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Immune-driven gene expression loss following intramuscular AAV delivery to non-human primates is only transient

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Recombinant adeno-associated virus (rAAV) vectors stand out as highly promising for *in vivo* gene transfer, particularly in targeting the skeletal muscle for treating muscular genetic diseases or secreting therapeutic factors. Despite the simplicity and efficacy of the established intramuscular (IM) route, it has been often associated with an immune-induced rapid loss of transgene expression, in particular in large animal models, and generally considered irreversible as a consequence of a cytotoxic elimination of transduced cells. Here, we report in a non-human primate model that transgene expression loss after IM delivery of an rAAV1 expressing an immunogenic protein is only transient, with the re-expression of the transgene lasting up to 5 years post-injection. We show that the recovery of transgene expression is due to persisting viral genomes in the injected muscles despite the detection of peripheral anti-transgene cellular immunity. Persisting genomes were observed in the presence of infiltrated mononuclear CD8 and CD4 T lymphocytes, among which we were able to detect FoxP3$^+$ regulatory cells. This is to our knowledge the first report of a transient immune-mediated loss of gene expression in a large animal model after rAAV delivery that should shed new light on the issue of rAAV vector immunogenicity.

INTRODUCTION

Recombinant adeno-associated virus (rAAV)-derived vectors hold great promise for long-term transgene expression after a single *in vivo* administration. They are being evaluated in a large number of preclinical and clinical studies. In addition, there are already six approved products in the European or US markets for the treatment of neuromuscular, metabolic, hemophilic, and retinal genetic diseases.[1–5] With a wide panel of clinical applications and the availability of rAAV musculotropic serotypes, gene transfer to the skeletal muscle is one of the most studied yet challenging target tissues. Administering rAAV vectors for the treatment of muscular dystrophies or the secretion of therapeutic factors already has been investigated and tested in patients using intravenous (IV) or intramuscular (IM) routes.[6–11] The first authorized rAAV market drug, Glybera, was an rAAV1 vector delivered IM to treat a genetic metabolic disease due to lipoprotein lipase deficiency.[7] More recently, a systemic rAAV product, Elevidys, has been approved for Duchenne myopathy by the US Food and Drug Administration.[4] The IM route of rAAV delivery is easy to implement, non-invasive, and considered to be a method of choice to secrete therapeutic factors from the muscle as reported in multiple preclinical studies.[12–14] Nevertheless, and despite these promising achievements, several reports in large animal models, including our own studies, have shown that IM-mediated rAAV gene expression can lead to a rapid transgene loss of expression.[15–21] This loss was correlated to a specific peripheral cellular immunity against the transgene product as well as an early muscle CD8 T cell infiltration at the site of vector administration. However, the follow-up in these studies was generally stopped at the time when transgene expression was lost, with the assumption that transduced cells were totally eliminated in an irreversible manner by CD8 T cells. The same assumption was made when transgene expression was lost following liver-targeted gene transfer,[22–24] despite the fact that the liver tissue environment differs from that of muscle and that distinct immune mechanisms in that case could lead to different outcomes.

In humans, the risk of transgene rejection following *in vivo* AAV delivery is possibly underestimated because most clinical trials were conducted under immunosuppression regimens or have excluded patients with null mutations. In animal models, it has been demonstrated that transgene rejection is stronger and more deleterious in the case of protein deletions.[17,25] More recent clinical data rather suggest that IM-mediated gene transfer can lead to *in situ* immune modulation or even tolerance.[10,26] In a clinical trial for alpha anti-trypsin deficiency, where an rAAV1 was administered via the IM route, a stable transgene expression was reported up to 5 years post-injection (pi) despite the detection of long-term T cell infiltrates in the muscle, as well as a positive transgene-specific interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) in one patient.[27,28] This study

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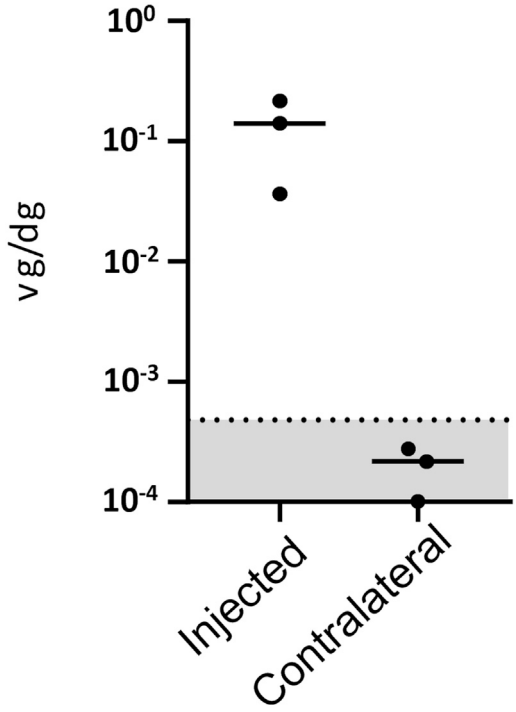

Figure 1. Persistent rAAV viral genomes in injected muscles despite a loss of transgene expression

Detection of viral genomes in injected muscles. Three primates (Mac1, Mac2, and Mac3) were injected with ssAAV2/1 rtTA/EPO in the left *tibialis anterior* at 1e11 vg/kg divided in 3–4 IM injection sites. A biopsy was performed in one muscle injection site and in the contralateral *tibialis* muscle at 26 months post-injection. After DNA extraction, viral genomes were measured by qPCR and expressed as viral genomes per diploid genome (vg/dg). The gray area indicates the limit of quantification of the PCR assay: 4.82e−4 vg/dg.

was among the first to question the ability of a peripheral immune response to efficiently eliminate AAV-transduced cells in the skeletal muscle. The presence of regulatory T cells (Tregs) specific to the viral capsid at the site of vector delivery was shown in muscle biopsies from treated patients at 1 and 5 years pi and demonstrated that muscle gene transfer can be tolerogenic in some settings despite the detection of a peripheral immunity.[10,29] In the meantime, the presence of exhausted CD8 lymphocytes expressing programmed cell death protein 1 (PD-1) in those biopsies was also demonstrated and could explain the absence of a deleterious immune response in this clinical trial. More recently, a study in non-human primates (NHPs; *Macaca fascicularis*) confirmed these findings and showed that rAAV delivery to the skeletal muscle results in persistent gene transfer that is mediated by the presence of both exhausted CD8 T cells and Tregs.[26]

Using an rAAV1 vector expressing the cynomolgus macaque reporter gene Erythropoietin (EPO) under the control of the reverse tetracycline-transactivator (rtTA)-based inducible Tet-On system, we have studied host immune responses in the muscle against the immunogenic transgene product rtTA.[15,18,20,30] We have shown that IM delivery of the vector in the NHP model results in an anti-rtTA peripheral cellular and humoral immune response. Following IM vector administration, a rapid loss of inducible EPO gene expression within the first 5–7 mpi was observed as shown in a previous cohort of three macaques described in Moreau et al.[18] We show a re-expression of the transgene that has persisted up to 5 years pi. This gene expression recovery is due to persisting viral genomes in the injected muscles despite the detection of infiltrated mononuclear CD8 and CD4 T lymphocytes; among these, we were able to detect FoxP3$^+$ Tregs. This is to our knowledge the first report of a transient immune-mediated loss of gene expression after rAAV delivery in a large animal model, which sheds new light on AAV vector immunogenicity.

RESULTS

Persisting rAAV viral genomes in injected muscles despite a loss of transgene expression

Using an rAAV1 vector expressing the reporter gene EPO under the control of the Tet-On inducible system, which is based on the expression of the rtTA transactivator, we have reported in our previous studies an early loss of EPO transgene inducible expression after IM vector delivery, correlated to the detection of anti-rtTA immunoglobulin G (IgG) antibodies and mononuclear cell infiltrates at the site of IM vector injection.[15,18,31] The assumption is that transduced cells are eliminated *in situ* by cytotoxic CD8$^+$ T cells after the immune response against the transgene product rtTA is mounted. Using the cohort of Moreau et al.[18] (referred to as Mac1, Mac2, and Mac3), which received 1e11 vector genomes (vg)/kg of rAAV-rtTA/EPO vector in 3 (Mac 1 and Mac 3) or 4 (Mac 2) IM sites of injection, we were unexpectedly able to detect persisting viral genomes in muscle biopsies performed in one IM injection site at 26 mpi (Figure 1). These persisting viral genomes at non-negligible values comprised between 3.6e−2 and 2.1e−1 vg/diploid genome (dg) were observed despite the loss of transgene expression within the first 5–7 months following vector delivery, correlated to the detection of anti-rtTA IgG circulating antibodies and IFN-γ cellular response (Figure S1; Tables S1 and S2).[18] In contrast, there were no viral genomes detected in the contralateral control muscles (Figure 1). Interestingly, we have observed the same findings in another macaque cohort (referred here to as K-Mac1, K-Mac2, and K-Mac3) using a different doxycycline (Dox) inducible gene transfer system based on the TetR-KRAB transactivator.[32] In this other cohort, we were indeed able to detect persistent viral genomes, similar to Mac1, Mac2, and Mac3 (at values between 5.1e−2 and 8.3e−1 vg/dg; Table S1) at the sites of IM vector injection, despite an early loss of inducible EPO transgene expression within the 6 first months (Table S1). Viral genomes were detected in comparable levels in all three macaques, including K-Mac1, that did not even respond to the first Dox induction.

Re-expression of the transgene despite its initial loss

In the presence of persisting viral genomes at the site of IM rAAV1 delivery, it is likely that the loss of transgene expression observed in Mac1, Mac2, and Mac3 may not be due to a complete elimination of transduced cells and may involve other immune mechanisms. We wanted to check whether a new induction could restore the loss of transgene expression. To do so, we administered additional Dox

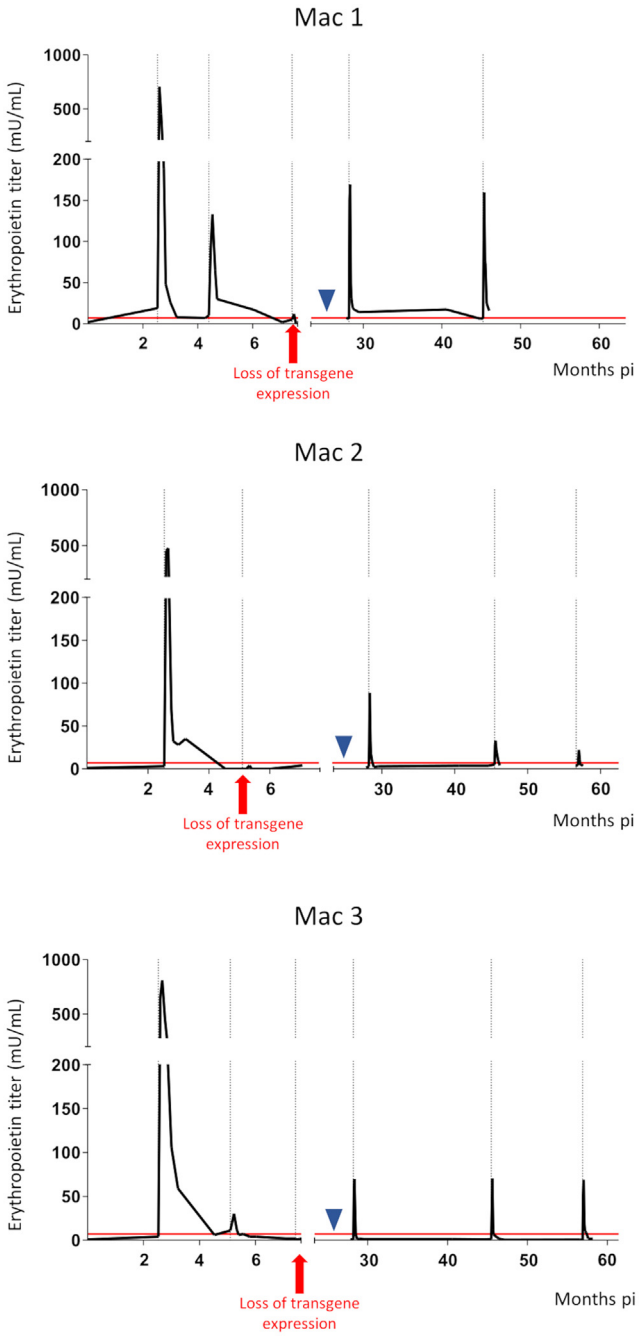

Figure 2. Re-expression of the transgene despite its initial loss

Follow-up of erythropoietin (EPO) levels after doxycycline induction cycles (dotted lines) in Mac1, Mac2, and Mac3 after IM rAAV1 delivery. The red line indicates the physiological basal level of EPO (7 mU/mL) established as the mean ± 2 SD of 182 measures of EPO in serum samples from 32 cynomolgus primates. The blue arrow indicates the time point at which the muscle biopsies were performed.

cycles after 2 years post-vector injection (at 28, 45, and 56 mpi). We were able to detect serum EPO peaks in the three primates that were found to be highly above physiological basal EPO levels (up to 24-, 47-, and 29-fold for Mac1, Mac2, and Mac3, respectively) (Figure 2). Nevertheless, it is worth noting that those levels of EPO protein secretion were found to be 3- to 8-fold lower than the levels measured during the first Dox induction (Figure 2). Thus, we were able to recover transgene expression after 2 years pi despite an initial loss, and this re-expression was maintained up to at least 5 years post-gene transfer. We next wanted to confirm that this long-term expression still originated from the injected muscles and not from untargeted biodistribution sites of the vector.

Transgene re-expression up to 5 years results from persisting viral genomes in injected muscles and not from untargeted tissues

To determine the site of transgene re-expression in the three macaques, we quantified viral genome numbers by quantitative PCR (qPCR) in the liver (right, caudal, and left lobes), in the injected *tibialis* muscle (3–4 IM injection sites), and in non-injected muscles (e.g., muscles from the injected limb, the contralateral *tibialis* muscle, and distant muscles from an upper limb in addition to the diaphragm) collected during the necropsy of animals at 5 years pi (Figure 3A). Viral genomes were detected mostly in the injected muscle in all three macaques at median values of 2.18, 1.33, and 2.38 vg/dg for Mac1, Mac2, and Mac3, respectively. Viral genomes were also detected in the liver at median values of 1.9e−3, 1.14e−2, and 8.2e−4 vg/dg, for Mac1, Mac2, and Mac3, respectively. Lower numbers (1,000- to 10,000-fold as compared to the site of IM injection) of viral genomes between 9e−5 and 4.84e−3 vg/dg were detected in non-injected muscles from the injected limb and from the contralateral limb. In the more distant muscles, viral genome numbers were even lower and below the quantification threshold of the qPCR, which was established at 4.76e−4 vg/dg.

The constitutive expression of the immunogenic rtTA transactivator is under the control of the muscle-specific desmin promoter. To confirm that EPO inducible transgene re-expression effectively originates from persisting viral genomes at the site of vector delivery, we next quantified rtTA transgene transcripts by quantitative reverse-transcription PCR in the same tissues as described above (Figure 3B). Interestingly, transgene transcripts were detected only in the injected *tibialis* muscles at non-negligible but inter-individual variable median relative quantity (RQ) values of 0.35, 2, and 8.85 for Mac1, Mac2, and Mac3, respectively. In all the other analyzed tissues, RQ median values were found below the quantification threshold established at 0.086, except for one liver lobe of Mac3 for which the RQ value was determined to be 0.53 (Figure 3B). These data confirm that the re-expression of EPO inducible transgene in our model is related to long-term persisting viral genomes at the site of muscular rAAV injection and not from untargeted tissues.

Detection of $CD4^+FoxP3^+$ T lymphocytes in the injected muscles

To better determine why the peripheral anti-rtTA cellular response measured by IFN-γ ELISpot at 3.5 mpi in our three macaques did not fully eliminate viral genomes at the site of vector IM injections (Table S2), we next analyzed host immune responses *in situ*. We first

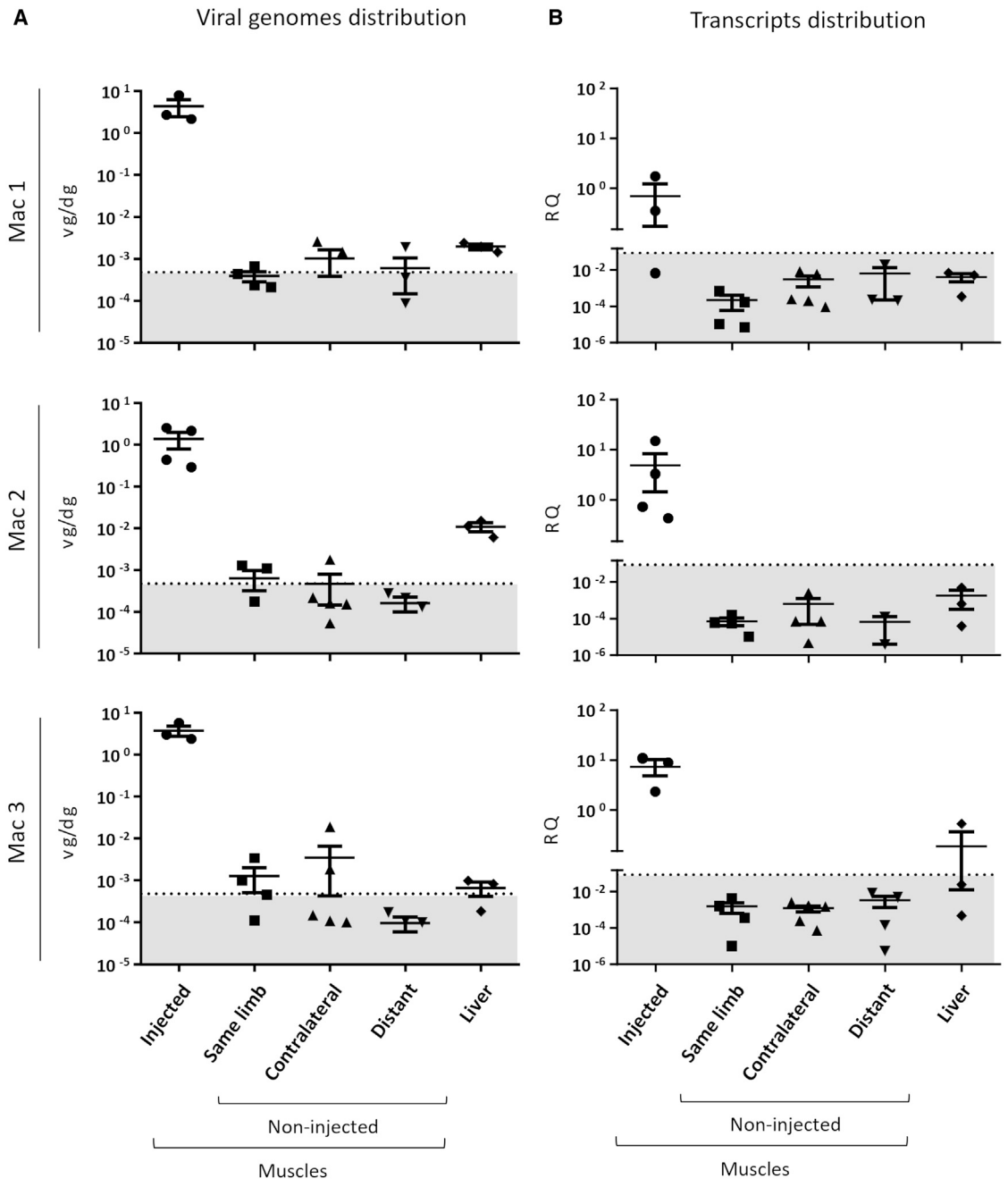

Figure 3. Transgene re-expression results from persistent viral genomes in injected muscles and not from untargeted tissues

Detection of viral genomes and transgene transcripts in the injected muscles. (A) Viral genomes were measured by qPCR in injected *tibialis*, non-injected muscles from the treated limb, non-injected muscles from the contralateral limb, and distant muscles (upper limb and diaphragm). Results are expressed as vg/dg. The gray area indicates the limit of quantification of the PCR assay: 4.82e−4 vg/dg. (B) Transgene transcripts were detected by quantitative reverse-transcription PCR (RT-qPCR) in the same tissues and expressed as RQ (relative quantity) to an endogenous gene. The gray area indicates the limit of quantification of the RT-qPCR assay: 8.68e−2.

performed hematoxylin-phloxine-saffron (HPS) colorations on paraffin-embedded sections of injected *tibialis* muscles and their contralateral controls. Infiltrated mononuclear cells were detected at 5 years pi in the three to four injected sites of the three primates, whereas no infiltrates were detected in the contralateral muscles (Figure 4). Comparable numbers of centrally nucleated fibers, a hallmark of muscle regeneration, were counted in the injected compared to the non-injected *tibialis* muscles (data not shown).

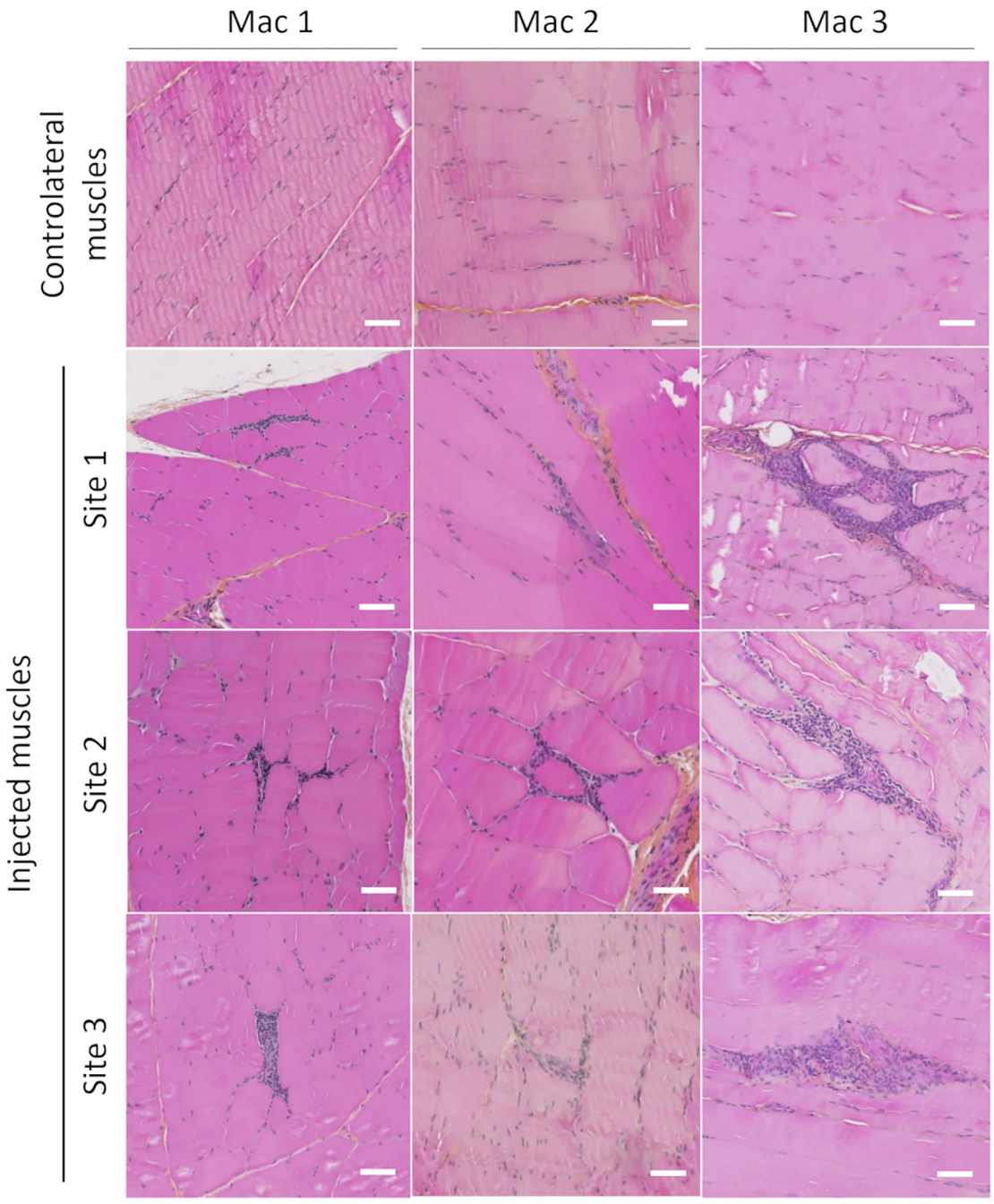

Figure 4. Detection of mononuclear cellular infiltrates in IM sites of vector delivery

Injected and contralateral *tibialis* muscles were collected at 5 years post-injection. Hematoxylin-eosin-saffron staining was performed on formalin-fixed paraffin-embedded muscle sections. Mononuclear infiltrated cells were observed in all IM injection sites of the vector. Scale bars: 50 μm.

We next performed an in-depth characterization of muscle-infiltrating cells by immunostaining and showed the presence of $CD3^+$, $CD4^+$, and $CD8^+$ cells (Figures 5 and 7). Because our data suggest an inefficient CD8 cytotoxic cellular response, we next performed a PD-1 staining as $CD3^+CD8^+PD\text{-}1^+$ exhausted T cells were previously described to be associated with an absence of a deleterious immune response in rAAV gene transfer studies.[26] Here, we were not able to detect $PD\text{-}1^+$ T lymphocytes, in contrast to a positive control consisting of a lymph node staining (Figure 5A) (CD8 staining not shown). We also performed CTLA4 staining as another T cell inhibitory

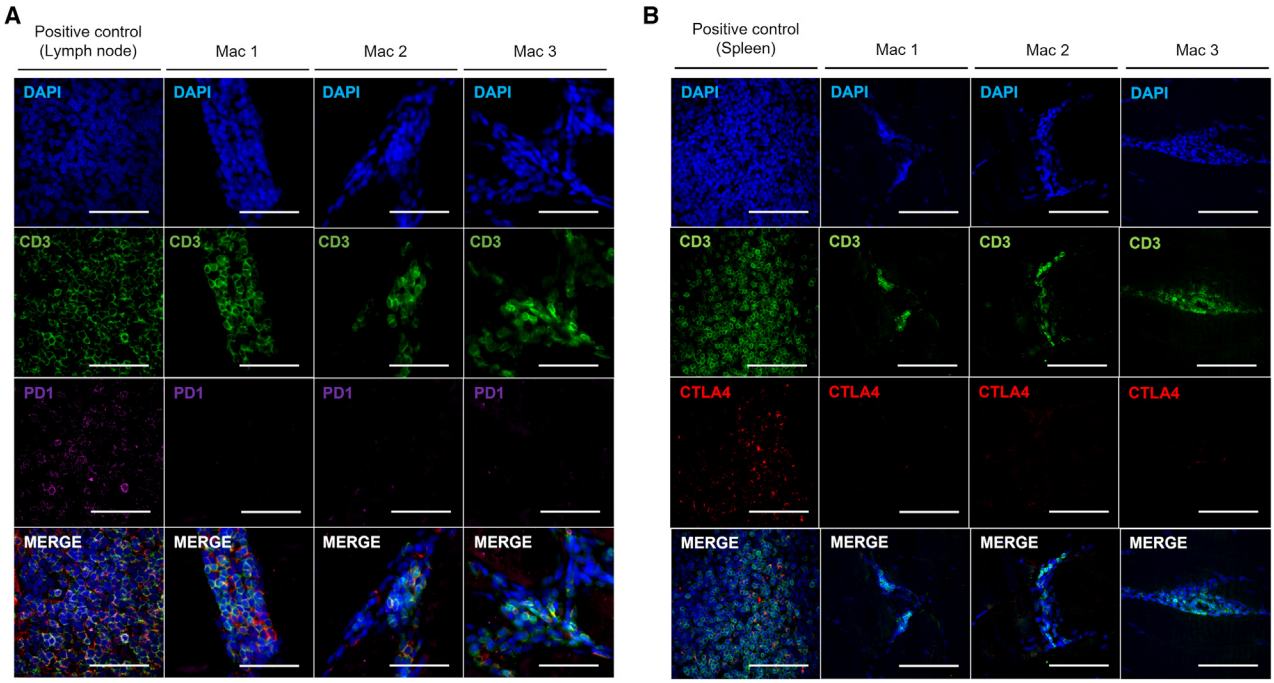

Figure 5. Absence of PD1$^+$ and CTLA4$^+$ exhausted T lymphocytes in cellular muscle infiltrates

Characterization of infiltrated cells in injected muscles on PFA-fixed paraffin-embedded muscles sections subjected to DAPI (blue), CD3 (green), CTLA4 (red) (A) and PD-1 (purple) (B) co-stainings. Positive control consists in PFA-fixed embedded-paraffin lymph node and spleen section subjected to the same co-stainings. (White scale bar: 50 μm).

marker,[33] but we were unable to detect CD3$^+$ CTLA4$^+$ T cells in the infiltrates, in contrast to a positive control (Figure 5B). Under normal conditions, skeletal muscle fibers do not constitutively express the major histocompatibility complex class I (MHC class I) protein.[34,35] Immune ignorance may partly explain why the transduced fibers are unable to present antigens to the effector cells. MHC-I staining was performed at the injection sites versus the contralateral muscles. We were able to detect upregulation of MHC-I expression only on muscular fibers at the injection sites (Figure 6). These data suggest that immune mechanisms other than ignorance or PD-1- and CTLA4-mediated T cell exhaustion are involved *in situ* to allow the long-term persistence of viral genomes.

Regulatory CD4$^+$FoxP3$^+$ T lymphocytes have also been described in muscle rAAV-based gene transfer studies where an immunomodulation with persisting transgene expression was reported.[21,29] Interestingly, we were able to detect at 5 years pi for all macaques, in each analyzed muscle-infiltrated area, CD4$^+$FoxP3$^+$ T cells, as shown in Figure 7.

DISCUSSION

Despite multiple promising achievements in the field of muscle rAAV gene therapy, several reports in large animal models, including our own studies, have shown that IM-mediated rAAV gene expression can lead to a rapid loss of transgene expression. This loss was generally correlated to an immune response against the transgene product.[15–17,19–21,36] The follow-up in these studies was generally stopped at the time when transgene expression was lost, with the assumption that transduced cells were totally eliminated in an irreversible manner by CD8 T cells. Here, using a rAAV1-rtTA/EPO vector, we report in an NHP model that the early loss of transgene expression can be only transient in the absence of any immunosuppressive regimen. Gene expression is indeed recovered from persisting viral genomes at the site of IM administration despite an early peripheral cellular immunity against the rtTA transactivator as reported for the three macaques in the supplemental data of Moreau et al.[18] The initial loss of transgene expression may not be related to a complete elimination of the transduced cells by a conventional cytotoxic immune response, but it may be related to other immune mechanisms. One possible mechanism that remains to be investigated in our study could be an inflammatory-induced epigenetic silencing of viral genomes during the first months of gene transfer. A type I IFN-dependent gene silencing was reported by Suzuki et al. using an adenoviral vector in the mouse model.[37] In this study, inflammatory signaling was not associated with transgene silencing with rAAV at either early or late time points, in contrast to our model. However, unlike our study, they administered the vector intravenously and analyzed gene expression in the liver. More recently, studies have shown that epigenetic regulation of rAAV genomes could be responsible for a loss of transgene expression.[38–40] In addition, a recent study in NHPs reported an early decrease in transgene expression in the liver, after which a stable but low level was maintained, likely due to integrated viral genomes.[36]

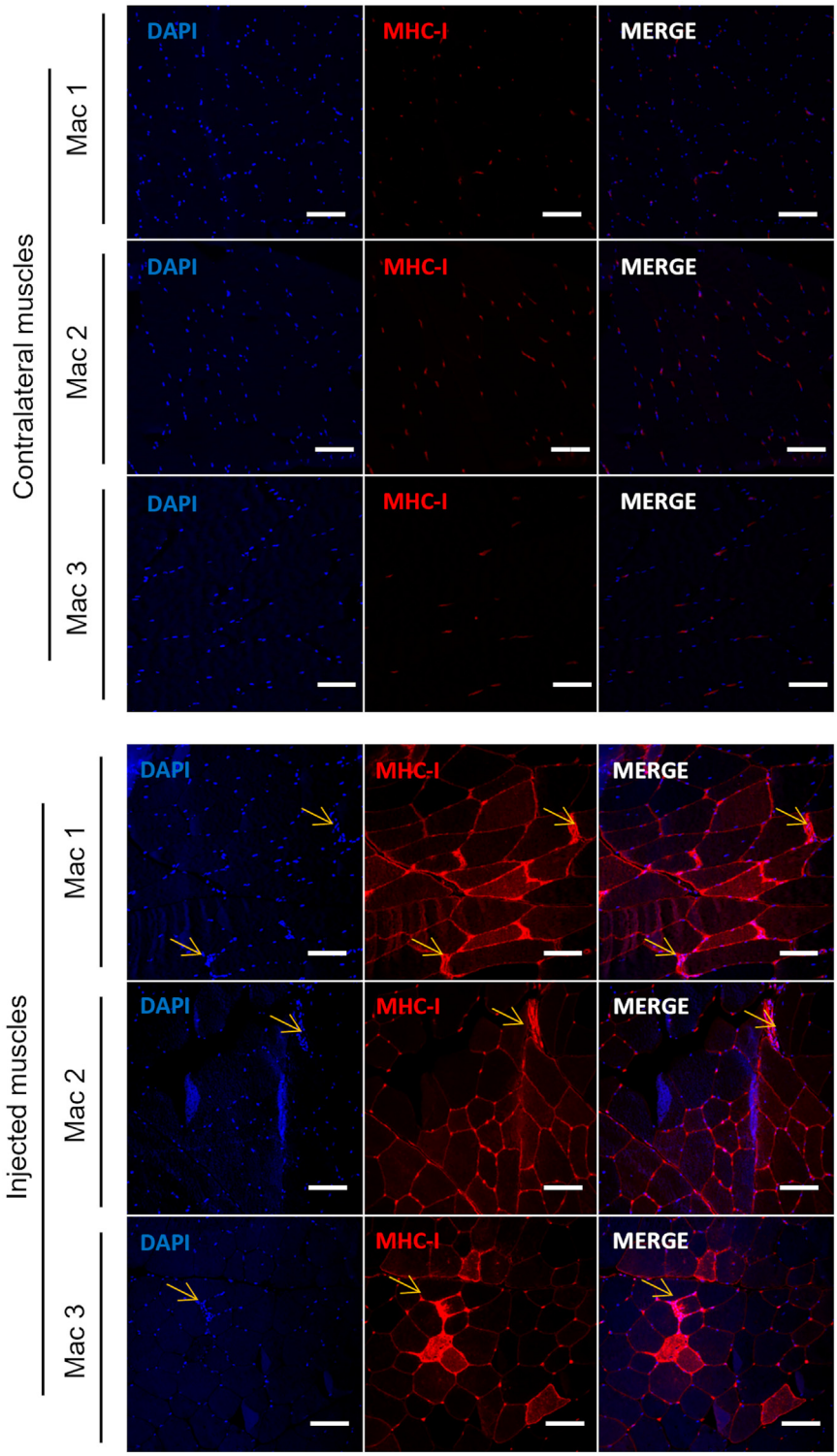

Figure 6. Detection of MHC class I$^+$ muscular fibers in injected tissues

Staining of MHC class I antigen on PFA-fixed paraffin-embedded muscle sections using anti-MHC class I (red) and DAPI (blue). The orange arrows correspond to immune infiltrates next to MHC class I$^+$ muscular fibers. Scale bars: 50 μm.

Because we have used the muscle-specific desmin promoter, which can be active in hepatic stellates and leaks in the liver,[20,41] we checked the site of gene expression recovery and demonstrated gene re-expression results, mostly from persisting viral genomes at the site of vector delivery. Only Mac3 showed weak expression in the liver (one lobe among three). After gene expression recovery, the levels of inducible EPO were found, but were relatively lower (3- to 8-fold) than during the early phase of gene transfer. However, EPO-induced peaks were largely above the physiological level of EPO measured in 182 control serum samples. This decrease in gene expression could be explained by an acute cytotoxic response that partially eliminated transduced cells during the early phase of gene transfer. In all three macaques, we were able to detect specific circulating IFN-γ-secreting T cells at 3.5 mpi[18] that only persisted in Mac1 with ELISpot assays negative for Mac2 and Mac3 at 18 mpi and 3.5 years pi (Figure S1; Table S2). However, an ongoing response may be responsible for the continuous drop of EPO expression observed in Mac2 despite the negative ELISpot assays (Figure 2). It is difficult to investigate this hypothesis as we have not been able to quantify the numbers of viral genomes in the injection sites during the first 6 months to compare them with the levels obtained at 26 mpi (muscle biopsies, Figure 1) and 5 years pi (necropsia, Figure 3). In addition, the levels of viral genomes in muscle biopsies at 26 mpi were found to be lower than those detected at necropsy. It is known that rAAV IM delivery leads to heterogenic muscle transduction,[20] which makes longitudinal transduction studies comparing muscular biopsies collected in different areas of the IM injection zone difficult.

In our model, a possible epigenetic chromatin remodeling of rAAV episomal or potentially integrated genomes could explain our observations and are under investigation.

The fact that a non-negligible number of viral genomes can persist for up to 5 years pi despite chronic CD8$^+$ cell infiltration suggests that rAAV did not induce a fully deleterious CD8$^+$ response. Similar to chronic viral infections, exhausted CD8$^+$

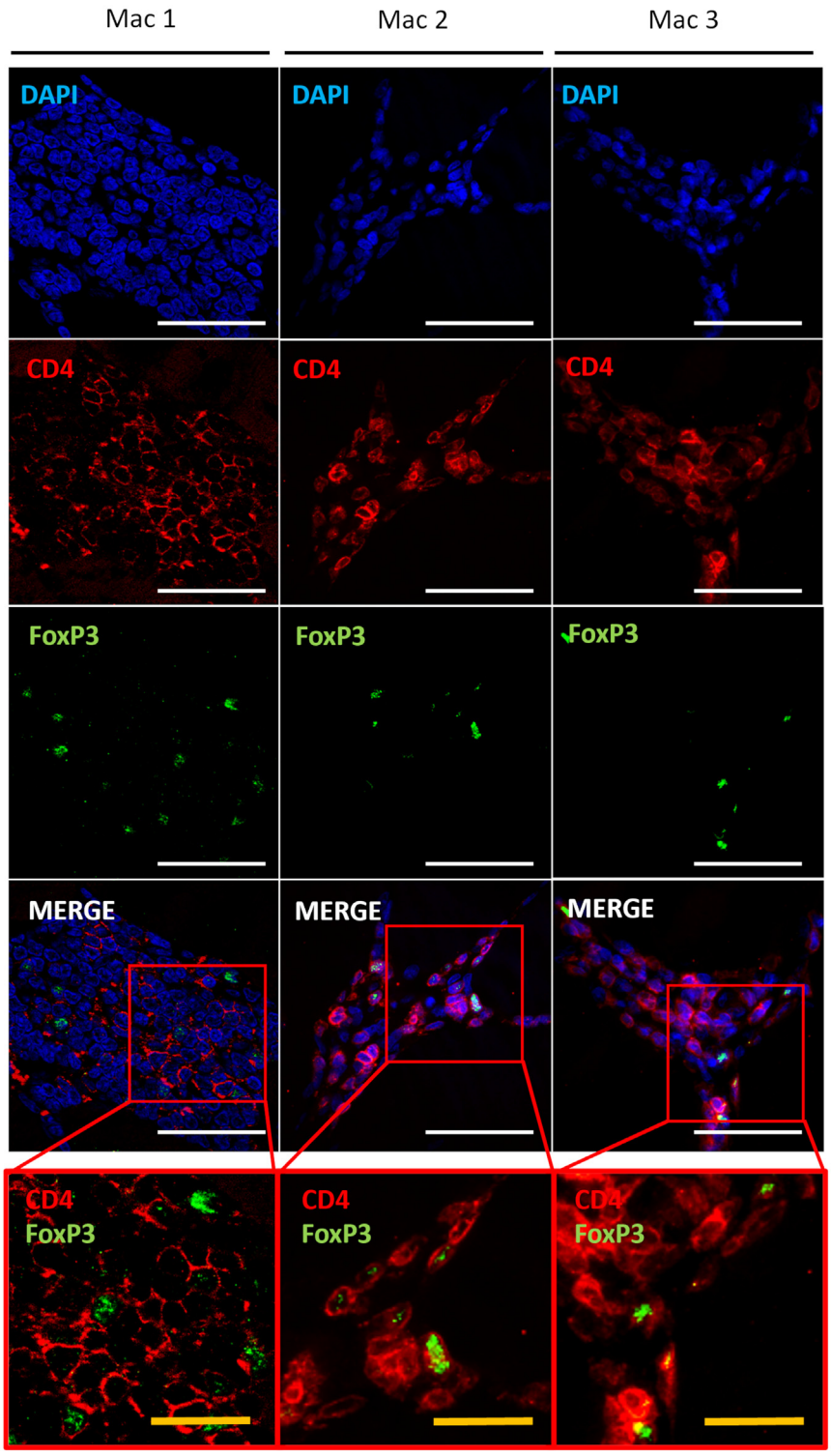

Figure 7. Detection of FoxP3$^+$CD4$^+$ T lymphocytes in cellular muscle infiltrates

Characterization of infiltrated cells in injected muscles on PFA-fixed paraffin-embedded muscle sections subjected to DAPI (blue), CD4 (red), and FoxP3 (green) co-staining. White scale bars: 50 μm. The red boxes correspond to an enlargement of the merge without visualization of DAPI, to show more precisely the CD4 membrane staining surrounding the Foxp3 intracellular staining. Yellow scale bars: 20 μm.

T cells were described in the context of rAAV gene transfer with impaired functionality such as low proliferation, low cytokine secretion, and expression of inhibitory molecules like PD-1.[26,42–44] Here, we did not detect PD-1 and CTLA4 inhibitory markers on infiltrated T cells (Figure 5), similar to another gene transfer model where the immunogenic GFP was expressed from an rAAV8.[21] We showed that the transduced muscle area expresses MHC class I molecules, which suggest that epitope presenting from muscle cells to the infiltrated T cells should be possible even if it is not demonstrated. Another possible mechanism for CD8$^+$ functional impairment is an antigen-driven programmed cell death. Such *in situ* silencing of cytotoxicity was described after rAAV delivery to the muscle[45,46] and remains to be investigated in our model. Furthermore, it is known that under normal conditions, skeletal muscle fibers do not constitutively express the MHC class I protein and are unable to present antigens to the effector cells.[34,35] As shown in Figure 6, the detection of MHC class 1 expressing muscular fibers at injected sites in close proximity to infiltrating T cells does not favor a possible immune ignorance mechanism allowing the long-term persistence of viral genomes.

Another immunomodulation mechanism is based on the recruitment of immunosuppressive cells like Tregs. It has already been described in previous rAAV preclinical models and in clinical studies for the treatment of enzymatic deficiencies where sustained transgene expression in the muscle was correlated to the presence of such immunomodulatory cells.[7,21,26,29,47] In the present study, we also observed infiltration of CD4$^+$FoxP3$^+$ Tregs up to 5 years pi (Figure 7), and this might contribute to the persistence of transgene expression. The specificity of those *in situ* Tregs is unfortunately difficult to assess. The long-term expression of the rtTA protein as shown by the quantification of transcripts could suggest that these infiltrated Tregs observed at 5 years pi are at least partially specific to the transgene product in our model,

similar to what has been shown for capsid-specific Tregs infiltrated in muscle.[29]

These data confirm again the emerging concept that the presence of muscle infiltrates after rAAV-mediated gene transfer to the skeletal muscle is not systematically deleterious. A non-conventional immune response can take place to allow persistent gene expression. More surprising, long-term gene expression can even be recovered after an initial gene silencing, as reported here. Nevertheless, there are still a number of questions to be addressed, in particular, the mechanisms involved in the short-term transient loss of transgene expression and the kinetics of events leading to persistent viral genomes. Also, the impact of the type of transgene and the viral genome sequence on these observations must be considered in future studies. The immunogenicity of AAV vectors in the muscle remains a special setting where a complex combination of parameters such as vector serotype, dose, mode of delivery, and transgene type can drive the host immune system from immunogenicity to an aberrant immune response or an immune tolerance, even in the context of an immunogenic method of delivery such as IM injection. In conclusion, IM administration of rAAV to the skeletal muscle should be revaluated in light of these findings and considered more in future clinical trials.

MATERIALS AND METHODS

A large part of this work was performed under the control of our quality management system, which is approved by Lloyd's Register Quality Assurance to meet the requirements of international management system standard ISO 9001:2008. It has been implemented to cover all activities in the laboratory, including research experiments and the production of research-grade viral vectors.

Vector production and transgene specificity

Recombinant AAV2/1 vector was manufactured by the Centre de Production de Vecteurs (CPV) vector core in the UMR1089 laboratory (https://umr1089.univ-nantes.fr/en/facilities-cores), as described by Toromanoff et al.[20] The rtTA/EPO vector plasmids express the cynomolgus macaque EPO complementary DNA (cDNA) under the control of the Dox-inducible Tet-On-cytomegalovirus promoter and rtTA-M2 under the control of the muscle-specific 1-kb human desmin promoter.[48] Vector titer (vg/mL) was determined by dot plot and qPCR.

Animal care and welfare and *in vivo* experiments

NHPs were maintained for experimentation after approval of the Institutional Animal Care and Use Committee of the University of Nantes and under the supervision of a doctor of veterinary medicine. Special attention was paid to the health and welfare of the animals. Three-year-old male (Mac2 and Mac3) and female (Mac1) NHPs were provided by BioPrim (Baziege, France). Cynomolgus monkeys were selected based on the absence of neutralizing antibodies to AAV1. *In vivo* procedures and euthanasia were performed as previously described by Moreau et al.[18] Briefly, animals were injected with a total vector dose of 1e11 vg/kg distributed over three to four injection sites along the left *tibialis anterior* muscle. Blood samples were collected under anesthesia with respect to physiological guidelines. Anesthesia was performed with an IM injection of 20 μg/kg medetomidine (Domitor, Pfizer, New York) associated with 8 mg/kg of ketamine (Imalgène, Rhone Mérieux, Lyon, France).

Follow-up of EPO expression

The induction protocol started 2 months after rAAV-rtTA/EPO vector injection and consisted of a 3-day Dox administration regimen, as described in Moreau et al.[18] The first three Dox induction cycles were performed at 2.5, 4, or 5 mpi (Mac1 or Mac2–Mac3, respectively) and 7.5 mpi. Three additional Dox induction cycles were then conducted at 28, 45, and 57 mpi (excluding Mac1). Circulating EPO levels were measured using Human Erythropoietin Quantikine IVD enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Physiological levels of circulating EPO were obtained from the titration of a total of 182 serum samples obtained from 32 different NHPs and were calculated as follows: mean of EPO protein level + 2 × standard deviation (SD).

Viral genome and transgene transcript detection by quantitative real-time PCR

Total genomic DNA and total RNA from tissues were extracted as previously described by Guilbaud et al.[30] All qPCRs were conducted using a StepOne Plus instrument (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA).

Vector genomes and transgene cDNA (after mRNA reverse transcription) were measured through the quantification of bovine growth hormone polyadenylation signal (BGHpA) sequence with the following primers: forward primer 5′-TCTAGTTGCCAGCCATCTGTTGT-3′, reverse primer 5′- TGGGAGTGGCACCTTCCA -3′, and TaqMan probe 5′(FAM)-TCCCCCGTGCCTTCCTTGACC-(TAMRA)3′. The BGHpA qPCR program was the following: initial denaturation for 20 s at 95°C, followed by 40 cycles of 1 s at 95°C and 20 s at 60°C. Quantities of vector genome were normalized by quantifying the endogenous NHP ξ-globine DNA using the following primers: forward primer 5′-TGGCAAGGAGTTCACCCCT-3′, reverse primer 5′-AATGGCGACAGCAGACACC-3′, and TaqMan probe 5′(FAM)-TGCAGGCTGCCTGGCAGAAGC-(TAMRA)-3′. The ξ-globine qPCRs were done using the following program: initial denaturation for 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 62°C. For each sample, Ct values were compared with those obtained with a standard dilution of control plasmid containing the BGHpA sequence.

The transgene cDNA was normalized through quantification of the endogenous hypoxanthine phosphoribosyltransferase (HPRT) with the following primers: forward primer 5′-GCTTTCCTTGGTCAGGCAGTA-3′, reverse primer 5′-TGGGAGTGGCACCTTCCA-3′ and the TaqMan probe 5′(FAM)-AATCCAAAGATGGTCAAGGTCGCAA-(TAMRA)3′. The HPRT qPCRs were done using the following program: initial denaturation for 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 62°C. Transcript detection was expressed in RQ of transcript levels as $RQ = 2^{-\Delta Ct}$, where $DCt = Ct_{transgene} - Ct_{endogenous}$.

Histopathological analysis and immunostaining

Muscles were sampled at sacrifice (>5 years post-injection) and embedded in paraffin after 4% paraformaldehyde (PFA) fixation. Transverse sections of muscles were colored with HPS according to standard histological protocols. Muscle slides were observed using a NanoZoomer Slide Scanner (Hamamatsu Photonics, Hamamatsu, Japan). Paraffin tissue sections were deparaffinized and rehydrated. Then, antigens were unmasked by boiling the section in ready-to-use citrate-based pH 6.0. Skeletal muscle sections were collected on slides, air dried, and fixed with 4% PFA (Thermo Fisher Scientific) for 10 min. All the sections were permeabilized in PBS containing 0.2% Triton X-100 for 10 min at room temperature (RT). Non-specific activity was blocked by incubating the sections for 45 min at RT in 10% NHP serum, 2% goat serum in PBS, and 5% BSA in PBS. Sections were then incubated overnight at 4°C in primary antibody diluted in PBS. Monoclonal mouse anti-human CD4 (1:50) (556614, BD Biosciences, Franklin Lakes, NJ) and monoclonal rat anti-human FoxP3 (1:50) (14-4776-82, Thermo Fisher Scientific) antibodies were used to detect Tregs. Monoclonal rat anti-human CD3 (1:200) (ab11089, Abcam, Cambridge, UK), monoclonal rabbit anti-human CD8 (1:200) (ab4055, Abcam), monoclonal mouse anti-human CTLA4 (1:100) (Sc-376016, Santa Cruz Biotechnology, Dallas, TX), and monoclonal mouse anti-human PD-1 (1:50) (329902, BioLegend, San Diego, CA) antibodies were used to detect exhausted T cells. Monoclonal mouse anti-human HLA-A (human leukocyte antigen-A), HLA-B, and HLA-C (1:200) (311402, BioLegend) was used to detect MHC class I antigen on muscle fibers. After washing with PBS, sections were incubated with secondary antibodies for 1 h at RT (1:300, Life Technologies, Carlsbad, CA), then with DAPI (1:500; Sigma, St. Louis, MO) to counterstain nuclei. Stained tissue sections were finally mounted using ProLong Gold medium (Life Technologies, Invitrogen) and observed with an A1 inverted laser scanning confocal microscope (Nikon, Tokyo, Japan).

DATA AND CODE AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplemental information.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.J., V.P., M.G., and O.A. Methodology, M.J., M.D., V.P., M.S., A.M., M.C.C., M.G., and O.A. Investigation, M.J., M.D., N.J., V.P., and J.L.D. Writing – original draft, M.J., M.G., and O.A. Writing – review & editing, M.J., V.P., M.G., and O.A. Supervision, O.A. Funding acquisition, V.P., M.G., and O.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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