora and other luminal antigens [4-6].

IBD include two major disorders: ulcerative colitis (UC) and Crohn's disease (CD). These disorders have distinct and overlapping pathologic and clinical characteristics [7]. UC is a relapsing non-transmural inflammatory condition that is limited to the colon [8]. Patients characteristically present with bloody diarrhoea, passage of pus, mucus, or both, and abdominal cramping [8]. CD is a relapsing, transmural inflammatory disease of the gastrointestinal (GI) mucosa that can involve the entire GI tract from the mouth to the anus [8]. Patients characteristically present with discontinuous involvement of various portions of the GI tract and the development of complications including strictures, abscesses, or fistulas [8]. IBD are associated with a considerable reduction in quality of life of the patients [9-11] and currently no curative treatment options are available. Conventional therapeutics cannot prevent complications in IBD and although novel treatment strategies, including TNF-neutralizing antibodies, have greatly increased the therapeutic armamentarium, many patients still have to undergo surgery [12]. For this reason, the development of new treatments to prevent initiation of inflammation and more important allow for long term remission is required.

Gene and cell therapy approaches are more and more considered in relation to the prevention of inflammation in the GI tract. Gene therapy consists of the insertion or alteration of genes within an individual's cells to treat disease. Cell therapy describes the process of introducing new cells into a tissue in order to treat a disease. Both approaches have been applied successfully in a clinical setting for a broad range of diseases either separately or together, including

early stage clinical development for IBD [13-23]. Here we discuss current progress in the field and future treatment prospects in the context of IBD.

Gene therapy as treatment for IBD

To facilitate the uptake and the expression of the transgene in the target cell, a vector is required. Vectors can be non-viral or viral. The choice of a safe and reliable vector that can mediate long term gene transfer to both dividing and non-dividing cells is of vital importance for a gene therapy approach. Although viral vectors are created from pathogenic viruses, they are modified in such a way as to minimize their pathogenicity. This usually involves the deletion of a part of the viral genome critical for viral replication. Such a virus can efficiently infect cells and has the potential for long term stable gene expression. In the gene therapy section of this review we will focus on viral vectors that have been used successfully in gene therapy applications in recent years [14, 15], are able to target the gut [24-32] and can therefore be considered for gene delivery in the GI tract, namely retroviral, lentiviral, adenoviral and adeno-associated viral vectors (for an overview see: **Table 1** or **Figure 1**).

For an overview of non-viral delivery methods to the intestine we recommend the review from O'Neill et al [33].

Retro- and lentiviral vectors

Retroviral vectors were used for the first time in a clinical setting over 20 years ago [34-36] and are among the most commonly used vectors in gene therapy. Retroviral particles require disruption of the nuclear membrane to gain access and therefore need cell division for entering the cell [37]. Retroviruses have been demonstrated to be able to transduce intestinal epithelial cells [24-26], although at a low efficiency. At the contrary, intestinal epithelial cells can be transduced efficiently by lentiviruses [27] which are a sub-class of retroviruses. The lentiviruses have an advantage over retroviruses as vectors in gene therapy because of their ability to transduce non-dividing cells [38, 39]. Furthermore the lentivirus did not induce mucosal damage or distribute beyond the

distal colon [27] and appeared therefore as a potential vector for gene delivery in the treatment of IBD.

However, a safety issue to be considered with both retro- and lentiviral vectors is their potential to integrate at many sites in the human genome [40, 41]. Those genomic integrations can result in insertional mutagenesis causing cancer development as has been observed in clinical trials [19, 42-44]. Even though significant improvements in lentiviral vector safety have been achieved in recent years [45], the concern for random integration remains and needs to be addressed [46, 47] before these vectors can be considered as safe tools for gene therapy applications in IBD.

Adenoviral vectors

Despite the fact that adenoviruses are pathogenic viruses and can cause morbidity, especially in immune-compromised patients [48], adenoviral vectors have been frequently used in gene therapy due to their broad tissue tropism and lack of integration into the host genome [49]. Gene therapy using adenoviral vectors has shown potential in the treatment of colitis in preclinical models [28-30]. For example a single systemic injection of an adenoviral vector carrying the Interleukin-10 (IL-10) transgene was sufficient not only to prevent the onset of colitis but also to induce clinical and histological remission in mice with established disease [29]. Additionally Schmiedlin-Ren and colleagues demonstrated that intestinal epithelial cells of IBD patients can be efficiently transduced *ex vivo* by adenoviral vectors [50]. All together, these results suggest that targeting of the inflamed intestine through the luminal route can be possible using adenoviral vectors [50].

However, hematologic and hepatic toxicities were observed in animal studies after injection with high vector doses [51-53], which imply that further development in generating a new type of adenoviral vector is necessary before considering clinical applications. Recently a gutted adenovirus, devoid of all viral coding sequences, was shown to induce less toxicity [54] after delivery. However, this finding, if promising for future therapeutic applications, needs further exploration.

AAV vectors

The non-pathogenic, replication-deficient adeno-associated virus (AAV) holds promise for gene therapy. The AAV vector has a good safety profile as it remains predominantly episomal [55]. In general, 99% of recombinant AAV are maintained as episomal copies [56], indicating a very low risk of insertional mutagenesis compared with retroviral vectors. Furthermore, AAV vectors are able to transduce both dividing and quiescent cells [57, 58] and were demonstrated to be effective as gene therapy vectors in several promising preclinical models for autoimmune and inflammatory disorders [59-66]. The therapeutic potential of the AAV as a vector in gene therapy has also been demonstrated in a clinical setting in recent studies [67-77].

AAV vectors were shown to be able to target the GI tract [31, 32] and long term transgene expression post AAV treatment was reported which in relation with the high turn-over of intestinal cells, suggests that transduction of the slow-dividing intestinal stem cells was achieved [31, 32]. However, no data are presently available about the treatment of experimental colitis with AAV vectors.

Cell therapy as treatment for IBD

Cell-based therapies aim to introduce new cells into a tissue in order to treat a disease and can permit the replacement of function [78], or restore the homeostasis of the immune system [79]. In the last 50 years hematopoietic stem cell transplantation has been developed as a curative option for inherited disorders and hematologic or lymphoid cancers [13, 80], leading the way toward innovative therapies for other illnesses. Recent results obtained from animal models and early human clinical trials in graft versus host disease but also CD showed that either regulatory T lymphocytes or mesenchymal stromal cells may be of clinical relevance for the treatment of IBD (for an overview see: **Table 2** or **Figure 1**).

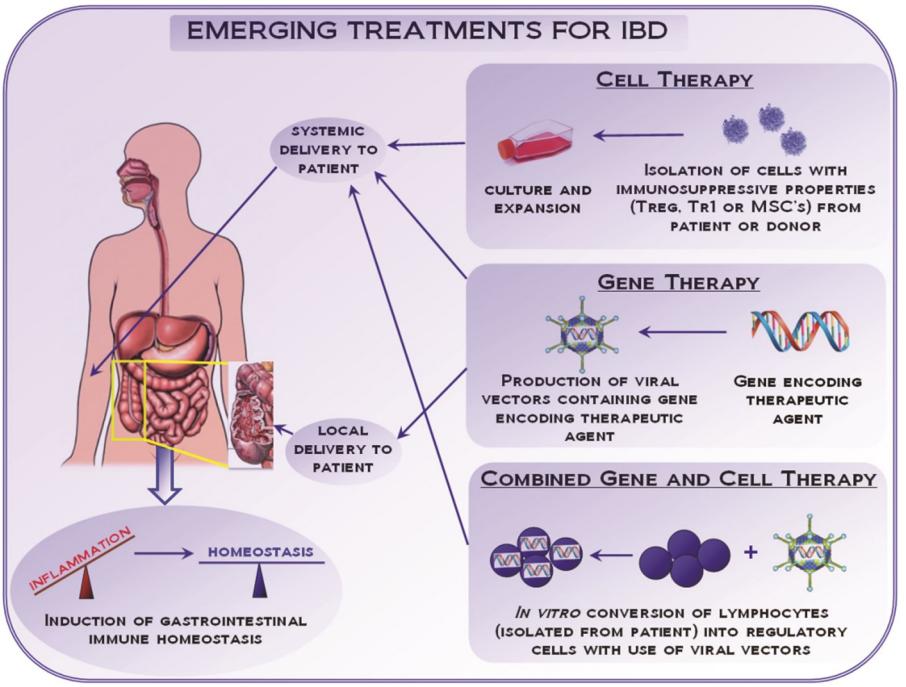

Figure 1. Emerging treatments for IBD. Overview of the gene and cell therapy based treatment strategies for IBD as discussed in this chapter. Treg: $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cell; Tr1: Type 1 regulatory T cell; MSC: Mesenchymal stromal cells

Table 1 Overview of the gene therapy based treatment strategies for IBD as discussed in this chapter

Viral vector	Gut targeting	Reference to gut targeting	Status of development	Reference for status of development
Retro- and Lentiviral	Yes	[24-27]	Not performed	N/A
Adenoviral	Yes	[28-30]	Preclinical	[28-30]
AAV	Yes	[31, 32]	Not performed	N/A

The status of development refers to research which has already been performed. AAV: Adeno-associated virus; N/A: Not Applicable

Table 2 Overview of the cell therapy based treatment strategies for IBD as discussed in this chapter

Cell type	Generated from	Reference for generating cell type	Status of development	Reference for status of development
Treg	Peripheral blood	[21, 101, 132-134]	Preclinical	[93, 96]
Tr1	Peripheral blood	[93, 135]	Phase I Clinical Trial	(Unpublished data, UEGW 2010-ABS-577)
MSCs	Adipose tissue/ bone marrow	[17, 22, 23]	Phase I Clinical Trial	[17, 22, 23]

The status of development refers to research which has already been performed. MSCs: Mesenchymal stromal cells; Treg: $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cell; Tr1: Type 1 regulatory T cell; UEGW: United Gastroenterology Week.

Table 3 Overview of the combined gene and cell therapy based treatment strategies for IBD as discussed in this chapter

Applied Strategy	Viral vector used	Reference for viral vector used	Status of development	Reference for status of development
Ex vivo generated Treg/Tr1	Retrovirus	[113, 115]	Preclinical	[113, 115]
T cell receptor transgenic Treg	Retrovirus	[116]	Precilnical	[116]

The status of development refers to research which has already been performed. Treg: $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cell; Tr1: Type 1 regulatory T cell.

Regulatory T lymphocytes

The immune system contains a population of T cells, called regulatory T lymphocytes that are specialized in immune suppression [81, 82]. Low level autoimmunity may occur in the intestine as a result of the presence of the microbial flora or auto-reactive T cells. Regulatory T lymphocytes are generated in the mesenteric lymph nodes and subsequently migrate and expand in the gut [83], thereby preventing progress to chronic autoimmune disease [84, 85]. These cells are able to suppress an immune response both by cell contact (*e.g.* killing or functional modulation of antigen presenting cells or effector T cells) and soluble factor dependent mechanisms (*e.g.* secretion of immunosuppressive cytokines or deprivation of cytokines necessary for the expansion/survival of responder T cells) [86, 87]. Antigen specific regulatory T lymphocytes have been described as having more therapeutic efficacy than polyclonal regulatory T cells [88-90]. In IBD the antigenic targets are not totally defined [6] and cell therapy would have to be restricted to polyclonal cells. However, regulatory T lymphocytes do not need to be antigen specific in order to suppress immune responses as a result of *bystander suppression* and *infectious tolerance* [91, 92]. These are general mechanism through which regulatory T lymphocytes are able to create a regulatory milieu *in vivo* [91, 92] and could introduce tolerance in IBD.

Regulatory T lymphocytes were shown to be effective in both the cure and the prevention of experimental colitis in multiple animal models [93-96]. It was shown for example that transfer of regulatory T lymphocytes into mice with colitis led to resolution of the lamina propria infiltrate in the intestine and reappearance of normal intestinal architecture [96]. Therefore regulatory T lymphocytes could be used as a therapeutic tool in IBD where their homeostasis is disturbed [97, 98].

Among the different T cells with suppressive activity the $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cell (Treg) [82] and the type 1 regulatory T cell (Tr1) [81] subsets are the most well-defined so far. The Tr1 is typically characterized based on the cytokine production profile ($IL\text{-}10^{high}$) [81] and Treg by the expression of the transcription factor Forkhead box p3 (FOXP3

in humans/Foxp3 in mice), which appears to function as the master regulator in their development and function [99, 100].

Treg and Tr1 have the potential to prevent or cure colitis [93-96] and a favourable safety profile in phase I clinical trials was demonstrated [21, 101]. Tr1 were shown to have a preliminary efficacy signal in patients in a phase I clinical trial for refractory CD (*unpublished data*, UEGW 2010-ABS-577). Currently the efficacy of Treg and Tr1 based cell therapy awaits further confirmation from phase II/III clinical trials but overall these results emphasize that both Treg and Tr1 are promising tools for therapeutic applications in IBD.

Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are non-haematopoietic stromal cells exhibiting multi-lineage differentiation capacity and the ability to mediate immunosuppressive and anti-inflammatory effects [102-105]. The exact mechanism by which MSCs suppress the immune system is not fully understood. It is known however that MSCs have immunosuppressive features in common with regulatory T lymphocytes, as for example preventing the maturation of antigen presenting cells (APCs) [102] or physically hinder T cells from contacting APCs [103]. Additionally it was shown that FOXP3 expression confers a greater immunosuppressive potential to MSCs [106].

MSCs can be isolated from various tissues [107-109] and were shown to ameliorate experimental colitis [110, 111]. In humans, MSCs obtained from adipose tissue induced healing in perianal fistulas in patients with CD [17]. Furthermore, in a phase I clinical trial, administration of autologous bone marrow -derived MSCs was shown to be safe and feasible in the treatment of refractory CD [22]. Additionally it was demonstrated that *ex vivo* expanded autologous bone marrow-derived MSCs are a safe and feasible approach for intrafistular injections in patients with CD [23]. These results [17, 22, 23] show potential and await further verification in phase II/III clinical trials which are currently being conducted.

Can gene and cell therapy overlap in the treatment of IBD?

The phenotype and function of lymphocytes can be modified using viral vectors, to create tools for a cell therapy approach in the treatment of autoimmune, and inflammatory disorders [112] and by consequent IBD [113-116]. It was shown that the *ex vivo* targeting of spleen derived $CD4^+$ T cells by a retroviral vector expressing IL-10 was able to generate Tr1 that prevented colitis in an experimental model of IBD [113].

Using the same approach, fully functional Treg were generated by transduction of T cells with a Foxp3 transgene. These cells were able to suppress autoimmunity and graft rejection *in vivo* [89, 115, 117, 118]. Furthermore, Hori and colleagues showed that the *in vitro* generated Treg prevented colitis in a mice model of IBD [115].

Additionally it was demonstrated that Treg can be efficiently transduced to express functional antigen-specific receptors [116]. Adoptive transfer of small numbers of these transduced Treg was associated with antigen-specific, dose-dependent amelioration of experimental colitis in mice (**Table 3**).

General considerations relevant for IBD

The route of therapeutic delivery is important when considering gene or cell therapy in relation with IBD. The mucus lining in the intestine is a barrier for gene transfer via the luminal route [119] and the clearance of viral particles by the liver represents a problem for the systemic delivery [120]. Nonetheless, as described above it has been shown that transduction via these routes is possible and that long term transgene expression can be achieved. Possible viral vectors, as for example the AAV based viral vectors seem to have the potential to transduce the GI tract, but the optimization of gene targeting to the gut needs to be further explored. This could be achieved by testing different AAV serotypes [121] or modifying the AAV capsid [122]. A promising method is the so called *DNA shuffling* method. DNA shuffling is a method whereby genes are rearranged to form hybrid genes with new properties [123]. This can

be done using polymerase chain reactions, as described by J. Cohen [123]. If this approach is used for genes encoding AAV capsid proteins it can allow for the development of cell type specific vectors [124] and thereby shows promise for creating a gut targeting AAV. Furthermore chemical redirection of the AAV capsid shows potential in engineering vectors with novel tissue tropisms [125]. Chemical engineering refers to a process whereby the amino acids on the surface of the AAV capsid are changed [125]. This method has proven to be successful in redirecting the AAV from liver to skeletal and cardiac muscle following systemic administration in mice [125] and could therefore have potential in directing the AAV to the GI tract.

Due to the presence of stem cells in the intestinal crypts [126] the gut is suggested to be an interesting target for therapeutic gene transfer. Every crypt in the intestine contains four to six independent stem cells [126]. Stem cells are believed to divide very rarely [126]. Therefore, these cells could have the potential to permit long term, stable transgene expression after transduction. It has been shown that intestinal stem cells can be transduced *in vitro* using a retroviral vector [127]. Long term transgene expression observed in the gut after AAV vector delivery in mice suggests that transduction of intestinal stem cells is possible *in vivo* [31, 32].

Future prospects

The knowledge about the pathophysiology of IBD is growing and it has become clear that significant genetic as well as phenotypic heterogenecity exists within both CD and UC [128]. These findings offer opportunities for more specifically targeted interventions. Gene or cell therapy based treatment strategies can be adapted and exclusively targeted at certain subgroups within the IBD patient population with characterized genetic defects linked to the impairment of their gut physiology.

Strategies to optimize gene therapy approaches include the use of a tissue specific promoter enabling site specific expression of a transgene. Recently, gut specific promoters have been described [129-131]. The A33-antigen promoter

for example strictly depends on the presence of the intestine-specific transcription factor Cdx1 which is essential for the unique intestinal expression pattern of the A33-antigen gene [129, 131]. Therefore this promoter is a promising candidate to induce intestine specific expression of a transgene [131].

Concluding remarks

IBD are a group of chronic inflammatory disorders most commonly affecting young adults and currently there is no curative treatment available. A gene therapy approach for the local expression of therapeutic agents in the gut or a cell therapy approach using regulatory T cells or mesenchymal stromal cells may offer an alternative treatment for gastrointestinal inflammation. Both gene and cell therapy approaches have shown promising results in preclinical models of IBD. Cell therapy approaches have been translated to a clinic setting and currently phase II/III clinical trials for the treatment of refractory CD are in progress. Concerning gene therapy, further development of viral vectors delivery to the gut as well as long term efficacy are still needed, but pre-clinical data are promising.

Overall, both gene and cell therapy have the potential to become important players in the next generation of therapeutic agents that will be aimed at unmet medical needs as those that exist in IBD.

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Chapter 2

Generation of stable, functional regulatory T lymphocytes *in vitro* within 4 days by a two step activation protocol

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Abstract

The significance of regulatory T cells in the control of tolerance shows promise for new therapeutic applications. Cell therapy with regulatory T cells has the potential to become a new patient tailored approach in the treatment of allergic, autoimmune or inflammatory disorders. Our objective was to define a new, straightforward protocol to generate sufficient numbers of inducible regulatory T cells *in vitro* applicable for future clinical trials.

With the use of a novel, two-step T cell activation protocol we generate *in vitro*, within 4 days regulatory T cells. The phenotype and functionality of those cells assessed by flow cytometry and MLR's were sustainable over a period of at least 30 days in culture. These cells could be applied either alone or together with naturally occurring regulatory T cells in the treatment of allergic, autoimmune or inflammatory disorders.

Introduction

Regulatory T cells (Treg) are key players in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation [1]. Therefore, strategies that aim for therapeutic tolerance induction may take advantage of the functions of Treg [2].

The transcription factor FOXP3 is generally considered as the master regulator in the development and function of Treg [3-5]. However FOXP3 expression can also be induced in human T effector cells which do not have suppressive activity [6]. Thus, FOXP3 expression is necessary, but not sufficient to identify functional Treg and other markers have to be taken in consideration as well [6].

Treg are able to suppress an immune response both by cell contact (*e.g.* killing or functional modulation of antigen presenting cells or effector T cells) and soluble factor dependent mechanisms (*e.g.* secretion of immunosuppressive cytokines or deprivation of cytokines necessary for the expansion/survival of responder T cells) [7]. They are considered to be an attractive tool for the treatment of a broad range of diseases [8-12]. Recently, different groups have been able to expand functional, naturally occurring regulatory T cells (nTreg) *ex vivo* [13-15]. In Phase I clinical trials a favorable safety profile was shown for nTreg in the treatment of acute graft versus host disease [16, 17]. However it was reported by the same group [18] that cellular therapy using Treg induced *in vitro* has several potential advantages over nTreg, including ease of isolation, increased number of the starting population, greater proliferation potential and reduced production costs [14]. Additionally multiple defects in nTreg in patients with autoimmune diseases have been described [19] which could prevent the use of these cells for the treatment of autoimmunity. This problem could be overcome by using Treg induced *in vitro*. These induced Treg (iTreg) like nTreg, have been shown to suppress auto-, and alloreactivity *in vivo* [18, 20]. Furthermore, it has been demonstrated that the physiologic generation of iTreg from naive T cells *in vivo*, is essential for establishing oral tolerance [21] and that iTreg generated *in vitro* can act in synergy with nTreg from the same donor to restore and maintain tolerance *in vivo* [22]. It is there-

fore postulated that iTreg could have an important, non-redundant role in establishing tolerance [21-23]. Consequently iTreg generated from $CD4^+CD25^-$ T cells *in vitro* have potential, either alone or together with nTreg, for the treatment of allergy, autoimmune or inflammatory disorders. This approach has been explored before, whereby stimulation with anti-CD3 was an important element [24, 25], as anti-CD3 up-regulates FOXP3 expression *in vitro* [26]. However, in contrast to nTreg FOXP3 expression seems to decline over time in iTreg [26] resulting in a loss of regulatory function. Apart from activation with anti-CD3 [24, 25], several other groups have used different strategies to generate iTreg *in vitro*, including use of TGFβ, all-trans-retinoic acid and the recently discovered regulatory cytokine IL-35 [27, 28]. All these technologies require more or less extensive cell handling and processing and it remains uncertain whether these methods will enable generation of sufficient iTreg under *Good Manufacturing Practices* (GMP).

We have established a new, simple and robust method to generate stable and functional iTreg from human $CD4^+CD25^-$ T cells *in vitro*. This method is composed of two steps of activation with an initial PMA/ionomycin activation step followed by anti-CD3 stimulation in presence of a low dose of Interleukin-2 (IL-2). This approach has the advantage of promoting the conversion of $CD4^+CD25^-$ T cells to a high number of functional iTreg and could be suitable for application under GMP. Furthermore, by making use of substances that are well known, simple to obtain and relatively low priced this protocol could be easy to employ in a routine laboratory setting.

Material and methods

Samples

Buffy Coats from healthy adults were obtained from the blood bank (Sanquin, the Netherlands). The study was reviewed and approved by the ethical board of Sanquin Blood Supply Foundation. Peripheral Blood Mononuclear Cells (PBMC's) were extracted using cell preparation tubes with sodium citrate (BD Vacutainer® CPT™, BD Biosciences).

Isolation and stimulation of $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells were obtained from PBMC's by means of negative selection using the naive $CD4^+$ T cells isolation kit (Miltenyi Biotec®) and CD25 microbeads (Miltenyi Biotec®) for additional CD25 depletion to ensure that no $CD4^+CD25^+$ cells remained, as described before [29]. Both isolation steps were performed according to the instructions of the manufacturer with the use of LD columns (Miltenyi Biotec®). Routine evaluation of the obtained $CD4^+$ $CD25^-$ population analyzed by flow cytometry showed a purity of >95%.

$CD4^+CD25^-$ T cells were re-suspended in X-vivo15 human medium (Lonza) containing 5% human serum (Lonza) at 2.5 x 10^5 cells/ml and subsequently activated with PMA (10 ng/ml, obtained from Sigma-Aldrich) and ionomycin (250 ng/ml, obtained from Sigma-Aldrich). After 48 h cells were washed twice with PBS, re-suspended in medium and activated with 10 μg/ml anti-human CD3 (Clone OKT3, eBioscience) in the presence or absence of IL-2 (50 U/ml, obtained from eBioscience). Cells were cultured in 96-well, flat bottom plates. IL-2 (50 U/ml) was added every 72 h.

Phenotypical characterization

Cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences) and CellQuest Pro software (BD Biosciences). For Treg staining the human regulatory T cell staining kit was used (PE-conjugated FOXP3, clone PCH101, FITC-conjugated CD4, clone RPA-T4 and APC-conjugated CD25, clone BC96) obtained from eBioscience. To assess the specificity of the PCH101 clone we performed FOXP3 staining with clone PCH101 (eBioscience), 259D/C7 (BD biosciences) and 3G3 (Miltenyi Biotec®) in parallel, according to the instructions of the manufacturer (experiments in triplicate, n=2, *data not shown*). Overall little (<5%), non significant differences were observed in our experimental setup and therefore we continued with PCH101 as this clone has been validated for FOXP3 detection [30].

Additional monoclonal anti-human antibodies used in this study were as follows: anti-CD127 (PerCP Cy5.5-conjugated, clone eBioRDR5), anti-CD4 (PE-conjugated, clone RPA-T4), anti-CD152 (CTLA-4, PE-conjugated, clone 14D3), anti-AITR/GITR (PE-conjugated, clone eBioAITR), anti-OX40

(CD134, FITC-conjugated, clone ACT35), anti-ICOS (APC-conjugated, clone ISA-3) and anti-IL-10 (APC-conjugated, clone JES3-9D7) obtained from eBioscience. The viability dye 7-AAD (BD Biosciences) and the scatter dot plot were used to exclude dead cells from analysis. For intracellular staining (FOXP3, CTLA-4 and IL-10), cells were fixed and permeabilized using the FOXP3 staining buffer set from eBioscience. The IL-10 staining was performed intracellularly, as it has been described before [29]. High expression of a marker is defined as fluorescence intensity greater than 100, as it has been described before [31]. Regulatory markers were followed over time up until 30 days in culture, with or without supplementation of a low dose of IL-2 (50 U/ml, every 72 h). As control, $CD4^+CD25^-$ T cells activated with PMA (10 ng/ml)/ionomycin (250 ng/ml) followed by IL-2 (50 U/ml) or anti-CD3 (10 μg/ml) followed by IL-2 (50 U/ml) were analyzed by flow cytometry after 9 days or after 30 days in culture.

Viability and proliferation

Viability of the cells was determined by staining with 7-AAD (BD Biosciences) followed by flow cytometry analysis. Cell number was determined by use of the NucleoCounter to assess proliferation rate in culture (NucleoCounter, Chemometec).

Functional characterization

Regulatory function was analyzed using Mixed Leukocyte Reactions (MLR's) with CFSE stained responder cells [32], described previously as a valuable tool to study suppressor T cells [33]. Shortly CFSE labelling: The CellTrace CFSE cell proliferation Kit (Molecular Probes, Invitrogen) was used to stain PBMC's from the same donor as the experimental cells according to the manufacturer's instructions. These cells are referred to as responder cells. A final working concentration of 5 μM CFSE was used per 5 x 10^6 PBMC's, to obtain a $CFSE^{high}$ stained responder population, as determined by flow cytometry using different titrations of CFSE. CFSE expression was routinely evaluated after staining by flow cytometry.

MLR

CFSE labelled PBMC's (responder cells) were cultured in 96-well round bottom plates at 2.5 × 10^4 cells/well with 5 × 10^4 cells/well irradiated allogenic PBMC's (as feeder cells) in the presence of varying amounts of experimental T cells. Autologous PBMC's as feeder cells for the autologous control, $CD4^+CD25^-$ T cells as negative control and rapamycin (100 ng/ml) as positive control were plated out to assess their effect on proliferation. Cell cultures were stimulated with 2 µg/ml anti-human CD3. All culture settings were performed in triplicate.

Experimental T cells, generated with the two-step activation protocol, were analyzed for their suppressive function in the described MLR's after 9 days in culture or after 30 days in culture. The MLR was set, after 5 days cells were harvested, washed with PBS and the CFSE signal was analyzed by flow cytometry. Routine evaluation during titration experiments showed that proliferating cells loose their $CFSE^{high}$ staining and become $CFSE^{low}$. Therefore suppression of $CFSE^{low}$ represents the suppression of proliferating responder cells compared to control (see: **Figure 4.B**), as described before [33]. Cell cultures were set with or without a 0.4 µm transwell permeable support system (Corning), in order to determine cell contact or cytokine dependence in the Treg functionality assay.

Results

Induction of a regulatory phenotype in $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells were activated with PMA/ionomycin and subsequently with anti-CD3, in the presence of IL-2 (50 U/ml). After 9 days in culture, the T cells from four different donors were analyzed by flow cytometry and 55% to 83% of the cells were shown to express the classical regulatory phenotype ($CD4^+CD25^{high}FOXP3^+$, **Figure 1.A**). Inter-donor variation was observed, but for every analyzed donor more than 50% of the $CD4^+CD25^-$ T cells differentiated into ($CD4^+CD25^{high}FOXP3^+$) Treg. We will refer to this inducible regulatory T cell population as 'TregPMA' cells.

In order to demonstrate the specificity and effectiveness of our two-step activation procedure, $CD4^+CD25^-$ T cells from three of the four donors were

activated with PMA/ionomycin followed by IL-2 or activated with anti-CD3 followed by IL-2, as control groups. The cells were analyzed by flow cytometry after 9 days in culture. Between 0.1% and 2% of the cells in the anti-CD3/IL-2 group and between 13% and 31% in the PMA/ionomycin/IL-2 group were shown to express the classical regulatory phenotype ($CD4^+CD25^{high}FOXP3^+$) after 9 days in culture (**Figure 1.B**).

Expression of regulatory markers by the TregPMA

High CD25 expression [34] and down-regulation of CD127 [35, 36] are established characteristics of the Treg phenotype in humans. Therefore expression of those markers on the surface of the TregPMA was monitored by flow cytometry. Furthermore, we analyzed the expression of CTLA-4, which is linked to Treg function [37, 38], glucocorticoid-induced TNF receptor (GITR), linked to Treg survival [1], ICOS, linked to Treg function [39, 40] and OX40, also known as CD134, which is linked to Treg homing and survival [41, 1]. Additionally, we analyzed the expression of IL-10, a regulatory cytokine that down-regulates the expression of T helper cell cytokines, MHC class II antigens, co-stimulatory molecules on macrophages and has been associated with Treg function [42].

The TregPMA demonstrate down-regulation of CD127 and up-regulation of CD25, FOXP3, IL-10, CTLA-4, GITR, ICOS and OX40 when compared to the PMA/ionomycin/IL-2 control group and to non-activated T cells (**Figure 2**).

To monitor the acquisition of the regulatory phenotype over time by the TregPMA in culture, the profile of expression of the established regulatory markers CD25, CD127, FOXP3 and IL-10 was analyzed by flow cytometry at different time points (**Figure 3**). A clear up-regulation of the Treg lineage transcription factor FOXP3 was observed over time, as well as of the markers CD25 and IL-10. CD127 was down-regulated, further confirming the regulatory phenotype [35, 36]. For these markers the PMA/ionomycin and anti-CD3 steps seemed to act synergistically to achieve the expression of a regulatory profile.

It has to be noted that the expression of CD4 at the cell surface was down-regulated after the PMA/ionomycin activation step, but was gradually restored after anti-CD3 activation, as it was described before [43, 44, 45].

The expression profiles, determined by flow cytometry of $CD4^+$, $CD25^{high}$, $CD127^{-/low}$ and $FOXP3^+$ demonstrate that within 4 days in culture the TregPMA obtain their regulatory phenotype (**Figure 3**).

Using the described TregPMA protocol we are able to generate a mean of 2.2 x 10^6 TregPMA cells/ml (SD 1.0 x 10^6; N=3) after 9 days in culture starting from 2.5 x 10^5 $CD4^+CD25^-$ T cells/ml. The mean cell viability was >95% as confirmed by both NucleoCounter and 7-AAD staining (*data not shown*).

TregPMA suppress the proliferation of responder cells *in vitro*

Since the main functional characteristic of Treg is the capacity to suppress the proliferation of responder cells *in vitro*, the capacity of the TregPMA to suppress the proliferation of responder cells was analyzed by MLR's, after 9 days in culture. A fixed number of CFSE labelled responder cells was co-cultured with a varying amount of TregPMA (responder cells to TregPMA ratio 1:0, 1:0.33, 1:0.67 and 1:1.67). The cells of three different donors were analyzed. Inter-donor variations were observed, but TregPMA always suppressed the proliferation of responder cells in co-culture with an efficacy between 25% and 43% (**Figure 4.A**). The observed effect was proportional to the number of TregPMA added in the assay, demonstrating a dose dependent inhibition. The separation of TregPMA from the responder cells, in a transwell assay, abrogates their suppressive function demonstrating contact-dependent suppression of the proliferation of responder cells (*data not shown*).

It has to be noted that the inhibition of proliferation was not due to depletion of nutrients or accumulation of waste products as in the negative control the addition of an equal amount of $CD4^+CD25^-$ T cells, instead of the TregPMA caused no inhibition of the CFSE division (**Figure 4.A and B**).

All together the obtained data show that the TregPMA suppress the proliferation of responder cells *in vitro* in a dose and contact dependent manner.

Phenotype and function of TregPMA are stable in long term culture

Since the sustainability of TregPMA phenotype and functionality over time is important for possible clinical applications [46], the TregPMA were kept in culture for 30 days and analyzed by flow cytometry and MLR's.

Expression of regulatory markers after long term culture

To monitor the sustainability of the regulatory phenotype over time of the TregPMA, the profile of expression of the regulatory markers, CD127, CD25, FOXP3, IL-10, CTLA-4, GITR, ICOS and OX40 was analyzed by flow cytometry after 30 days in culture. After following our two-step protocol, the TregPMA of two different donors demonstrated a sustainable down-regulation of CD127 as well as up-regulation of CD25, FOXP3, IL-10, CTLA -4, GITR, ICOS and OX40 when compared to the PMA/ionomycin/IL-2 control group and to non-activated T cells (*data not shown*).

Stable regulatory function of the TregPMA after 30 days in culture

The capacity of the TregPMA, after 30 days in culture, to suppress the proliferation of responder cells was analyzed by MLR's. The TregPMA from two different donors cultured for 30 days were shown to inhibit the proliferation of responder cells (mean inhibition up to 40%). Therefore it can be concluded that the regulatory function of the TregPMA is maintained over time in culture.

IL-2 contributes to FOXP3 transcription through activation of Stat5 and is thereby of importance both for Treg homeostasis and for maintaining their suppressive program [47]. Therefore we cultured the generated TregPMA in the presence or absence of IL-2 and examined their regulatory properties. Cells of two different donors were analyzed by flow cytometry and it was determined that 65% and 70% of the TregPMA express FOXP3 when cultured in the presence of a low dose of IL-2, while less than 5% of cells express FOXP3 without IL-2 supplementation. Furthermore, the TregPMA, cultured in the absence of exogenous IL-2, were not able to suppress the proliferation of responder cells in MLR's (*data not shown*).

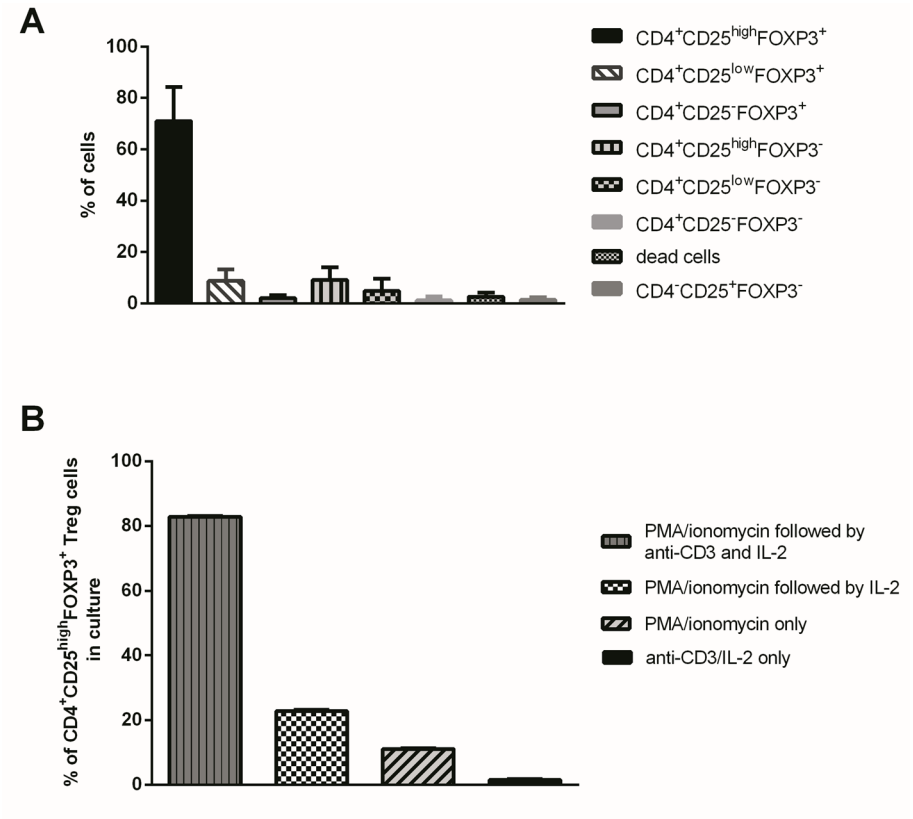

Figure 1. CD4$^+$CD25$^-$ T cells acquire a regulatory phenotype

A. CD4$^+$CD25$^-$ T cells expressing the regulatory phenotype CD4$^+$CD25^{high}Foxp3$^+$, activated with our two-step protocol, after 9 days in culture. Experiments were performed in triplicate with four different donors. Mean of 71.06% (SD 13.27, N=4) of the CD4$^+$CD25$^-$ T cells acquired a regulatory phenotype (CD4$^+$CD25^{high}Foxp3$^+$). Results are presented as means +/- standard deviation (SD) of all four donors.

B. CD4$^+$CD25$^-$ T cells expressing the regulatory phenotype CD4$^+$CD25^{high}Foxp3$^+$ after activation with our two-step protocol as compared to control groups. Experiments were performed in triplicate with three different donors. One representative experiment is shown. Depicted data are means +/- standard deviation (SD).

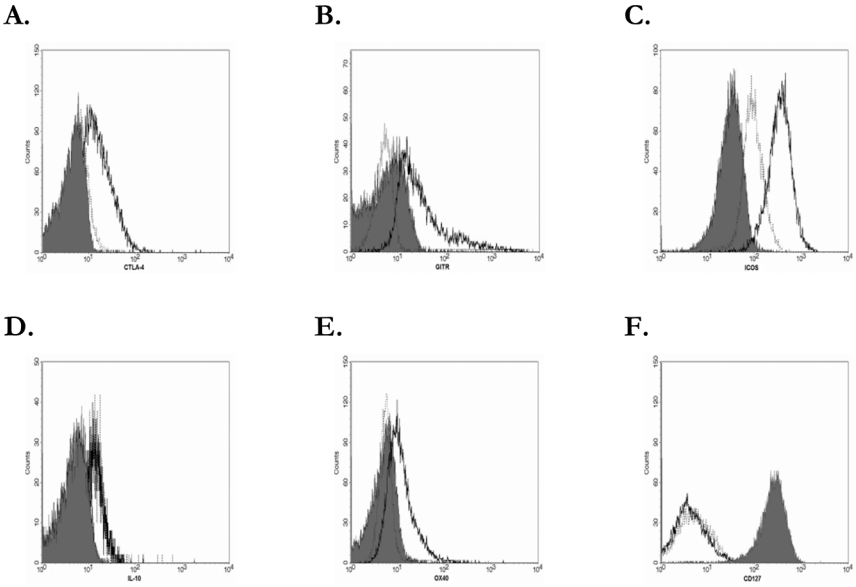

Figure 2. The TregPMA up-regulate CTLA-4, GITR, ICOS, OX40, IL-10, CD25, and FOXP3 and down-regulate CD127 as compared to control groups
TregPMA (black continuous line), show up-regulation of markers CTLA-4 (**A**), GITR (**B**), ICOS (**C**), OX40 (**D**), IL-10 (**E**), CD25 (**F**) and FOXP3 (**G**) and down-regulation of CD127 (**H**) after 9 days in culture as compared to not activated T cells (grey, filled in) and compared to PMA/ionomycin/IL-2 alone (non filled in, dotted line) control groups.
Experiments were performed in triplicate and with three different donors. One representative experiment is shown.

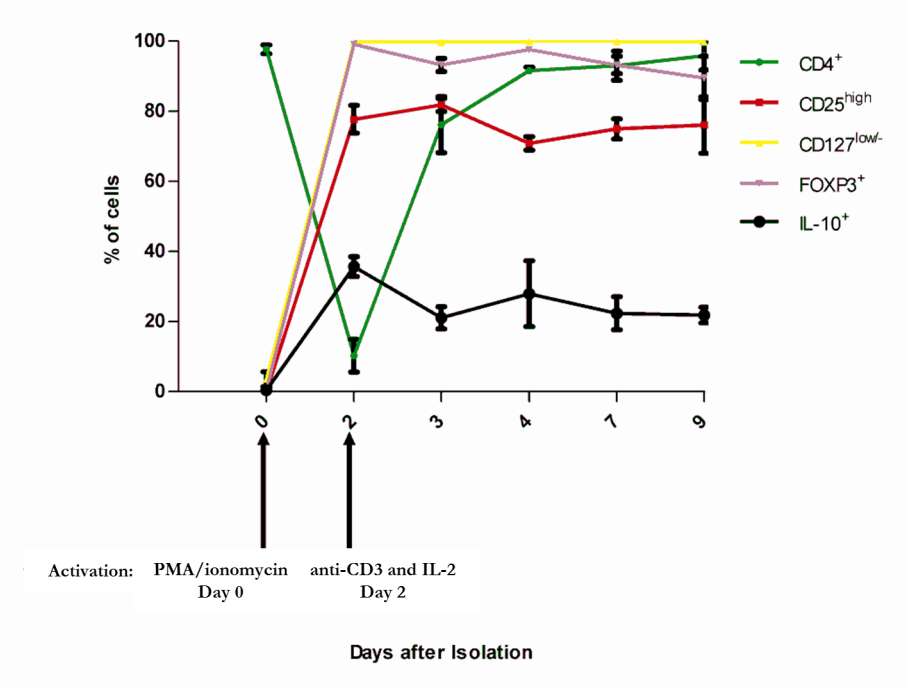

Figure 3. The TregPMA expression profile of $CD4^+$, $CD25^{high}$, $CD127^{low/-}$, $FOXP3^+$ and $IL\text{-}10^+$ the first 9 days in culture

TregPMA were analyzed at day 0, 2, 3, 4, 7 and 9 after activation. The marker CD127 was down-regulated, the markers CD25, FOXP3 and IL-10 were up-regulated and we show a synergy over time with the expression of FOXP3. Experiments were performed in triplicate and with three different donors. Results are presented as means +/- standard deviation (SD) of all three donors.

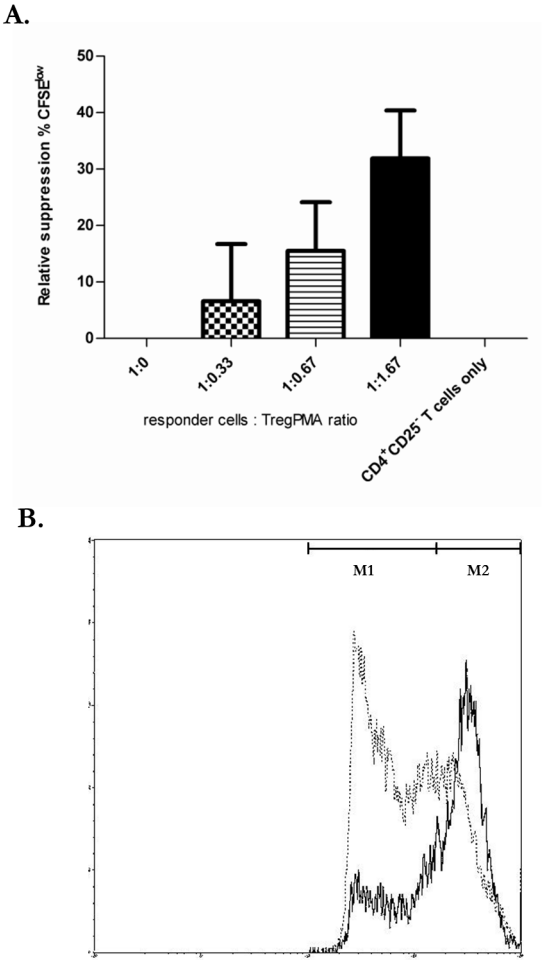

Figure 4. The regulatory phenotype of the TregPMA correlates with suppressive activity *in vitro*

A. TregPMA, after 9 days in culture suppress the proliferation of responder cells in response to irradiated allogenic feeder cells in a CFSE based MLR. Suppression of $CFSE^{low}$ represents the suppression of proliferating responder cells as compared to control groups. Overall we show a mean inhibition up to 31.87% (SD 9.38, N=3). $CD4^{+}CD25^{-}$ T cells caused no inhibition of the CFSE division. Experiments were performed in triplicate. Results are presented as means +/- standard deviation (SD) of all three donors.

B. Histogram of one representative flow cytometry experiment

The dotted line is the control group without Treg, showing the overall proliferation ($CFSE^{low}$, represented in M2). The continuous line represents the experimental group, with 1:1.67 TregPMA added showing less proliferation of responder cells (non-proliferating cells in M1).

Discussion

This paper describes a new and simple method to generate *in vitro*, within 4 days, stable and functional $CD4^+CD25^{high}FOXP3^+CD127^{-/low}$ iTreg. This approach could be easily adapted to clinical application under *Good Manufacturing Practices* (GMP) and thereby allow the preparation of a sufficient quantity of cells to enable use of iTreg for both prevention and subsequent therapy [48]. Other techniques to generate iTreg have been reported and are available [27, 28]. However, the protocol described in this paper presents a straightforward and robust alternative, by making use of substances which are well known, easy to obtain and relatively low priced.

Our protocol consists of two steps. The first step is the activation of $CD4^+CD25^-$ T cells with PMA/ionomycin. This combination provides a potent stimulation allowing us to bypass the T cell receptor activation essential for Treg development and prevent the emergence of $CD4^-CD8^+$ cells in the culture [49]. PMA activates protein kinase C [50] while ionomycin is a Ca^{2+} mobilizing agent [51]. This combination has shown to up-regulate CD25 on T lymphocytes [49] and a high CD25 expression is a marker of the Treg phenotype [34]. Additionally, it was demonstrated that Ca^{2+} signaling is required for the development and function of Treg [52, 53]. Therefore we expected that raising the intracellular levels of Ca^{2+} in $CD4^+CD25^-$ T cells using ionomycin would have a synergistic effect with PMA in starting a regulatory developmental program in $CD4^+CD25^-$ T cells. The expression profiles, analyzed by flow cytometry, after activation with PMA/ionomycin, show a clear up-regulation of the Treg lineage transcription factor FOXP3, as well as of the markers CD25 and IL-10. Conform to the regulatory phenotype [35, 36], CD127 was down-regulated.

The second step in our protocol is a step of activation with anti-CD3 in the presence of a low dose of IL-2. This second stimulation is needed to maintain the regulatory developmental program, since the initial up-regulation of FOXP3 expression, as well as the expression of CD4 on the cell surface declines in time after only PMA/ionomycin stimulation. Anti-CD3 was used for this purpose since it has been shown to maintain and expand Treg [54, 55]

and restore CD4 expression [45]. We show here that, for the established regulatory markers, $CD25^{high}$, $CD127^{-/low}$, and $FOXP3^{+}$ [34-36] the PMA/ionomycin and anti-CD3 steps seem to act synergistically to achieve the expression of a regulatory profile within 4 days in culture. Furthermore, the anti-CD3 activation step in our protocol restores the CD4 expression, which is down-regulated after the PMA/ionomycin activation step.

IL-2 contributes to FOXP3 transcription through activation of Stat5 and is thereby of importance both for Treg homeostasis and for maintaining their suppressive program [47]. Therefore we cultured the generated TregPMA in the presence or absence of IL-2 and examined their regulatory phenotype and function. We demonstrate that the expression of regulatory markers and capability of suppressing proliferation of responder cells *in vitro* are dependent on exogenous IL-2. Furthermore we show that in the presence of IL-2 the phenotype and function are stable over a long period of time.

Conclusions

Generating sufficient numbers of iTreg from human PBMC's and preserving their regulatory function for clinical application still proves to be difficult. In this paper we describe a protocol which permits to induce a stable regulatory phenotype and function in $CD4^{+}CD25^{-}$ T cells *in vitro*, within 4 days. We present a good alternative to previously established methods [27, 28] by making use of substances which are well known, simple to obtain and relatively low priced. Therefore, our two-step activation protocol could be easily adapted to routine laboratory settings, for future clinical applications, using iTreg either alone or together with nTreg in the treatment of allergic, autoimmune or inflammatory disorders.

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Chapter 3

Murine CD4(+)CD25(-) cells activated in vitro with PMA/ ionomycin and anti-CD3 acquire regulatory function and ameliorate experimental colitis *in vivo*

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Abstract

Background

Induced regulatory T (iTreg) lymphocytes show promise for application in the treatment of allergic, autoimmune and inflammatory disorders. iTreg cells demonstrate advantages over natural Treg (nTreg) cells in terms of increased number of starting population and greater potential to proliferate. Different activation methods to generate iTreg cells result in iTreg cells that are heterogeneous in phenotype and mechanisms of suppression. Therefore it is of interest to explore new techniques to generate iTreg cells and to determine their physiological relevance.

Methods

Using phorbol myristate acetate (PMA)/ionomycin and anti-CD3 activation of $CD4^+CD25^-$ cells we generated *in vitro* functional $CD4^+CD25^+$ iTreg (TregPMA) cells. Functionality of the generated TregPMA cells was tested *in vivo* in a mouse model of inflammatory bowel disease (IBD) - CD45RB transfer colitis model.

Results

TregPMA cells expressed regulatory markers and proved to ameliorate the disease phenotype in murine CD45RB transfer colitis model. The body weight loss and disease activity scores for TregPMA treated mice were reduced when compared to diseased control group. Histological assessment of colon sections confirmed amelioration of the disease phenotype. Additionally, cytokine analysis showed decreased levels of proinflammatory colonic and plasma IL-6, colonic IL-1 β and higher levels of colonic IL-17 when compared to diseased control group.

Conclusions

This study identifies a new method to generate *in vitro* iTreg cells (TregPMA cells) which physiological efficacy has been demonstrated *in vivo*.

Introduction

T regulatory (Treg) lymphocytes are a cellular component of the immune system that suppresses immune responses of effector cells. As a result of their ability to control immune responses and to sustain systemic immune balance, they have the potential to prevent allergic, autoimmune and inflammatory disorders [1-10] as well as to be an adjuvant therapy for chronic and acute graft versus host disease [11].

The safety profile of Treg cells has been established in phase I clinical trials [11, 12] demonstrating that Treg cells are a suitable candidate for therapeutic purposes. However a major limitation to the clinical use of natural Treg (nTreg) cells is their low availability as they represent only a small percentage of the peripheral circulating $CD4^+$ T cell population. In order to overcome this issue, several groups have developed different methods to expand nTreg cells *in vitro* when keeping their functionality. Generally the technologies are complex, time-consuming and the plasticity of nTreg cell lineage in artificial environment during *ex vivo* expansion can lead to loss of their suppressive activity [13]. Furthermore their stage of differentiation makes their expansion *in vitro* a difficult process [14].

In vitro induced T regulatory (iTreg) cells represent a good alternative to nTreg cells since they have been reported to have similar functionality in *in vivo* setup. Additionally iTreg cells show advantages over nTreg cells in terms of increased number of starting population and greater potential to proliferate [15]. Therefore it is of importance to investigate and explore new approaches to generate iTreg cells.

Based on the activating and stimulating properties of phorbol myristate acetate (PMA)/ionomycin and anti-CD3 on T cells [16, 17] we developed a new method to generate iTreg cells *in vitro*, which we refer to as 'TregPMA' cells. The functionality of the TregPMA cells was assessed *in vivo* in a mouse model of experimental colitis.

Methods

Mice

BALB/C and C.B.-17 SCID mice (8-10 weeks) were obtained from Harlan and maintained in specific pathogen-free conditions. Mouse experiments were approved by the local animal welfare committee (University of Amsterdam).

Generation of regulatory T lymphocytes (TregPMA cells)

Splenocytes were isolated from BALB/C mice. $CD4^+$ $CD25^-$ T cells were isolated from splenocytes by means of negative selection using the mouse "$CD4^+$ T Cell Isolation Kit" followed by "CD25 MicroBead Kit" (Miltenyi Biotec). Cells were seeded at day 0 at 1 x 10^5/well into anti-CD3e (0.5 μg/well, clone 145-2C11, eBioscience) coated 96-well flat bottom plates (Costar) and cultured in X-VIVO 15 medium (Lonza) at 37°C in a 5% CO_2 incubator. On day 1, cells were activated with PMA (10 ng/ml) and ionomycin (250 ng/ml) and at day 2, 3 and 4 supplemented with 20 U/well of IL-2 (eBioscience). On day 5, prior to use, cells were harvested and treated with "Dead Cell Removal Kit" (Miltenyi Biotec).

Flow Cytometry Analysis

Cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences). For Treg cell staining the mouse "Regulatory T cell Staining Kit" was used which consisted of anti-mouse CD4 FITC (clone RM4-5), anti-mouse CD25 APC (clone PC61.5) and anti-mouse/rat Foxp3 PE (clone FJK16). Additional monoclonal anti-mouse flow cytometry antibodies used in this study were as follows: anti-mouse CD152 (CTLA-4) APC (clone UC10-4B9), anti-mouse GARP PE (clone YGIC86), anti-mouse/rat CD278 (ICOS) FITC (clone C398.4A) and anti-mouse CD134 (OX-40) APC (clone OX86). All antibodies were obtained from eBioscience.

Induction of CD45RB Transfer Colitis Model and treatment with TregPMA cells

Chronic colitis was induced in C.B.-17 SCID mice by intraperitoneal (IP) injection of $CD45RB^{high}$ cells (4 x 10^5) isolated from normal BALB/C mice

splenocytes (positive control group). Mice that received CD45RB^{high} cells in combination with CD45RB^{low} cells were protected from disease development (negative control group). The treatment group received CD45RB^{high} cells in combination with *in vitro* generated TregPMA cells (TregPMA cell treated group). The amount of TregPMA cells injected per mouse was 1.2 x 10^6.

Monitoring development of colitis

The primary read-out to assess the development of colitis was the body weight loss. Mice were weighed three times a week. Body weight loss was determined by percentage of weight loss from base line body weight.

After sacrifice, colon was excised and longitudinally divided into 2 parts both of which were rolled up: one was used for preparation of paraffin embedded samples while the other was snap frozen in liquid nitrogen. Disease activity index (DAI) was calculated by combining the scores applied to weight loss (0: <1%; 1: 1-5%; 2: 5-10%; 3: 10-15%; 4: >15%), stool consistency at sacrifice (0: normal; 1: loose droppings; 2: loose stools, colon filled with feces; 3: loose stool, feces only near caecum, 4: empty bowel) and rectal bleeding (0: negative; 2: positive; 3: gross bleeding) divided by three.

Histology

The half divided colon tissue was fixed in 4% paraformaldehyde and embedded in paraffin. The colon tissue was cut in 5 μm sections and stained with haematoxylin and eosin for histological scoring. An experienced pathologist blinded to experiment inspected microscopically all sections and graded them on a scale 0 to 4 looking at: mononuclear and polymorphonuclear infiltrate, goblet cell depletion, crypt loss, epithelial hyperplasia, presence of ulcerations and manifestations of crypt abscesses.

Cytokine analysis

Frozen colon tissue was crushed with the use of CryoPrep™ System (Covaris) and resuspended in ice-cold PBS (pH 7.2) containing complete protease inhibitor (Roche). Homogenates were then centrifuged at 15000 x g for 5 min at C and the supernatants were stored at -80°C until the cytokine assay. Prior to

cytokine analysis total concentration of the protein in supernatants was determined with the use of Bradford assay (Biorad). Colonic tissue and plasma levels of IL-1β, IL-6, IL-10, IL-17 and TNF-α were analyzed using a mouse cytokine magnetic bead-based multiplex assay (Biorad) according to the manufacturer's instructions. Additionally TGF-β levels were analyzed with Bio-Plex Pro TGF-β assay (Biorad) according to manufacturer's instructions.

Statistical analysis

Data were analyzed statistically and graphed using Prism 5.0 (GraphPad Software). Changes in mice weekly body weight, body weight at sacrifice, disease activity index (DAI), histological score and cytokine levels are shown as mean ± standard deviation (SD) and analyzed by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Results

$CD4^+CD25^-$ T cells acquire a regulatory T cell phenotype through PMA/ionomycin/anti-CD3 mediated activation

In order to generate regulatory T cells, $CD4^+CD25^-$ cells from Balb/C mice were activated overnight on 96-well plate coated with anti-CD3. Subsequently PMA/ionomycin and IL-2 were added to the cell culture. After 5 days in culture, the cells were analyzed for their phenotype by flow cytometry. Between 85.26% and 91.85% of the cultured cells were shown to up-regulate and co-express the CD4 and CD25 markers (mean 91.4 ± 5.9%, n=3, **Figure 1.A, C**). The absence of PMA/ionomycin activation step on $CD4^+CD25^-$ cells resulted in significant decrease in cell viability (data not shown) which confirms the necessity of additional activation with PMA/ionomyin.

Additionally, we analyzed the expression of Foxp3, CTLA-4, GARP, ICOS and OX-40 markers which are known to be associated with a T regulatory cell phenotype and function [18-22]. All tested markers were found to be up-regulated in ~30% of the $CD4^+CD25^+$ T cells after activation (a representative result is shown in **Figure 1.D, Figure 2.A, B, C, D**).

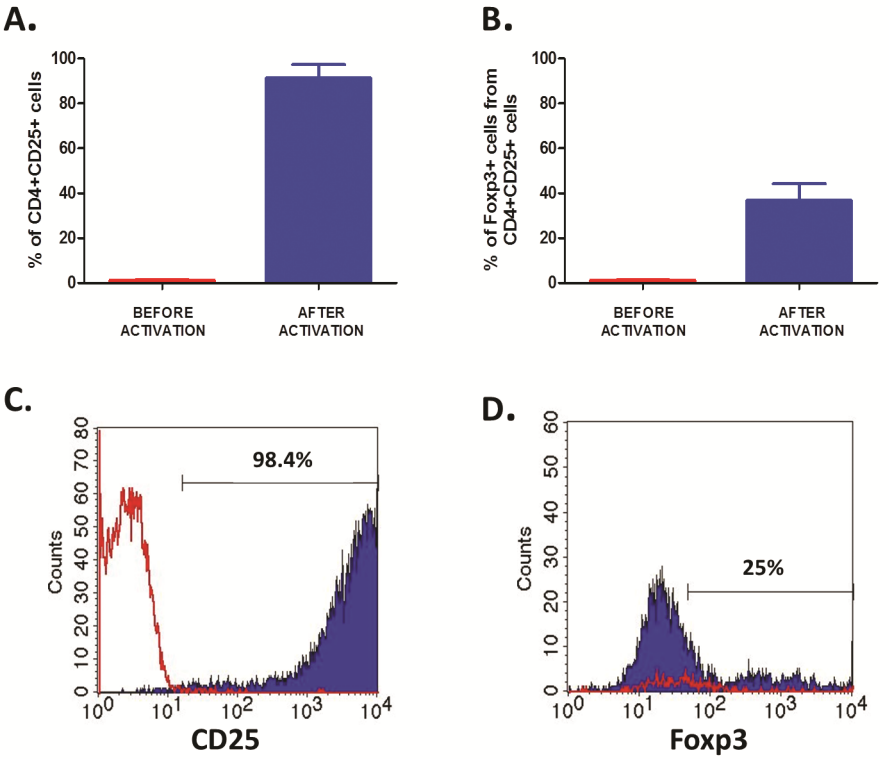

Figure 1. $CD4^+CD25^-$ cells acquire regulatory phenotype- $CD4^+CD25^{high}Foxp3^+$. After activation with PMA/ionomycin/anti-CD3 and 5 days in culture, the $CD4^+CD25^-$ T cells were analyzed by flow cytometry and 85.26% to 91.85% (mean: 91.4 ± 5.9%, n=3) of the cells were shown to co-express CD4 and CD25 marker (**A**) and mean of 36.86 ± 7.46% of the cells acquire the classical regulatory phenotype- $CD4^+CD25^{high}Foxp3^+$ (**B**). Three independent experiments were performed (**A, B**). Representative overlays of CD25 histograms(**C**) and Foxp3 (**D**) before (in red) and after (in blue) PMA/ionomycin/anti-CD3 activation and 5 days in culture. One representative experiment is shown. Foxp3 histogram was gated on $CD4^+CD25^+$ cell population (**D**).

Similarly to previously reported data with iTreg cells generated *in vitro* [23], the cellular level of Foxp3 expression in the cells activated with PMA/ionomycin/ anti-CD3 appears to be low (**Figure 1.B, D).**

We will refer to the cells treated with PMA/ionomycin/anti-CD3 activation (iTreg cell population) as 'TregPMA' cells.

TregPMA cells ameliorate the disease phenotype in a colitis transfer mouse model

IBD was induced in C.B-17 SCID mice by transfer of $CD45RB^{high}$ cell population from BALB/C mice (positive control group) and was prevented by co-transfer of $CD45RB^{low}$ cell population (negative control group) [24-27].

The read-out parameters of the development and progression of the colitis in the transfer mouse model are body weight loss and inflammation of the intestinal tissue.

The positive control group developed colitis and presented a relative decrease of initial body weight 86.88 ± 5.06% (n=9) at sacrifice which was significantly lower than the negative control that reached 110 ± 3.29% (n=10) of the initial body weight. The TregPMA cell treatment (injection of TregPMA cells together with $CD45RB^{high}$ cells) reduced significantly the body weight loss to 99.18 ± 7.17% (n=10) as compared to the positive control group (**Figure 3.A, B**).

The Disease Activity Index (DAI), which is an indicator of colonic inflammation, reflecting weight loss, stool consistency and presence of rectal bleeding was determined at sacrifice. The DAI was significantly lower in mice treated with TregPMA cells (1.18 ± 0.55) when compared to the positive control group (2.04 ± 0.35), confirming the amelioration of the disease phenotype (**Figure 3.C**).

Intestinal inflammation was further determined by histopathological analysis of colon tissue post mortem which includes evaluation of mononuclear and polymorphonuclear cell infiltrate, goblet cell depletion, loss of crypts, epithelial hyperplasia and manifestations of ulcerations and abscesses [24-27].

A significant reduction of inflammation was observed in the colon tissue of the TregPMA cell treated mice when compared to the positive control group (**Figure 3.D**). The spread of intestinal inflammation was less extensive and no ulcerations were observed in the TregPMA cell group (**Figure 4.B**) which was similar to the negative control group (**Figure 4.A**) while in the diseased control group they were commonly present (**Figure 4.C**).

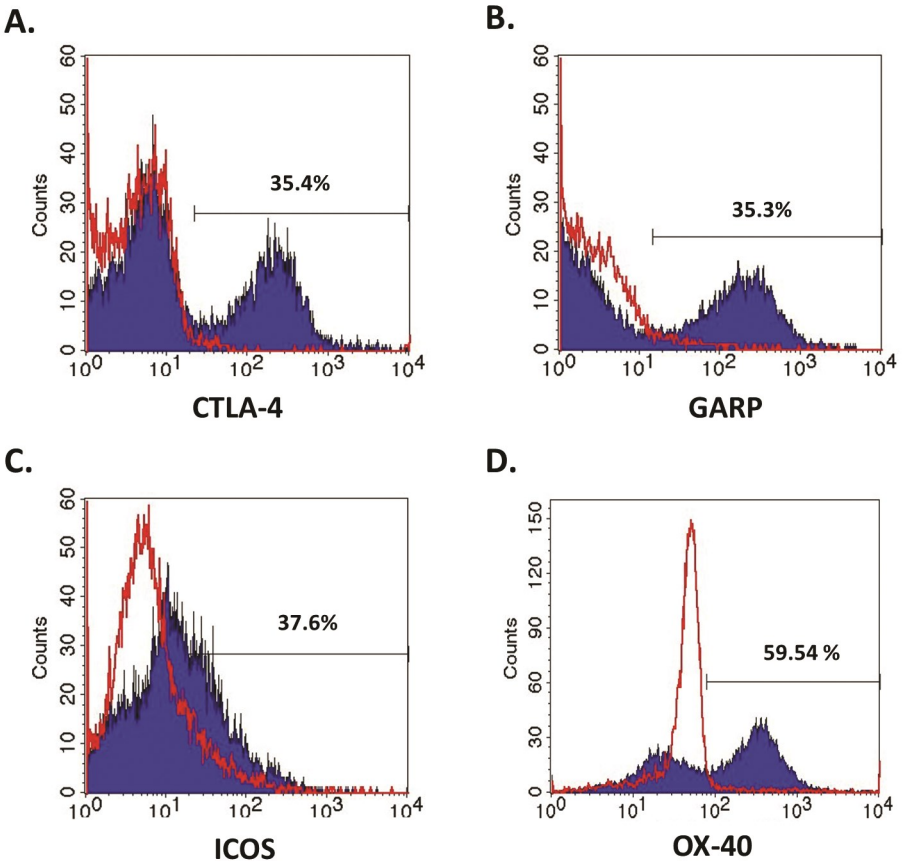

Figure 2. CD4+ CD25- cells up-regulate markers associated with regulatory phenotype. TregPMA cells express the markers CTLA-4 (**A**), GARP (**B**), ICOS (**C**) and OX40 (**D**) associated with a regulatory phenotype and function. Representative histogram overlays are shown. In red CD4+CD25- T cells at time 0 and in blue TregPMA cells after following PMA/ionomycin/anti-CD3 activation and 5 days in culture.

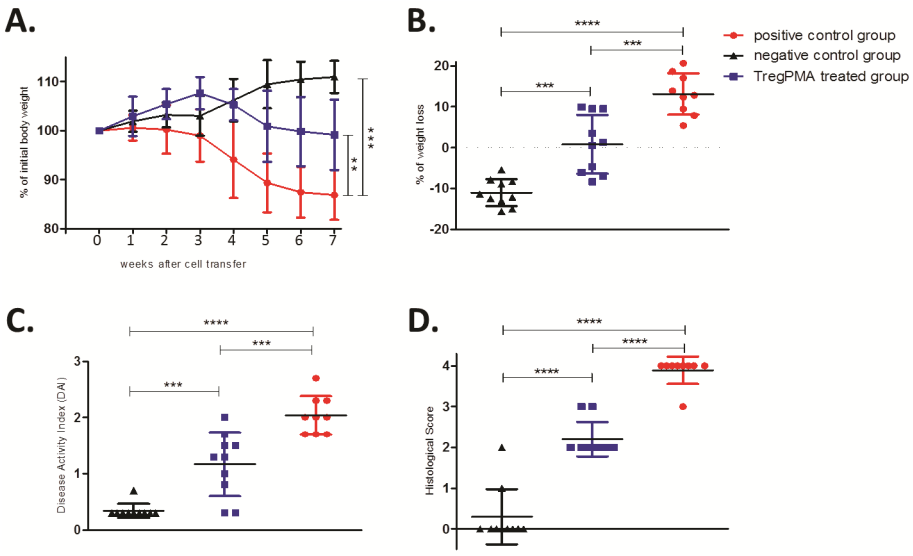

Figure 3. Amelioration of the disease phenotype in TregPMA cell treated mice.
Mice that were injected with CD45RB^{high} cells only developed colitis (positive control group, n=9) while the mice that were injected with CD45RB^{high} and CD45RB^{low} cells together remained healthy (negative control group, n=10). The experimental group was injected with CD45RB^{high} and TregPMA cells (n=10). Disease progression was assessed by changes in weekly body weight (**A**) as well as macroscopic and microscopic scores at day of sacrifice which comprises of Disease Activity Index (**C**) and Histological Score (**D**). TregPMA cell treated mice showed to have reduced weight loss over time (**A**) and at the time of sacrifice (**B**) when compared to positive control. The change of weight is expressed as the mean percentage of initial weight per group ± SD. Disease Activity Index (DAI), calculated from combining scores applied to weight loss, stool consistency at sacrifice and rectal bleeding divided by three, shows a significant difference between groups (**C**) as well as Histological Score of the H&E stained colon tissue sections (**D**). The data were analyzed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Figure 4. Histopathology of mouse colon.
Representative whole-colon photographs of H&E stained tissue sections of negative control mouse (**A**), TregPMA cell treated mouse (**B**) and positive control mouse with ulceration manifestation (**C**).

Figure 5. Cytokine levels in mouse colon homogenates and plasma.
Colonic tissue homogenates and plasma levels of IL-1ß (**A, D**), IL-6 (**B, E**) and IL-17 (**C, F**) of negative control group (n=10), TregPMA cell treated group (n=10) and positive control group (n=9) were analyzed using a mouse cytokine magnetic bead-based multiplex assay. TregPMA cell treatment has shown to suppress increased levels of colonic IL-1ß (**A**), IL-6 (**B**) and plasma IL-6 (**E**) in CD4^{+}CD45RB^{high} CD25^{-} cell transferred colitic mice. The data were analyzed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

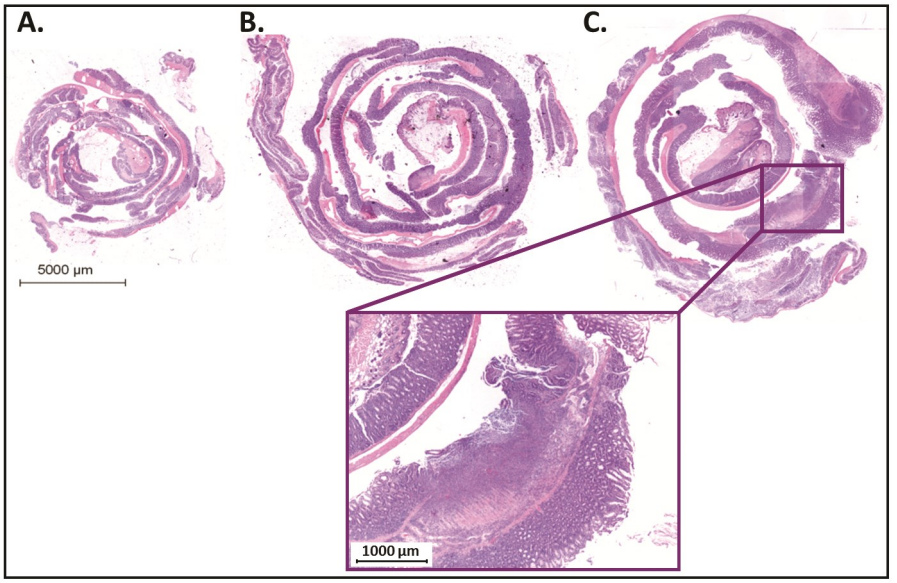

Figure 4. Histopathology of mouse colon.

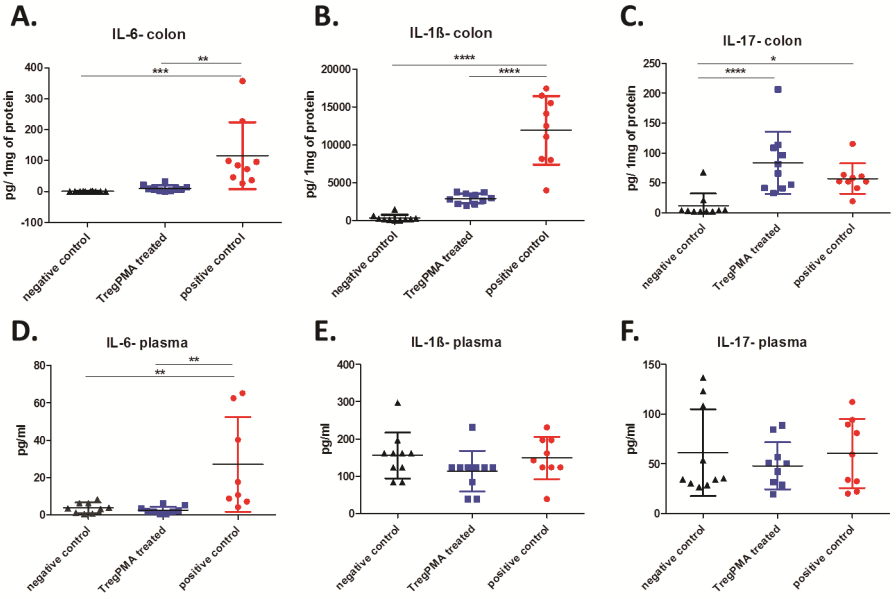

Figure 5. Cytokine levels in mouse colon homogenates and plasma.

Proinflammatory cytokine production in colon and in plasma of TregPMA cell treated mice is reduced

We next examined the influence of TregPMA cell treatment on the production of pro-inflammatory cytokines IL-6, IL-1β and TNF-α that have been previously described to be increased in CD45RB transfer colitis mouse model [28, 29]. The level of IL-10 and TGF-β that have been shown to have a regulatory effect in experimental colitis were also analyzed [30, 31]. Additionally the level of IL-17 cytokine that have been described to have both pro- and anti-inflammatory effect in IBD was measured [30, 32-35].

The level of IL-6 was found to be decreased in TregPMA cell treated group in both colon homogenates and plasma at the day of sacrifice when compared to the positive control group (**Figure 5.A, D**). IL-1 β level was also found to be reduced after TregPMA cell treatment in colon homogenates but this decrease was not reflected in plasma (**Figure 5.B, E**).

Elevated levels of IL-17 were found in colon tissue homogenates of TregPMA cell treated mice as well as in positive control group. However, the TregPMA cell treated group had higher level of colonic IL-17 than the positive control (**Figure 5.C**). No significant difference between groups was observed in the levels of IL-17 expression in plasma (**Figure 5.F**).

TNF-α, IL-10 and TGF-β levels in both colon homogenates and plasma showed no significant differences between groups (data not shown).

Discussion

In this article we describe the generation of iTreg cells (TregPMA cells) *in vitro* from $CD4^+CD25^-$ cells by PMA/ionomycin/anti-CD3 activation based method. Several other groups have explored different strategies to generate iTreg from naive T cells *in vitro*, including use of TGF- β, all-trans-retinoic acid and the recently discovered regulatory cytokine IL-35 [36, 37]. However, all these methods are more or less impeded with their own limitations [16]. Therefore, there is need for improvement in this field and our group designed and ex-

plored a new method of *in vitro* iTreg cells (TregPMA) generation which we report in this manuscript.

Mechanistically, PMA/ionomycin provides a potent stimulation allowing us to bypass the T cell receptor activation essential for Treg cell development and it prevents the emergence of $CD4^{-}CD8^{+}$ cells in the culture [17]. PMA activates protein kinase C [38] while ionomycin is a Ca^{2+} mobilizing agent [39]. This combination has been shown to up-regulate CD25 on T lymphocytes [17] and a high CD25 expression is a marker of the Treg cell phenotype [40]. Additionally, it was demonstrated that Ca^{2+} signaling is required for the development and function of Treg cells [41, 42]. Therefore we expected that raising the intracellular levels of Ca^{2+} in naive T cells using ionomycin would have a synergistic effect with PMA in starting a regulatory developmental program in $CD4^{+}CD25^{-}$ cells. Additionally a low dose of IL-2 was used in cell culture since IL-2 is important for Treg cell homeostasis and maintaining their suppressive survival program [43] while anti-CD3 which is also present in the cell culture has been shown to maintain and expand Treg cells [44], however use of only anti-CD3 and IL-2 for *in vitro* $CD4^{+}CD25^{-}$ cell stimulation resulted in low cell viability and poor regulatory markers expression which indicates that additional stimulation with PMA/ionomycin is needed.

The TregPMA cells up-regulate CD25, and ~30% of the $CD4^{+}CD25^{+}$ T cells up-regulate CTLA-4, GARP, ICOS, OX-40 and Foxp3 which are important markers associated with regulatory cell phenotype and function [18-22, 45, 46]. Interestingly, the level of Foxp3 expression in TregPMA cells is quite low. Although Foxp3 expression is considered to be a main characteristic of Treg cells, Treg cells are recognized to be a heterogeneous cell population. Especially, it has been reported that $CD4^{+}CD25^{+}$ $Foxp3^{-}$ cell population also can present regulatory function *in vitro* and *in vivo* [23].

The functionality of the TregPMA cells has been demonstrated *in vivo* by their potential to ameliorate the disease phenotype in a CD45RB transfer colitis mouse model. It cannot be fully excluded that the amelioration of the disease phenotype observed in the group of mice treated with the TregPMA cells does not partially involve cell types, other than regulatory, present in the cell

suspension injected. Indeed, high numbers of CD45RB^{high} cells (6 x 10^6/ mouse) have been shown to reduce the severity of the disease in a mouse model of colitis [47]. However, in our study, the total number of cells injected (1.2 x 10^6/mouse) was significantly lower and the amelioration of the severity of the disease was observed in all of the treated animals (n = 10) against 3 out of 5 in the mentioned study. All together, these data suggest that the observed protective effect is mostly related to TregPMA cells.

The disease amelioration was accompanied by a decrease of the pro-inflammatory IL-1 β levels in the colon tissue which reflect the severity of experimental colitis [28]. Such a decrease in IL-1 β was previously reported by Hirano and Kudo after treatment of CD45RB transfer colitis mouse model with both dexamethasone and anti-tumor necrosis factor-α (anti-TNF-α).

IL-1 β stimulates the production of the pro-inflammatory cytokine IL-6 [48] which has been described to be up-regulated in mouse model of IBD [49] and in human ulcerative colitis or Crohn's disease patients [50]. Simultaneously with the decrease of IL-1β in our study, IL-6 levels were found to be reduced in both colonic homogenates and plasma samples after treatment with TregPMA cells.

The concentration of IL-17 was found to be more elevated in colon homogenates of TregPMA cell treated mice than in the positive control group. Although the role of this cytokine in the initiation or pathogenesis of IBD is controversial, a protective function of IL-17 in the gut of CD45RB transfer colitis model has been previously noted [30, 33, 51]. Several groups have reported that both IL-17-/- CD45RB^{high} and IL-17R-/- CD45RB^{high} cells induce a more aggressive disease phenotype than wild type CD45RB^{high} cells [30, 51]. Accordingly, our results show that an increased IL-17 level also correlated with less severe intestinal inflammation and lack of ulcerations.

IL-10 and TGF- β cytokine level in both mouse plasma and colon homogenates show no significant differences between experimental groups at the time of experiment termination (week 7). However, it does not exclude the possible influence of those cytokines on prevention of the colitis induction at earlier time point.

The cells that have been found mostly responsible for pathogenesis of CD45RB transfer model of IBD are Th1 cells. Recently it has been shown that IL-17 blocks the Th1 cell development *in vivo* via IL-17R on naive $CD4^+$ T cells [30, 51]. In the TregPMA cell treated group we also observe elevated level of IL-17 which correlates with disease amelioration. IL-17 can be produced by many types of cells among which are also T regulatory cells [52]. Therefore we are hypothesizing that *in vivo* disease amelioration by TregPMA cells is IL-17 mediated. As follow-up to this study, physiological relevance of TregPMA cells and their exact molecular mechanism of action needs to be determined.

Conclusions

Induced regulatory T cells are promising tools for therapeutic applications in IBD [49] as well as in other autoimmune disorders and are of great scientific interest and many techniques to generate inducible Treg cells from naive T cells have been developed and described [8, 36, 37, 54, 55]. Different activation methods to generate iTreg cells result in iTreg cells that are heterogeneous in phenotype. The *in vitro* PMA/ionomycin/anti-CD3 activation of $CD4^+CD25^-$ T cells has proven to generate functional iTreg cells (TregPMA cells) but the *in vivo* physiological relevance of TregPMA cell signaling needs to be further investigated.

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Chapter 4

Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis

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Abstract

Aim

To explore the anti-inflammatory potential of adeno-associated virus-mediated delivery of Tregitope 167 in an experimental colitis model.

Methods

The Trinitrobenzene sulfonate (TNBS) model of induced colitis was used in Balb/c mice. Subsequently after intravenous adeno-associated virus-mediated Tregitope delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 days later. Control groups included mice not treated with TNBS (healthy control group) and mice treated by TNBS only (diseased group). At the time of sacrifice colon weight, the disease activity index and histology damage score were determined. Immunohistochemical staining of the colonic tissue was performed to assess the cellular infiltrate and the presence of transcription factor Forkhead Box-P3 (Foxp3). Thymus, mesenteric lymph nodes, liver and spleen tissue were collected and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of $CD4^+$ T cell and regulatory T cell associated markers.

Results

The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice. In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice (*P* value <0.01). The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index and a decreased colon weight as compared to the diseased control group (*P* value <0.01 and <0.001 respectively). Immunohistochemical staining of the colonic tissues for $CD4^+$ showed that inflammatory cell infiltrates were present in TNBS treated mice with or without administration with Tregitope 167 and that these cellular infiltrates consisted mainly of $CD4^+$ cells. For both TNBS treated groups $CD4^+$ T cell infiltrates were

observed in the sub-epithelial layer and the lamina propria. $CD4^+$ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the Tregitope 167 treated group. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167. Furthermore, the Foxp3 and glycoprotein A repetitions predominant (GARP) markers were significantly increased in the $CD4^+$ T lymphocyte population in the thymus of the mice pre-treated with adeno-associated virus serotype 5 (cytomegalovirus (CMV) promoter-Tregitope 167), as compared to lymphocyte populations in the thymus of diseased and the healthy control mice (*P* value <0.05 and <0.001 respectively).

Conclusion

This study identifies adeno-associated virus-mediated delivery of regulatory T-cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.

Introduction

Inflammatory Bowel Diseases (IBD) are inflammatory diseases that affect mostly young adults [1, 2]. Although the precise pathogenesis has not been identified, it is generally accepted that IBD result from inappropriate mucosal immune system responses against intestinal flora and other luminal antigens [3 -5]. IBD are associated with a reduction in quality of life [6-8] and no curative treatments are available.

Despite the fact that novel treatment strategies, including tumor necrosis factor (TNF)-neutralizing antibodies, have greatly expanded the therapeutic armamentarium, these therapeutics do not prevent complications in IBD and many patients still have to undergo surgery [9]. New treatment strategies that would prevent the initiation of inflammation and enable long-term remission would improve the lives of millions of individuals who are affected by IBD world-wide [10, 11].

Recently, biological therapies that target immune pathways have emerged as a new therapeutic approach for the treatment of immune-mediated diseases. They include administration of monoclonal antibodies against inflammatory cytokines [12] and those that influence immune responses such as certain small molecules, Helminths and stem cells [10, 13, 14]. Since IBD are immune -mediated diseases, these biological therapies are highly promising treatment approaches and have the potential to achieve mucosal tolerance and long-term remission in IBD [10, 12-14]. Here, we introduce regulatory T-cell epitopes (Tregitopes) [15, 16] as novel biological agents that could create new possibilities for the regulation of inflammation and postulate that Tregitopes, delivered by adeno-associated virus (AAV), could be developed as a new therapeutic modality for the treatment of IBD.

Tregitopes are a set of putative regulatory T cell epitopes present in the immunoglobulin G molecule, which have been identified by using computational epitope mapping [15, 16]. Tregitope sequence 167 (Tregitope 167) and an additional sequence (Tregitope 289) were synthesized and shown to bind to multiple Major Histocompatibility complex (MHC) class II molecules and to sup-

press immune response when co-administered with an antigen. Tregitopes 167 and 289 were also shown to expand natural occurring regulatory T (nTreg) cells and to induce a regulatory phenotype and function in peripheral T (iTreg) cells [15, 16]. Harnessing the potential of Treg cells activated or induced by Tregitopes to regulate pathological immune responses in IBD may reduce the requirement for systemic immunosuppressive therapies. However, the use of immunomodulatory peptides in clinical applications for IBD so far have shown that the *in vivo* delivery of these peptides for therapeutic purposes is hindered by difficulties in obtaining sufficient and stable peptide concentrations [17-19]. Therefore, novel means for stable delivery of regulatory peptides have to be explored. Adeno-associated viruses (AAV) present a good safety profile and have been shown to be effective as gene delivery vectors in the clinic for the treatment of a broad range of diseases [20-22]. Therefore, AAV-mediated delivery represents an attractive approach to deliver the immunomodulatory Tregitope peptides.

In the present study, the potential of AAV-mediated gene therapy for the therapeutic delivery of Tregitope 167 was explored. Systemic AAV-mediated administration of Tregitope 167 was shown to ameliorate the clinical and histo-pathological severity of Trinitrobenzene sulfonate (TNBS) induced inflammatory colitis in mice. Hence, AAV-mediated delivery of regulatory T-cell epitopes appears to be a promising novel therapeutic approach for the treatment of IBD and could represent an alternative or adjunct to the use of immunosuppressive drugs.

Materials and methods

AAV vector production and characterization

Mouse Tregitope cDNA was synthesized (Integrated DNA Technologies, IDT, Inc) according to the published corresponding sequence [15, 16] and cloned into the plasmid pCH110 [23] under the control of the cytomegalovirus (CMV) promoter. The Woodchuck hepatitis virus post-transcriptional enhancer (WPRE) was incorporated behind the Tregitope 167 cDNA to further optimize gene expression [24]. The AAV vector, AAV5 (CMV-Tregitope 167)

was produced according to a technology adapted from A. Negrete and R.M. Kotin [23]. The AAV batch was purified with an AVB sepharose column using the ÄKTA explorer system (GE Healthcare). After purification, the concentration of AAV vector genomes copies (gc/ml) was determined at 9 x 10^{13}gc/ml by Taqman qPCR amplification. The biological infectivity of AAV5 (CMV-Tregitope 167) was demonstrated *in vitro* by PCR amplification of the "CMV-Tregitope 167" DNA fragment (product size 402bp) on DNA isolated from HEK293T transduced with AAV5 (CMV-Tregitope 167). Primers designed and synthesized for Tregitope 167 and the CMV promoter were used.

Induction of colitis and study design

Balb/c mice (males, age 6-8 weeks) were obtained from Harlan Laboratories, the Netherlands. The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). Colitis was induced in mice by administration of TNBS (Trinitrobenzene sulfonate, TNBS, Sigma-Aldrich), as described previously [25]. The general procedure is summarized in **Figure 1**.

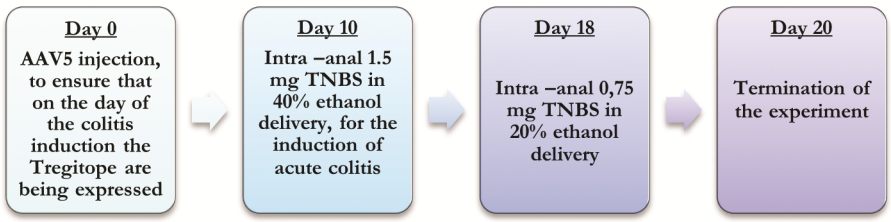

Figure 1. Schematic overview of the Trinitrobenzene sulfonate (TNBS) induced colitis model. Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group).

Mice were injected intravenously with either PBS or AAV5 (CMV-Tregitope) 10 days before acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol. Consecutively, a second TNBS treatment (0.75 mg in 20% ethanol) was done 8 days after the first TNBS treatment as described

precedently [25]. Mice not treated with TNBS (healthy control group) and mice treated with TNBS only (diseased control group) were used as references to monitor colitis development. A concomitant sham AAV control vector was not used in this study as this control has been shown to be equivalent to saline control [26, 27]. Even though AAV-mediated gene transfer leads to the development of neutralizing antibodies against the vector capsid [28], preventing vector re-administration, no inflammatory responses against the AAV capsid were documented in *in vivo* gene transfer mice models using AAV vectors [27, 29].

Assessment of Inflammation

The body weights of the mice were recorded daily, and wasting disease progression was expressed by the percentage of weight loss as compared to body weight at the day of initiation of TNBS treatment (**Figure 2**). Animals were withdrawn from the study when their weight loss was >25% of their original body weight. At the time of sacrifice, colons were collected and presence of loose stool and visible fecal blood was assessed.

At the time of sacrifice, a composite score (disease activity index, DAI) was established as described previously [25]. Body weight loss was scored on a scale of 0–4 (0, <1%; 1, 1%-5%; 2, 5%-10%; 3, 10%-15%; 4, >15%). Loose stool was scored on a scale of 0–4 (0, normal; 1, loose droppings; 2, loose stools, colon filled with feces; 3, loose stool, feces only near cecum; 4, empty bowel). Visible fecal blood was scored on a scale of 0–4 (0, negative; 2, positive; 4, gross bleeding). The DAI consists of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 as described previously [25].

Colon tissue weights were recorded and used as an indicator of disease-related intestinal wall thickening. Increased colon weight has been shown to correlate with increased colon inflammation [25]. Colons were first divided longitudinally into two parts: one part was immediately frozen in liquid nitrogen for protein extraction and cytokine level determination, while the second part was stored in formalin and embedded in paraffin for immunohistological evaluation. Blood was collected by orbital puncture immediately following sacrifice

and plasma was separated by centrifugation (5000 rpm for 5 min). Plasma samples were stored at −80°C until analysis.

Histological analysis

Colonic segments were fixed in 10% formalin overnight and thereafter stored in 70% ethanol before embedding in paraffin. Tissue sections (7 μm thick) were stained with haematoxylin for histology scoring. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells as described previously [30]. The percentage of area involved and crypt loss were scored on a scale of 0–4 (0, normal; 1, <10%; 2, 10%; 3, 10%–50%; and 4, >50%). Erosions were defined as 0 if the epithelium was intact, 1 if the lamina propria was involved, 2 if ulcerations involved the submucosa, and 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0–3 (0, absent; 1, weak; 2, moderate; and 3, severe) [30]. A certified pathologist scored all the tissue sections (blinded analysis).

Immunohistochemistry

Colon tissues sections of 7 μm were aceton-fixed and stained with rat anti-mouse (rαm) CD4 (1:100, BD550278), rαm CD8a (1:50, BD550281), rαm CD19 (1:50, BD550284), rαm Foxp3 (1:100, eBiosciences14-5773-82) and rαm F4/80 (1:500). Prior to anti-rat biotin conjugated secondary antibody (1:50, BD51-7605kc) and streptavidin-HRP (BD) incubations, endogenous peroxidases were blocked by incubation with 0.3% H_20_2 for 20 minutes. After 5 min of DAB staining (BD), the sections were counter-stained with haematoxylin, dehydrated and mounted in entallan.

Flow cytometry

Thymus, mesenteric lymphoid nodes, liver and spleen tissue were collected upon sacrifice. Cell suspensions obtained from each of the tissue samples were prepared using 40 μm cell strainers (BD Biosciences) and stained for T cell surface markers CD4 (clone RM4-5, eBioscience), CD8 (Clone 53-6.7, Miltenyi) and Treg cell surface markers GARP and CD25 (clone YGIC86 and

clone PC61.5 respectively, both eBioscience) as well as for the intracellular Treg cell marker Foxp3 (clone FJK16, eBioscience). The analysis was performed by flow cytometry (FACSCalibur, BD Biosciences).

Statistical analysis

The results are presented as means (+/- standard deviation (SD) or standard error of the mean (SEM), where appropriate). Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 1 way ANOVA, followed by Dunn's post hoc test for multiple comparisons.

Results

We investigated the potential for AAV5-mediated delivery of regulatory T-cell epitopes to prevent the development of TNBS induced colitis. Mice treated intra-rectally with TNBS in ethanol developed a severe illness as reflected in the progressive body weight loss over time and an increase in disease activity index, histology damage score and mucosal inflammatory parameters at the time of sacrifice.

Tregitope 167 delivery protects against TNBS colitis development

Development of colitis in the TNBS mice model is strongly associated with wasting disease [31]. Daily weight determination is therefore important to determine colitis severity and is indicative of differences in colitis development between experimental groups [31]. Animals were withdrawn from the study when their weight loss was >25% of their original body weight.

The body weight of the mice was monitored daily after the first TNBS treatment as an indication of the severity in the colitis development between experimental groups (**Figure 2**). TNBS treated mice that were pre-administered with Tregitope 167, showed a body weight that increased over time and was comparable to the weigh gain of untreated healthy control mice (**Figure 2**). The Tregitope 167 treated mice gained an average of 4% over their initial body weight at the time of sacrifice (**Figure 3.A**). In contrast, the mice treated with TNBS alone (no Tregitope) developed colitis, and lost 4% of their initial body weight at the time of sacrifice (**Figure 3.A**).

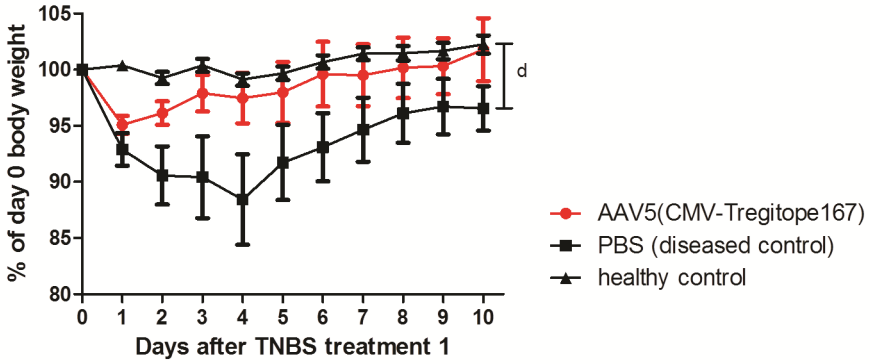

Figure 2. Adeno-associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 ameliorates Trinitrobenzene sulfonate induced colitis development over time. Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery, acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second TNBS treatment (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight. Animals were withdrawn from the study when their weight loss was >25% of their original body weight. Overall for the healthy controls, n = 9; AAV5 (CMV-Tregitope 167) treated, n=7; diseased controls, n=6, were included in the analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn's post hoc test for multiple comparisons. Data are presented as means +/- SEM of all the mice. ^{d}P value <0.0001 between PBS (diseased control group) and both AAV5 (CMV-Tregitope 167) treated and the healthy control group. PBS: Phosphate-buffered saline; TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.

Increases in colon weight, as well as in the disease activity index are both indicative of colonic inflammation and were determined at the time of sacrifice. The body weight increase that had been observed in the mice pre-treated with Tregitope 167 was substantiated by a lower disease activity index (**Figure 3.B**) and a decreased colon weight (**Figure 3.C**) as compared to the diseased control group (*P* value <0.01 and <0.001 respectively).

The histology damage score was performed on HE stained tissue sections. The score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. The histological scoring showed that the AAV5 (CMV-Tregitope 167) pre-treated mice presented a decreased severity of colitis as compared to the diseased control group (**Figure 3.D**) as a result of lower levels of inflammation, namely decreased cellular infiltrations, little crypt loss and the absence of erosions and ulceration (**Figure 4**).

The TNBS induced colitis model is characterized by the local infiltration of $CD4^+$ T cells in the intestinal mucosa [32]. Immunohistochemical staining of the colonic tissues for $CD4^+$ showed that, at the day of sacrifice, inflammatory cell infiltrates were present in TNBS treated mice with or without administration with Tregitope 167 and that these cellular infiltrates consisted mainly of $CD4^+$ cells. For both TNBS treated groups $CD4^+$ T cell infiltrates were observed in the sub-epithelial layer and the lamina propria. $CD4^+$ T cell infiltrates were also present in the muscularis mucosa layer of the diseased control mice, but were absent in the AAV5 (CMV-Tregitope 167) treated group (**Figure 5**).

Increase of regulatory markers expression in the intestinal mucosa and thymus of mice administered with Tregitope 167

The reported ability of Tregitopes to both activate and induce Treg cells led us to further assess the presence of Treg-cell associated markers in the colonic tissues.

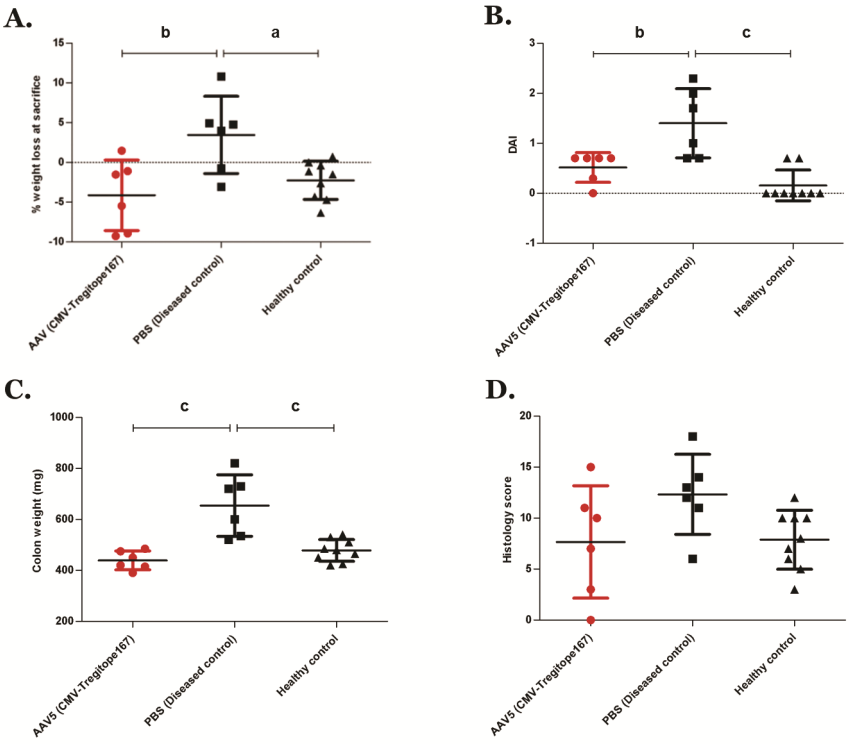

Figure 3. Adeno-associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 alleviates colonic inflammation as determined at the day of sacrifice. Mice were injected intravenously with either PBS or AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167). 10 d after AAV-mediated Tregitope delivery acute colitis was initiated by intra-rectal administration of 1.5 mg TNBS in 40% ethanol followed by a second treatment with TNBS (0.75 mg in 20% ethanol) 8 days later. Control groups consisted of mice not treated by TNBS (healthy control group) and mice treated by TNBS only (diseased control group). Disease progression was assessed by changes in daily body weight as well as macroscopic and microscopic scores on the day of sacrifice. **A.** Animal body weight change on day 20 upon sacrifice. The values of body weight are expressed as a percentage of body weight on the day of the first TNBS treatment; **B.** Macroscopic disease score. The disease activity index (DAI) consist of a combination of body weight loss, loose stool and visible fecal blood scores divided by 3 at the day of sacrifice; **C.** Assessment of colonic weight upon sacrifice as an index of disease-related intestinal wall thickening; **D.** Histological grading of colonic colitis scores. The histology damage score was calculated using the following criteria: percentage of area involved, number of follicle aggregates, edema, fibrosis, erosion/ulceration, crypt loss, and infiltration of mononuclear and polymorphonuclear cells. Individual mice are depicted; from the AAV5 (CMV-Tregitope 167) pre-treated group an outliner was removed. Animals were withdrawn from the study when their weight loss was >25% of their original body weight. Overall for the healthy controls, n = 9; AAV5 (CMV-Tregitope 167) treated, n=6; diseased controls, n=6, were included in the analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn's post hoc test for multiple comparisons. Data are presented as means +/- SD of all the mice. P value <0.05 (a), P value, <0.01 (b), P value <0.001 (c) *vs* PBS (diseased control group).

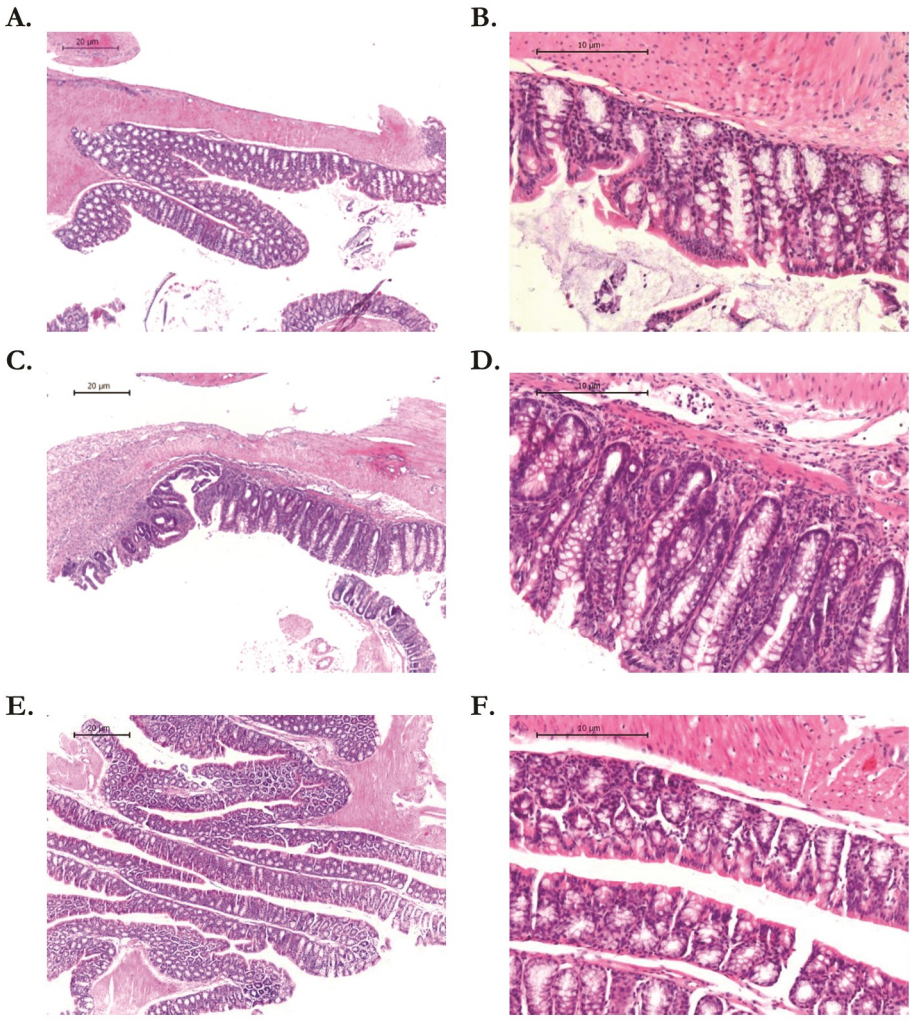

Figure 4. Hematoxylin and eosin-stained paraffin section from colon tissue.
A, B: Healthy control mice; **C, D**: Diseased control mice; **E, F**: AAV5 (CMV-Tregitope 167) pre-treated mice. Histological evidence that AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) ameliorates TNBS-induced pathology. Images depicted of an HE-stained paraffin section of a representative mouse colon from each group at the moment of sacrifice. The images of the diseased control (**C, D**) demonstrated acute inflammation: elongated villi, abundant transmural cellular infiltrate, erosions and crypt loss as compared to both the AAV5 (CMV-Tregitope 167) pre-treated and healthy control mice. TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.

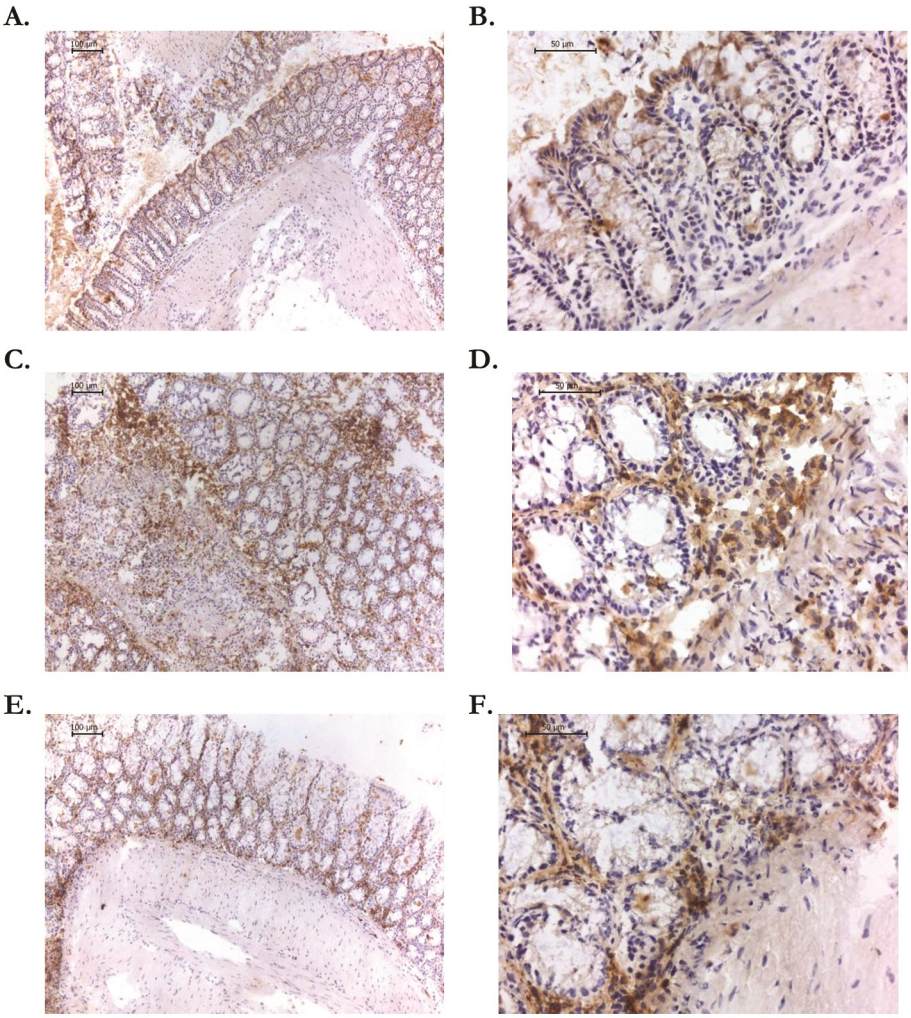

Figure 5. Immunohistochemistry pictures depicting CD4 staining colon tissue.
A, **B**: Healthy control mice; **C**, **D**: Diseased control mice; **E**, **F**: AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) pre-treated mice. Specific immunohistochemical staining showed inflammatory cell infiltrates present in TNBS treated mice with or without administration with Tregitope 167 consisted of CD4 positive cells, localized in the sub-epithelial layer, in the lamina propria (**C-F**) and for the diseased control also in the muscular layer (**C**, **D**). Depicted are representative data from a single mouse. TNBS: Trinitrobenzene sulfonate; AAV: Adeno-associated virus.

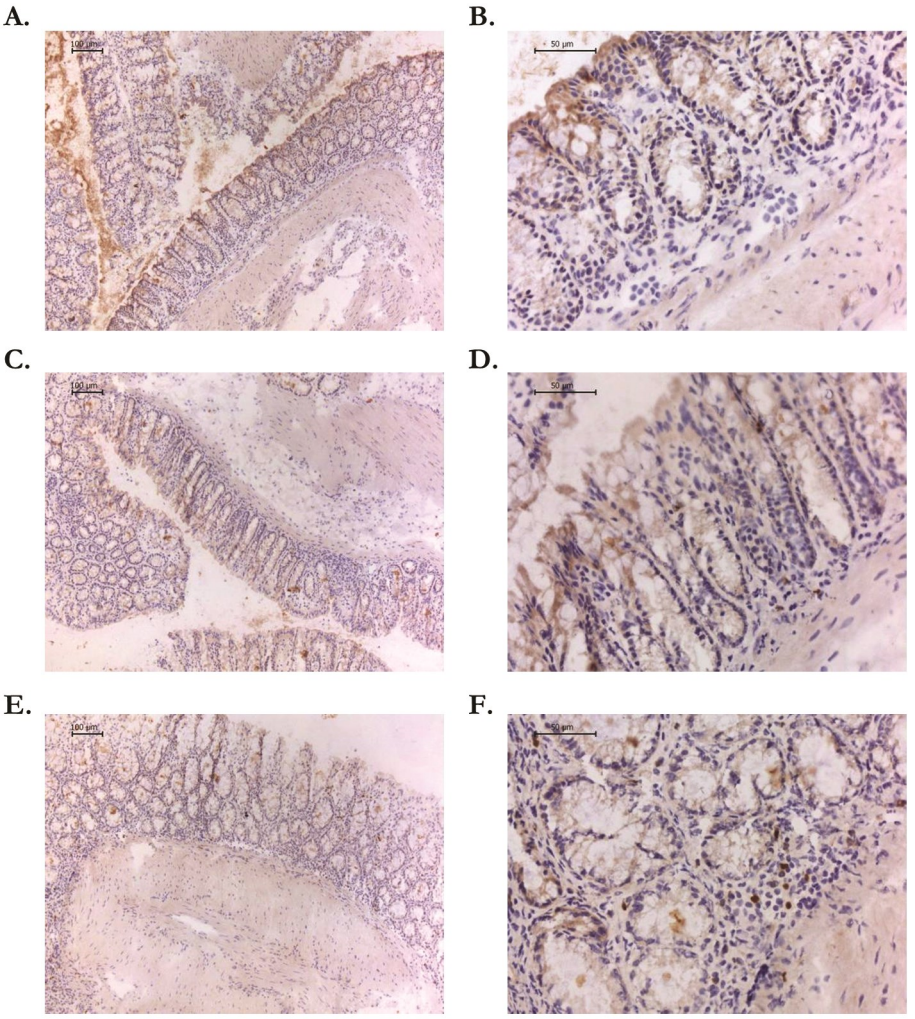

Figure 6. Immunohistochemistry pictures depicting Forkhead Box-P3 staining colon tissue. A, B: Healthy control mice; **C, D**: Diseased control mice; **E, F**: AAV5 (cytomegalovirus (CMV) promoter-Tregitope 167) pre-treated mice. Colon tissue were prepared and assessed by immunohistochemistry for the expression of the transcription factor Foxp3 as a marker for regulatory T cells. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (**E, F**). Foxp3 positive cells were rarely present in the colon of healthy and diseased control mice (**A-D**). Depicted are representative data from a single mouse. Foxp3: Forkhead Box-P3

Colon tissues were prepared and the presence of transcription factor Foxp3 was assessed by immunohistochemistry, so as to determine whether regulatory T cells were present in the peri-colonic infiltrates [33]. Numerous Foxp3 positive cells were detected in the lamina propria and sub-epithelium of the colon sections from mice treated with Tregitope 167 (**Figure 6**). Foxp3 positive cells were absent or sporadic in the colon of healthy and diseased control mice (**Figure 6**).

Thymus, mesenteric lymph nodes, liver and spleen tissue were collected at the time of sacrifice and the corresponding lymphocyte populations were further assessed by flow cytometry analysis for the expression of the CD4 T cell surface marker and the Treg cell associated markers Foxp3 [34], CD25 [35] and GARP [36, 37]. The Foxp3 and GARP markers were significantly increased in the $CD4^+$ T lymphocyte population in the thymus of the mice pre-treated with AAV5 (CMV-Tregitope 167), as compared to lymphocyte populations in the thymus of diseased and the healthy control mice (*P* value <0.05 and <0.001 respectively, **Figure 8**). $CD4^+$ thymic lymphocyte population (mean ± SD, 11% ± 2%, $n=6$) co-expressed Foxp3 and GARP in the thymus of AAV5 (CMV-Tregitope 167) pre-treated mice as compared to the thymic lymphocyte population of diseased (mean ± SD, 7% ± 3%, $n=6$) and the healthy control groups (mean ± SD, 6% ± 2%, $n=9$), respectively (**Figures 7**). Both the relative and absolute number of Foxp3 expressing T cells were expanded in the thymus after AAV5 (CMV-Tregitope 167) pre-treatment (**Figure 9**). No significant differences in the expression of Foxp3 and GARP in the lymphocyte populations of the mesenteric lymph nodes, liver and spleen were identified.

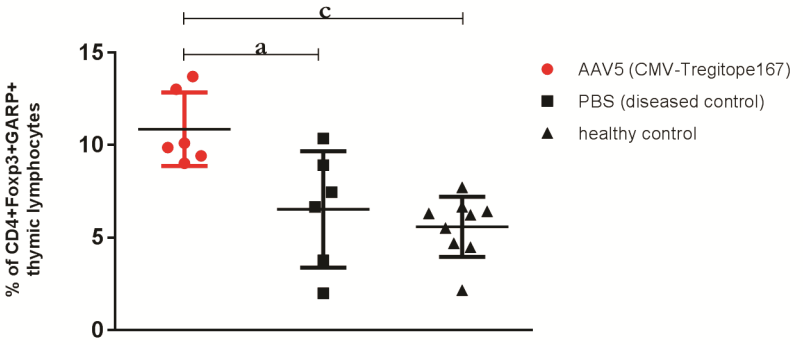

Figure 7. Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 induces Foxp3 and GARP expression in the CD4 positive thymic lymphocyte population. Thymus tissue was collected upon sacrifice. Cells suspensions were prepared and stained for the following markers: CD4, GARP and Foxp3 before analysis by flow cytometry (FACSCalibur, BD Biosciences). Depicted are percentages of CD4 positive, Foxp3 positive and GARP positive thymic lymphocytes. Individual mice are depicted; for the AAV5 (CMV-Tregitope 167) pre-treated group one mouse did not have a thymus and therefore n=6 mice were included in this analysis. The data were analyzed using a 1 way ANOVA, followed by Dunn's post hoc test for multiple comparisons. Data are presented as means +/- SD of all the mice. *P* value <0.05 (a), *P* value, <0.01 (b), *P* value <0.001 (c) *vs* PBS (diseased control group) or healthy control.

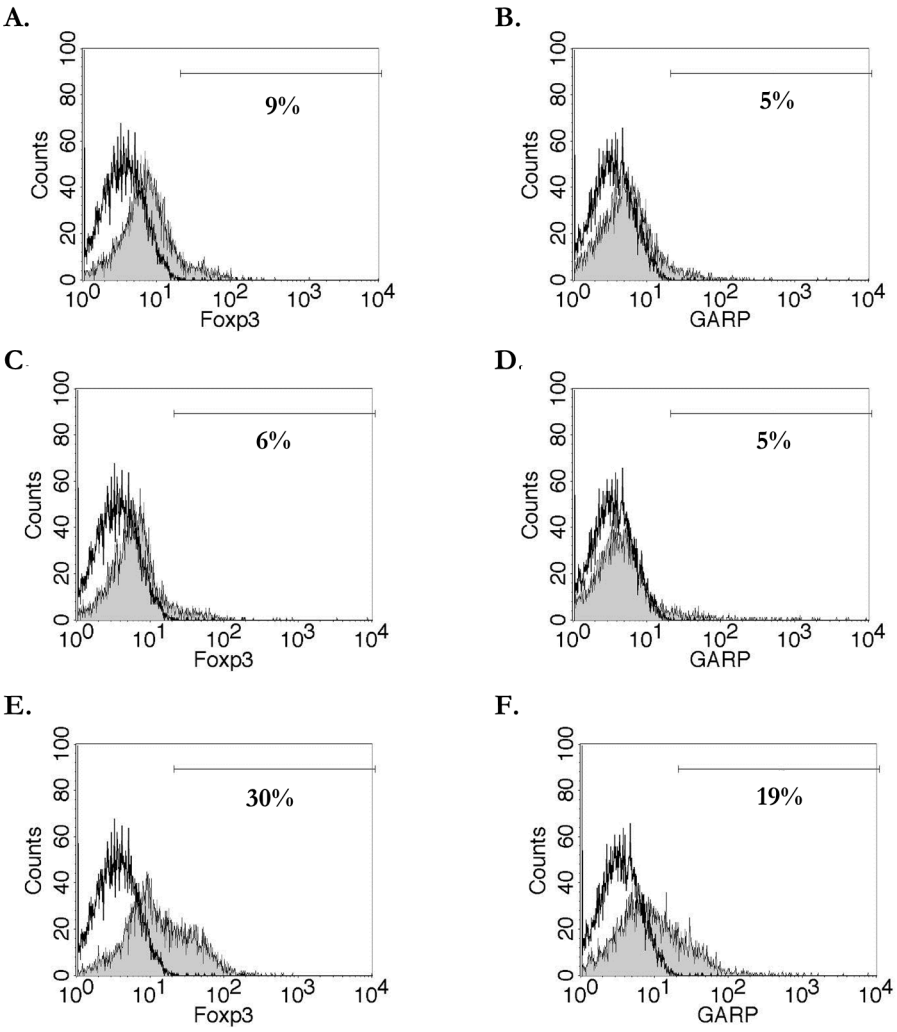

Figure 8. Adeno associated virus serotype 5 mediated delivery of regulatory T-cell epitope 167 induces Forkhead Box-P3 and GARP expression in the CD4 positive thymic lymphocyte population. Thymus tissue was collected upon sacrifice. Cells suspensions were prepared and stained for the following markers: CD4, GARP and Foxp3 before analysis by flow cytometry (FACSCalibur, BD Biosciences). A: Histogram Foxp3 cell count: healthy control; C: Histogram Foxp3 cell count: diseased control; E: Histogram Foxp3 cell count: AAV5 (CMV-Tregitope 167) pre-treated group (grey, filled in) versus unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse; B: Histogram GARP cell count: healthy control; D: Histogram GARP cell count: diseased control; F: Histogram GARP cell count: AAV5 (CMV-Tregitope 167) pre-treated group (grey, filled in) versus unstained control (black continuous line). Gating was done on the CD4 positive thymic lymphocyte population. Depicted are representative data from a single mouse.

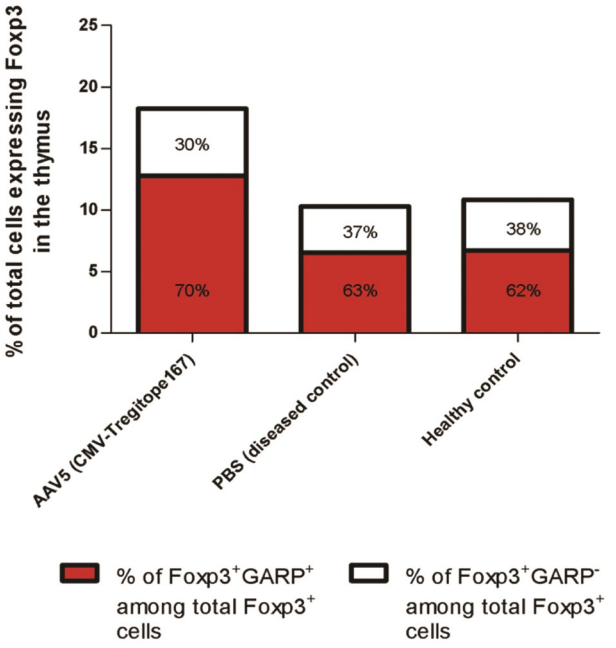

Figure 9. Percentage of Foxp3 expressing T cells in the thymus. The percentage of Foxp3 expressing cells in the thymus was higher when in the group that was pre-treated with AAV5(CMV-Tregitope).

Discussion

Curative treatment approaches for Crohn's disease and ulcerative colitis represent a significant unmet medical need. Regulatory T (Treg) cells are key players in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation [33, 38]. Therefore, novel strategies that aim for therapeutic tolerance induction and leverage Treg cells are currently being explored [39]. In the present study, the potential for AAV-mediated delivery of an immunomodulatory peptide (Tregitope 167) was investigated.

In this study, we demonstrate that the systemic AAV-based delivery of Tregitope 167 has the potential to prevent the development of fulminant colitis in a TNBS-induced model of IBD. Tregitope 167 was used in our study as its binding affinity for the MHC molecule in Balb/c mice is superior to Tregitope 289 (De Groot, manuscript submitted for publication). The significant de-

crease of colonic inflammation in the Tregitope 167 pre-treated mice was reflected in an overall weight gain and substantiated by a decreased disease activity index, colon weight and histology damage score at the time of sacrifice. Additionally, there was less mucosal inflammation in the AAV (CMV-Tregitope 167) pre-treated mice. This therapeutic benefit corresponded with increases in the relative number of T cells expressing regulatory T cell markers in the colon tissues and among thymic lymphocytes of the Tregitope 167-treated mice.

IBD patients do not present defects in regulatory T cell function or phenotype [40-42] and by consequence are more likely to benefit from therapies aiming at inducing and expanding Treg cells than patients affected by other autoimmune diseases. Tregitope 167 has the potential to both activate nTreg cells and induce iTreg cells [15, 16] and may be suitable as a novel therapeutic agent for IBD. However, the use of immunomodulatory peptides in clinical applications for IBD has been hindered by difficulties associated with the systemic delivery of the therapeutic peptides in sufficient quantity and concentration to the target tissues [17-19]. AAV has proven to be both effective and safe as a gene therapy delivery vector in the clinic [20-22]. Therefore, AAV-mediated delivery of Tregitopes was explored in this study. The AAV serotype 5 (AAV5) was used since pre-existing immunity to AAV5 in humans has been shown to be low [28, 43]. Our data demonstrate that AAV5-mediated delivery may be an efficient approach for stable administration of Tregitopes *in vivo*. Further studies will need to be performed to determine the duration of the immunological tolerance that is evoked by induction and activation of Treg cells.

Regulatory T (Treg) cells are considered to be essential in the counter-regulation of inflammatory reactions and Foxp3 is considered as a marker of the regulatory phenotype [34, 44, 45]. Staining for Foxp3 in mice pre-treated with Tregitope 167 revealed the presence of Foxp3 positive cells in the lamina propria and sub-epithelium of the colon sections. Additionally, expression of both Foxp3 and GARP were increased in the thymic $CD4^+$ T lymphocyte population in mice pre-treated with AAV5 (CMV-Tregitope 167), indicating an increase in activated regulatory T-cells [34, 36, 37, 44, 45]. The presence of

higher numbers of activated regulatory T cells corresponded with the prevention of fulminant intestinal inflammation *in vivo* in this TNBS model of IBD.

No increase in the Treg cell populations was observed in the mesenteric lymph nodes, liver and spleen in the current study. We hypothesize that this could be due to the duration of the experiment and the timing of the Treg cell evaluation. In other mouse models such as the model of spontaneous encephalomyelitis, the *de novo* generation of Treg cells was initiated intrathymically, and was followed by Foxp3 induction in peripheral tissue at later time points [46].

Tregitopes are T cell epitopes naturally located in immunoglobulins that bind to multiple MHC Class II alleles and induce Treg cell responses. We have demonstrated that antigen presenting cells (APCs) present Tregitopes to nTreg cells, engage feedback mechanisms promoting a tolerogenic APC phenotype, induce Treg cell expansion, and modulate antigen-specific effector T cell responses (Cousens and De Groot, manuscript submitted for publication). Proportions of APC expressing MHC II, CD80, and CD86 are suppressed, consistent with reported effects of Intravenous immunoglobulin (IVIG) [47] and of the immunoglobulin (Ig) G-derived peptide hCDR1 [48]. The basic mechanism of Tregitope tolerance induction is currently proposed to be as follows: 1) APC present Tregitopes to nTreg cells, 2) nTreg cells are activated to proliferate, 3) nTreg cells provide tolerogenic feedback signals to APC, modulating the APC phenotype, and 4) nTreg cells and tolerogenic APC together suppress antigen-specific T cell responses (Cousens and De Groot, manuscript submitted for publication).

A limitation of the colitis model used in this study was the acute necrotizing enterocolitis, occurring in the first 3 days after the first TNBS treatment, a presentation of disease which is unrelated to IBD [25]. Therefore the surviving number of mice, included in the analysis was lower than anticipated, which, for some analysis, conflicted with statistical analysis of the data. As a consequence, a large variability in the colon mucosa cytokines levels was observed after TNBS induced experimental colitis and prevented an accurate analysis of those parameters. Therefore, further development of AAV mediat-

ed delivery of Tregitope 167 in different experimental models of inflammatory disease will be necessary to confirm the obtained results.

In summary, our data provide preliminary evidence supporting the potential use of AAV-based Tregitope delivery as a therapy for the treatment of IBD. Further investigations will permit to define the mechanism by which Tregitope exert their immune regulatory properties, the duration of the effect, the ability of Tregitopes to reduce disease that has already been established and their eventual impact on systemic immunity.

Overall, this study identifies AAV-mediated delivery of regulatory T cell epitope 167 as a novel anti-inflammatory approach with the capacity to decrease intestinal inflammation and induce long-term remission in IBD.

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Part II

Chapter 5

Introduction Part II

Overcoming AAV immunogenicity

I AAV vectors

Adeno-associated (AAV) vectors are used to achieve therapeutic gene delivery for the treatment of genetic and chronic disorders. Recombinant AAV vectors contain an expression cassette with a transgene of choice flanked by two non-coding viral inverted terminal repeats (ITR's) enclosed in a capsid which is composed of three proteins: VP1, VP2 and VP3. When AAV enters the cell, its genome is converted into double-stranded, transcriptionally active DNA, which predominantly persists in a non-integrated episomal form [1, 2]. These vectors efficiently transduce a wide variety of tissues and can provide the long term expression of the delivered gene after a single administration. Importantly, AAV vectors have not been associated with any pathology in humans and are replication-defective. AAV-based gene delivery has been successfully employed in treatment of genetic disorders in preclinical studies as well as in clinical trials [3-6]. The clinical studies included hundreds of patients and indicate an excellent safety record of AAV vectors for gene therapy in humans. The different safety aspects of AAV for the use in humans have recently been summarized elsewhere [7]. Immune responses have been assessed in clinical trials by measuring systemic and local cytotoxic reactions as well as neutralizing antibodies (NAB) against AAV and/or the expressed therapeutic protein [3, 5, 8-17]. The immunogenicity data reported so far show that immune responses against AAV capsid proteins can vary and are influenced by the target organ, route of delivery and dosing schedule.

II Cellular immune responses against AAV vectors

The first observation of a cellular immune response induced by AAV gene therapy occurred in patients with Hemophilia B who were treated with an AAV2 vector to deliver human coagulation factor IX [14, 18]. In this study, a cell mediated immune response to AAV2 capsid was reported, which was measured in parallel with a loss of transgene expression. Similar observations were reported in a more recent clinical study with AAV serotype 8 for FIX

delivery to the liver of Hemophilia B patients, when two patients receiving the highest vector dose required a short course of glucocorticoids which normalized serum aminotransferase levels and prevented the loss of transgene expression [6]. Whereas in patients with Hemophilia B a correlation between the induction of a $CD8^+$ T cell response towards the AAV capsid proteins and a loss of transgene expression was observed, this does not seem to be an issue in case of the intramuscular AAV vector delivery. In a clinical study in patients with α-1 antitrypsin deficiency in which the gene for α-1 antitrypsin was delivered by an AAV1 capsid, cellular immune responses were found against the capsid proteins from day 14 on in all subjects. However, the functional activities of those T cells, as well as the biological effects thereof are not clear since the expression of the transgene was sustained at sub-therapeutic levels in all subjects. These data suggest that the cellular immune responses to the AAV capsid did not eliminate the transduced cells [8]. Similarly, systemic and local cellular immune responses induced by intramuscular injection of alipogene tiparvovec did not impact on clinical efficacy and safety [4].

III Managing humoral immune responses against AAV capsid

A major challenge for successful tissue targeting of AAV vectors in patients is the presence of circulating neutralizing antibodies (NAB) against AAV vector capsid. There is little knowledge about the antigenic structures of AAV capsids and how exactly they interact with the antibodies that are raised against them. Nevertheless, antigenic epitopes have been identified and described for AAV capsids of serotypes 2 and 8 [19-21].

NAB can be present in the patient's blood prior to therapy due to naturally acquired infections with the wild type AAV virus (pre-existing NAB) pre-existing NAB against AAV are currently an exclusion criteria for participation in clinical trials that use AAV vectors. This is a major problem, because, depending on the AAV serotype, the reported prevalence of serotype-specific

pre-existing anti-AAV NAB in humans is considerable and there is a significant difference between AAV serotypes (**Figure 1**) [22].

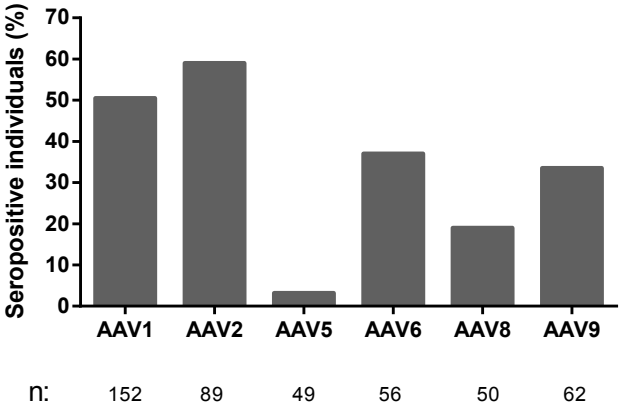

Figure 1. Adapted from Boutin et al. (Human Gene Therapy 2010). Prevalence of neutralizing factors in serum against AAV types 1, 2, 5, 6, 8 and 9, n indicates the number of serum samples tested.

Anti-AAV NAB are also formed after first administration of AAV vector gene therapy. Those NAB may reach high titers and have been reported to persist after treatment.

Although AAV-mediated expression generally is long-lived, re-administration may be necessary, during the lifetime of the patient, the naturally occurring turnover of cells might lead to loss of AAV transduced cells that episomally express DNA encoding the therapeutic protein, resulting in a decrease of the production of therapeutic protein. Repeated AAV treatment might also be needed if the initial treatment does not result in expression of sufficient therapeutic protein. In both cases, re-administration of the same AAV is thought not to be possible due to the presence of circulating NAB formed after the first administration. Those antibodies totally block the transduction and no efficacy of the treatment can be observed [1, 23].

In order to address these issues, numerous approaches have been explored to overcome or circumvent humoral immune responses directed at AAV vectors.

A. Cross administration of different natural AAV serotypes

Twelve AAV serotypes have been identified to date in humans and over 100 serotypes in nonhuman primates [24]. All serotypes studied present antigens that induce production of neutralizing antibodies (NAB). A potential strategy to avoid interference of NAB with primary or secondary AAV-based gene delivery is the use of AAV vector serotypes with different antibody reactivity profiles but similar affinity for the target tissue. When choosing AAV vector serotypes for such "cross-administration" approaches two pivotal aspects should be carefully considered.

Firstly, as would be predicted, homology between AAV serotypes might influence the possibility of NAB cross-reactivity [22, 25, 26]. Indeed, for closely related AAV serotypes 1 and 6 in *in vitro* studies, cross –neutralization of NAB from those serotypes was observed. AAV4, on the other hand, which is a divergent serotype, was only neutralized by the anti-AAV4 NAB from the spectrum of anti-AAV1-6 NAB, while AAV5 serotype showed to be inhibited by anti-AAV1, 2, 3, 5, and 6 NAB but not by anti-AAV4 NAB [26]. Additionally AAV7 and AAV8 have minimal cross-reactivity to other serotypes.

Secondly, different AAV serotypes have divergent tissue tropisms and different transduction efficiencies [27]. As a consequence, depending on the target tissue, the choice of alternative AAV serotypes might be limited.

Cross-administration approaches have proven to be successful in non-human primates, when AAV2/5 vector was administered intravenously in animal that had pre-existing anti-AAV8 NAB. In contrast, AAV8 vector administration in an animal with pre-existing anti-AAV8 NAB did not result in therapeutic protein expression [28]. Effective cross-administration strategy *in vivo* without a significant cross-inhibitory effect was also observed after cross-administration of AAV1, 2 and 5 serotypes in mouse skeletal muscle [29].

Although this approach has proven to be successful in the research setting, it should be noted that changing serotypes, also changes the drug from regulatory point of view and this would necessitate separate costly drug approval procedures for every serotype used.

B. Genetic AAV capsid

Extensive efforts have been directed towards the development of new artificial, less immunogenic AAV capsid variants. Conceptually, genetic modification of the regions that contain immunogenic AAV capsid epitopes could be an approach to escape pre-existing NAB binding and neutralization of modified AAV capsid. One of such strategies involves targeted modifications of identified antigenic regions of AAV capsid [21]. Such rational design was for instance investigated by Huttner at al. who demonstrated that insertion of different ligands at position 587 in AAV2 VP1 protein allow the transduction of cells in the presence of anti-AAV2 NAB [30]. Another group successfully employed a directed evolution strategy to generate large AAV2 mutant capsid library followed by high-throughput selection process to isolate mutants which evade NAB formation [31].

Even though the AAV capsid engineering methods are promising, none of the currently identified AAV capsid mutants are completely resistant to NAB and accumulated mutations in AAV capsid may negatively affect efficiency of gene delivery. For example, AAV vector production, purification, stability, infectivity and tissue tropism might be affected. Finally, the immunogenicity of the new AAV capsids should be carefully investigated to assess whether they would be suitable for re-administration purposes.

C. Chemical modifications of the AAV capsid

Chemical polymers are used for steric stabilization of drug carrier systems such as lipoplexes, nanoparticles, and liposomes. Chemical polymers are coating materials that form a protective hydrophilic layer that limits the interaction with blood components. Coated nanoparticles are not efficiently absorbed by macrophages and therefore show reduced immune responses. The best characterized protein stabilizer material is polyethylene glycol (PEG) and its use has been approved in several therapeutic products. PEG is generally non-toxic and it extends the half-life of proteins with a reduction of immune responses. The attachment of chemical ligands to AAV capsid could potentially protect the AAV capsid from binding of NAB. Several groups have tested AAV capsid coating with PEG and other polymers [32-34] but this has resulted in a

moderate protection of AAV capsid against NAB at best, and in some cases the modification caused impaired AAV infectivity [34].

Hence, chemical conjugation of polymers to AAV capsids remains challenging and needs to be further developed. The current technology is not satisfactory as it does not fully prevent immunogenicity and may cause a loss of AAV infectivity.

D. Physical removal of circulating anti-AAV NAB

Plasmapheresis is an extracorporeal blood component separation technique which is currently used in the clinic to remove high-molecular-weight substances such as autoantibodies, immune complexes, cryoglobulins, and bacterial lipopolysaccharides [35]. Plasmapheresis may be used to remove antibodies from the bloodstream, thereby preventing them from binding to their targets. Due to the fact that plasmapheresis does not interfere with antibody production, the therapeutic effect generally is only transient. Plasmapheresis was used as a strategy to lower anti-AAV NAB, and different groups have reported promising results. Nonetheless, plasmapheresis by itself seems to be an option only for patients that have relatively low anti-AAV NAB titers [36, 37].

Physical contact between anti-AAV NAB in blood and injected AAV vector can be also prevented by specific delivery technologies. For delivery of AAV vectors to the liver in individuals with NAB, specific portal vein injection strategies were developed. The first approach involves portal vein branch flushing with saline that is directly followed with AAV8 vector injection. The second method is based on injection of the vector into the portal vein with the use of balloon catheter. Both of the methods proved to be similarly effective, but second is considered to be safer [38, 39]. This approach needs to be further investigated in order to determine the possibility of its application when high titers of NAB are present.

E. Immunosuppression

Immune suppression (IS) is used in the clinical practice to reduce or prevent immune response in organ transplantation and to treat autoimmune diseases. IS regimens are based on combinations of drugs such as glucocorticoids, anti-

proliferative and antimetabolite agents, calcineurin inhibitors, rapamycin inhibitors, and immune cell depleting or nondepleting monoclonal antibodies. Immunosuppressive drugs have many side effects and treatment with them increases susceptibility to opportunistic infections or chance of cancer occurrence.

For reduction of anti-AAV NAB titers B cell targeting drugs, as bortezomib or anti-CD20 monoclonal antibody have been investigated. Bortezomib, which is a proteasome inhibitor that is approved for the treatment of multiple myeloma, can eliminate both short- and long-lived plasma cells by activation of the terminal unfolded protein response [40]. Unfortunately the bortezomib-induced reduction in the levels of anti-AAV NAB was not sufficient to allow for re-administration of AAV vector. This limitation was related to residual anti-AAV NAB levels and bortezomib's inability to completely deplete memory B cells that are re-activated upon AAV vector re-administration (Karman 2010). The use of anti-CD20 antibody (rituximab) alone [41] or in combination with cyclosporine A [42] also showed only partial efficacy in lowering the anti-AAV NAB titer.

Novel IS approaches can specifically target $CD4^+$ T cells. Targeting of $CD4^+$ T cells in mice with non-depleting anti-CD4 antibody and cyclosporine A at the time of AAV delivery resulted in inhibition of the primary induction of anti-AAV NAB and it allowed for efficient re-administration of the same AAV serotype [43]. This IS strategy has to be further investigated for efficacy and safety in non-human primates before possible clinical application in AAV-based gene therapy. It also remains unknown whether this approach will prove to be effective in case of already established anti-AAV NAB titers (pre-existing anti-AAV NAB) which at the moment exclude many patients from AAV-based gene therapy.

F. Adsorption of NAB against AAV capsid

The principle of antibody adsorption was first demonstrated in gene therapy studies with adenovirus studies in which antibodies directed against adenoviral capsid were depleted from rabbit serum with the use of chromatography columns containing bound capsid proteins [44]. Such specific antibody depletion

proved to facilitate the transduction with adenovirus [45]. A similar principle of antibody adsorption was also studied in AAV-based gene delivery in mice by Scallan et al. who demonstrated that the presence of empty AAV capsids, which acted as decoys, significantly reduced the neutralization of AAV by anti -AAV NAB [46]. Mingozzi et al. have recently shown in mice and non-human primates that injection of therapeutic AAV vector together with empty AAV capsids allows liver transduction in the presence of even high titers of anti-AAV NAB. An additional factor in their experimental setup was the use of a mutant empty AAV capsid that cannot enter target cells but can adsorb anti-AAV NAB [47].

IV Handling cellular and humoral immune responses against transgene product

Next to the immune responses against the AAV capsid protein, cellular and humoral immune responses can be induced against the protein encoded by the transgene and it can cause a limited efficacy of the treatment [48]. The development of specific immune responses directed against the transgene product has been shown to be highly dependent on route of administration, AAV vector serotype, AAV vector dose, tissue specificity of the promoter and clinical profile of the patient.

Even though, promising pre-clinical experimental data have been obtained when targeting the liver [49-54], immune responses against the transgene product were reported in case of intramuscular AAV-based gene delivery which resulted in a limited efficacy of the treatment [55-57]. 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 pre-clinical animal studies [58, 57]. Similarly, in the clinical trial for treatment of Duchenne's muscular dystrophy study in which children were injected intramuscularly with AAV-minidystrophin, immune responses against minidystrophin were observed and expression of the transgene product was lost over time [59, 60].

To circumvent the appearance of immune responses against transgene product several approaches can be considered.

A. Immune suppression

Clinical strategies to avoid immune responses directed at expressed proteins include the use of IS regimens. This approach proved to be efficient in pre-clinical studies using intramuscular AAV gene delivery. For instance, treatment with cyclophosphamide at the time of AAV administration and subsequently twice a week for next 6 weeks resulted in a sustained partial correction of haemophilia B in a null mutation dog model [61]. Another promising outcome was obtained when using immunosuppression with a combination of anti-thymocyte globulin (ATG), cyclosporine (CSP) and mycophenolate mofetil (MMF) which resulted in long-term expression of canine micro-dystrophin in skeletal muscle after intramuscular AAV-based delivery into canine X-linked muscular dystrophy model.

Although immune suppression may have some efficacy, in order to ensure long-term durability of protein expression, immune-modulating therapeutic approaches should facilitate tolerance induction towards the transgene product. Importantly, some IS regimens interfere with the induction or maintenance of tolerance towards the transgene. For example, it has been reported that administration of MMF, sirolimus and daclizumab resulted in a decrease of the Treg population which correlated with formation of inhibitory antibodies against human FIX while none of those was observed when the 2-drug IS consisting of MMF and sirolimus was used [62].

Many different IS regimen combinations for attenuation of transgene-directed immunity in AAV-based gene therapy have been tested in pre-clinical animal models with quite promising results [42, 43, 63, 64]. However, some IS drugs have significant side-effects and lack antigen-specificity [65], and it is desirable to be able to exclude those drugs from future IS approaches in gene therapy.

B. Alternative AAV vector delivery methods

An approach that has been recently developed and might be an attractive alternative to IS regimens when targeting the muscle with AAV-based gene de-

livery are regional intravenous (RI) deliveries instead of intramuscular (IM) injections. Toromanoff et al. have demonstrated that IM injections of AAV in non-human primates are frequently associated with presence of inflammatory infiltrates and the destruction of transduced myofibers necessitating the use of IS. In contrast, RI AAV delivery resulted in a stable transgene expression without immunosuppressive treatment. It seems that the local vector distribution in the skeletal muscle is a key factor that triggers the immunogenicity after IM delivery, and this route of administration results in a high number of vector genome copies per cell. On the other hand, RI route of AAV delivery resulted in homogenous and wide spread vector distribution and lower vector genome copies per cell [66]. Arruda et al. have demonstrated similar results in canine skeletal muscle for FIX after RI delivery by AAV2 vector [67].

C. Tissue-specific promoter use

Avoiding the overexpression of the transgene product in non-target tissues and especially in antigen presenting cells (APCs) is a very important method to avoid transgene directed immune responses. Tissue-specific promoters have been demonstrated to have an important role in reducing the immune responses to the transgene products in pre-clinical animal models, especially in muscle directed AAV-based gene therapy for muscular dystrophy [68, 69].

D. B-cell mediated tolerance induction

B lymphocytes can act as antigen presenting cells (APCs) and via this pathway they may induce of antigen-specific tolerance [70, 71]. Genetically modified B cells have proven to be excellent tolerogenic APCs in several animal models. Expression of peptide-immunoglobulin fusion proteins by B cells can induce tolerance towards the fused peptide and this results in reduced cellular and humoral immune responses against that peptide upon immunization in CFA [72-74]. Therefore, transgene product specific tolerance induction using autologous B-cell gene therapy could be a potential strategy to avoid transgene-directed immune responses in AAV-based gene therapy.

E. Exploiting the endogenous microRNA machinery for regulation of transgene-directed immune responses

MicroRNAs are small non-coding RNAs that are endogenously expressed in a tissue-specific manner and play an important role in maintaining their functions and differentiation [75, 76]. They are also important in innate and adaptive immunity as they control differentiation of various immune cell subsets and their functions [77-79].

MicroRNAs are capable of post-transcriptional regulation of gene expression when they bind to their specific target sequence. Modification of viral vector cassettes with microRNA targets is the latest pursuit to regulate gene expression in gene therapy. A recently explored strategy to avoid immune responses against transgene products in gene therapy took advantage of the activity of mir-142-3p, which is a miRNA specifically expressed in antigen presenting cells (APCs). Incorporation of mir-142-3p target sequences within a transgene sequence has been shown to mediate inhibition of transgene expression in haematopoietic lineage cells, including APCs in both *in vitro* and *in vivo* setup [80]. The use of mir-142-3p target sequences prevented immune responses towards the transgene product in mice when a lentiviral vector was used for gene delivery targeting the liver [81, 82]. Additionally, our group provided evidence that humoral and cellular immune responses against the transgene product can be efficiently reduced by use of mir-142-3p target sequences in AAV-based intramuscular gene delivery [83]. Boisgerault et al. also applied that strategy in intramuscular AAV-based gene delivery settings and confirmed our findings [84].

V Concluding remarks

Significant progress has been made in the use of AAV vectors in human gene therapy and the first AAV vector product for treatment of lipoprotein lipase deficiency received market authorization in Europe [85, 86]. Immune responses observed during AAV gene therapy trials in humans do not appear to be a safety risk. However, for future indications, host immune responses against AAV vector capsid and the transgene product will need to be more effectively addressed. There is a need for the development of safe and effi-

cient strategies that would allow treatment of patients with pre-existing NAB against AAV capsids, re-administration of AAV vectors in case transgene expression is lost due to natural turnover of the transfected cells and that prevent or treat possible immune responses against the transgene product.

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Chapter 6

Successful re-administration of adeno-associated virus (AAV) vectors: cross administration of AAV serotypes 5 and 1

Anna Majowicz and Valerie Ferreira

Introduction

Adeno-associated virus (AAV) vector based gene therapy has proven to be very promising for the treatment of numerous genetic and inflammatory disorders [1, 2]. It has to be considered that re-administration of the therapy might be necessary due to the possibility of decrease of AAV-mediated gene expression overtime as a result of the natural turnover of transduced cells. This point is particularly valid when considering the treatment of life-long diseases.

The major challenge to achieve a successful re-administration is the presence of circulating neutralizing antibodies (NAB) directed against AAV capsids which are developed after the first administration of AAV vectors. Those neutralizing antibodies that recognize viral capsid proteins do not allow repeated gene transfer with AAV of the same serotype [3, 4, 5] Therefore, strategies that would permit a repeated gene delivery need to be developed.

In order to avoid this problem different AAV vector serotype with different antibody reactivity profiles and similar affinity to the target tissue could be used for the re-administration. Such serotype switching strategy is referred to as cross administration. Cross administration of AAV serotypes for the delivery of therapeutic protein has a great advantage over the alternative approach that involves use of immunosuppressive regimens. The immunosuppression strategy can lead to serious side effects and might not be applicable in all clinical settings depending on patient treatment history and health condition. It has been reported that AAV serotypes 1 and 5 could be used sequentially for re-administration in the muscle as no significant inhibitory cross-reaction were reported *in vivo* in muscle and *in vitro* in hepatic cell lines [3, 6]. In the present study we explored *in vivo* the efficacy of sequential intravenous administration of AAV serotype 5 and 1 for AAV re-administration in the liver.

Material and Methods

Ethics statement

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

AAV production

The AAV vectors batches (AAV5-hAAT-SEAP, AAV5-LP1-hFIX, AAV1-hAAT-eGFP and AAV1-LP1-hFIX) were produced in insect cells according to a technology adapted from R. M. Kotin [7]. The AAV vector 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.

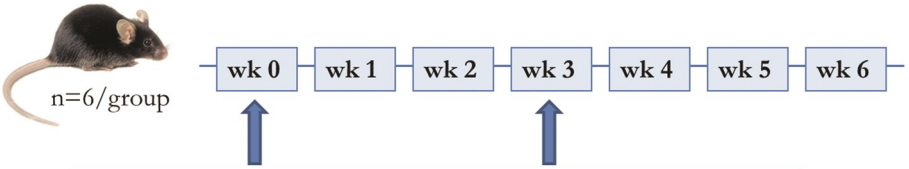
Animal Experiments

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

In the first experiment mice (n=6/group) were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV1-hAAT-eGFP or PBS was performed at week 3 (**Figure 1**).

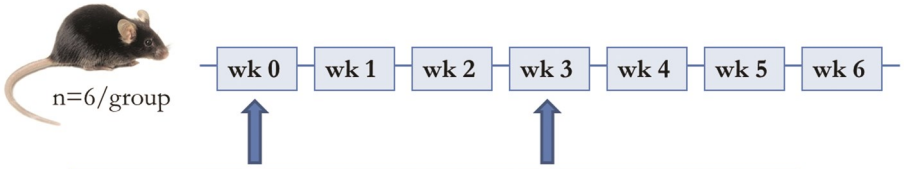
In the second experiment mice (n=6/group) were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV5-LP1-hFIX or PBS was performed at week 3 (**Figure 2**). The dose of all the AAV batches that were injected was 1.46×10^{13}gc/kg and injection volume was 10 µl/g.

For both experiments, blood was collected weekly by submandibular vein puncture in tubes containing sodium citrate. Plasma was isolated after centrifugation for 20 min at 2500 g at 4°C and stored at -80°C until further analysis. All mice were sacrificed at week 7. Liver tissues were collected and snap frozen in liquid nitrogen with or without pre-fixation in picric acid and stored at -80°C until further analysis.



Groups	1st intravenous injection	2nd intravenous injection
1.	PBS	PBS
2.	PBS	AAV1-LP1-hFIX
3.	PBS	AAV1-hAAT-eGFP
4.	AAV5-hAAT-SEAP	PBS
5.	AAV5-hAAT-SEAP	AAV1-LP1-hFIX
6.	AAV5-hAAT-SEAP	AAV1-hAAT-eGFP

Figure 1.
Scheme of the first experimental setup; n=6/group.



Groups	1st intravenous injection	2nd intravenous injection
1.	PBS	PBS
2.	PBS	AAV1-LP1-hFIX
3.	PBS	AAV5-LP1-hFIX
4.	AAV5-hAAT-SEAP	PBS
5.	AAV5-hAAT-SEAP	AAV5-LP1-hFIX
6.	AAV5-hAAT-SEAP	AAV1-LP1-hFIX

Figure 2.
Scheme of the second experimental setup; n=6/group.

Assessment of transgenes expression

Human FIX expression was measured in plasma of mice with the use of FIX ELISA kit (VisuaLize™ FIX Antigen Kit, Affinity Biologicals™$_{INC}$).

SEAP expression was measured in mouse plasma with the use of chemiluminescent "SEAP Reporter Gene Assay" (Roche).

GFP expression was assessed by post mortem fluorescent microscopy of liver tissue sections of mouse livers that were fixed in picric acid upon harvesting.

Assessment of anti-AAV5 and anti-AAV1 antibody levels

Levels of anti-AAV5 antibody in mouse plasma was measured by an anti-AAV5 specific ELISA. Practically, MaxiSorp® flat-bottom 96-well plates (ThermoScientific) were coated with AAV5 capsid and anti-AAV5 antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO).

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 capsid and anti-AAV1 antibody level in samples was detected with 1:1000 rabbit anti-mouse Immunoglobulins/HRP (DAKO).

NAB assay against AAV5 and AAV1 capsid

HEK293T cells were seeded in 96-well plates (Corning) that were pre-coated with 0.25% poly L-lysine at a density of 1 x 10^5cells/well in 100 µl of DMEM with 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated overnight at 37°C in 5% CO_2 water jacket incubator. Medium was then removed and the following mix was added: AAV5-CMV-GFP for anti-AAV5 NAB assay or AAV1-CMV-GFP for anti-AAV5 NAB assay with heat-inactivated plasma sample in a total volume of 100 µl of DMEM without phenol red and 1% Penicillin/Streptomycin. The mix was incubated for 1 hour at 4°C prior to addition on the cells. Medium of the HEK293T cells was removed by aspiration, and then the mix was added and incubated for 16-20 h at 37°C. Serial dilutions of test plasma that were prepared were: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800. As a positive control, cells without plas-

ma addition in the mix were analyzed. After 16-20 h, cells were rinsed with PBS, collected after trypsinization and fixed in PBS, 2% Formaldehyde, 1% BSA. GFP expression of the cells was analyzed by flow cytometry (FACScalibur, Becton Dickinson) in channel FL1 at a wavelength of 530 nm. The analysis was performed with the Cellquest Pro software. The percentage of inhibition was calculated related to GFP expression measured in AAV HEK293T infected cells (no inhibition, 100% expression). Plasma dilutions causing a 50% reduction of GFP expression when compared to positive control, were determined (EC50 determination after sigmoidal curve fit in GraphPad Prism software).

Results

No cross reactivity of antibodies raised against AAV5 and AAV1 capsids was observed.

Mice were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV5 -LP1-hFIX or PBS was performed at week 3. In order to determine the total anti-AAV antibody levels and anti-AAV neutralizing antibody (NAB) levels plasma samples of injected animals were analyzed.

Total anti-AAV5 antibodies were detected in the plasma of all the mice that were injected with AAV5 vectors, the levels of antibodies increased over time until week 3 and remained stable until the end of the experiment (AAV5-hAAT-SEAP and AAV5-LP1-hFIX) (**Figure 3.A**). Similarly, all the mice that were injected with AAV1-LP1-hFIX developed antibodies against AAV1 with an increase until week 3 after injection was performed (**Figure 3.B**). As expected, anti-AAV1 antibodies were not detected in plasma of mice that were injected with AAV5-hAAT-SEAP alone, AAV5-LP1-hFIX alone or PBS (**Figure 3.B**) as well as anti-AAV5 antibodies were not detected in plasma of mice that were injected with AAV1-LP1-hFIX alone or PBS (**Figure 3.A**).

Overall, those results demonstrate the absence of cross reactivity between total AAV5 and AAV1 antibodies in our assay. In case of two sequential injections with AAV5-based vectors, a low level of recognition of AAV1 capsids

by anti-AAV5 antibodies was observed, which is probably due to general enhancement of immune system responses (**Figure 3.B**).

In order to determine the potential of the measured total antibodies to neutralize AAV transduction, neutralizing antibodies assays were performed for both AAV5 and AAV1 serotypes. The neutralizing antibody (NAB) titers against AAV5 capsid were similar in all the mice injected once with AAV5-hAAT-SEAP or AAV5-LP1-hFIX. The anti-AAV5 NAB titers were slightly higher in the animal group that was injected twice with AAV5 (AAV5-hAAT-SEAP followed by AAV5-LP1-hFIX) (**Figure 4.A**).

Similarly, neutralizing antibody (NAB) titer against AAV1 capsid raised in plasma of mice that were injected with AAV1-LP1-hFIX was measured. No NAB antibodies against AAV1 capsid were detected in the mice groups that were injected with AAV5-hAAT-SEAP alone, AAV5-LP1-hFIX alone or PBS (**Figure 4.B**).

Our Results demonstrate the absence of cross reactivity between NAB against AAV5 and AAV1 capsids.

Stable dual gene expression after sequential intravenous administration of AAV5- and AAV1-mediated gene delivery

Mice were injected intravenously (iv) with AAV5-hAAT-SEAP or PBS at week 0. The second intravenous administration with AAV1-LP1-hFIX, AAV1 -hAAT-eGFP, AAV5-LP1-hFIX or PBS was performed at week 3. In order to determine the expression of SEAP and hFIX protein, plasma of injected animals was collected weekly, while the eGFP was assessed by post mortem fluorescent microscopy of liver tissue sections.

SEAP expression was detected in the plasma of all the mice injected with AAV5-hAAT-SEAP (**Figure 5.A, 6.A**). This expression increased from week 0 to week 2 and was stable until sacrifice at week 7. The mice that received AAV1-LP1-hFIX in second injection at week 3 showed raising expression of hFIX protein in plasma from week 4 which stabilized at week 5 and remained stable until sacrifice. At the opposite, the mice which received AAV5-LP1-hFIX in second injection did not express detectable level of FIX in the plasma

(**Figure 5.B**). It should be noted that the expression level of hFIX protein that appeared after injection with AAV1-LP1-hFIX was not influenced by the prior injection with AAV5-hAAT-SEAP as the same level of FIX was measured in the control group that received PBS in first injection and AAV1-LP1-hFIX in second injection (**Figure 5.B.**).

The expression level of hFIX protein in plasma of mice injected with AAV5-LP1-hFIX was higher than in plasma of mice injected with AAV1-LP1-hFIX. This observation confirms previous publications reporting that AAV5 transduces the liver more efficiently than AAV1. However, the level of hFIX protein measured in the mouse plasma after delivery with AAV1 vector is above therapeutic level which indicates that AAV1 serotype could also be efficient for gene delivery in the liver.

Successful cross administration of AAV5 and AAV1 vector was also achieved with combination of others expression cassettes: AAV5-hAAT-SEAP and AAV1-hAAT-eGFP (week 3) when both SEAP (**Figure 6.A**) and eGFP (**Figure 6.B.III**) were expressed by the injected animals.

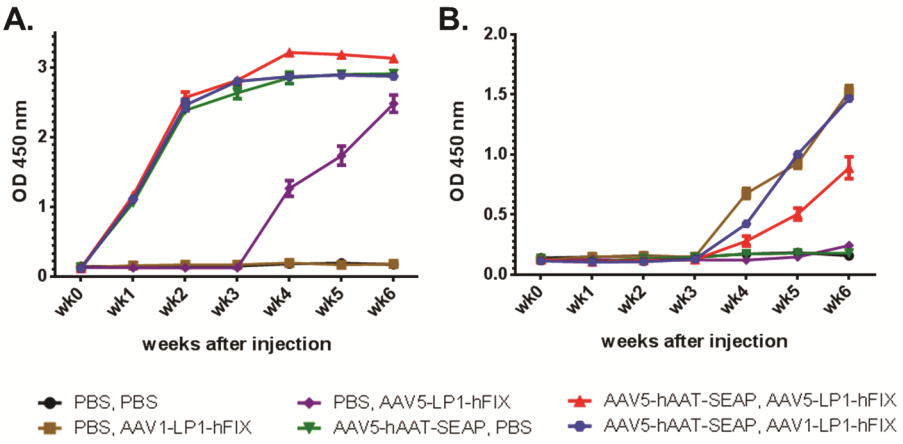


Figure 3. Total anti-AAV5 (A) and anti-AAV1 (B) antibody level in mouse plasma. Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

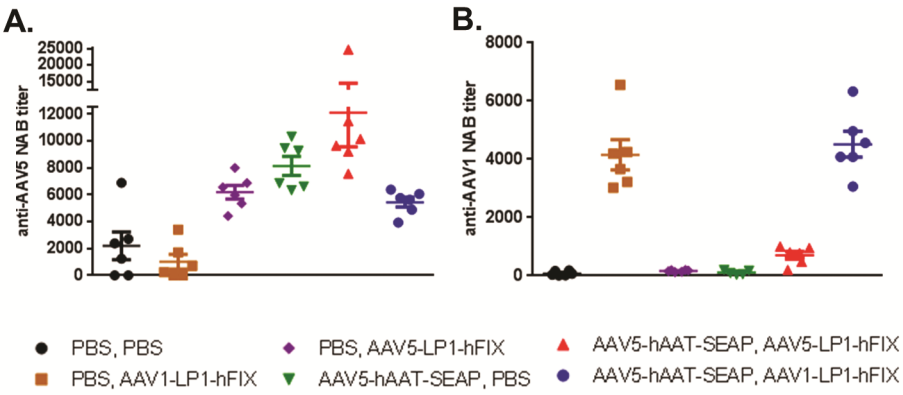


Figure 4. Anti-AAV5 (A) and anti-AAV1 (B) NAB level in mouse plasma at week 6. Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

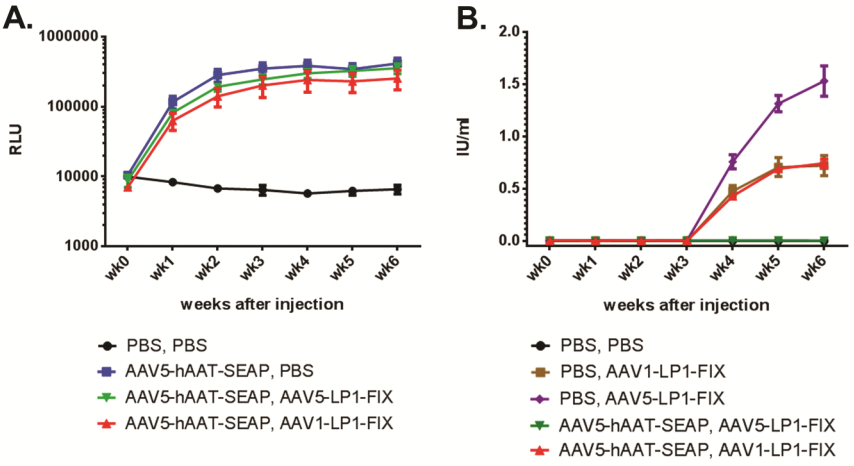


Figure 5. Expression of SEAP (A) and FIX protein (B) in mouse plasma overtime.
Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS, AAV1-LP1-FIX or AAV5-LP1-FIX at week 3; n=6/group.

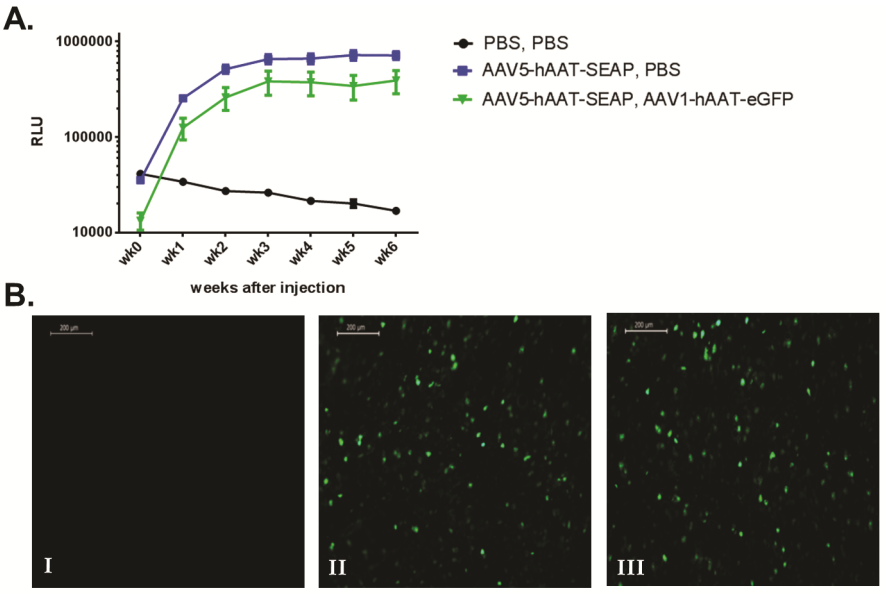


Figure 6. Expression of SEAP measured overtime in mouse plasma (A) and expression of eGFP in representative mouse liver sections (B).
Mice received intravenous injections of PBS or AAV5-hAAT-SEAP at week 0 and second intravenous injection of PBS or AAV1-hAAT-eGFP at week 3; n=6/group. B: **I**- PBS, PB group; **II**- PBS, AAV1-hAAT-eGFP group; **III**- AAV5-hAAT-SEAP, AAV1-hAAT-eGFP group; RLU- Relative Luminescence Units.

Discussion

The study presented in this chapter demonstrates the feasibility of re-administration of AAV-based vectors when using sequentially the serotypes 5 and 1 for liver targeting. We have shown that the anti-AAV neutralizing antibodies (NAB) were not cross reactive between the serotypes 5 and 1 and that a high level of gene expression was observed after sequential delivery of reporter genes from both AAV5 and AAV1 vectors. In contrast, the re-administration of the same serotype (AAV5) was unsuccessful due to the total inhibition of secondary AAV5 transduction by anti-AAV5 NAB. Our data confirms the previously reported *in vitro* study where the cross-reactivity and neutralization mechanisms between AAV1 and AAV5 serotypes were examined and no cross-reactivity between them was demonstrated [5].

The cross administration approach with AAV5 and AAV1 appears to be efficient for hepatic gene transfer as a high level of gene expression was measured for both reporter genes used (SEAP and hFIX). It should also be noted that despite the fact that hFIX protein expression level after delivery by the AAV1 serotype is lower than after delivery with AAV5 vector, it remains above the defined therapeutic level. All together, these data indicate that AAV1 serotype is also a good candidate for liver targeting. However, further evaluation of the efficiency of AAV5 and AAV1 cross administration for liver targeting is necessary and non-human primate studies need to be performed in order to evaluate the future clinical relevance of such approach. Despite the fact that this approach has proven to be successful and could be a good option for many patients, changing serotypes, also changes the drug from regulatory point of view and that implies separate costly drug approval pathways for every serotype used and that certainly is a limiting factor.

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Chapter 7

Use of immunosuppressive regimens to reduce humoral immunogenicity generated by primary AAV vector delivery

Anna Majowicz, Sander van der Marel and Valerie Ferreira

Abstract

Adeno-associated virus (AAV) vector-based gene therapy has proven to be effective in both clinical and pre-clinical settings. However, a successful re-administration of the therapy remains a challenge due to development of neutralizing antibodies (NAB) against the AAV capsid after the first administration. Therefore, exploration of strategies to minimize existing anti-AAV NAB to a level that would allow re-administration is of great interest.

In this study we evaluated the potential of a combined therapy of bortezomib and anti-CD20 monoclonal antibody, to reduce the pre-existing humoral immunity to AAV in mice.

The combination of bortezomib and anti-CD20 failed to reduce high anti-AAV1 NAB titer to a level that would permit re-administration of AAV1. In contrast, this immunosuppressive treatment prevented immune response against the immunogenic protein (ovalbumin) delivered as a transgene by AAV1.

Introduction

Recombinant adeno-associated viral vectors (AAVs) show great promise for gene therapy in a variety of different genetic disorders [1- 3]. AAV vectors have an excellent safety profile [4, 5] and were shown to mediate a stable therapeutic transgene expression in several non-clinical studies [1, 6-9] and more recently in clinical studies [10-13]. The majority of the transgenic proteins are expressed from episomal, double stranded DNA [14]. This characteristic raises the possibility that transgene expression will decline overtime with the natural turnover of transduced cells. Therefore, repeated administration may become necessary to maintain expression of the transgenic protein at therapeutic levels. The major challenge in a successful re-administration of AAV vectors, is the presence of neutralizing antibodies (NAB) that develop after the first administration. Those neutralizing serotype-specific antibodies directed towards the viral capsid proteins are reducing efficient gene transfer with rAAV of the same serotype [15, 16]. Therefore, the generation of a humoral immune response is limiting the use of the "vector of choice" more than once which is a potential concern for life–long chronic disorders for which re-administration might have to be considered. Hence, strategies to decrease existing anti-AAV NAB titers need to be explored.

Bortezomib, a selective inhibitor of the 26S proteasome [17, 18] has been shown to significantly decrease AAV specific humoral immune responses after AAV-based gene delivery in mice [19]. However, this inhibition was only partially effective and insufficient to allow subsequent re-administration with a recombinant AAV vector of the same serotype [19]. This limitation was shown to be due to the combination of residual antibody levels and the inability of bortezomib to completely deplete the memory B cells [19].

In this study, we explored the potential of a immune suppression protocol for which bortezomib was combined with an anti-CD20 monoclonal antibody, targeted against pre-B cells, mature B cells and memory B cells [20, 21], to reduce level of AAV specific NAB. The efficacy of the combined immunosuppressive regimen was challenged by injecting mice with AAV1 carrying an expression cassette for ovalbumine (OVA).

Materials & methods

Study Design

C57BL/6 mice (males, age 6-8 weeks) were obtained from Harlan Laboratories, the Netherlands (n=6 per group). The experimental protocol was approved by the ethical committee for animal welfare of the AMC (Academic Medical Center, Amsterdam, the Netherlands). The general procedure is summarized in **Figure 1.** Mice received intramuscular injection of AAV1-CMV-OVA at day 0, at 1 x 10^{13}gc/kg or PBS/5% sucrose. Blood was drawn weekly and at sacrifice. Treatment was initiated with three different experimental regiments. A group receiving mono-therapy with bortezomib (velcade) at day 28, 33 and 36, a group receiving mono-therapy with anti-CD20 antibody (eBioscience, clone: AISB12) at day 22 and 27, a group receiving poly-therapy with both bortezomib (day 28, 33 and 36) and anti-CD20 (Day 22 and 27). Anti-CD20 was administered intraperitoneal at 100 µg/mouse; bortezomib was administered intravenously at 0.75 mg/kg. The control groups were mice injected with PBS and mice injected with AAV1-CMV-OVA only.

AAV vector production and characterization

AAV vector (AAV1-CMV-OVA) was produced in insect cells according to a technology adapted from R. M. Kotin R.M. Kotin [24]. It was 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.

Virus neutralizing assay

HEK293T cells were seeded in 96-well plates (Corning) that were pre-coated with 0.25% poly L-lysine at a density of 1 x 10^5cells/well in 100 µl of DMEM with 10% FBS and 1% Penicillin/Streptomycin. Cells were incubated overnight at 37°C in 5% CO_2 water jacket incubator. Medium was then removed and the following mix was added: AAV5-CMV-GFP for anti-AAV5 NAB

assay or AAV1-CMV-GFP for anti-AAV5 NAB assay with heat-inactivated plasma sample in a total volume of 100 µl of DMEM without phenol red and 1% Penicillin/Streptomycin. The mix was incubated for 1 hour at 4°C prior to addition on the cells. Medium of the Hek293T cells was removed by aspiration, and then the mix was added and incubated for 16-20 h at 37°C. Serial dilutions of test plasma that were prepared were: 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, and 1:12800. As a positive control, cells without plasma addition in the mix were analyzed. After 16-20 h, cells were rinsed with PBS, collected after trypsinization and fixed in PBS, 2% Formaldehyde, 1% BSA. GFP expression of the cells was analyzed by flow cytometry (FACScalibur, Becton Dickinson) in channel FL1 at a wavelength of 530 nm. The analysis was performed with the Cellquest Pro software. The percentage of inhibition was calculated related to GFP expression measured in AAV HEK293T infected cells (no inhibition, 100% expression). Plasma dilutions causing a 50% reduction of GFP expression when compared to positive control, were determined (EC50 determination after sigmoidal curve fit in GraphPad Prism software). Plasma samples were considered to have neutralizing activity if the lowest plasma dilution inhibited vector transduction by at least 50%, as described before [25, 26, 27, 28].

Assessment of anti-OVA antibody level

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-HRP (DAKO).

Statistical analysis

Results are presented as means (+/-standard error of the mean (SEM). Statistical analyses were performed using Prism 5.0 (GraphPad). Data were analyzed using a 2 way ANOVA, followed by Bonferroni post hoc test for multiple comparisons.

Results & Discussion

Experimental setup

Mice were injected intramuscularly with 1 x 10^{13}gc/kg of AAV1-CMV-OVA or PBS. All the mice were followed for 21 days (3 weeks) for the development of NAB against the AAV1 capsid proteins and antibodies against OVA transgene product. From day 22, immunosuppressive (IS) therapy was initiated for 2 weeks, mono-therapy with bortezomib or anti-CD20, and combination-therapy with bortezomib and anti-CD20 (**Figure 1**). The drugs were administered as follows, three times intravenously at a dose of 0.75 mg/kg for the bortezomib (day 28, 33 and 36 of the experiment) and two times intraperitoneally at a dose of 100 μg for anti-CD20 (day 22 and 27 of the experiment).

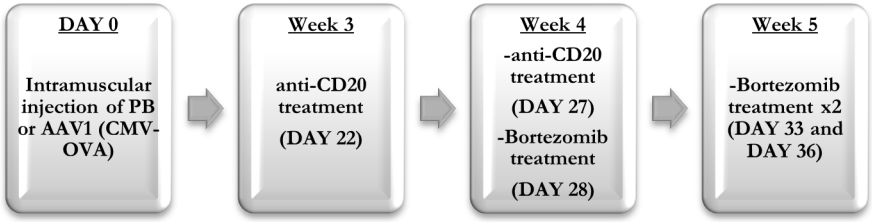

Figure 1. Scheme of the experimental setup. Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0, at 1x10^{13}gc/kg or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody).

Humoral response against OVA

To assess the influence of bortezomib and anti-CD20 on the humoral immune response against the OVA transgene, the levels of specific anti-OVA antibodies generated, were assessed over time (**Figure 2.A-C**). The data obtained show a significant inhibitory effect of bortezomib (67.19 % on day 28 and 71.18 % on day 35) (**Figure 3.A, B**) and anti-CD20 (61.51% on day 28 and 44. 38 % on day 35) (**Figure 3.A, B**) on the humoral response raised against the OVA protein when compared to the control without IS. No signif-

icant additive inhibitory effect on anti-OVA antibody level was observed when anti-CD20 and bortezomib treatment were combined (**Figure 2.C**). The inhibitory effect of the IS treatment on the humoral response was transient as the antibodies level increased at day 42 of the experiment (**Figure 2.A-C**).

Humoral response against AAV1- Neutralizing antibodies (NAB) against the AAV1 vector capsid

To determine the effect of bortezomib and anti-CD20 on the levels of neutralizing antibodies against the AAV1 viral capsid proteins, a neutralizing antibody assay was performed on mice plasma samples obtained at days 0, 14, 28, 42, 56 and 84 (**Figure 4**). After bortezomib administration (days 28, 33 and 36), a decrease was observed in the levels of the NAB titers against the AAV1 capsid at days 42 (13.6%), 56 (26.3%) and 84 (38.7%) when compared to the control without IS (**Figure 4.A**). After anti-CD20 administration (days 22, and 27), a decrease was observed in the levels of the NAB titers against the AAV1 capsid (day 84) when compared to the control without IS (35.5%) (**Figure 4.B**). The delayed response to the anti-CD20 antibody treatment is consistent with the mechanism of action of this antibody [22]. The combination of both IS drugs did not show an additive effect on the reduction of anti-AAV1 NAB levels (**Figure 4.C**). The effect of the IS treatment on the humoral response was sustained until the end of the observation period as the neutralizing antibodies levels remained low until day 84 for both bortezomib and anti-CD-20. However, the anti-AAV NAB titer obtained after treatment (a titer of 2592) was not low enough to perform re-administration, as previously reported [19].

We conclude therefore that a combined treatment with bortezomib and anti-CD20 antibody when a high titer (~3550) of anti-AAV1 NAB has already been established cannot reduce the level of those antibodies sufficiently to permit re-administration. Therefore, other combination of treatments that include additional cellular targets will be explored in the future.

Safety

No weight loss or signs of illness were observed in the mice receiving the immunosuppressive drugs alone or in combination.

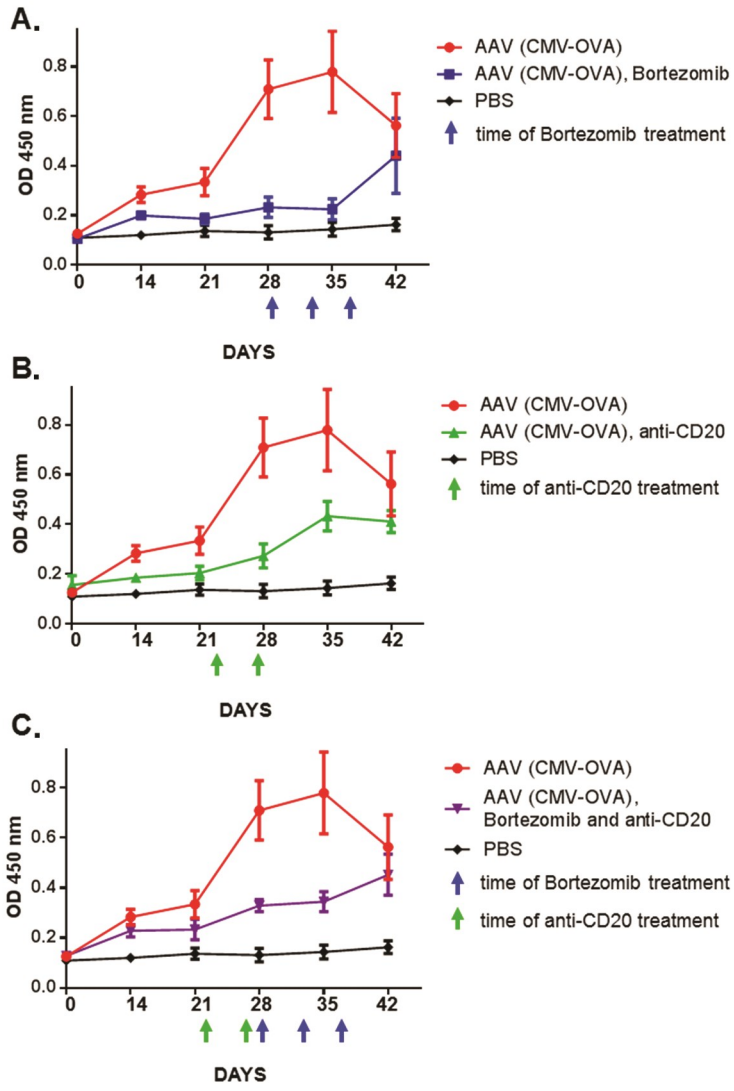

Figure 2. Immune suppressive regimens reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery

Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0, at $1x10^{13}$gc/kg or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of anti-OVA antibodies was monitored overtime in plasma of mice treated with Bortezomib (**A**), anti-CD20 (**B**) or Bortezomib and anti-CD20 (**C**).

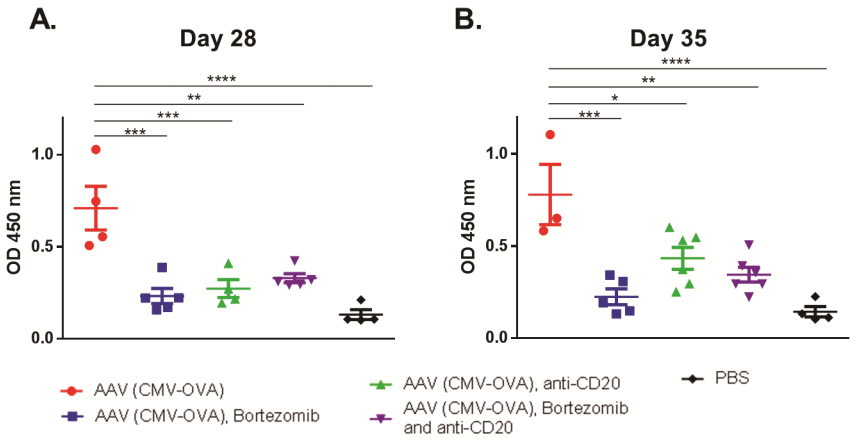

Figure 3. Immune suppressive regimens reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery.
Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0 or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of anti-OVA antibodies was monitored overtime after treatment with Bortezomib, anti-CD20 or both on day 28 (A) and 35 (B) of experiment. The data were analysed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test. *P<0.05, **P<0.01, ***P,0.001, ****P<0.0001.

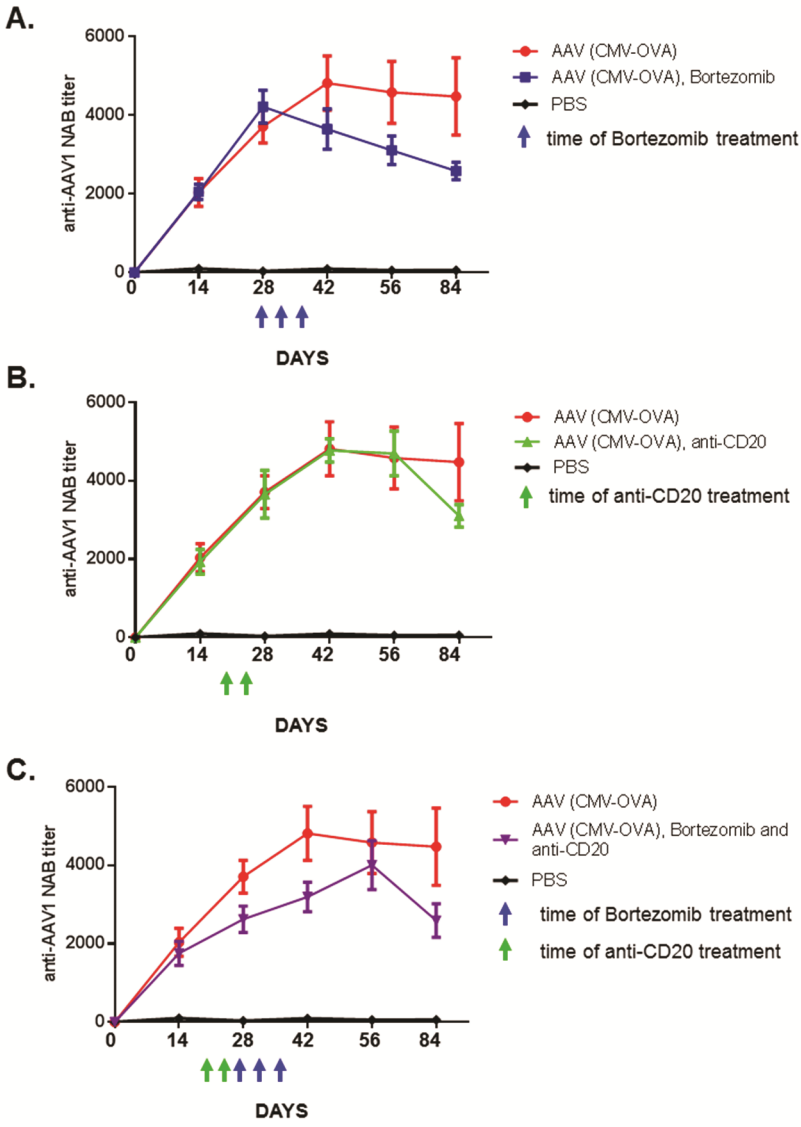

Figure 4. Immune suppressive regimens reduce neutralizing antibody (NAB) titers against AAV1 raised after intramuscular delivery of AAV1.
Mice received intramuscular injection of AAV1 (CMV-OVA) at day 0 or PBS/5% sucrose. Treatment was initiated with three different experimental regimens (Bortezomib, anti-CD20 antibody and with both Bortezomib and anti-CD20 antibody). Development of nAb titer agaist AAV1 was monitored overtime in plasma of mice treated with Bortezomib (**A**), anti-CD20 (**B**) or combined Bortezomib and anti-CD20 (**C**).

It has been described previously that a dose of 1 mg/kg of bortezomib was associated with toxicity in 15% of the animals treated [19] suggesting a narrow therapeutic window of this drug. We report that administration of a 0.75 mg/kg dose of bortezomib either combined with anti-CD20 (100 μg per injection) or not did not appear to induce toxicity in mice.

Additionally thymus, spleen, bone marrow and PBMCs were collected at sacrifice, stained for B and T cell markers and analyzed with the use of flow cytometry. We did not observe any significant differences in expression of CD19, CD25 or CD138 markers (*data not shown*) which demonstrate the absence of long term effects on the adaptive immune system.

Conclusions and future prospects

Overall, this study shows that a combination-therapy of bortezomib with an anti-CD20 monoclonal antibody was not sufficient to lower a high titer of pre-existing antibodies against the AAV1 capsid to a level that will allow an efficient AAV1 re-administration. The same drugs alone or in combination were, in contrast, efficient in preventing immune response against the immunogenic protein (OVA) delivered as a transgene by AAV1. This observation can be related to the differences in the kinetic and mechanism of development of the humoral immune response against the AAV capsid and the transgene product delivered by AAV [23].

The two immunosuppressive molecules used in this study, bortezomib and anti-CD20, are mainly targeting pre-B cells, mature B cells and memory B cells [20, 21]. Additional cellular inhibitors, as T cell inhibitors, may be required in order to bring down the humoral response to the AAV1 capsid. Further studies are needed in order to evaluate the efficacy of combination treatment that targets both B and T cells population as well as the therapeutic benefit in relation to different NAB levels.

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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.

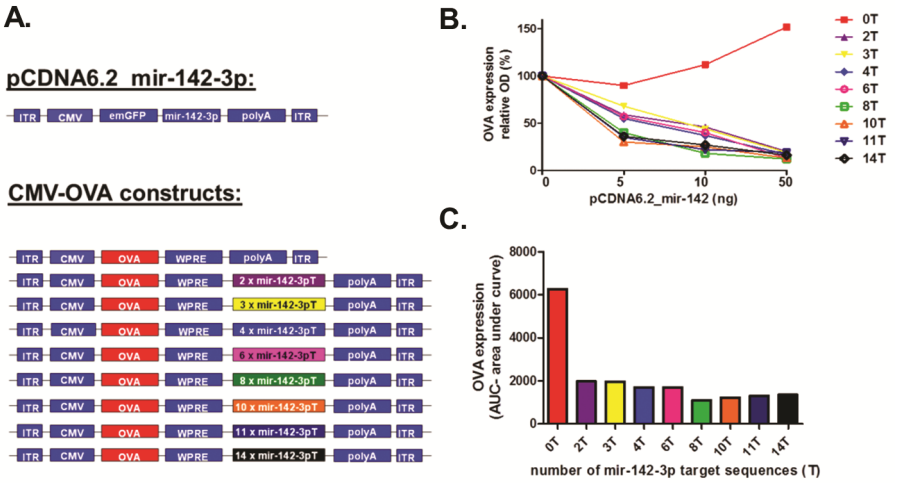

Figure 1. Inhibition of OVA protein expression from CMV-OVA-WPRE plasmid constructs containing mir-142-3p target sequences upon *in vitro* co-transfection with mir-142-3p expressing plasmid (pCDNA6.2_mir-142-3p). A: Mir-142-3p expressing construct, CMV-OVA-WPRE construct and CMV-OVA- WPRE constructs 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.

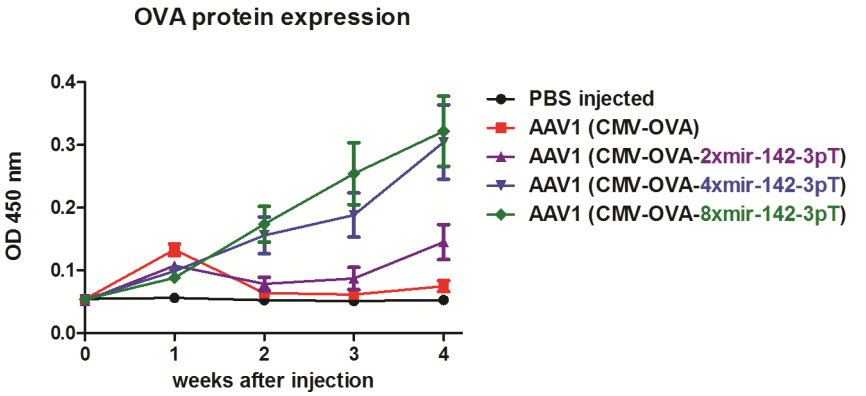

Figure 2. Sustained OVA protein expression level correlates proportionally to the number of mir-142-3p target sequences incorporated after OVA transgene sequence. Mice were injected with PBS, AAV1-CMV-OVA ($1x10^{14}$gc/kg), AAV1-CMV-OVA-2xmir-142-3p ($1x10^{14}$gc/kg), AAV1-CMV-OVA-4xmir-142-3p ($1x10^{14}$gc/kg)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

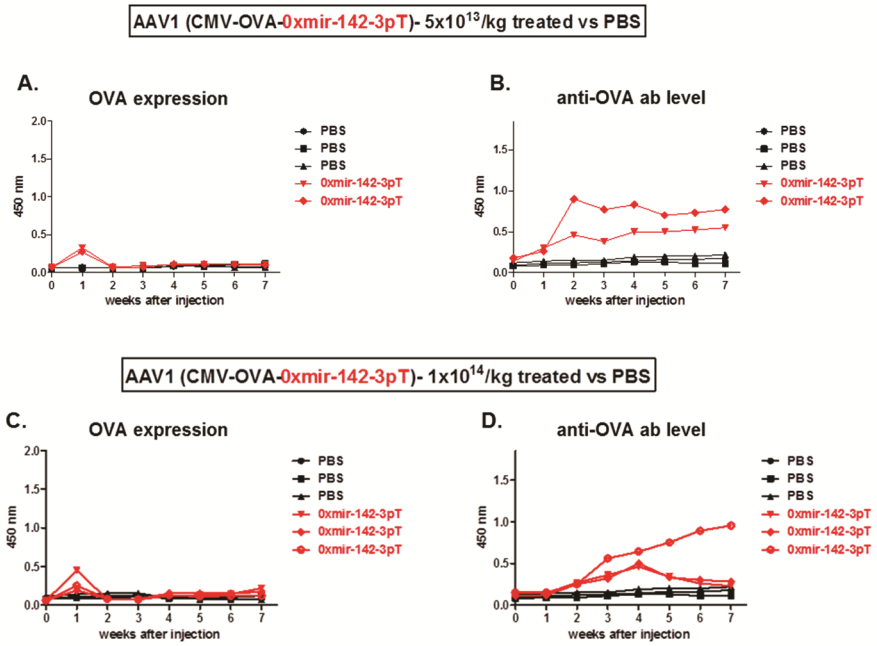

Figure 3. Loss of OVA protein expression that correlates with raising anti-OVA antibody levels following AAV1 intramuscular delivery. Expression of OVA protein after intramuscular delivery of $5x10^{13}$ and $1x10^{14}$ gc/kg of AAV1-CMV-OVA raises atweek1, although it is totally lost in subsequent weeks (**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.

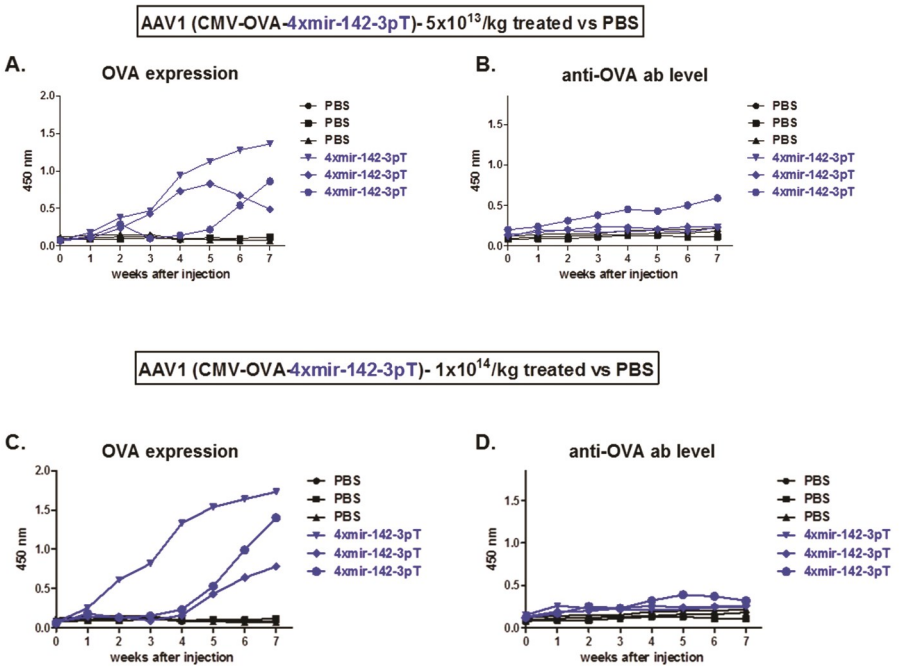

Figure 4. Mir-142-3p target sequences reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery. Intramuscular delivery of AAV1-CMV-OVA-4xmir-142-3p shows sustained expression of OVA protein (**A, C**) which correlates with low levels of anti-OVA antibody (**B, 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.

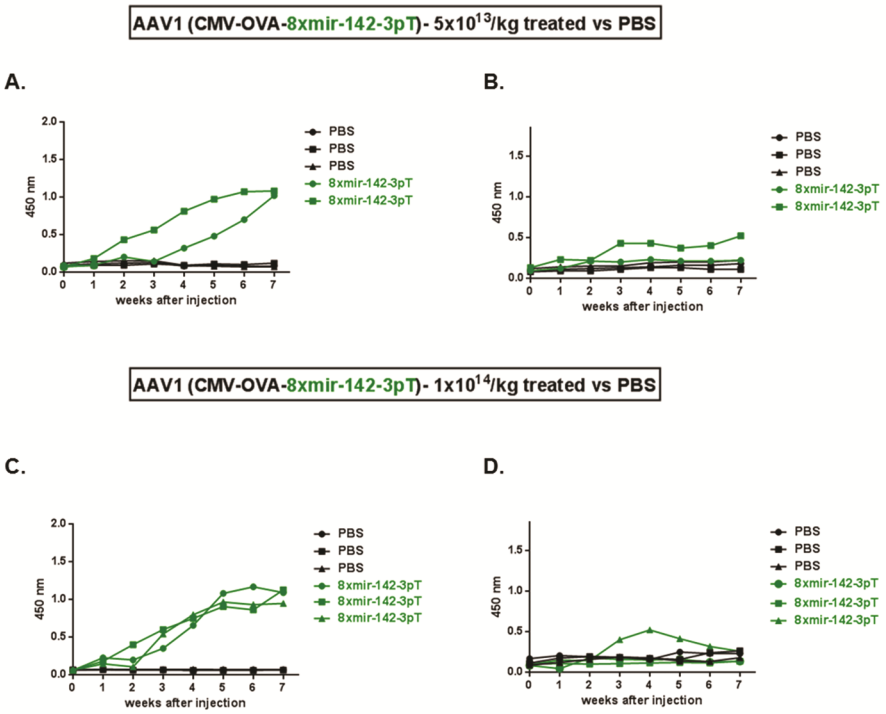

Figure 5. Mir-142-3p target sequences reduce specific humoral immune responses against OVA protein following AAV1 intramuscular delivery. Intramuscular delivery of AAV1-CMV-OVA-8xmir-142-3p shows sustained expression of OVA protein (**A, C**) which correlates with low levels of anti-OVA antibody (**B, 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.

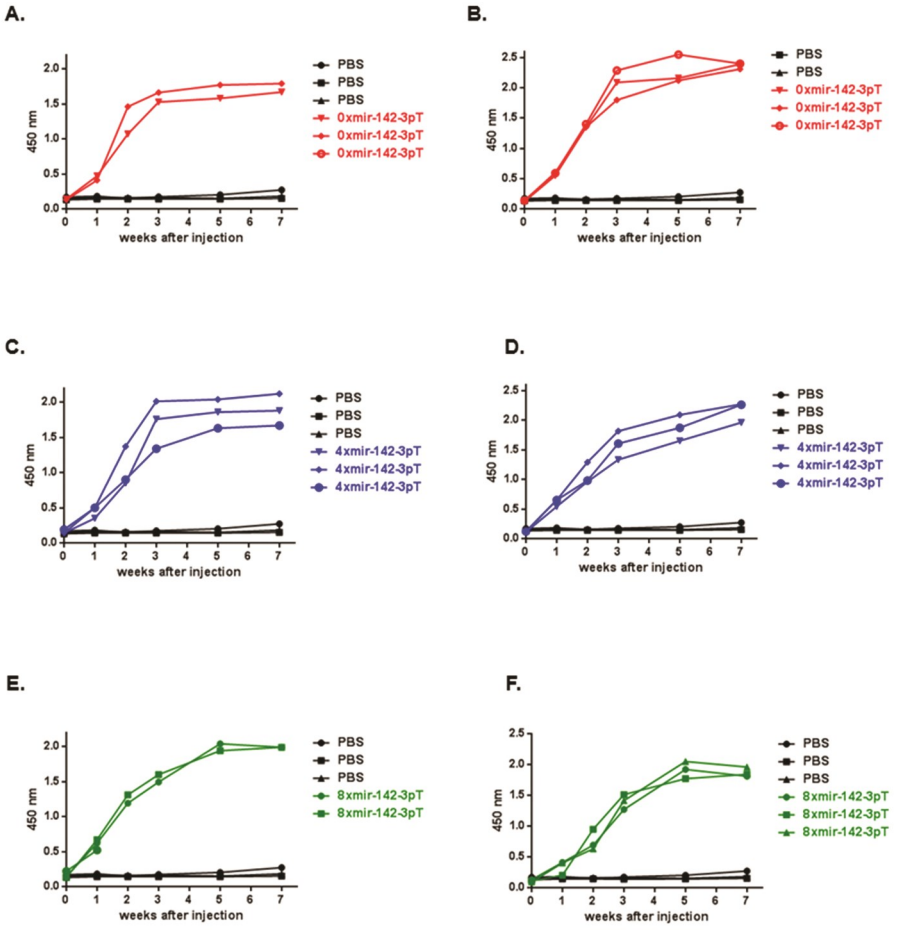

Figure 6. Total anti-AAV1 antibody levels after intramuscular delivery of different AAV1 batches at $5x10^{13}$gc/kg (A, C, E) and $1x10^{14}$gc/kg (B, D, F) dose. Anti-AAV1 antibody level remains the same in all AAV1 injected groups (**A-D**). Black lines on the graphs represent PBS injected control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

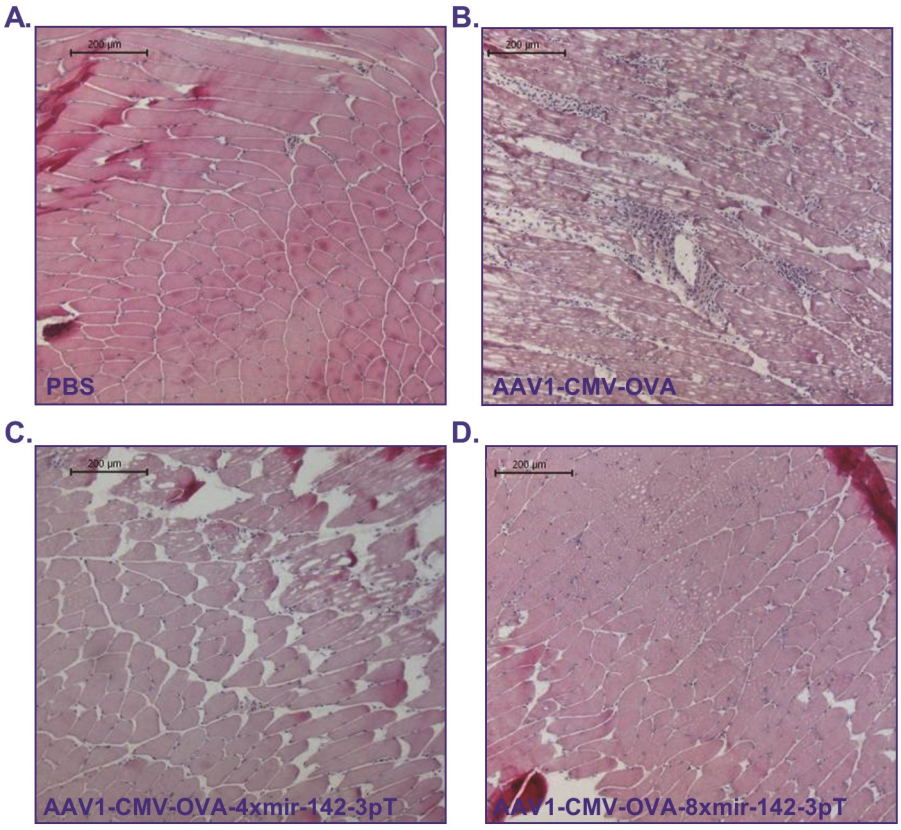

Figure 7. Haematoxylin and Eosin staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

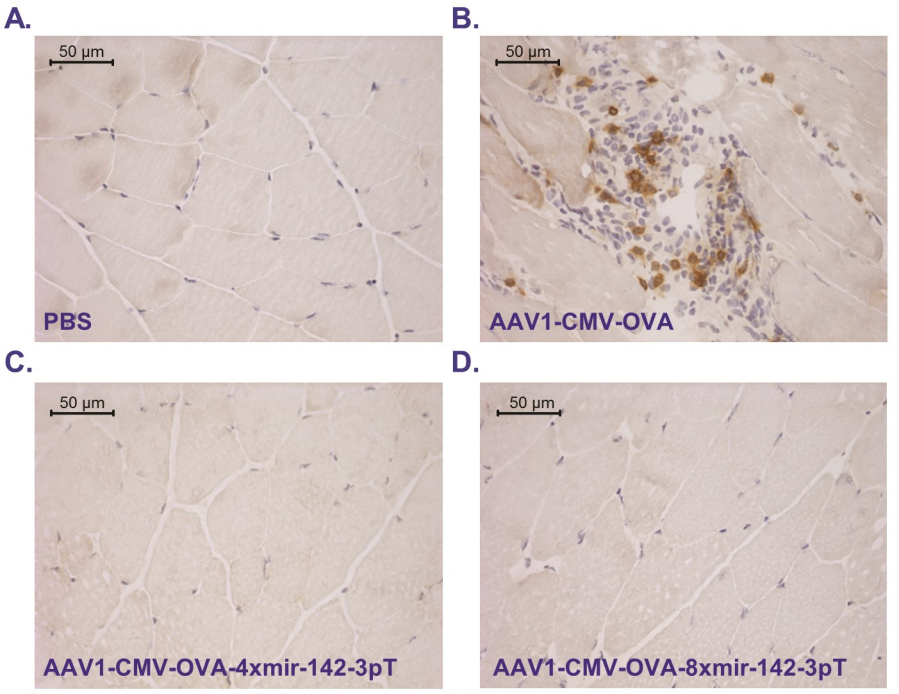

Figure 8. Anti-CD8 staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

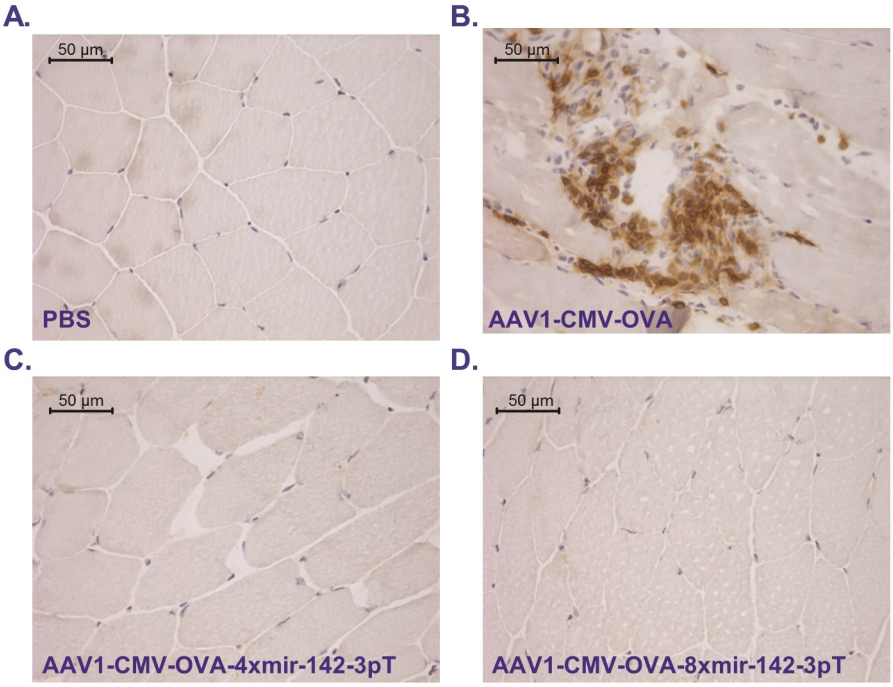

Figure 9. Anti-CD4 staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

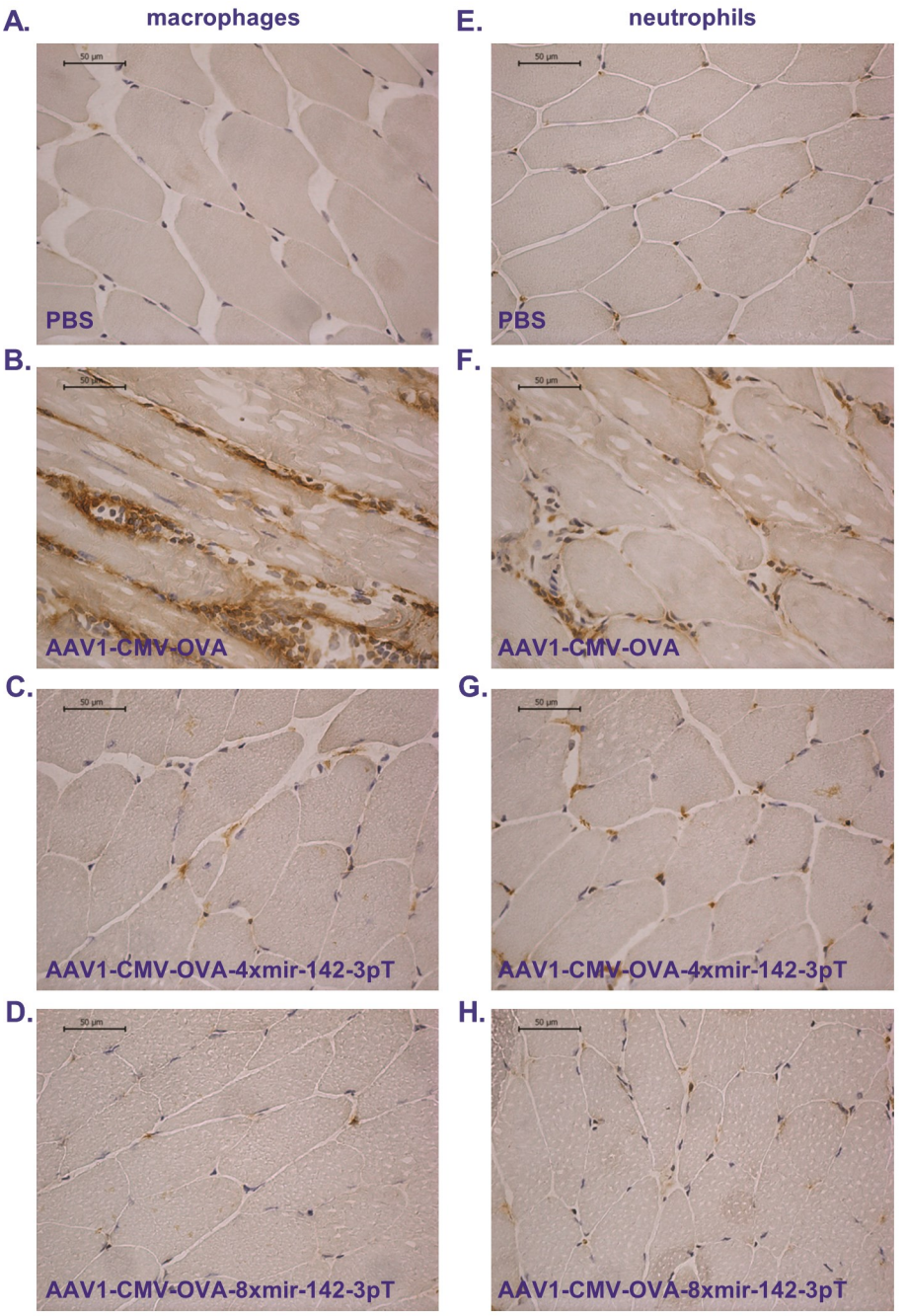

Figure 10. Anti-macrophage and anti-neutrophil stainings of mouse muscle tissue after intramuscular delivery of PBS (**A, E**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B, F**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C, G**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D, H**).

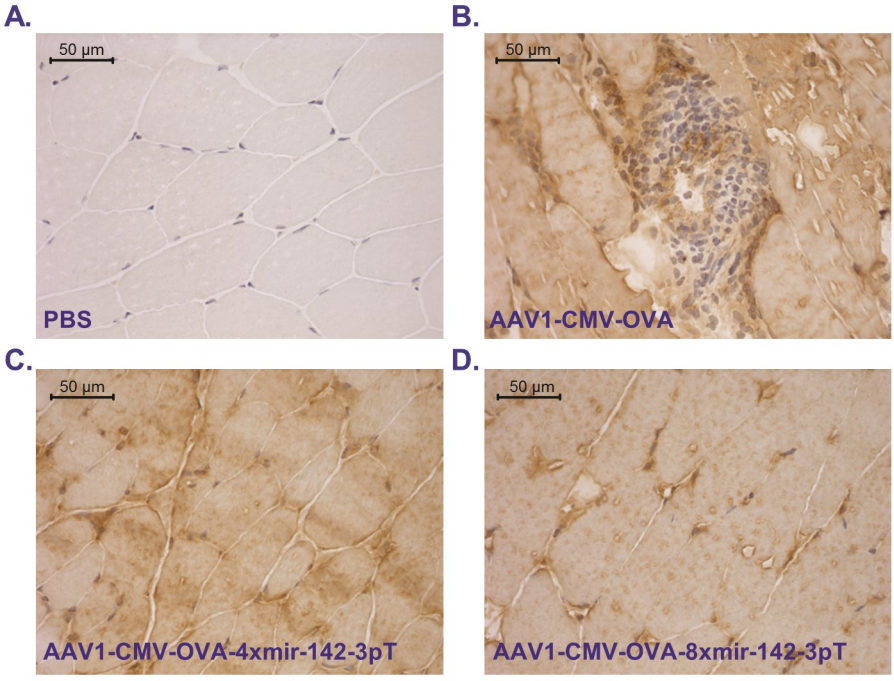

Figure 11. Anti-OVA staining of mouse muscle tissue after intramuscular delivery of PBS (**A**), AAV1-CMV-OVA ($1x10^{14}$gc/kg) (**B**), AAV1-CMV-OVA-4xmir-142-3pT ($1x10^{14}$gc/kg) (**C**) or AAV1-CMV-OVA-8xmir-142-3pT ($1x10^{14}$gc/kg) (**D**).

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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Chapter 9

General Discussion

Lack of tolerance against "self" antigens or "self" commensal bacteria leads to autoimmune and inflammatory disorders. Current treatment of those diseases is symptomatic, not specific and unfortunately often not effective. Recent advances in the fields of immunology, cell biology, genetics and bioinformatics led to identification of new therapeutic targets and tools for treatment of autoimmune and inflammatory disorders. One of these novel prevention and treatment strategies is induction of immune tolerance. This thesis focuses on inducing immune tolerance by regulatory T (Treg) cells generated *in vitro* by activation of naive T cells, or *in vivo* by adeno-associated virus (AAV)- mediated delivery of immunomodulatory peptides (Part I). We also explored novel methods for tolerance induction with the aim to prevent unwanted immune responses directed at AAV vector capsid or immunogenic transgene product in the setting of gene therapy (Part II).

Part I Summary

Treg cells are a cellular component of the immune system that is specialized in suppressing immune responses of effector cells. They can be divided into naturally occurring, thymus-derived Treg (nTreg) cells and induced Treg (iTreg) cells which develop outside the thymus under specific conditions. Treg cells are responsible for sustaining homeostasis of the immune system, and deficiency of Treg in the system is generally associated with severe inflammatory disease states. Treg can be induced ex vivo and have proven to be safe and efficient in clinical trials for graft versus host disease [1, 2]. Treg mediated immune tolerance may also be employed as a treatment for diseases with an autoimmune or inflammatory background [3-8]. We have studied the latter in this thesis and used as *in vivo* model of inflammatory and autoimmune diseases two murine models of Inflammatory Bowel Disease (IBD) (CD45RB transfer and trinitrobenzene sulfonate mouse models). These models serve as mouse analogs of two chronic inflammatory disorders in humans, Crohn's disease and ulcerative colitis. There is no definite, curative treatment available for those conditions and patients require lifelong symptomatic management.

Although the exact etiology of the IBD is unknown, it is thought to originate as a result of genetic and environmental factors that lead to inappropriate immune responses against dietary or bacterial flora antigens present in the gut lumen. Those unwanted immune responses are specifically associated with activation of Th17 or Th2 cells and the inability to shut down the resulting immune-mediated inflammation. Clearly, inflammatory control by regulatory T cell therapy is an attractive treatment strategy for these disease states. A major limitation to the use of nTreg cells is availability as they represent only a small percentage of the peripheral circulating $CD4^+$T cell population. In order to overcome this issue, several groups have developed various methods to expand nTreg *in vitro* while keeping their functionality. Generally, the technologies are complex, time-consuming and the plasticity of nTreg cells in artificial environment during *ex vivo* expansion may lead to loss of their suppressive activity. Additionally their relative mature stage of differentiation makes expansion *in vitro* a difficult process.

In **Chapter 2** we describe a new simple method to generate stable and functional human iTreg cells *in vitro*, providing a simple alternative to previously reported techniques. We applied this protocol to murine cells as reported in **Chapter 3** and demonstrated the functionality of the generated iTreg cells, *in vivo* by their potential to ameliorate the disease phenotype in a CD45RB transfer colitis mouse model. The iTreg cells can also be induced *in vivo* by a variety of immunomodulatory peptides such as for instance cationic host defense peptides that have been successfully employed for treatment of inflammatory and autoimmune diseases. Among those peptides are recently discovered regulatory T cell epitopes (Tregitopes) that are derived from immunoglobulin G (IgG). Tregitopes, which are stimulators of $CD4^+CD25^+Foxp3^+$ T regulatory cell (Treg) expansion. The anti-inflammatory potential of Tregitope 167 and Tregitope 289 has been previously reported [9- 11], but a limiting factor of this treatment is the achievement of stable, therapeutic levels of the immunomodulatory peptides. A solution to this problem could be a gene therapy approach that would provide stable peptide expression after the delivery of the gene that encodes the peptide of interest and the most promising vector that can provide long term expression of the delivered gene after single administra-

tion is adeno-associated virus (AAV). AAV-mediated gene therapy has been effective and safe in preclinical studies as well as in several clinical trials [12-15]. In **Chapter 4**, we report the development of an AAV-based approach to deliver the anti-inflammatory Tregitope 167 peptide. Tregitope 167 transgene was delivered intravenously by an AAV vector in the trinitrobenzene sulfonate (TNBS) mouse model of IBD and this resulted in decreased intestinal inflammation. Hence, tolerance induction using Treg might be a future prospect for inducing immune tolerance in autoimmune and inflammatory diseases.

Part II Summary

A main concern with AAV-based gene therapy is the presence of pre-existing neutralizing antibodies (NAB) against AAV due to naturally occurring asymptomatic infections with the wild type virus or due to prior treatment with an AAV vector. Those anti-AAV NAB can inhibit transduction upon first administration in case of pre-existing immunity or upon re-administration with the same AAV vector serotype [16-19]. As an alternative to repeated delivery of the same AAV serotype, cross-administration, which is the sequential use of different AAV serotypes, may be considered. AAV serotypes 5 and 1 have been shown to have no significant inhibitory cross-reaction. In **Chapter 6** we have demonstrated that AAV serotypes 5 and 1 can be used sequentially for re-administration in the liver with no significant inhibitory cross-reaction observed. A non-human primate experiment is in preparation to confirm the data obtained in mice.

Cross administration of different AAV serotypes for re-administration of therapeutic gene, might not always be feasible, as different AAV serotypes [17] have different tissue tropisms. Therefore, a careful selection of appropriate AAV serotypes is required when employing this approach for a specific target tissue.

Another option to avoid formation of NAB against AAV vectors would be modifying the AAV vector capsid to exclude viral epitopes which induce immune response upon presentation to the immune cells [20]. Finally, the most

common and widely applied approach to inhibit immune responses is use of immunosuppressive drugs [21]. Our group has investigated the influence of bortezomib and anti-CD20 alone or in combination therapy on NAB against AAV capsid formation (in press). We describe this approach in **Chapter 7**. This approach clearly reduced immune responses, but the effect was short-lived which indicates that in order to reach satisfying and long-term results extended treatment regimens will be necessary.

Another obstacle that needs to be overcome in AAV-based gene therapy is the appearance of immune responses against the expressed protein which might result in loss of therapeutic gene expression [22-26]. MicroRNA, mir-142-3p, which is specifically expressed in antigen presenting cells (APCs) may be used as a novel approach to avoid transgene directed immunogenicity. Incorporation of mir-142-3p target sequences within a transgene sequence has been shown to prevent of mRNA and protein expression in haematopoietic lineage cells, including APCs in both *in vitro* and *in vivo* setup [27]. The use of mir-142-3p target sequences prevented immune responses towards the transgene product in mice when a lentiviral vector was used for gene delivery targeting the liver [28, 29]. Furthermore, our group provided evidence that both humoral and cellular immune responses against the transgene product can be efficiently reduced by use of mir-142-3p target sequences in AAV-based intramuscular gene delivery and these experiments are summarized in **Chapter 8**.

Conclusions and future perspectives

The major achievements reported in this thesis are the identification of two novel approaches to generate regulatory T cells with the capacity to ameliorate inflammatory response and to restore immune tolerance. Additionally, in relation to AAV-mediated gene delivery approach to induce tolerance, we developed new strategies to prevent specific immune responses to the transgene product or to the adeno-associated virus (AAV) vector capsid. Initially, we provided the basis for further clinical development of cell therapies that involve Treg cells for the treatment of autoimmune and inflammatory diseases

by developing approaches to generate both in vitro and in vivo Treg cells. Next, we reported a functional approach to reduce the immune responses against the transgene product after intramuscular delivery by an AAV vector. This strategy could be applied in any AAV vector- based gene therapy targeting the muscle where there is a risk of immune responses against transgene product. We are currently pursuing further research to evaluate the impact of mir-142-3pT regulated AAV gene delivery on the normal miRNA profile in the muscle tissue.

We also investigated the feasibility of cross-administration of AAV vectors, as an approach to avoid the problem of formation of neutralizing antibodies (NAB) against AAV capsid following primary delivery. Such antibodies may interfere with AAV vector transduction upon re-administration of the same serotype. We showed that AAV5 and AAV1 could be sequentially delivered and the NAB against the capsids of those AAV vectors do not cross-react. Hence, this is an attractive approach for therapeutic protein re-administration. Our study was performed in a mouse model and should be confirmed in non-human primates before its possible translation to the human patients.

We subsequently studied the effect of immune suppressive regimens on neutralizing antibody (NAB) formation against the AAV capsid. Bortezomib and/or anti-CD20 treatment did not lower the anti-AAV NAB level to a value that would permit the re-administration of AAV vector. Therefore, there is a need for further studies that would include longer treatment time, dose-finding and the introduction of additional immunosuppressive therapeutics that would influence not only the B but also the T cell population.

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Addendum

Samenvatting

Inductie van immuun tolerantie in inflammatoire darmziekten en tegen adeno-geassocieerde virussen (AAV)

Dit proefschrift bestaat uit twee delen. **Deel I** is gericht op de ontwikkeling van nieuwe strategieën voor het behandelen van inflammatoire darmziekten (IBD). In een experimenteel IBD model, werd een immuun tolerantie bereikt door gebruik te maken van *in vitro* gegenereerde regulatoire T cellen. Ook werd, met behulp van een AAV vector, een methode ontwikkeld om regulatoire T cellen *in vivo* te induceren. Deze inductie van regulatoire T cellen ging gepaard met het onderdrukken van ontsteking in een experimenteel IBD model.

Het is mogelijk dat een immuunreactie ontstaat tegen de AAV capside, of tegen het AAV transgen. Dergelijke reacties zouden succesvolle gentherapeutische toepassingen in de weg staan. Het onderzoek beschreven in **Deel II** van dit proefschrift is gericht op tolerantie inductie voor deze factoren om behandeling met de AAV vector als onderdeel van een gentherapie strategie, efficiënter te maken.

Deel I

Inflammatoire darmziekten zijn aandoeningen die gepaard gaan met chronische ontstekingen in het maagdarmkanaal en waarvoor geen curatieve behandelingen beschikbaar zijn. Behandelstrategieën, die gebruik maken van cel-, of gentherapie, zijn in recent onderzoek succesvol gebleken in het onderdrukken van ontsteking en zodoende hoopgevend voor de behandeling van IBD. In dit verband is de regulatoire T cel (Treg) een veelbelovende celpopulatie. Treg cellen zijn in staat om immuun reacties te doen afnemen en aldus balans in het maagdarmkanaal te doen terugkeren. Een probleem is dat de beschikbaarheid van natuurlijke Treg (nTreg) cellen erg beperkt is. Ook is het fenotype profiel niet stabiel genoeg voor ontwikkelingen *ex vivo*. Voorts laat de Treg cel zich niet makkelijk vermenigvuldigen *in vitro*. Daarom biedt het *in vitro* genereren van regulatoire T cel vanuit naïeve T cellen een goed alternatief voor de toekomst om deze obstakels te overwinnen. Op het moment zijn diverse strategieën beschikbaar om Treg te induceren *in vitro*. Echter, ieder van deze strategieën kent

zijn beperkingen en het is tot op heden nog niet gelukt om *in vitro* geïnduceerde Treg cellen te gebruiken in de kliniek. Het doel van deel I van dit proefschrift was zodoende om een nieuwe methode te ontwikkelen om Treg cellen *in vitro* te kunnen produceren. **Hoofdstuk 2** beschrijft een activatie protocol, waarmee op een eenvoudige wijze, Treg cellen worden gegenereerd vanuit naïeve T cellen *in vitro*. Deze Treg (TregPMA) cellen, remmen de proliferatie van geactiveerde leukocyten in een zogenaamde *mixed leukocyte reaction* (MLR), op een dosis afhankelijk wijze. In **Hoofdstuk 3** wordt aangetoond dat dit protocol ook werkzaam is voor naïeve muis T cellen en dat de gegenereerde TregPMA cellen functioneel zijn *in vivo*, in een experimenteel IBD model. In **Hoofdstuk 4** wordt gebruik gemaakt van een strategie om Treg cellen te induceren *in vivo*. Met behulp van een AAV vector wordt een eiwit in een experimenteel IBD model tot expressie gebracht, te weten een Treg epitoop (167). Met behulp van deze AAV vector kon de ontsteking in de darm worden verminderd. Bovendien ging dit gepaard met een toename van het aantal Treg cellen in de behandelde groep muizen.

Deel II

Een belangrijk obstakel voor gentherapie met behulp van op AAV gebaseerde virale vectoren, is een humorale immuun reactie tegen de AAV capside. Neutraliserende antilichamen (NAB), die ontstaan als reactie op blootstelling aan "wild type" AAV of na behandeling met een recombinant AAV vector, voorkomen herhaalde blootstelling aan de AAV en maken zodoende een volgende gentherapie behandeling minder efficiënt. **Hoofdstuk 6** beschrijft een methode waarbij het alternerend toedienen van AAV serotype 1 en serotype 5, zogenaamde readministratie, wel mogelijk maakt. Daarnaast beschrijft **Hoofdstuk 7** diverse immuun suppressie strategieën, die de humorale immuunreactie tegen de primaire AAV vector doen afnemen. Een bijkomende zorg voor gentherapie met behulp van op AAV gebaseerde virale vectoren is de mogelijkheid dat een immuunreactie tegen het transgen product ontstaat, hetgeen zou kunnen leiden tot verlies van therapeutisch potentieel van het gentherapie product. **Hoofdstuk 8** beschrijft een methode, die gebruik maakt van mir-142-3p expressie om immuunreacties tegen het transgeen product te voorkomen.

Streszczenie

Adresowanie problemów tolerancji immunologicznej w nieswoistym zapaleniu jelit oraz transferze genów przy pomocy wirusów towarzyszących adenowirusom (AAV)

Obecna praca doktorska składa się z dwóch części, które dotyczą problemów tolerancji immunologicznej w nieswoistym zapaleniu jelit oraz transferze genów przy pomocy wirusów towarzyszących adenowirusom. Badania opisane w **Części I** dotyczą rozwoju strategi leczenia nieswoistego zapalenia jelit. Osiągnęliśmy indukcję tolerancji immunologicznej w mysim modelu nieswoistego zapalenia jelit przy użyciu limfocytów T regulatorowych (Treg) uzyskanych poprzez specyficzną aktywację limfocytów T naiwnych *in vitro*. Ponadto, wykazaliśmy również, iż limfocyty Treg są indukowane *in vivo* poprzez AAV dostarczające specyficzny gen i przywracają jelitową tolerancję immunologiczną. W odniesieniu do stosowania wektorów AAV, w **Części II** niniejszej pracy opisane są badania, które mają na celu zaadresowanie problemów dotyczących indukcji tolerancji immunologicznej wobec kapsydów wektorów AAV oraz transgenów dostarczonych za pomocą wektorów AAV.

Zarys Części I

Rozwój nowych strategii leczenia chorób nieswoistego zapalenia jelit, które są uważane za grupę chorób autoimmunologicznych, ma duże znaczenie, gdyż obecnie nie ma dla tych chorób skutecznej terapii. Terapia komórkowa oraz terapia genowa były ostatnio wykorzystywana do badań, których celem była inhibicja stanu zapalnego przewodu pokarmowego. Treg mają zdolność tłumienia odpowiedzi immunologicznej oraz utrzymania centralnej równowagi immunologicznej. Dzięki temu, limfocyty Treg, mogą zapobiegać powstawaniu stanów zapalnych poprzez indukcję tolerancji immunologicznej. Głównym ograniczeniem dla terapeutycznego użytkowania naturalnych limfocytów Treg (nTreg) jest ich mała dostępność oraz niestabilny fenotyp podczas ich ekspansji ex vivo. Dlatego też zastosowanie indukowanych limfocytów Treg (iTreg) jest dobrą alternatywą. Obecnie, istnieje kilka technik służących do wytwarzania limfocytów iTreg, jednakże każda z tych technik ma pewne ograniczenia. W związku z tym, wprowadzenie nowych, ulepszonych technik do wytwarzania

limfocytów iTreg, jest bardzo interesujące. Celem eksperymentów opisanych w **Rozdziale 2** było stworzenie nowego, prostego sposobu wytwarzania *in vitro* funkcjonalnych i stabilnych limfocytów iTreg z ludzkich $CD4^+CD25^-$ komórek. Wygenerowane limfocyty iTreg (TregPMA) okazały się być funkcjonalne *in vitro* w mieszanej hodowli limfocytów (MLR), gdyż tłumiły proliferację komórek biorcy w sposób zależny od dawki. Protokół generacji limfocytów TregPMA *in vitro* został również zastosowany z użyciem komórek mysich. Jest to opisane w **Rozdziale 3**. Funkcjonalność wygenerowanych limfocytów iTreg została zobrazowana poprzez złagodzenie zapalenia jelita w mysim modelu nieswoistego zapalenia jelita.

Pośród niedawno podejmowanych badań naukowych, które mają na celu wygenerowanie limfocytów iTreg są badania, które użytkują terapię genową i komórkową. W **Rozdziale 4,** zbadana została metoda indukcji limfocytów Treg *in vivo* poprzez wprowadzenie do komórek pewnego genu. Dostarczenie regulatorowego epitopu 167 limfocytów T (Tregitope 167) za pomocą wektora AAV, wykazało indukcję limfocytów Treg *in vivo* i złagodzenie eksperymentalnego zapalenia jelita. To badanie wykazało, że dostarczenie przeciwzapalnego Tregitope za pomocą wektora AAV powoduje indukcje tolerancji immunologicznej przez limfocyty Treg, co w konsekwencji może być użyte do leczenia chorób o podłożu autoimmunologicznym i zapalnym.

Zarys Części II

Główną przeszkodą w dostarczaniu genów za pomocą wektora AAV jest humoralna odpowiedź immunologiczna skierowana przeciw białkom kapsydu AAV, która pojawia się po pierwszej infekcji wektorem AAV. Przeciwciała neutralizujące skierowane przeciw kapsydom AAV blokują ponowną transdukcję tym samym serotypem AAV. W **Rozdziale 6** zademonstrowaliśmy na mysim modelu, iż można skutecznie dostarczyć gen z użyciem wektora AAV serotypu 5 a następnie ponownie dostarczyć inny gen z użyciem wektora AAV serotypu 1, gdyż przeciwciała neutralizujące skierowane przeciw białkom kasydów wektora AAV serotypu 5 nie hamują transdukcji za pomocą wektora AAV serotypu 1. W **Rozdziale 7** zbadaliśmy zdolność różnych kombinacji środków immunosupresyjnych do obniżenia poziomu krążących we krwi

przeciwciał neutralizujących przeciwko kapsydom wektorów AAV, które pojawiają się po pierwszym podaniu wektora AAV. Celem tego badania było określenie strategii leczenia immunosupresyjnego oraz ram czasowych, w których obniżenie poziomu przeciwciał neutralizujących przeciwko kapsydowi wektora AAV umożliwi ponowne podanie wektora AAV.

Innym problemem w terapii genowej przy zastosowaniu wektorów AAV jest możliwość odpowiedzi immunologicznej skierowanej przeciw produktom transgenów. Ta reakcja immunologiczna może doprowadzić do utraty ekspresji terapeutycznego transgenu. Dlatego też, istnieje zapotrzebowanie na określenie metod, które doprowadzą do indukcji tolerancji immunologicznej wobec białka transgenu. W **Rozdziale 8**, opisaliśmy możliwość zastosowania sekwencji targetowych mir-142-3p w celu uniknięcia reakcji immunologicznej przeciw produktowi transgenu, po domięśniowych dostarczeniu wektora AAV.

Curriculum Vitae

Anna Majowicz was born on 16th of August 1983 in Krosno, Poland. She lived in Rzeszów (Poland) where in 2002 she completed High School (I Liceum Ogólnokształcące). In October 2002 she moved to Kraków (Poland) where she started Biology studies at Jagiellonian University. In 2007 she completed the studies and obtained MSc in Biology title. In February 2008 she arrived in Amsterdam (The Netherlands) where she started an internship at Amsterdam Molecular Therapeutics (AMT, currently uniQure). After completing the internship, she was enrolled in a PhD program at Leiden University Medical Center while working in Immunology group at AMT/uniQure. The work presented in this thesis was performed at AMT/uniQure. Following the end of her PhD thesis she started working as a Junior Scientist in Immunology group at uniQure.

List of publications

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List of abbreviations

AAV	Adeno-associated viruses
Ab	Antibody
APC	Antigen presenting cell
ATP	Adenosine-5'-triphosphate
QA/QC	Quality assurance/ Quality control
CCMO	Centrale Commissie Mensgebonden Onderzoek
CD	Crohn's disease
cDNA	Coding deoxyribonucleic acid
CMV	Cytomegalovirus
CRP	C reactive protein
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DAI	Disease activity index
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment, antigen binding region
Fc	Fragment, crystallizable region
FBS	Fetal bovine serum
Foxp3	Forkhead Box-P3
Gc	Genome copy
GI	Gastro-intestinal
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein

GMP	Good Manufacturing Practices
HE	Haematoxylin and eosin
HEK293T	Human Embryonic Kidney 293 cells transformed by DNA from human adenovirus type 5
IBD	Inflammatory bowel disease(s)
ICOS	Inducible co-stimulator
Ig	Immunoglobulin
IL	Interleukin
IS	Immuno-suppression
iTreg	Induced regulatory T cell
IVIG	Intravenous immunoglobulin
Max	Maximum
MHC	Major Histocompatibility complex
Min	Minimum
miRNA	Micro RNA
MLR	Mixed lymphocyte reaction
MSC	Mesenchymal stromal cells
NAB	Neutralizing antibodies
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
nTreg	naturally occurring regulatory T (cells)
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PMA	Phorbol-12-myristate-13-acetate
qPCR	Quantitative Polymerase Chain Reaction
rAAV	Recombinant adeno-associated viruses

R&D	Research and Development
RLU	Relative luminescence unit
RNA	Ribonucleic acid
SD	Standard deviation
SEM	Standard error of the mean
TCR	T cell receptors
T1D	Type 1 diabetes
TNBS	Trinitrobenzene sulfonate
TNF	Tumor Necrosis Factor
Teff	Effector T (cells)
Treg	Regulatory T (cells)
Tregitope	Regulatory T cell epitope
TregPMA	iTreg cells induced by PMA/ionomycin/antiCD3/IL-2
UC	Ulcerative colitis
WPRE	Woodchuck hepatitis virus post-transcriptional enhancer
Wk	Week