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Regulatory and exhausted T cell responses to AAV capsid

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Abstract

Recombinant adeno-associated viruses are quickly becoming the preferred viral vector for viral gene delivery for the treatment of a wide variety of genetic disorders. However, since their use in a clinical trial targeting Hemophilia B patients 10 years ago, immune responses to AAV capsid appear to have hampered some of the early clinical gene transfer efficacy. Indeed, AAV-based gene transfer has been shown to reactivate capsid-specific memory T cells which have correlated with a decline in AAV transduced tissue in some patients. Importantly, clinical trials have also shown that this reactivation can be quelled by administering time-course taper of glucocorticoid steroids before or after dosing. More recently, two clinical studies have shown that AAV gene transfer is not only able to induce a deleterious immune response, but it can also result in the initiation of a tolerance to AAV capsid mediated by regulatory T cells and exhausted T cells. In this article, we review clinical trials describing immune responses to AAV, as well as the mechanisms responsible for immune tolerance in chronic infections and how it could apply to AAV-based gene transfer. A better understanding of both cytotoxic and tolerogenic immune responses to recombinant AAV will lead to safer gene transfer protocols in patients.

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Introduction

Adeno-associated virus (AAV) is an apathogenic parvovirus belonging to the *dependoviradae* family. In its wildtype form, AAV is known to infect a wide swath of the human population at an early age, usually as co-infection with adenovirus. However, to this date there is not a reported case of pathologic event caused or related to wildtype AAV infection. This natural tolerance for AAV suggests that it has evolved a protein capsid and an efficient and elegantly compact DNA genome that is not in itself highly immunostimulatory; and, thereby, this virus tends to persist in its host rather unperturbed. While the vector does reliably elicit a humoral response as evidenced by the seroprevalence of the human and other animal populations, it has the ability to persist in its host with little to no evidence of an effective cytotoxic T-cell response. It is this naturally-evolved immune stealth and its genetic simplicity that has made AAV a successful and promising viral vector for gene therapy. Initially recombinant AAV (rAAV) was described as non-immunogenic vector due to their inefficiency at transducing antigen-presenting cells (APC).[1] As the rAAV field matured and the experimental setting moved on from mice to large animal models as well as humans, it was quickly demonstrated that rAAV delivery could actually trigger immune responses to AAV capsid and/or transgene.

Indeed, AAV vectors had been considered as non-immunogenic viral vectors until a clinical trial on hemophilia B patients described a cytotoxic immune response to AAV capsid mediated by CD8+ T cells.[2, 3] Despite a proof-of-concept of persistent expression in studies with factor IX (FIX)-deficient mice[4] and dogs[5], it was only in humans that Manno *et al.*[2] first appreciated the transient expression of FIX. This loss of FIX expression was related to an asymptomatic elevation of transaminases and detection of AAV2 capsid-specific T cells secreting IFNγ between 4 and 6 weeks after dosing. A second clinical trial in hemophilia B patients by Nathwani *et al.*[6], using an AAV8 vector has also shown an immune response marked by a transient elevation in transaminases and positive IFNγ ELISpot responses to the capsid. However unlike the AAV2 trial described by Manno *et al.* in this case transgene expression was diminished but sustained. They reported an elevation of transaminases between 7 to 10 weeks post-dosing in addition to a transient cellular immune response to AAV capsid. Importantly this trial had as part of its clinical protocol the use of a brief immune suppressive regimen which is thought to have quelled the immune response to the capsid evidenced by a return to basal transaminase levels and persistence of transgene expression. More recently, two clinical trials were initiated by Spark Therapeutics and uniQure companies with rAAV encoding a codon-optimized FIX. In the Spark Therapeutics trial, 9 patients were injected

intravenously (IV) with a proprietary rAAV capsid designated as SPK-9001 encoding a high activity FIX (Padua mutant) at a dose of 5e11 viral genome (vg)/kg and have shown persistence of transgene expression above the FIX therapeutic threshold up to 12-31 weeks (31.8% of normal FIX level) in the absence of any immunosuppressive regimen except for 2 out of 9 patients who showed asymptomatic liver enzyme elevation and developed immune response to AAV capsid (**Table 1**).[7] In the uniQure trial, two cohorts of 5 patients were injected with a rAAV5 encoding for a codon-optimized FIX at doses of 5e12vg/kg and 2e13vg/kg. In comparison to the Spark Therapeutics trial, they have reported persistence of transgene expression above the FIX therapeutic threshold up to 6 months but at lower levels (5.1 to 13% of normal FIX level depending on the dose) despite much higher vector doses and in the absence of any immunosuppressive regimen except for 3 out of 10 patients (**Table 1**).[8] The results of these two studies suggest that, at least in the context of hemophilia B optimization of the expression cassette, the dose and/or the choice of AAV serotype can influence the initiation of an expression limiting immune response.

Finally and contrary to the results obtained by Manno *et al*. and Nathwani *et al*. in hemophilia B patients, two clinical trials have shown that rAAV gene transfer may not only be reactivating cytotoxic T cell responses but is also able to initiate a regulatory T cell response. This was first described by Mueller *et al.*[9] in a phase 2b trial for alpha-1 antitrypsin deficiency (AATD) and later confirmed in a clinical trial with lipoprotein lipase- (LPL)[10] deficient patients. In both cases patients received intramuscular (IM) injections of a rAAV1 which led to the finding of infiltrated regulatory T cells (Tregs) *in situ*. These studies have highlighted a new research area on AAV immunology including the understanding of Treg activation and T cell exhaustion.

Regulatory T cells

The importance of CD4+CD25+ T cells in tolerance was described in 1995 when mice depleted in CD25+ T cells showed T cell self-reactive responses and elicited autoimmune diseases.[11] Since then, the exploration of these commonly called regulatory T cells has expanded, and they are now considered a central mechanism of immune regulation.

Two major subsets of Tregs have been described: the natural Treg (nTreg) and induced Treg (iTreg). Natural Treg are differentiated in the thymus and educated on self-antigens, whereas iTregs develop in the periphery and are generated from naive T cells populations.[12] Subtypes of Tregs are characterized but are not limited to expression of CD4, CD25,

CTLA-4, HLA-DR or CD39 and downregulation of CD127 IL-7 receptor on mouse and/or human Tregs.[13-17] In addition to extracellular markers, intracellular markers such as the FoxP3 (forkhead box P3) transcription factor has been described as the most specific Treg marker in the thymus and in periphery. Foxp3 has shown its importance in the thymic development and suppressive activity of regulatory T cells. Indeed, the last point was supported by data suggesting that its expression in naïve T cells confers regulatory phenotype and function to those cells.[18-20] Another marker that has been recently identified is Helios, a member of the Ikaros transcription factor family that is expressed in natural and activated Tregs and is also involved in Treg suppressive activities.[21, 22]

During the past years, *in vitro* and *in vivo* studies have demonstrated that Tregs can mediate tolerance by interacting with cells in an APC-dependent or -independent manner, but also through the secretion of regulatory cytokines (**Figure 1**). Tregs are able to interact with the CD8+ effector T cells by preventing proliferation and IFNγ secretion by CD8+ T cells without any interaction with APC.[23] They can also induce effector T cell death through the granzyme and perforin-dependent pathways.[24-26] In some cases, the immune regulation can be APC-dependent; Tregs have shown the ability to prevent dendritic cell (DC) maturation *in vitro* through down-regulation of CD80/CD86 costimulatory receptor expression by affecting the activation of effector T cells.[27-29] Moreover, regulatory T cells are also able to decrease the time of interactions between the CD4+ T cells and DC *in vivo*, as shown by 2-photon laser-scanning microscopy.[30, 31] Finally, the cytokine environment plays an important role in immune regulation. In fact, the regulatory cytokines: IL10, TGF-β or IL35 have shown the ability to convert naïve T cells to regulatory T cells by activating, for example, the Foxp3 pathway in these cells.[32-34]

Despite the fact that regulation mechanisms are not completely understood, regulatory T cells play a major role in peripheral tolerance maintenance and autoimmune disease prevention. However, several studies have demonstrated that the induction or presence of preexisting Treg cells can also prevent effective virus clearance especially during chronic infections.

Regulation mechanisms in chronic infections

Regulatory T cell responses were initially described as regulators in a context of self-reactive antigen modulation in the thymus and autoimmune diseases. However, as mentioned above they have also been shown to be deleterious in animal

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models of chronic viral infection. In fact, in some cases, chronic viral infections are associated with tolerance to viral antigens mediated by Tregs which in turn leads to uncontrolled viral replication. It is thought that, in these cases, Tregs suppress the activity of antigen-specific CD4+ T cells through secretion of IL10 and TGF-β; this mechanism has been widely observed in patients infected by HIV (human immunodeficiency virus) receiving antiretroviral therapy as well as patients with chronic HBV (hepatitis B virus) or papillomavirus infections.[35-37] Moreover, suppressive assays have shown that Tregs suppress effector T cells by inhibiting their proliferation and IFNγ secretion. Their depletion leads to an increase of IFNγ secretion after PBMC restimulation *in vitro*. Interestingly, Treg induction is not the only mechanism responsible for immunoregulation in chronic infections. In fact, HCV (hepatitis C virus) chronic infected patients have presented induction of Tregs secreting IL10 and TGF-β[38] but also T cell exhaustion. This exhaustion was revealed by a lower frequency of specific cytotoxic T cells in long-term HCV-seropositive patients.[39] Exhausted T cells have been first described in a lymphocytic choriomeningitis virus mouse model where activated virus-specific CD8 T cells without effector function were detected.[40] These cells were actually expressing activation markers associated with TCR signaling such as CD69 after antigen restimulation *in vitro* but were unable to elicit cytotoxic cytokine secretion such as IFNγ leading to an unresolved viral infection.

To date, three different pathways involving exhaustion have been described: these include exhaustion due to interactions with the regulatory cells, exhaustion due to influence of the cytokine milieu or by activation of inhibitory receptors (**Figure 2**). Whereas it is still unclear whether Tregs directly affect T cell exhaustion, it is clearly established that involvement of inhibitory receptors like PD-1 and LAG3 can drive T cell exhaustion. Indeed, several studies have shown the reversal of the exhaustion by *in vitro* blockade of these inhibitory pathways.[41, 42]

Altogether, these results have shown the importance of Treg and exhausted T cells in modulating immune response in chronic infections. Results described in mouse studies using rAAV as a viral vector or vaccine have led us to question whether AAV-based gene transfer is not mirroring a chronic viral infection and whether it could induce tolerance mechanism. CD8+ T cell exhaustion was actually described after IM AAV in a mouse model where CD8+ T cells failed to secrete IFNγ and bound annexinV at the site of injection, suggesting they received a signal for activation-induced death.[43] Others studies using rAAV as a vaccine to HIV-1 epitopes have also suggested a T cell exhaustion after administration.[44, 45] Despite that a transgene-specific cytotoxic T cell response was induced after the first exposure to

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rAAV, and the cells failed to proliferate after a booster immunization with an adenoviral vector encoding for the same antigen due to their inability to differentiate into memory T cells. It turns out these cells were also expressing inhibitory markers including PD-1 and CTLA-4 suggesting exhaustion. More recently, exhausted CD8 cells in addition to Tregs have been described in clinical trials using AAV vectors.[9, 10]

First evidence of immune tolerance to recombinant AAV in clinical trials

In 2011 Flotte *et al*. reported that in AATD patients injected IM with rAAV1 (6e12vg/kg) expressing AAT presented with an IFNγ^+ ELISpot immune response to AAV capsid without loss of transgene expression 3 months after dosing.[46] In 2013, Mueller *et al*. published a follow-up to this clinical study showing the first evidence of Treg activation in response to AAV capsid after gene transfer in humans.[9] AATD patients have been IM injected with an AAV1 vector encoding for the AAT protein. Patients injected with the highest dose of vector (6e12vg/kg) presented with a transient elevation of creatine kinase at 30 days post-injection and a partial decrease of transgene expression.[46] However, the AAT expression persisted for more than 1 year after dosing despite the detection of cellular infiltrates in the injected muscle. The characterization of muscle biopsy sample by immunofluorescence staining and analysis bisulfite-specific PCR of the methylation of TSDR (Treg-specific demethylated region) showed that ~10% of the T-cells in the biopsy material was actually composed of Tregs (CD4+CD25+FoxP3+). Moreover, an *in vitro* restimulation assay revealed that the patients had peripheral Tregs that were reactivated when stimulated with AAV1-capsid peptides, demonstrating they were AAV1 capsid-specific. The presence of the Helios marker on peripheral AAV specific Tregs suggests that this population is natural Treg. Another important finding in that study was that the IM injection of AAV1 may be mimicking a chronic infection. While unexpected it was reported that intact AAV1 capsids were still present in the biopsy material at both 3 months and 1 year post-administration. In fact, much like would be expected during a chronic infection the muscle biopsies showed high levels of staining for PD-1 and PD-L1 markers *in situ*, suggesting initiation of a T cell exhaustion. More recently this was confirmed in a update on this trial where the authors described sustained expression of AAT protein 5 years after dosing with continued evidence of infiltrated Treg and more importantly the presence of exhausted CD8 T cells *in situ*, all concomitant with a persistent IFNγ positive ELISpot to AAV capsid.[47]

The AAV1 trials by Flotte *et al.* for the first time have shown that a tolerogenic response could be initiated after IM AAV-based gene transfer in humans. However, the mechanism remains to be established whether it is due to the dose, the transgene, and/or the administration route. There is some evidence to suggest that the mode of delivery and transgene could not be the only factors inducing this tolerance phenomenon because a previous clinical trial targeting LPL-deficient patients injected intramuscularly with a rAAV1 showed a loss of gene transfer efficacy after 18 to 31 months post dosing[48] probably related to an IFNγ immune response to AAV1 capsid (**Table 1**).[49] On the other hand, the same clinical trial performed more recently on patients transiently immunosuppressed described a continued transgene expression based on increased chylomicron turnover at 52 weeks and positive biopsies and showed evidence of infiltrated Treg *in situ* (**Table 1**).[10]

Consequences of immunosuppressive treatment in AAV-based gene transfer

Since a deleterious immune response to AAV capsid was described in patients in 2006, efforts were made to develop new strategies preventing this phenomenon. One of the strategies established was the use of immunosuppressant drugs. These protocols were first tested in animal models and inspired from immunosuppressive protocols developed to prevent graft rejection in transplantation and have shown mixed results. More recently glucocorticoid steroid immunosuppression has also been used in the context of human trials and has shown promising results after recombinant AAV gene transfer.

Immunosuppression protocols in animal models after AAV-based gene transfer.

Preclinical studies have shown the importance of immunosuppressant choice and combination. In one study, an ATG (anti-thymocyte globulin), cyclosporine, and mycophenolate mofetil (MMF) cocktail inducing T cell depletion, blocking transcription of cytokine genes and targeting activated T cells and primary antibody response respectively was used in Duchenne muscular dystrophy (DMD) dogs injected IM with an AAV6 vector encoding for the canine microdystrophin.[50] This cocktail allowed transgene expression persistence even after treatment interruption in areas without cellular infiltrates and these results were then confirmed in the same model after injection in several muscles; transgene expression was maintained until 1 year post dosing after immune suppressive treatment was stopped.[51]

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On the contrary, the immune suppressive treatment might sometimes have a negative impact on gene transfer efficacy as described in a non-human primate (NHP) study. NHPs received an AAV2 vector encoding for FIX and were assigned to 3 groups receiving no immunosuppression, a combination of MMF, sirolimus (a potent inhibitor of antigen-induced proliferation of T cells and B cells) and daclizumab and a combination of MMF and sirolimus 1 week prior to dosing and maintained 10 weeks after liver-directed gene transfer. Surprisingly, the animals on 3-drug immunosuppressive (IS) regimen developed high titer inhibitory antibodies to FIX whereas monkeys receiving the vector alone or the 2-drug IS regimen did not and had sustained FIX expression. The 3-drug regimen also had a negative impact on antibodies to AAV2 capsid by showing higher antibody titer than monkeys receiving no IS or the 2-drug IS regimen where the titers were the lowest. In this study, the 3-drug IS regimen did not only show a boosting effect on the B-cell response but also a deleterious effect on the cellular immune response through a dramatic drop of Treg percentage. It was not the case when they receive the same regimen without the daclizumab. This phenomenon was explained by the fact that this drug is an antagonist of the CD25 receptor highly expressed on Tregs.[52]

These studies highlight the importance of optimizing the immune suppressive regimens before protocol translations to humans. Immunosuppressive treatments have shown the ability to prevent immune responses to AAV capsid in humans affected by muscular dystrophies or metabolic disorders.

Use of immune suppressive drugs and/or regimen in AAV-based gene transfer clinical studies

The first evidence of the efficiency of immune suppressive drugs on immune response to AAV capsid in patients was described in 2011. Hemophilia B patients were injected with an AAV8 vector encoding for codon-optimized FIX transgene at different doses. On the contrary of preclinical studies described above or clinical trials cited below, the administration of immunosuppressive drug was not initially planned and occurred because patients receiving the highest dose (2e12 vg/kg) have shown an elevation of liver transaminases after 7 to 10 weeks post vector administration as already described.[2] These patients received a transient immune suppression based on injection of prednisolone during at least 4 weeks, and then until transaminases returned to baseline. This corticosteroid treatment was justified by its efficiency in severe autoimmune hepatitis where cytotoxic T lymphocytes (CTL) are directed to hepatocytes. This transient immune suppression led to resolution of elevated ALT level (range 2 to 35 days) and disappearance of capsid-

specific T cell response demonstrating the effect of prednisolone on cellular immune response to AAV capsid occurring after gene transfer.[6] The last update was published in 2014, and the patients are still expressing the FIX 3 years after gene transfer.[53] Given that this was not a controlled clinical trial with two arms to explore the effectiveness of the prednisolone on curbing the immune clearance of AAV transduced hepatocytes it is yet to be determined if this intervention can be applied broadly.

The efficacy of this immunosuppressive regimen was also described in clinical trials targeting neuromuscular disorders however in this context they were designed to prevent or suppress immune responses to the transgene protein product and not necessarily to the AAV capsid. Patients suffering of muscular dystrophies are generally treated with glucocorticoids known to inhibit inflammation-associated molecules such as cytokines and chemokines, and prednisolone treatment in these patients has shown improvement of motor function and increased ambulation up to 1-3 years. However, the mechanism remains unknown.[54] The prednisolone was also used in two different clinical trials in LGMD (limb-girdle muscular dystrophy) and DMD patients to prevent cellular immune responses. The LGMD patients were off glucocorticoid treatment during the trial and 3 months before gene therapy. They received a boost of methylprednisolone 4 hours prior to dosing (2mg/kg IV) to prevent inflammation due to the needle. In patients injected with 3.25e11 vg of an AAV1 vector, no IFNγ response to AAV capsid was detected, despite CD4+ and CD8+ cell detection *in situ* and transgene expression having been observed for 3 months.[55] Regarding the immune response to the transgene, a clinical trial was performed on DMD patients using IM injection of an AAV1 vector encoding for the minidystrophin while patients underwent a standard glucocorticoid therapy and received methylprednisolone prior to dosing, as described previously. Surprisingly, 2 out of 6 patients showed an IFNγ cellular immune response to the dystrophin protein. These cells were already detected before injection, suggesting that these patients had a pre-existing immunity to the dystrophin probably due to the expression of this protein in revertant muscle fibers.[56] Thus, this immunosuppressive treatment does not seem to prevent the reactivation of specific-T cells to the transgene. The authors did not describe the cellular immune response to the capsid in this study.

More recently and in a different patient population, a transient prednisolone treatment resolved the episode of transaminase elevation in addition to a high IFNγ response to AAV9 capsid in spinal muscular atrophy (SMA) patients, which appeared 2 to 3 weeks after IV administration[57] (**Table 1**) as already described in hemophilia B patients.

All these trials described a persistence of transgene expression with a transaminase elevation episode resolved and a loss of IFNγ-secreted T cells detection after transient immunosuppressive treatment. However, the mechanism(s) responsible for induction of tolerance to AAV capsid, and in some cases to the transgene remain to be explored. Only one study in LPL deficient-patients has shown the evidence of Treg induction after AAV gene transfer associated to immune suppressive regimen.

As described earlier, the first clinical trial performed on LPL deficient-patients receiving an AAV1 vector encoding the LPL protein has shown an elevation of creatine kinase correlated to a dose-dependent activation of a capsid-specific T cell response limiting the duration of transgene expression.[49] The results led to the development of a new clinical trial including an immune suppressive regimen pre- and post-vector administration. The vector used in this trial was the first approved by the EMA (European Medicines Agency). The regimen consisted in administration of cyclosporine and MMF starting before injection and continued for 12 weeks in addition to a bolus of methylprednisolone 30 min before dosing. This led to a long-term transgene expression and clinical improvement of the metabolic disorder. Regarding the immune response to AAV capsid, patients have shown a transient IFNγ positive response to AAV capsid without any clinical consequences. As already described after IM injection, the patients presented infiltrated Treg (CD4+ FoxP3+) cells *in situ* in addition to CD8+ cells. In accordance to the AAT clinical trial results where T cells are expressing PD-1 and PD-L1 regulatory markers ([9] and unpublished data), the infiltrated CD8+ T cells do not seem to present characteristics of cytotoxicity due to absence of expression of Granzyme B and Fas ligand. A further study regrouping analysis of exhausted T cell markers would be interesting to characterize the mechanism of tolerance involved.

Conclusion and future prospects

In the past decade, immunological research on AAV vectors has evolved considerably. Before translation to humans, rAAVs were considered as non-immunogenic vectors due to their low ability to transduce APC.[1] Now, we know that they are able to trigger an innate immune response through TLR (toll-like receptor) signaling pathways[58] but also transduce DC *in vitro*[59-61] and *in vivo.*[62] This parameter could play a major role in the initiation of the immune response or reactivation of the pre-existing humoral and/or cellular immunity[63, 64] after gene transfer. Previous studies showed the DC transduction efficiency is dependent of AAV serotype and genome; self-complementary vectors are more efficient than

single-stranded vectors and rAAV serotype 1, 2 and 6 are described to transduce murine[59] and human monocyte- or $CD34^{+}$-derived DC[60, 61] whereas serotype 8 presents a lower tropism to DC at least *in vitro*.[65] This difference of tropism for DC between the different serotypes and the genome conformation (ss versus sc vector) could explain why some serotypes are more immunogenic or on the opposite more tolerogenic than others. Further investigation of DC phenotype or functional maturation after rAAV transduction would be helpful to understand mechanisms involved in immune response initiation or reactivation and to modulate immune responses as described by Pandya *et al.* in an anti-tumor vaccine context.[66, 67] Despite that immune activating properties of rAAV are much reduced relative to other viral vectors like adenoviral or non-viral vectors.

A number of strategies have emerged to prevent adaptive immune responses to antigens such as the capsid and the transgene product by transient immune modulation. The duration of these treatments were based on studies suggesting that rAAV capsid do not persist more than 6 to 8 weeks, depending on serotypes, after gene transfer.[68] However, past and recent studies have demonstrated that AAV capsid can be detected up to 6 years after gene transfer depending on the administration route. Studies on NHP have shown that 6 years after subretinal injections, rAAV2 and rAAV5 capsids were still detected in the retina.[69] More recently, Mueller *et al.* described the AAV1 capsid persistence in the muscle 1 year after IM injection.[9] These observations support that AAV vector gene transfer could mimic a chronic infection due to the epitope persistence *in situ*. This hypothesis is also supported by the detection of Tregs and exhausted T cells *in situ* as described in two different clinical trials.[9, 10] However, the mechanisms initiating the regulation are still unknown; to date, we cannot conclude whether the persistent antigen exposure leads to T cell exhaustion and/or Treg initiation or whether the Treg are initiating the exhaustion. Moreover, the administration route cannot be the only factor inducing the immune regulation either. Indeed, in LPL clinical trials, regulatory T cells were observed when an immunosuppressive regimen is administered and in the AAT context we cannot exclude the influence of the transgene itself. AAT is actually described to help promoting immune regulation; this protein is described to promote IL10 production by DC and to facilitate Treg expansion in mouse models.[70] Another way to promote tolerance to AAV capsid would be the use of molecules targeting DC maturation and/or Treg initiation. Nowadays, the only immune suppressive treatment administered in patients injected with a rAAV was glucocorticoids prior to or post dosing. Further studies using molecules altering DC maturation and differentiation such as FLT3[71] and GSK-3[72] inhibitors and Wtn[73] in a β-catenin-dependent or -independent signaling pathways or drugs promoting Treg induction like rapamycin[74] would deserve to be investigated in a rAAV-based gene transfer context.

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Along this review, we have seen the administration route seems to play an important role in immune response modulation to AAV capsid but additional factors are described to modulate the immune response initiation after gene transfer, including the AAV serotype, the promoter (ubiquitous versus tissue-specific), and/or the dose as illustrated in **Table 2**; some AAV serotypes are described to be more tolerogenic than others[65] and the dose also appears important, as observed in several clinical trials experimenting a dose-escalation. Most of the time, the low or intermediate doses do not lead to an enzyme elevation or to an immune response to the AAV capsid. However, the main limitation—therapeutic level—is not reached at the lower doses. Finally, contrary to the clinical trial in hemophilia B patients reported by Manno *et al.*, subsequent trials at a lower (5e11 vg/kg, Spark Therapeutics) or equivalent dose (5e12 vg/kg, uniQure) and in absence of any suppressive regimen have resulted in patients expressing 31.8 to 5.1% of normal FIX level respectively, and most of them do not present any elevation of ALT/AST transaminases and/or cellular immune response to AAV capsid. Clearly there is a broad variability in immune responses mounted by patients and to date there is not a clear predictor of which patents will mount an expression limiting response or not. Given the complexity of the human immune system and immense diversity of the TCR and MHC repertoire of each individual it may take some time before we can properly predict which patients are likely to present with self-limiting AAV capsid immune response. The field has been fortunate that while the immune response to AAV are far more complex than initially appreciated (i.e. involving Tregs, CTL and exhausted T cells) in the clinical setting it remains one that is amenable thus far to immune modulation with prednisolone and rather mild when placed in the context of other gene therapy approaches.

In conclusion, during the past decade, this field has considerably evolved, and we are now able to observe long-term transgene expression in patients when immune responses to capsid and/or transgene are avoided or under control. However, cytotoxic as well as tolerogenic immune response initiation still need to be investigated and understood in order to develop safer gene transfer strategies.

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Author Disclosure Statement

C.M. is an inventor on various patents that may be entitled to royalty payments in the future. J.M.W. is an advisor to REGENXBIO, Dimension Therapeutics, and Solid Gene Therapy, and is a founder of, holds equity in, and has a sponsored research agreement with REGENXBIO and Dimension Therapeutics; in addition, he is a consultant to several biopharmaceutical companies and is an inventor on patents licensed to various biopharmaceutical companies. G.G. declares no competing financial interests.

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Figure legends

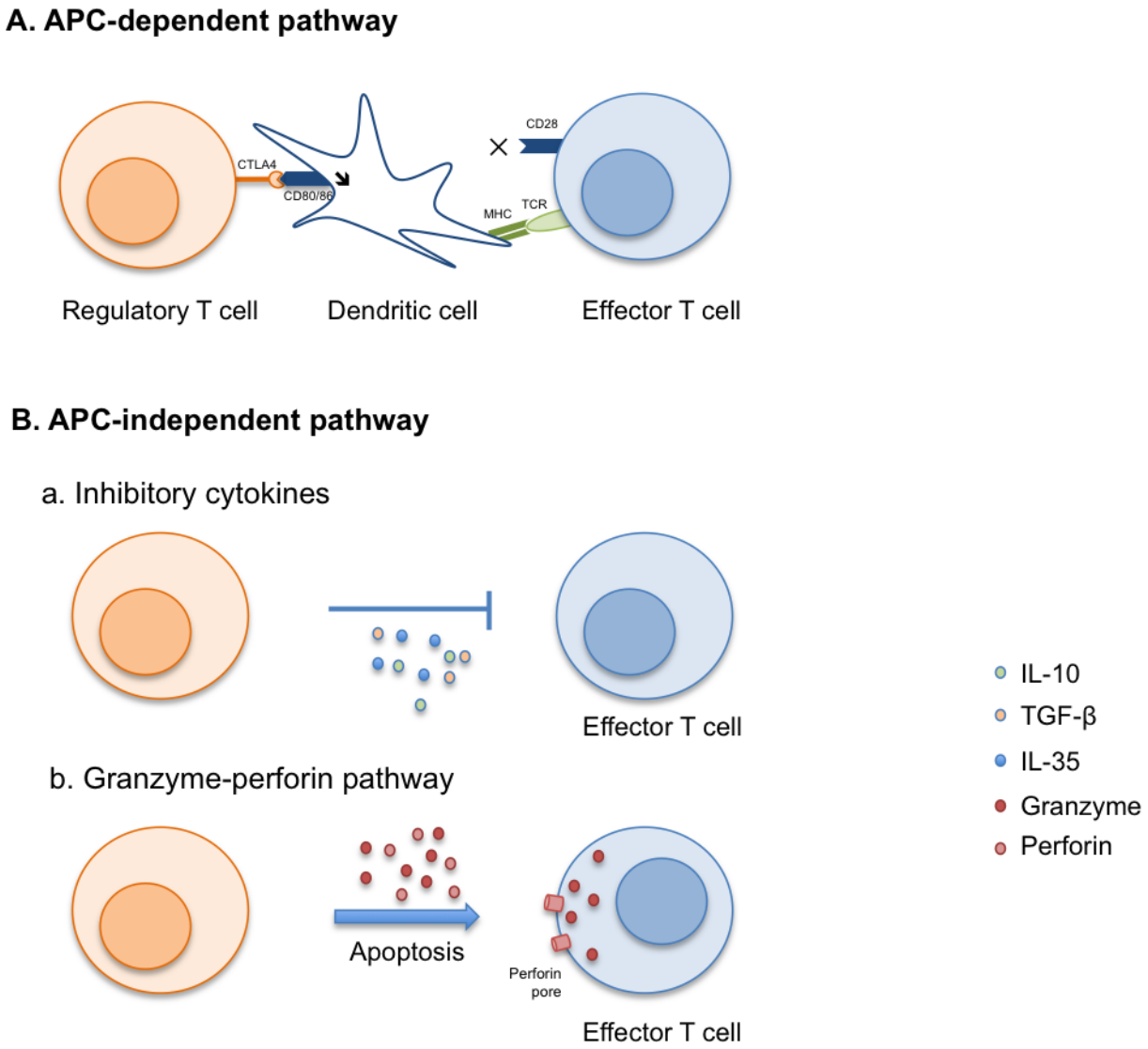

Figure 1. Mechanisms used by regulatory T cells to mediate tolerance. Tolerance can be mediated by APC-dependent (**A**) or independent (**B**) pathways. **A.** Regulatory T cells are able to prevent DC maturation through the downregulation of CD80/86 costimulatory receptor required for effector T cell activation. **B.** Cytokines secreted by Tregs are able to prevent effector T cell proliferation and IFNγ secretion (**a**) but also to induce cell death mediated by the granzyme/perforin pathway (**b**).

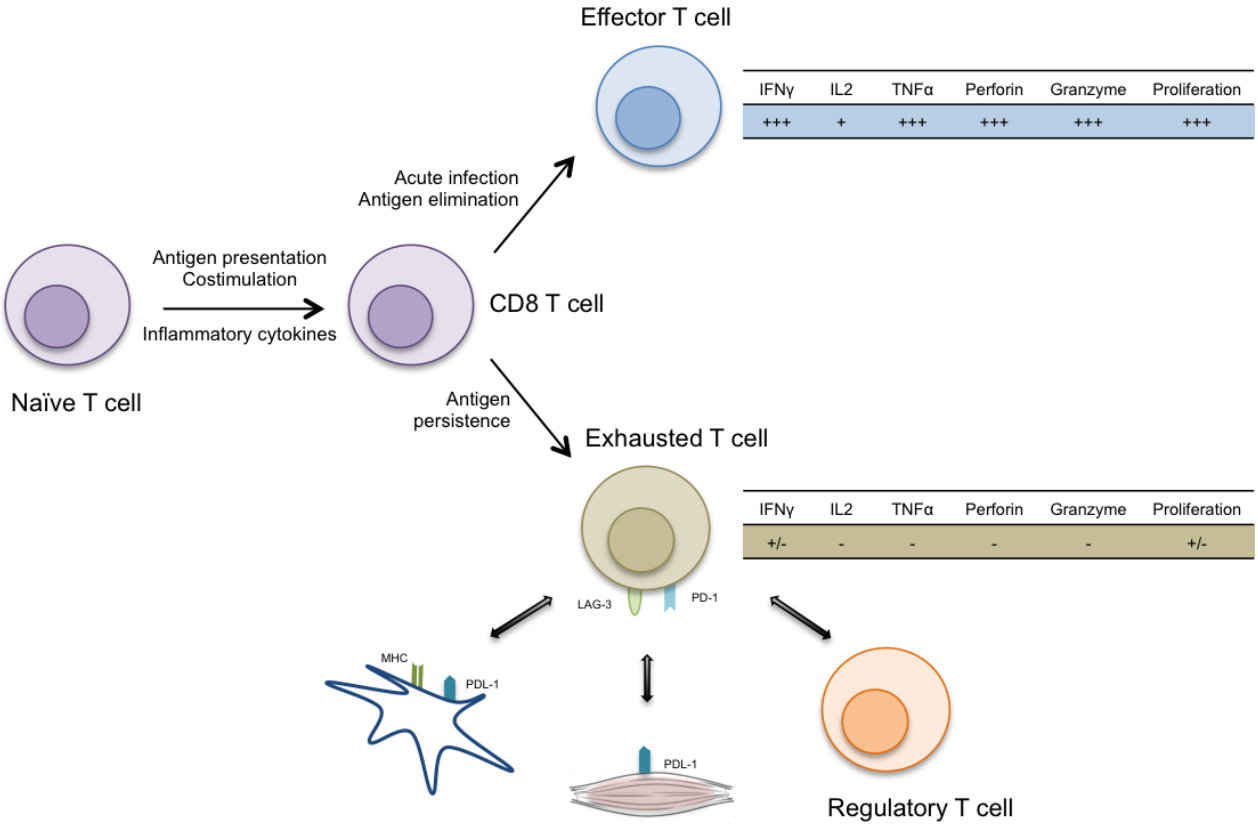

Figure 2. Mechanisms leading to T cell exhaustion. During infection, T cells are primed by antigen, costimulation signals, and inflammatory cytokines, and they differentiate into effector T cells. These cells show a cytotoxic (IFNγ, IL2 and TNFα secretion) and cytolytic (perforin and granzyme release) phenotype and a high capacity of proliferation (top panel). During chronic infection and/or recombinant AAV-mediated gene transfer, antigens persist *in situ*. T cells progressively lose their effector functions leading to T cell exhaustion. This exhaustion is driven by different pathways: interactions between regulatory receptors (PD-1 and LAG-3) and their ligands expressed by dendritic cells and/or target cells and interactions with immunoregulatory cell types such as Tregs (bottom panel).

Gernoux_Table1

Disease	rAAV serotype	Expression cassette	Mode of delivery	Dose	Immunosuppressive treatment	IFNγ response to rAAV capsid	Enzyme elevation	Transgene expression	References
Alpha1-antitrypsin deficiency	ssAAV1	CB-AAT	Intramuscular injection	6e12 vg/kg	None	+	Transient (CK)	Sustained but below the therapeutic concentration	Flotte *et al*. 2011[46]
Hemophilia A	ssAAV5	HLP-FVIIIco	Intravenous infusion	6e13 vg/kg	Transient, starting after dosing	+	+ (ALT)	Sustained, up to 28 weeks	Biomarin[75]
Hemophilia B	ssAAV2	APOE-HCR-SERPINA1-FIX	Hepatic artery	2e12 vg/kg	None	+	+ (ALT/AST)	Transient	Manno *et al*. 2006[2]
	scAAV8	LP1-hFIXco	Intravenous infusion	2e12vg/kg	Transient (8 to 12 weeks time-course after transaminase elevation)	+	Transient (ALT/AST)	1 to 6% of normal FIX level (up to 3 years)	Nathwani *et al*. 2014[53]
	N/A designated as SPK-9001	Padua mutant FIX	Intravenous infusion	5e11 vg/kg	None, except 2 out of 9 who developed immune response to AAV capsid (tapering course of corticosteroids)	- except for 2 patients	None, except 2 patients with liver enzyme elevation	31.8% of normal FIX level, up to 12-31 weeks	Spark Therapeutics[7]
	AAV5	LP1-FIXco	Intravenous infusion	5e12 vg/kg	None, except 1 out of 5 patient treated with prednisolone (7 weeks tapering course, 10 weeks after dosing)	-	None, except 1 patient with transient ALT	5.1% of normal FIX level, up to 6 months	uniQure[8]
				2e13 vg/kg	None, except 2 out of 5 patients treated with prednisolone	-	None, except 2 patients with transient ALT	7 to 13% of normal FIX level, up to 6 months	
Lipoprotein lipase deficiency	ssAAV1	CMV-LPL	Intramuscular injection	3e11 vg/kg	None	+	Transient (CK)	Transient, triglycerides return to baseline 10 weeks after dosing	Mingozzi *et al*. 2009[49]
	ssAAV1	CMV-LPL	Intramuscular injection	1e12 vg/kg	Transient (12 weeks time-course starting shortly before dosing)	- except for 2 out of 5 patients	None	Sustained, viral genome detected up to 52 weeks	Gaudet *et al*. 2013[76] Ferreira *et al*. 2014[10]
Spinal muscular atrophy	scAAV9	CB-SMN	Intravenous infusion	6.7e13 vg/kg 2e14 vg/kg	Transient (short-term treatment after dosing)	+	Transient	CHOP-INTEND score improved and survival increased	Mendell *et al*. 2016[57]

Table 1. Clinical trials using recombinant AAV and implications of a capsid-specific immune response on gene transfer efficacy.
ALT: Alanine transaminase; AST: Aspartate transaminase; CK: Creatine kinase; co: codon-optimized; ss: single-stranded; sc: self-complementary

Gernoux_Table1

Site of administration	rAAV serotype	Indication	Promoter	Dose	Antibody response	T cell response	Regulatory T cell induction	References
CNS	ssAAV2	Canavan disease	NSE	1e12 vg	- (7/9)	n/a	n/a	McPhee *et al.* 2006[77]
	AAVrh10	MPSIIIA	PGK	7.2e11 vg	-	n/a	n/a	Tardieu *et al.* 2014[78]
Eye	ssAAV2	LCA2	CBA	1.5e11 vg	+ (2/3, transient)	-	n/a	Simonelli *et al.* 2010[79]
Liver	ssAAV2	Hemophilia B	Serpina1	8e10 vg/kg	+	-	n/a	Manno *et al.* 2006[2]
				4e11 vg/kg	+	-	n/a	
				2e12 vg/kg	+	+	n/a	
	AAV5	Hemophilia B	LP1	5e12 vg/kg	n/a	- (4/5)	n/a	uniQure[8]
				2e13 vg/kg	n/a	- (3/5)	n/a	
	scAAV8	Hemophilia B	LP1	2e11 vg/kg	+	-	n/a	Nathwani *et al.* 2014[53]
				6e11 vg/kg	+	-	n/a	
				2e12 vg/kg	+	+	n/a	
	scAAV9	SMA	CB	6.7e13 vg/kg	n/a	+	n/a	Mendell *et al.* 2016[57]
	SPK-9001	Hemophilia B	not specified	5e11 vg/kg	n/a	- (7/9)	n/a	Spark Therapeutics[7]
Muscle	ssAAV1	AATD	CB	6e11 vg/kg	+	+	n/a	Flotte *et al.* 2011[46]
				1.9e12 vg/kg	+	+	n/a	Mueller *et al.* 2013[9]
				6e12 vg/kg	+	+	+	
		LGMD2D	MCK	3.25e11 vg	+	-	n/a	Mendell *et al.* 2009[55]
		LPLD	CMV	1e11 vg/kg	+	+ (2/4)	n/a	Mingozzi *et al.* 2009[49]
				3e11 vg/kg	+	+ (2/4)	n/a	
			CMV	1e12 vg/kg	+	-	+	Ferreira *et al.* 2014[10]
	ssAAV2	AATD	CB	2.1e12 to 6.9e13 vg/kg	+	-	n/a	Brantly *et al.* 2006[80]
		Hemophilia B	CMV	2e11 to 1.6e13 vg/kg	+	-	n/a	Manno *et al.* 2003[81]

Table 2. Factors involved in immune responses to AAV capsid in clinical trials.
ss: single-stranded; sc: self-complementary; CNS: Central nervous system; MPSIIIA: Mucopolysaccharidosis Type IIIA; LCA2: Leber's congenital amaurosis type 2; SMA: Spinal muscular atrophy; AATD: α1-antitrypsin deficiency; LGMD2D: Limb-girdle muscular dystrophy type 2D; LPLD: lipoprotein lipase deficiency; n/a: not analyzed.