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THERAPY

Muscle-Directed Delivery of an AAV1 Vector Leads to Capsid-Specific T Cell Exhaustion in Nonhuman Primates and Humans

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With the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) approvals for Zolgensma, Luxturna, and Glybera, recombinant adeno-associated viruses (rAAVs) are considered efficient tools for gene transfer. However, studies in animals and humans demonstrate that intramuscular (IM) AAV delivery can trigger immune responses to AAV capsids and/or transgenes. IM delivery of rAAV1 in humans has also been described to induce tolerance to rAAV characterized by the presence of capsid-specific regulatory T cells (Tregs) in periphery. To understand mechanisms responsible for tolerance and parameters involved, we tested 3 muscle-directed administration routes in rhesus monkeys: IM delivery, venous limb perfusion, and the intra-arterial push and dwell method. These 3 methods were well tolerated and led to transgene expression. Interestingly, gene transfer in muscle led to Tregs and exhausted T cell infiltrates *in situ* at both day 21 and day 60 post-injection. In human samples, an in-depth analysis of the functionality of these cells demonstrates that capsid-specific exhausted T cells are detected after at least 5 years post-vector delivery and that the exhaustion can be reversed by blocking the checkpoint pathway. Overall, our study shows that persisting transgene expression after gene transfer in muscle is mediated by Tregs and exhausted T cells.

INTRODUCTION

The adeno-associated virus (AAV) is a dependovirus belonging to the parvovirus family. Its 4.7-kb genome carries 2 *Rep* and *Cap* genes flanked by 2 inverted terminal repeats (ITRs) and is packaged into an icosahedral protein capsid composed of 3 proteins: VP1, VP2, and VP3 at a 1:1:10 ratio. Despite its low encapsidation capacity, its genetic simplicity and low immunogenicity have made it a promising tool for gene transfer in the past decades. With the recent FDA and EMA approvals for Zolgensma, Luxturna, and Glybera, recombinant AAVs (rAAVs) are now, more than ever, considered efficient and successful viral vectors for the treatment of inherited disorders. Its variety of serotypes offers a large spectrum of abilities to transduce tissues, including the liver,[1,2] muscle,[3–6] central nervous system (CNS),[7–9] and retina,[10,11] as demonstrated in preclinical studies targeting metabolic,[12–16] neuromuscular,[17–20] neurodegenerative,[21,22] and retinal[23–25] disorders. Interestingly, certain serotypes of AAV capsids elicit more robust immune responses than others, with AAV8 being less immunoreactive and AAVrh32.33 being more immunoreactive in mouse models.[26,27] Furthermore, when protocols were translated to patients, limits imposed by the host immune system have emerged. Indeed, the study of T cell-mediated immune responses to AAV capsids in patients have shown that, after gene transfer, memory T cells can be reactivated and can lead to transduced cell clearance.[28] The first evidence was an elevation of transaminases related to a cellular immune response characterized by interferon (IFN)γ-secreted T cells in response to AAV capsid peptide libraries.[28–30] Studies on pre-existing immunity to AAV have shown that exposure to wild-type AAV2 occurs early in life,[31] suggesting that gene transfer past 3–18 years of age using AAV vectors can potentially result in the re-activation of these memory T cell pools. Currently, one way to efficiently manage the cytotoxic immune response and restore gene expression post-vector delivery is transient corticosteroid-based immunosuppression.[29,32]

Elevation of serum enzymes is not always evidence of a deleterious immune response resulting in loss of transgene expression. In a clinical trial targeting the muscle in the absence of any immunosuppression, Flotte et al.[33] showed persistent alpha1-antitrypsin (AAT) protein expression in serum, despite (1) an elevation of serum creatine kinase and (2) the detection of IFNγ-secreting cells to AAV1 capsid in peripheral blood mononuclear cells (PBMCs). This was explained by the presence of muscle-infiltrated regulatory T cells (Tregs) 1 year post-vector delivery in addition to AAV capsid persistence *in situ*.[34] Tregs were also detected in lipoprotein-lipase-deficient (LPLD) patients[35] after intramuscular (IM) gene transfer, suggesting that cellular immune response to rAAV does not lead exclusively to cytotoxicity and gene transfer failure but can also result in immune tolerance. Altogether, these data

Table 1. Experimental Groups

Vector	Route	Dose	Animal No.	Serum rhAAT-cMyc Concentration at Endpoint (μg/mL)
ssAAV1-CB-rhAAT-cMyc	IM	6e12 vg/kg	RA1598	12
			RA1683	50
			RA0764	51
ssAAV1-CB-rhAAT-cMyc	VLP	6e12 vg/kg	RA1567	92
			RA0770	116
			RA0332	61
ssAAV1-CB-rhAAT-cMyc	IAPD	6e12 vg/kg	RA1562	20
			RA1709	17

rhAAT, rhesus alpha1-antitrypsin; ss, single-stranded; CB, chicken β-actin promoter; IM, intramuscular; VLP, venous limb perfusion; IAPD, intra-arterial push and dwell; vg/kg, viral genomes per kilogram.

highlight that (1) several factors can manage the nature of the T cell response, including the AAV serotype, the dose, the administration route, the transgene, and/or the promoter;[36] and (2) the nature of the tolerance mechanism and its kinetic are still not clearly established.

Here, we studied the mechanisms responsible for persisting transgene expression after gene transfer in muscle in rhesus monkeys by comparing three modes of delivery: IM; a high-volume venous limb perfusion (VLP), also called hydrodynamic intravenous delivery;[37,38] and an intra-arterial push and dwell (IAPD) method. To better characterize the mechanisms involved, we focused on Tregs and exhausted T cells. We performed an *in situ* immune cell analysis shortly after vector dosing (days 21 and 60) instead of analyzing peripheral immune responses. We showed that both Tregs and exhausted T cells might be responsible for the absence of a cytotoxic immune response after IM injection. Because we already had evidenced that IM injection may induce an AAV-specific Treg response in humans at least 1 year post-vector delivery, we focused on the functionality of exhausted T cells in AAT-deficient (AATD) patients. In the present study, we assessed the functionality of these cells by blocking the PD1 pathway and showed an increase of IFNγ secretion when the checkpoint pathway is blocked, demonstrating a capsid-specific T cell exhaustion.

Our results suggest that the absence of a deleterious T cell response to the AAV capsid is mediated by Tregs and exhausted T cells and occurs early after gene transfer to muscle and may offer a new way to interrogate *in situ* immune responses to the AAV capsid. Importantly, these responses occurred in both humans and non-human primates without either steroid therapy or any other form of immune modulation.

RESULTS

Experimental Design, Transgene Expression, and Cellular Immune Response

Rhesus macaques were injected with a single-stranded (ss)AAV1-CB-AAT vector at a dose of 6e12 viral genomes per kilogram (vg/kg), corresponding to the highest dose injected in AATD patients

(ClinicalTrials.gov: NCT01054339).[33] Three different methods of delivery were tested: IM (n = 3), VLP (n = 3), and IAPD (n = 2) (Table 1).[39] As previously described by our group,[39] the procedures are well tolerated, and the vector biodistribution analysis showed a broader distribution when the vector is delivered by VLP compared to IM injection. To track protein expression and differentiate endogenous AAT from recombinant AAT, a c-Myc tag was added to the recombinant rhesus AAT (rhAAT) sequence. The rhAAT-cMyc fusion protein expression was measured at the mRNA level by qRT-PCR and at the protein level in serum by a semiquantitative immunoblot over time.[39] Our results show that the protein expression was detected in all groups, with a higher protein expression in the VLP group: up to an average of 90 μg/mL at 60 days post-injection. The level of protein expression is respectively VLP > IM > IAPD (Table 1).[39] As expected, all the animals seroconverted after rAAV administration, as illustrated by detection of anti-AAV1 neutralizing antibodies (NAbs) and immunoglobulin G (IgG) (Table 2). In contrast to results obtained in the phase II AATD clinical trial, these animals showed sporadic and transient elevation in serum creatine kinase or liver transaminases (alanine aminotransferase [ALT]/aspartate aminotransferase [AST]). IFNγ secretion was measured by ELISpot on PBMCs prior to dosing and at necropsy. Only one animal (RA0332, VLP group) showed a positive cellular immune response to AAV1 capsid protein at day 60 post-injection (Table 2),[39] despite evidence of cell infiltrates *in situ*. Analysis of cell infiltrates in the injected limbs reveals that IM vector injection leads to a higher cell infiltration than the VLP and IAPD administration routes (Figure 1).

Activated Tregs Are Detected in Muscle after IM Injection in Nonhuman Primates

To date, two studies have shown that AAV1 IM injection can lead to Treg infiltration in injected muscles in AATD and LPLD patients.[34,35] To verify that Tregs can be detected in injected or perfused muscles post-AAV1 administration in non-human primates, muscle biopsies were performed at day 21 post-vector administration, and muscles were harvested at necropsy (day 60). After tissue dissociation, samples were analyzed for the presence of regulatory T cells *in situ*. This analysis was performed by flow cytometry. Regulatory T cells are defined as CD3+CD4+Helios+Foxp3+ cells (Figures 2A and S1A for isotype controls), and activated Tregs are defined as Tregs expressing CD25. For each nonhuman primate, the injected or perfused muscle and a contralateral muscle were analyzed at days 21 and 60 after dosing (Figures 2B and 2C; Table S1). Our results show that all animals that were injected IM have infiltrated Tregs at day 21 (7%–15% of CD4+ T cells), and these cells are still detected 60 days post-dosing (7%–8% of CD4+ T cells) (Figures 2B and 2C; Table S1). Some of the animals injected via VLP or the IAPD method had infiltrated CD4+ T cells (Figure 2B), but none of these cells were Tregs (Figure 2C). These results were then confirmed by immunohistochemistry. Since no Tregs were detected by flow cytometry after VLP and IAPD deliveries, we focused on muscle tissue from the IM group (Figures 2D and S2A for negative control). Our data confirm that Tregs are present in the muscle and suggest that these cells

Table 2. Humoral and Cellular Immune Responses to AAV Capsid

Route and Animal No.	Anti-AAV1 IgG Concentration (μg/mL)			Anti-AAV1 NAb Titer			IFNγ Secretion to rAAV1 Capsid
	Pre-dose	Day 21	Day 60	Pre-dose	Day 21	Day 60	
IM							
RA1598	ND	18.2	76.4	<1:10	1:640	1:1,280	−
RA1683	0.6	NA	1,080.2	1:10	1:1,280	1:20,480	−
RA0764	ND	30.0	530.1	<1:10	1:1,280	1:10,240	−
VLP							
RA1567	ND	91.2	139.7	<1:10	1:2,560	1:5,120	−
RA0770	ND	55.4	170.4	<1:10	1:1,280	1:5,120	−
RA0332	ND	51.3	159.7	1:10	1:1,280	1:5,120	+
IAPD							
RA1562	ND	19.8	120.4	<1:10	1:160	1:1,280	−
RA1709	0.3	32.0	241.3	<1:10	1:320	1:1,280	−

IM, intramuscular; VLP, venous limb perfusion; IAPD, intra-arterial push and dwell; NAb, neutralizing antibodies; ND, not detected, NA, not available; −, no response; +, positive response.

play a role in maintaining transgene expression *in situ* after IM vector administration in nonhuman primates.

Evidence of Exhausted T Cells in Injected Limb after Gene Transfer in Muscle in Nonhuman Primates

Regulatory T cells are not the only mediator of immune regulation. As we described in an AATD patient 1 year and 5 years after vector delivery,[34,40] exhausted T cells can also be involved in AAV capsid tolerance. As performed for the Tregs, we analyzed by flow cytometry the muscle tissues for the presence of exhausted T cells at day 21 and day 60 after vector administration (Figure 3). Exhausted T cells are defined as CD3+CD8+PD1+ cells (Figures 3A and S1B for isotype controls) and activated exhausted T cells are defined as CD3+CD8+PD1+ CD69+ cells. For each nonhuman primate, the injected or perfused muscle and a contralateral muscle were analyzed at both time points (Figures 3B and 3C; Table S2). Our results show that all animals that were injected IM have infiltrated exhausted T cells at day 21 (39%–48% of CD8+ T cells), and these cells are still detected (47%–87% of CD8+ T cells) (Figure 3C; Table S1) and express the CD69 activation marker (Table S3) 60 days post-dosing. Some of the animals injected by VLP or IAPD method have infiltrated CD8+ T cells expressing the PD1 marker as well (Figures 3B and 3C). These results were then confirmed by immunohistochemistry, as illustrated in Figure 3D (Figure S2B for negative control). Altogether, our data suggest that T cell exhaustion might also be involved in maintaining transgene expression after muscle-directed gene transfer.

Persistence of AAT Expression after Gene Transfer Is Driven by T Cell Exhaustion in AATD Patients

Our AATD clinical trial has shown that, despite an IFNγ immune response to AAV capsid, the transgene expression is maintained for years after gene transfer, suggesting an unresponsiveness toward the AAV capsid. Indeed, *in situ* analysis has shown that Tregs and exhausted T cells are present up to 5 years post-dosing in injected

muscle.[34,40] Because several studies have shown the reversal of the exhaustion by *in vitro* blockade of these inhibitory pathways, we have developed an assay to verify that functional exhausted T cells are involved in persisting AAT expression in the patient still expressing the transgene 5 years post-vector administration. First, we confirmed by ELISpot that the PBMCs were still secreting IFNγ in response to AAV capsid peptides but also in response to AAV1 immunodominant peptides described by Hui et al.[41] (Figure 4A; Table S4). Then, PBMCs were restimulated *in vitro* for 48 h with the AAV1 immunodominant peptides with or without PD1/PDL1 blocking antibodies or isotype controls. An IFNγ-ELISpot assay was performed and showed an increase in IFNγ secretion when blocking the PD1/PDL1 pathway (Figure 4B). The cell relative reactivity was assessed and showed that the IFNγ secretion increased by 44% when the checkpoint pathway is blocked, compared to the reference condition (stimulation with AAV1 peptides only; Figure 4C). Altogether, these data suggest that the lack of a cytotoxic T cell response to rAAV1 capsid is, indeed, mediated by exhausted T cells.

DISCUSSION

Despite the evidence of efficacy of AAV vectors for the treatment of inherited diseases, some limitations imposed by the host immune system, such as cellular immune responses to AAV vector and/or transgene, persist. Depending on the administration route, the dose, and/or the AAV serotype, gene transfer can result in a deleterious immune response[30,42–44] or tolerance.[34,35] Because immune tolerance initiation after AAV-based gene transfer in muscle remains unknown, we studied the mediators involved in persisting transgene expression after rAAV1 injection in muscle in rhesus monkeys by comparing three modes of delivery (IM, VLP, and IAPD) and in an AATD patient who was injected IM with an AAV1 vector . We showed that both Tregs and exhausted T cells are detected early after muscle-directed gene transfer and are involved in modulating immune response to AAV capsid in nonhuman primates and humans.
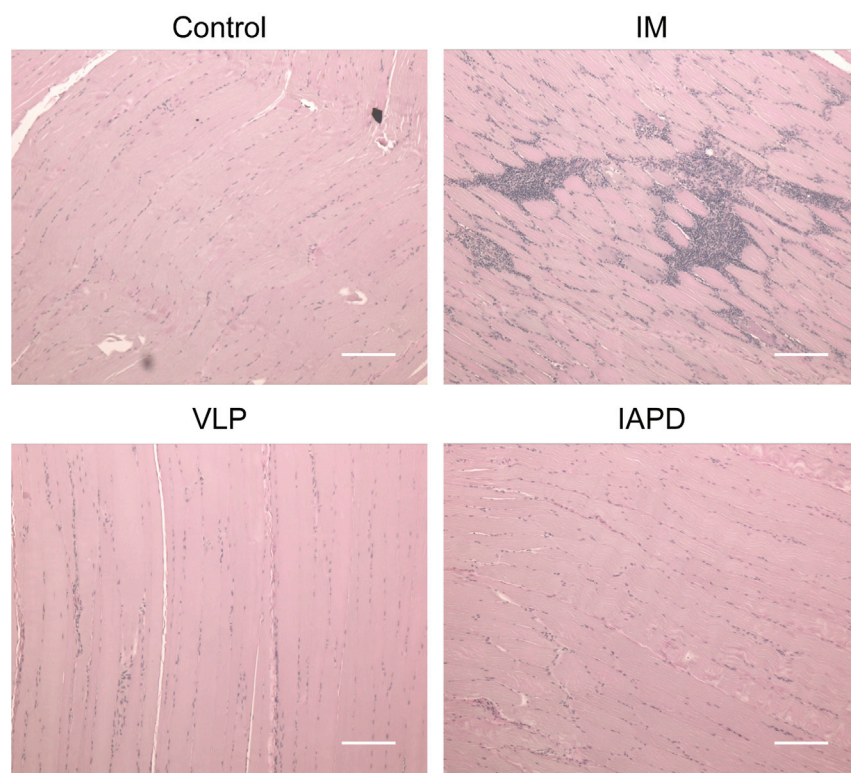
Control

IM

VLP

IAPD

Figure 1. Detection of Cellular Infiltrates in the Injected Muscle or Perfused Limb after rAAV1 Dosing
Nonhuman primates were injected IM (n = 3), VLP (n = 3), or IAPD (n = 2) with a ssAAV1 vector (6e12 vg/kg). Muscles were harvested 60 days after dosing. Infiltrated cell populations were analyzed by hematoxylin and eosin (H&E) staining in a control monkey, quadriceps receiving an IM injection (RA1683), VLP perfused quadriceps (RA0770), and IAPD perfused quadriceps (RA1709). Each illustration is representative of each group. Scale bars, 200 μm.

In this study, we confirmed in nonhuman primates that the injection of an AAV1 encoding for the AAT protein in muscle leads to a sustained transgene expression for up to 60 days with no evidence of an IFNγ-positive response to the capsid. This is expected, since a CD8 T cell-mediated cellular immune response to the capsid had not been anticipated in preclinical models[16,38,45] and had been described for the first time in hemophilia B patients treated with a rAAV2.[30] Although a capsid-specific immune response is not detected in the periphery (PBMCs), we analyzed and characterized the inflammation *in situ*. Our data confirmed that the IM delivery results in higher inflammation than VLP and IAPD deliveries.[38,46] So far, IM delivery has been described as immunogenic with the induction of antibodies to the transgene and loss of transgene expression. In this study, gene transfer in muscle leads to sustained transgene expression explained by the presence of Tregs and exhausted T cell infiltrates *in situ*. In our model, Tregs were detected only after IM injection, whereas exhausted T cells were detected in animals that were injected IM but also via VLP (2/3) and IAPD (1/2) deliveries but only by flow cytometry and at a lower frequency. These findings in nonhuman primates led us to analyze the same cell population in humans after gene transfer in muscle. In AATD patients, we have shown that, after 5 years post-vector delivery, Tregs and exhausted T cells are still detected in muscle.[40] Here, we focused on exhausted T cell properties. These cells are usually found in chronic diseases and are described to express PD1 receptor and to lose their ability to secrete cytotoxic cytokines such as IFNγ, interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α).[47] In the present

study, we show that AAV vectors can actually induce T cell exhaustion in AATD patients that can be reversed by immune checkpoint inhibitors. Altogether, our data suggest that both Tregs and exhausted T cells are involved in modulating the immune response to the AAV capsid. More importantly, our results indicate that these mechanisms seem to occur early after gene transfer, as soon as day 21 post-dosing, and last up to 5 years post-vector delivery.

Surprisingly, in our model, the absence of a detrimental immune response is observed when the vector is injected in the muscle. In gene transfer protocols, the liver has more often been described as a tolerogenic organ, as illustrated in animal model.[48–50] However, a systemic injection has shown limits due to a capsid-specific cellular immune response observed in high-dose patient groups injected with either rAAV2,[28] rAAV8,[29] or rAAV9[32] and was explained by the presentation of AAV-capsid-derived antigen on major histocompatibility complex class I (MHC I) expressed on hepatocytes.[51] The fact that it was not predicted in animal models can be due to the pre-existing immunity to AAV capsid in humans.[30,52,53] Even if monkeys are natural hosts of AAV, their pre-existing capsid-specific T cells differ from those of humans, with an effector phenotype compared to a memory phenotype in humans, and also have more restricted function: human T cells produce mainly IFNγ and IL-2 cytokines, while nonhuman primate T cells produce mainly IL-2.[54]

In contrast to liver, where capsid clearance presumably occurs up to 16 weeks after gene transfer depending on vector serotype,[55] in muscle, the AAV vector seems to be trapped in muscle for years after IM injection.[34] This results in a potential persistent exposure of T cells to capsid antigens that might be presented by antigen-presenting cells mimicking a chronic disease and explaining the induction of Tregs and exhausted T cells. Indeed, both cell types have shown the ability to mediate tolerance in the case of chronic diseases due to long duration of antigen exposure.[56–59] The initiation of a regulatory response can also be explained by the requirement of a certain concentration of epitopes to induce an active tolerance in opposition to ignorance.[60,61] In the case of a rAAV1 IM delivery, the vector stays
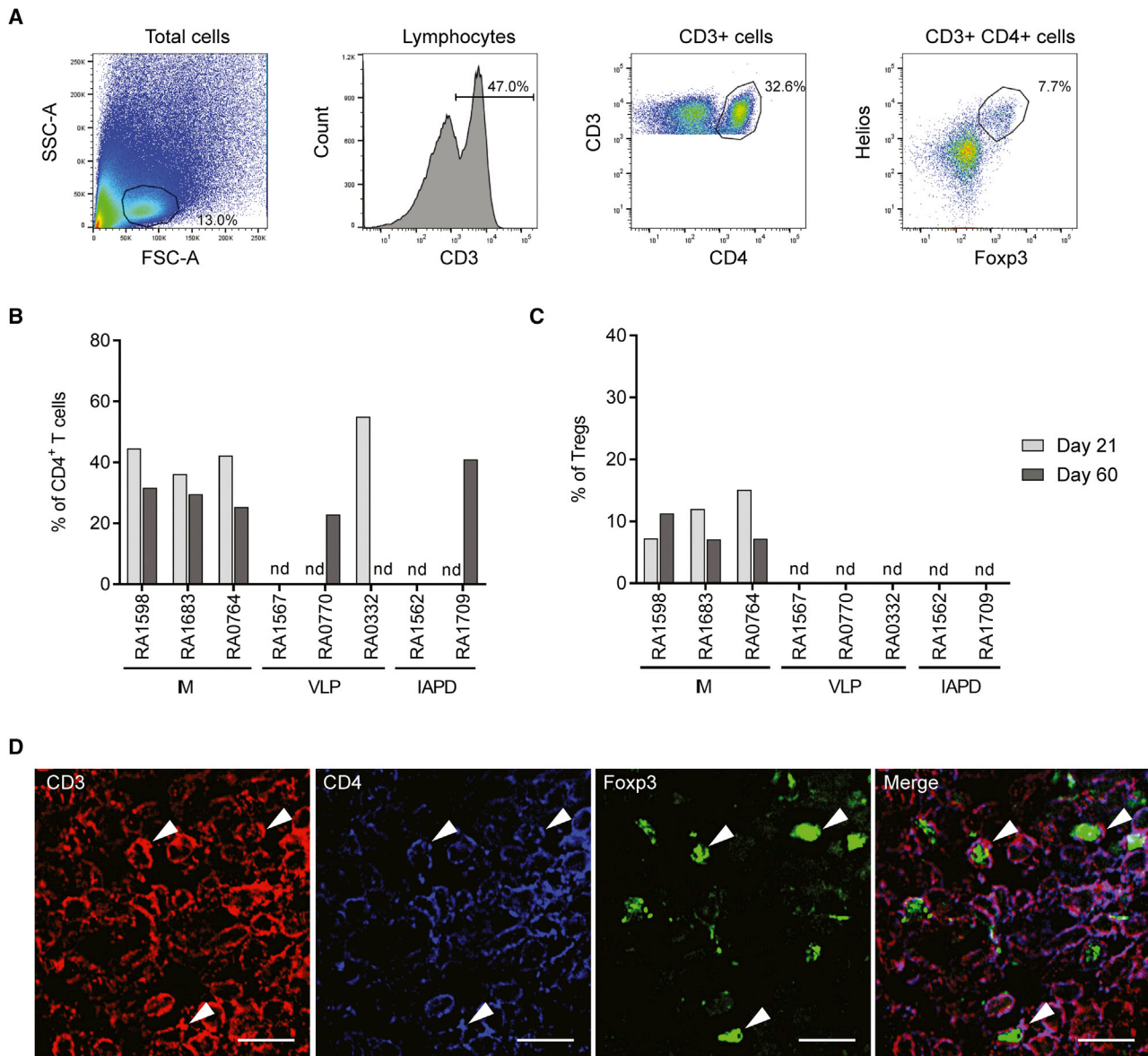
Figure 2. Regulatory T Cells Infiltrate the Muscle after rAAV1 IM Injection

Nonhuman primates were injected IM, VLP, or IAPD with an ssAAV1 vector (6e12 vg/kg). Muscles were harvested 21 and 60 days after dosing and were dissociated as described in the Materials and Methods section. Regulatory T cell population was analyzed by flow cytometry. (A) Gating strategy. RA1683, right quadriceps (injected muscle), 60 days after dosing. T cells are gated as CD3+ cells in the lymphocyte subset, CD4 T cells are gated as CD3+ CD4+ cells in the CD3+ subset, and Tregs are gated as Helios+Foxp3+ cells in the CD3+ CD4+ subset. (B and C) Percentage of CD4 T cells (B) and Tregs (C) in injected muscle or perfused limb (right quadriceps) at day 21 and day 60 after dosing. nd, not detected. (D) Detection of Tregs by immunohistochemistry in injected muscle after IM delivery (RA0764, day 60 post-injection). Scale bars, 10 µm. SS, side scatter; FSC, forward scatter.

concentrated at the site of injection, as illustrated by the vector biodistribution data: the same dose is administered via IM, VLP, or IAPD delivery, but the vector biodistribution is different, with a less concentrated and more diffuse distribution in the injected limb after VLP or IAPD delivery.[37–39,62–64] Tregs can also mediate T cell exhaustion,[65] but this does not seem to be the case here. Indeed, both cell types are detected early post-injection; more

importantly, exhausted T cells are detected after VLP and IAPD delivery, whereas Tregs are not.

In this study, we were able to correlate results obtained in monkeys to those obtained with patients, but our data confirm that monkeys do not completely mimic the immune response to the AAV capsid observed in humans. These observations highlight the complexity
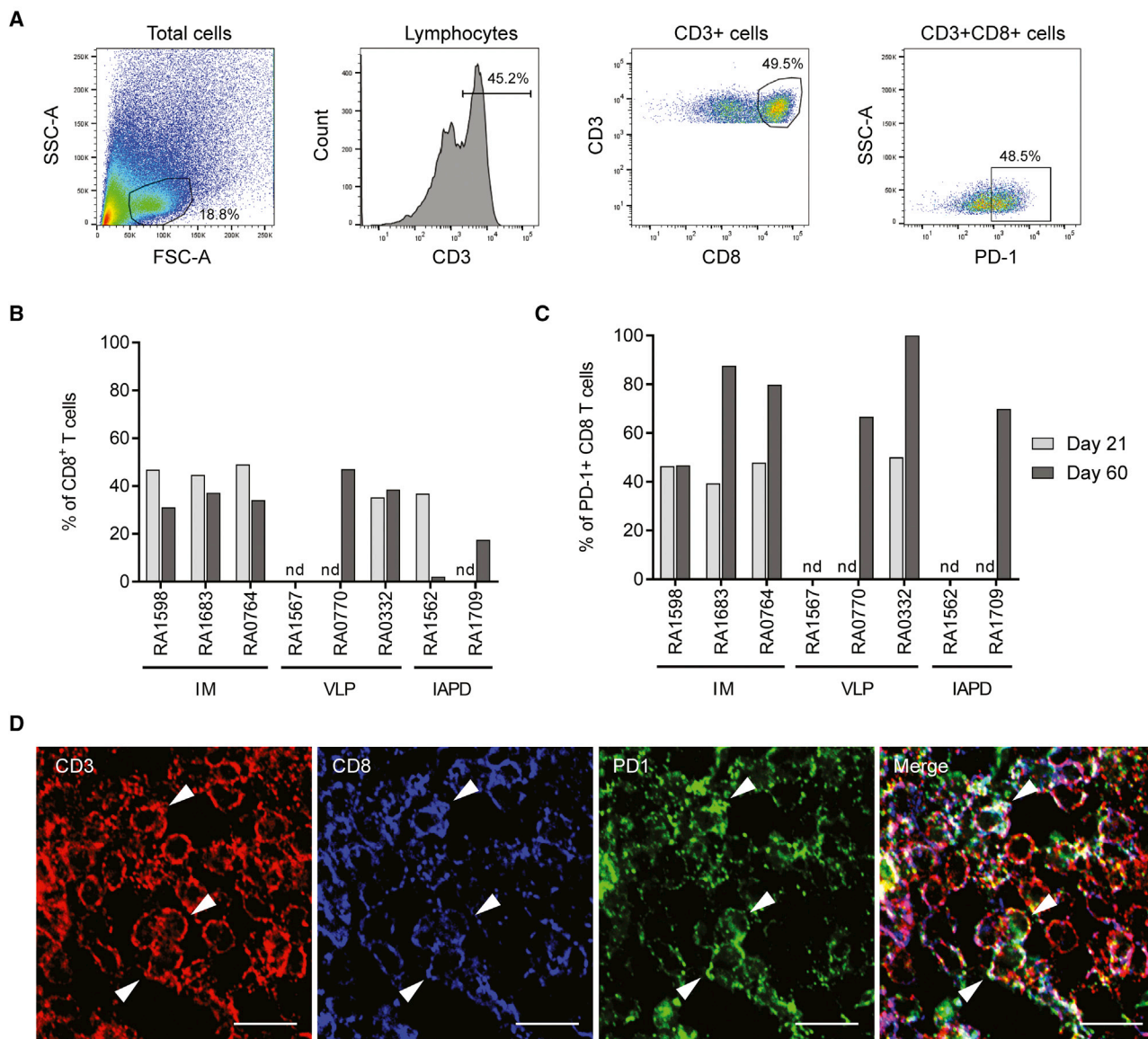
Figure 3. Exhausted T Cells Infiltrate the Muscle after rAAV1 Administration

Nonhuman primates were injected IM, VLP, or IAPD with an ssAAV1 vector (6e12 vg/kg). Muscles were harvested 21 and 60 days after dosing and were dissociated as described in Materials and Methods. The exhausted T cell population was analyzed by flow cytometry. (A) Gating strategy. RA1683, right quadriceps (injected muscle), 60 days after dosing. T cells are gated as CD3$^+$ cells in the lymphocyte subset, CD8 T cells are gated as CD3$^+$ CD8$^+$ cells in the CD3$^+$ subset, and exhausted T cells are gated as PD-1$^+$ cells in the CD3$^+$ CD8$^+$ subset. (B and C) Percentages of CD8 T cells (B) and exhausted T cells (C) in injected muscle or perfused limb at day 21 and day 60 after dosing. nd, not detected. (D) Detection of exhausted T cells by immunohistochemistry in injected muscle after IM delivery (RA0764, day 60 post-injection). Scale bars: 10 μm.

of the human immune system that cannot be completely understood with animal models, including those of nonhuman primates, which differ in their immune repertoire and reactivity to AAV capsids. Nevertheless, our study shows that, if analysis is performed *in situ*, we may observe similarities between our two models.

In summary, we showed that AAV vector delivery in muscle mediates T cell exhaustion and/or Treg response without preventing the formation of anti-AAV IgG or NAbs, which are critical for AAV readminis-

tration.[66–68] One major point to take into consideration is that these results were obtained when gene transfer is made in healthy muscles in opposition to some previous preclinical and clinical trials where the IM delivery was used to treat neuromuscular disorders, such as Duchenne muscular dystrophy (DMD)[42] or limb-girdle muscular dystrophy type 2D (LGM2D).[69] In these models, the immunogenicity of the IM route might be enhanced because of the inflammatory environment caused by the disease itself.[70,71] A good alternative to the IM delivery would be the VLP route, which still leads to T cell exhaustion and
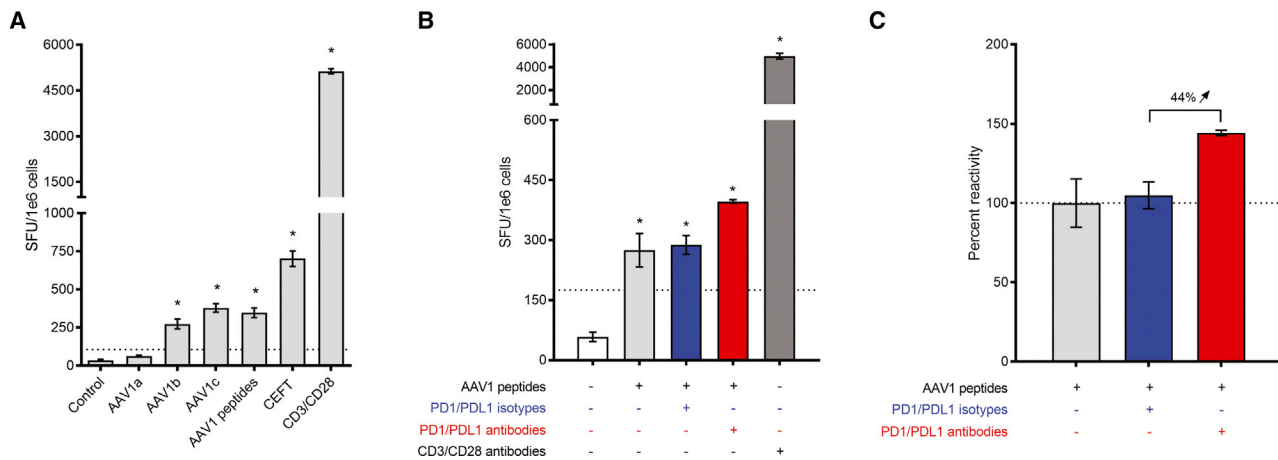
Figure 4. PD1/PDL1 Blocking Leads to Exhaustion Reversal in Patient PBMCs

PBMCs were isolated 5 years post-injection. (A) PBMCs were stimulated *in vitro* with overlapping peptides (15-mers overlapping by 10 aa) spanning the VP1 capsid protein sequence or AAV1 immunodominant peptides. (B) PBMCs were stimulated *in vitro* with AAV1 immunodominant peptides for 48 h in the presence or absence of PD1/PDL1 antibodies or isotype controls. For each experiment in (A) and (B), the negative control consisted of unstimulated cells (medium), whereas CD3/CD28 stimulation was used as a positive control for cytokine secretion. Responses were considered positive when the number of spot-forming units (SFUs) per 1e6 cells was >50 and at least 3-fold higher than in the control condition (dotted line); asterisks denote positive response. (C) IFNγ secretion fold change expressed as percent reactivity relative to the AAV1 peptide stimulation condition. Each condition was performed in triplicate. Error bars represent SEM.

provides higher transgene expression. Other alternatives can also be explored, such as modulating the dose or using less prevalent[72–74] or less immunogenic[27,75] AAV serotypes or even capsid-modified rAAV.[76] Indeed, the transduction efficiency of dendritic cells (DCs) depends on AAV serotype and/or genome conformation (ss versus self-complementary)[27,77–79] and might explain why some serotypes are more immunogenic or, conversely, more tolerogenic than others. In the case of capsid-engineered vectors, we can imagine that the capsid circumvents natural pre-existing immunity. Actually, in recent preclinical and clinical trials using engineered capsids, the immune response does not occur as frequently as in previous trials.[80]

In conclusion, our data show that rAAV gene transfer may result in the absence of a deleterious T cell response to the capsid, and at least two cell populations are involved: Tregs and exhausted T cells. These findings, in addition to the characterization of immune responses in seronegative and seropositive patients,[53] will help in developing safer gene transfer protocols and the establishment of new immune modulatory strategies.

MATERIALS AND METHODS
Vector Production
Nonhuman Primate Study
Vector design and manufacturing has been previously described by Gruntman et al.[39] Briefly, the CB-rhAAT-cMyc expression cassette has been packaged into an ssAAV1 capsid using the herpes simplex virus 1 (rHSV1) system, concentrated by tangential flow, exchanged to Ringer's buffer, and filtered through a 0.2-μm filter. The vector was manufactured by Applied Genetic Technologies(AGTC).

AATD Phase II Clinical Trial
The rAAV1-CB-hAAT vector used in the AATD clinical trial has been previously described.[33,81] Briefly, the rAAV1-CB-hAAT vector has been made using the rHSV1 system and purified by chromatography followed by AVB Sepharose affinity chromatography.

Study Design
Nonhuman Primate Study
Rhesus monkeys with a NAb titer to AAV1 at or below 1:10 were selected. The NAb titer was determined by the University of Massachusetts Gene Therapy Vector Core using an *in vitro* assay. At study day 0, three groups of animals were injected with 6e12 vg/kg of an ssAAV1-CB-rhAAT-cMyc by IM (n = 3), VLP (n = 3), or IAPD (n = 2) delivery. At study day 21, blood was drawn, and muscle biopsies were collected. At study day 60, animals were euthanized; blood and tissues were collected for evaluation. All the animal work was done under the approval and supervision of the Lovelace Respiratory Research Institute Animal Care and Use Committee.

The injection procedures are detailed by Gruntman et al.[39] Briefly, the IM injection procedure consisted of four 0.5-mL injections in each quadriceps and gastrocnemius muscles in the right hindlimb.

The VLP animals received a volume of 50 mL/kg of the rAAV1 vector diluted in Lactated Ringer's solution (LRS). An intravenous catheter was placed into the saphenous vein of the right pelvic limb. Then, a tourniquet was placed around the level of the proximal thigh. The vector was infused over 5–10 min. After infusion, the tourniquet remained tight for 15 min and then was released.

Animals in the IAPD group received a volume of 12.5 mL/kg rAAV1 vector diluted in LRS. An incision was made in the lower anterior thigh of the right pelvic limb, and the superficial femoral artery and vein were dissected and isolated with silk suture. Arterial and venous access was obtained with sheath catheters. The arterial and venous balloon catheters were then placed through their respective sheaths. After a pre-flush with LRS (5 mL/kg), the ssAAV1-CB-rhAAT-cMyc vector was infused in a volume of 12.5 mL/kg through the arterial catheter sheath port. After infusion, the vector solution was allowed to dwell for 15 min. Then, a post-flush of 5 mL/kg LRS was injected into the arterial catheter. After the infusion had been completed, the balloons were deflated, and the catheters were removed.

AATD Phase II Clinical Trial

The study design was previously reported by Flotte et al.[33] This study is registered with ClinicalTrials.gov (ClinicalTrials.gov: NCT01054339) and was conducted under an IND (investigational new drug) with the approval of the University of Massachusetts Medical School and Cincinnati Children's Hospital institutional review boards and institutional biosafety committees and in accordance with the tenets of the Declaration of Helsinki. This study was conducted as an open-label, non-randomized, multicenter, sequential, 3-arm, phase II clinical trial to determine the safety and efficacy of IM administration of a rAAV1-CB-hAAT vector. Nine subjects (three per cohort) received IM doses of rAAV1-CB-hAAT (6e11, 1.9e12, or 6e12 vg/kg body weight). For the present study, blood was collected on subject 308 (high-dose cohort) at 5 years post-dosing.

NAb Assay

Sera sampled prior to dosing, day 21, and at necropsy were heat inactivated for 30 min at $56°C$. AAV1-CMV-LacZ (10^9 gc/well) was incubated with 2-fold serial dilutions of samples for 1 h at $37°C$, 5% CO_2. The mixture was added to Huh7 cells (1e5 cells) previously infected with an adenovirus (100 particles per well) and incubated for 18–22 h at $37°C$, 5% CO_2. Cells were washed and developed with the Galacto-Star Kit (Applied Biosystems). Luminescence was measured with a luminometer. The NAb titers are expressed as the highest dilution that inhibited β-galactosidase expression by at least 50% compared to a negative mouse serum control.

Anti-AAV1 IgG ELISA

Anti-AAV1 IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Plates were coated with 5e9 vg of rAAV1 vector in coating buffer (carbonate-bicarbonate, 0.5 M [pH = 9.6]) at $4°C$ overnight. The plates were blocked with PBS-Tween 0.1% for 1 h at $37°C$. Monkey sera were serially diluted and incubated for 2 h at $37°C$. Plates were then incubated with an anti-human IgG horseradish peroxidase (HRP) antibody (A80-104P, Bethyl Laboratories) for 1 h at room temperature (RT). Substrate (tetramethylbenzidine [TMB]; BD Biosciences) was added, and plates were incubated for 7 min at RT. The reaction was stopped using 2 N H_2SO_4. Plates were read in a spectrometer (VersaMax, Molecular Devices) at 450 nm.

H&E Staining

Skeletal muscle paraffin sections (8 μm) were collected on slides. H&E staining was performed as per standard histological protocols using formolin-fixed and paraffin-embedded muscle sections. The slides were observed using a Leica DM5500B microscope.

Muscle Dissociation

Muscle biopsies and muscles were harvested 21 and 60 days after AAV vector injection. Tissues were cut into approximately 5-mm³ pieces and incubated in RPMI media supplemented with DNase I (62.5 U/mL; Zymo Research) and collagenase (0.25 mg/mL; Roche) for 2 h at $37°C$. Cell suspension was homogenized and filtered using a 70-μm cell strainer before staining for flow cytometry analysis.

Flow Cytometry

Regulatory T cells and exhausted CD8+ T cell populations were analyzed using CD3/CD4/Helios/FoxP3/CD25 and CD3/CD8/PD1/CD69 markers, respectively. Extracellular staining consisted of cell incubation on ice for 30 min. Cells were then washed twice with 1× PBS and re-suspended in 1× PBS supplemented with 0.5% fetal bovine serum (FBS) and 2 mM EDTA. FoxP3 staining and Helios intracellular staining were performed according to the manufacturer's recommendations (anti-human FoxP3 Flow Kit, BioLegend). Cells were acquired using a BD FACS LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (v.10.1; Tree Star).

Immunohistochemistry

Skeletal muscle cryosections (5 μm) were collected on slides, air dried, and fixed with 3% paraformaldehyde (PFA) (Thermo Scientific) for 3 min. Saturation was performed in 10% goat serum (S-1000, Vector Laboratories) and 5% BSA (BP1600-100, Thermo Scientific) for 45 min at RT. Then, sections were incubated in blocking buffer antibodies overnight at $4°C$. Polyclonal rabbit anti-human CD3 (1:100) (A0452, Dako), monoclonal mouse anti-human CD4 human (1:100) (317402, BioLegend), monoclonal rat anti-human CD8 (1:100) (MCA351GT, Bio-Rad), monoclonal mouse anti-human FoxP3 (1:100) (14-4777-82, Invitrogen), and monoclonal mouse anti-human PD1 (1:100) (329902, BioLegend) antibodies were used to detect Tregs (CD3+CD4+FoxP3+) and exhausted T cells (CD3+CD8+PD1+). After washing with PBS, sections were incubated with goat anti-rabbit Alexa Fluor 555-conjugated antibody (1:500) (A32732, Invitrogen) combined with goat anti-mouse (A21242, Invitrogen) or goat anti-rat (1:500) (A21247, Invitrogen) Alexa Fluor 647-conjugated antibodies or with goat anti-mouse Alexa Fluor 488-conjugated antibody (1:500) (A21121, Invitrogen) for 1 h at RT. Nuclei were counterstained with DAPI for 5 min at RT (0.001 mg/mL) (10236276001, Sigma Aldrich). Finally, slides were mounted with Clear-Mount (17985-12, Electron Microscopy Sciences). Images were captured using a Leica DM5500B microscope and Leica Application Suite AF v.3.1.0 build 8587 imaging software.

IFNγ ELISpot Assay

PBMCs were isolated 5 years post-injection, and the IFNγ ELISpot assay was performed according to the manufacturer's

recommendations (Human IFN-γ ELISpot[BASIC], MABTech). Briefly, PBMCs were stimulated *in vitro* for 48 h with overlapping peptides spanning the AAV1 capsid VP1 sequences and divided into 3 pools (2 μg/mL, 15-mers overlapping by 10 aa, Mimotopes, Mulgrave, VIC, Australia) or stimulated with the AAV1 immunodominant peptides (5μg/ml, Mimotopes, Mulgrave, VIC, Australia; Table S4). A negative control consisted of unstimulated cells (medium only), whereas CEFT (JPT Peptide Technologies, Berlin, Germany) and CD3/CD28 stimulation were used as a positive control for cytokine secretion. Spot number was determined using an iSpot ELISpot Reader ELR068IFL (AID) and analyzed with AID ELISpot Reader software v.6.0. Responses were considered positive when the number of spot-forming units (SFUs) per 1e6 cells were >50 and at least 3-fold higher than in the control condition.

PD1/PDL1 Blocking Assay

The PD1/PDL1 assay was conducted as described in the previous IFNγ ELISpot Assay section, except that PBMCs were stimulated *in vitro* for 48 h with the AAV1 immunodominant peptides (5 μg/mL, Mimotopes, Mulgrave, VIC, Australia; Table S4) with either no antibodies or 1 μL/100 μL cells of PD1/PDL1 antibodies (BioLegend) or their respective isotypes (BioLegend).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.01.004.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

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