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# 5 years of successful inducible transgene expression following locoregional AAV delivery in nonhuman primates with no detectable immunity

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Key words: AAV, gene transfer, Locoregional delivery, non-human primate, Immunogenicity, long-term follow-up.

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## Abstract

Anti-transgene immune responses elicited after intramuscular (IM) delivery of recombinant Adeno-Associated Viruses (rAAV) have been shown to hamper long-term transgene expression in large animal models of rAAV-mediated gene transfer. To overcome this hurdle, we and others described an alternative mode of delivery of rAAV vectors in nonhuman primate muscles: the locoregional intravenous route of administration (LR). Using this injection mode, we previously reported in cynomolgus monkeys a persistent inducible transgene expression for at least 1 year under the control of the tetracycline-inducible TetON system, with no immunity against the rtTA transgene product. The present study shows the long-term follow-up of these animals. We report that LR delivery of a rAAV2/1 vector allows long term inducible expression up to at least 5 years post-gene transfer with no any detectable host immune response against the transactivator rtTA despite its immunogenicity following IM gene transfer. This study shows for the first time a long-term regulation of muscle gene expression using TetON-inducible system in a large animal model. Moreover, these findings further confirm that rAAV LR delivery route is efficient and immunologically safe, allowing long-term skeletal muscle gene transfer.

## INTRODUCTION

Over the last decade, recombinant Adeno-Associated Virus (rAAV)-based gene therapies have shown great promises for the treatment of genetic disorders, with numerous studies evolving from proof-of-concepts to clinical trials or even Marketing Authorization Application (MAA) in Europe or Biologic Licence Application (BLA) in US<sup>1-4</sup>. Nevertheless, immune responses directed against the vector capsid or the transgene product remain potential limiting factors to long-term gene transfer efficiency, even though their impact on transgene expression can be difficult to understand<sup>5-7</sup>. For several years, we've been taken particular interest in the impact of anti-transgene immune responses triggered after muscle-directed rAAV-mediated gene transfer<sup>8-10</sup>. Skeletal muscle is the target tissue of choice for gene therapy of numerous neuromuscular diseases and metabolic disorders<sup>11</sup>. In the context of rAAV-mediated gene transfer, muscle-directed gene therapy strategies have first relied on the direct intramuscular (IM) delivery of the viral vector. Indeed, this administration route is non-invasive, easily transposable into clinic, and long-term transgene expression has been obtained upon IM delivery of rAAV in large animal models as well as in humans<sup>8,11-14</sup>. However, in large animal models, numerous cases of immune responses directed against the transgene product have been reported, all leading to the loss of transgene expression that can be attributed to the activation of transgene-specific CD8+ T lymphocytes<sup>8,15-17</sup>. In clinical trials, the limited number of available data seems to indicate that the impact of anti-transgene immune responses on rAAV-mediated gene transfer efficiency is not as predictive as animal models led us to expect<sup>13,18</sup>. Indeed, their occurrence seems not to be systematically correlated to the loss of transgene expression, although consensual conclusions are hard to draw considering the wide diversity of clinical settings (pathological state and pre-medication of the patients, immunosuppressive regimens administered before or during clinical trials, genetics and vector dosing)<sup>19</sup>. In addition, in the majority of trials, patients with null mutations and/or a high risk of transgene rejection were generally excluded.

Several key features of rAAV-mediated gene transfer, likely to influence the occurrence of immune responses directed against the transgene product, have now been identified. Among which, we find the vector serotype, the dose, the genome conformation, the immunogenicity of the transgene product and the route of vector administration<sup>20</sup>. In an effort to circumvent the risks of anti-transgene immune responses following rAAV IM delivery, and to allow the transduction of larger muscle territories upon a single injection of an rAAV vector, we and others have developed an alternative mode of muscle-directed vector administration: the locoregional intravenous (LR) route<sup>10,21–24</sup>. The LR administration route has shown in large animal models (dogs and non-human primates (NHP)) that, on top of being well tolerated, it allows a more diffused transgene expression in all muscles of the perfused limb (when compared to IM administration), thanks to an increased extravasation of vector particles into the muscles. We also previously reported a persisting gene transfer (for at least one year post-perfusion) after LR delivery of an rAAV2/1 vector expressing an inducible Epo reporter gene under the control of the TetON system that is based on the expression of the rtTA transactivator. This persisting gene regulation was observed in the absence of any immunosuppressive regimen in all 3 cynomolgus monkeys, whereas transgene expression did not exceed 3 months in IM-injected NHPs<sup>10</sup>. Additionally, although we observed anti-AAV2/1 directed antibodies in these LR-injected animals, we did not detect any humoral response directed toward the transgene product or cellular infiltrates in muscle biopsies.

In the present study, we document the long-term follow-up of these 3 LR-perfused NHPs over a period of 5 years. We characterized vector biodistribution and level of transgene expression, as well as the state of anti-transgene immune responses during this extended period. In all animals, we observed a persistent and stable TetON inducible transgene expression in the muscles of the perfused limb for at least 5 years post-rAAV2/1 LR injection. This persisting transgene expression wasn't associated with any humoral or cellular responses directed against the rtTA transgene product, neither in the periphery nor in the perfused muscles with no cellular infiltrates. To our knowledge, this is the first time that the TetON inducible system was proven to be efficient over a long period of time in a large animal model of muscle gene transfer. In previous studies, the TetON rtTA-based

system was systematically rejected by the host immune system when the vector was administered to the muscle or systemically, with a rapid loss of inducible gene expression<sup>8,25–27</sup>. Our findings strongly suggest that LR delivery of rAAV vectors is efficient and immunologically safe, in contrast to IM injection. It confirms the critical impact of vector mode of delivery on host immunity.

## MATERIALS AND METHODS

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

### **Vector Production**

Recombinant AAV2/ vector was produced as described in Toromanoff *et al.*<sup>10</sup>.

### **Animal care and welfare**

Nonhuman primates (*Macaca Fascicularis*) 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 Degree (DVM). Special attention was paid to the health and welfare of animals. Blood samples were collected regularly to follow biochemical and hematological parameters, in conformity with physiological guidelines. More precisely, primates were anesthetized with intramuscular injection of 20 µg/kg medetomidine (Domitor®; Pfizer, Paris, France) associated to 8 mg/kg of ketamine (Imalgène®; Rhone Merieux, Toulouse, France). For each Epo protein expression induction, 20, 10 and 10 mg/kg of doxycycline (ELERTE, France) were administrated *per os* at day 1, 2 and 3 respectively. At the end of the protocol, euthanasia was performed with intravenous injection of sodium pentobarbital (Dolethal®, Vétoquinol, France) after 0.1 mg/kg morphine-induced analgesia.

### ***Analysis of secreted Erythropoietin***

As described in our previous studies<sup>8,10,25</sup>, serum cynomolgus Epo protein levels were measured by enzyme-linked immunosorbent assay (Quantikine IVD kit, R&D system) according to manufacturer's procedures. Physiological variation levels of serum Epo protein were obtained from titration of a total of 182 serum samples obtained from 32 different NHP and calculated as follow: mean of Epo protein level + 2\*Standard Deviations.

### ***Quantitative real-time PCR analysis***

Total genomic DNA from total tissues was extracted using the Genra Puregene kit (Qiagen) and Tissue Lyser II (Qiagen) according to manufacturers' procedures. Total RNA was obtained from lysis of tissues by Tissue Lyser II and using TRIzol<sup>®</sup> (Thermo) and chloroform (Sigma)-based extraction. Total RNA was then treated with RNase-free DNase I from the TURBO DNA-free<sup>™</sup> kit (Thermo) to eliminate DNA contamination. 500 ng of RNA were reverse transcribed using the M-MLV reverse-transcriptase kit (Thermo). Vector genome DNA and transgene cDNA were measured through quantification of Bovine Growth Hormone polyadenylation signal (BGH-pA) localized downstream the rtTA sequence<sup>25</sup> using a StepOne Plus instrument (Thermo). The primers and TaqMan probe used are: forward primer 5'-TCTAGTTGCCAGCCATCTGTTGT-3' ; reverse primer 5'-TGGGAGTGGCACCTTCCA-3' and BGH-pA probe 5'(6 FAM)- TCCCCGTGCCTTCCTTGACC-3' TAMRA. The BGH-pA quantitative PCR was done using the following program: initial denaturation 20 sec at 95°C followed by 45 cycles of 1 sec at 95°C and 20 sec at 60°C.

For vector genome quantification, data were normalized by quantifying the endogenous NHP  $\epsilon$ -globin DNA using the following primers: forward primer: 5'-ACATAGCTTGCTTCAGAACGGT-3' ; reverse primer : 5'-AGTGTCTTCATCCTGCCCTAAA-3' and  $\epsilon$ -globin probe: 5' (6 FAM)-TGCAGGCTGCCTGGCAGAAGC-3' TAMRA. The  $\epsilon$ -globin quantitative PCR was performed with the program: initial denaturation 20 sec at 95°C followed by 45 cycles of 3 sec at 90°C and 30 sec at 60°C. For each sample, Ct values were compared with those obtained with plasmid (containing either the BGH-pA sequence or the  $\epsilon$ -globin sequence) standard dilutions.

The reverse-transcribed mRNA measurement were normalized by quantifying the endogenous NHP HPRT1 reverse transcribed mRNA, using the following primers to target the HPRT1 sequence: forward primer 5'- GCTTCCTTGGTCAGGCAGTA-3' ; reverse primer 5'-TGGAGTCCTTTTACCAGCA-3' ; and HPRT1 probe 5' (6 FAM) AATCCAAAGATGGTCAAGGTCGCAA-3' TAMRA. The HPRT1 quantitative PCR was done using the following program: initial denaturation 20 sec at 95°C followed by 40 cycles of 3 sec at 95°C and 30 sec at 62°C. The Ct results obtained for the transgene and transcripts were normalized with HPRT values using  $RQ=2^{-\Delta Ct}$ .

#### ***Follow-up of anti-TetR humoral immune responses***

Detection of serum anti-rtTA antibodies was conducted using an enzyme-linked immunosorbent assay (ELISA) as previously described<sup>28</sup>. Briefly, Nunc MaxiSorp P96 plates (Sigma) were coated with recombinant rtTA protein (5µg/mL, Proteogenix). After washing steps and saturation, sera were added in each well at various dilutions (twofold dilutions from 1/10 to 1/20480) and incubated 2 hours at 37°C. Following 1 hours at 37°C incubation of (HRP)-conjugated anti-rhesus IgG (Cliniscience), revelation was performed using 2,2',3,3',5,5'-Tetramethylbenzidine (TMB, BD OptEIA, BD Biosciences). Absorbances of duplicate samples were read at 450 nm with a correction at 570 nm on a Multiskan Go reader (Thermo Scientific). Threshold of positivity was determined using 21 negative sera obtained from naïve NHPs as mean of optic density for each dilution + 2\*SD. IgG titers for experimental animals were defined as the last serum dilution whose optic density remained above the threshold.

#### ***Follow-up of anti-TetR cellular immune responses***

The anti-TetR Immune cellular response was evaluated with an IFN $\gamma$  ELISpot assay as previously described<sup>28</sup>. Briefly, 2E5 thawed PBMC were stimulated with 5 rtTA peptides pools covering the rtTA sequence (overlapping peptide library 15 *per* 10 mers, Sigma) into human anti-IFN $\gamma$  (MabTech, France) pre-coated polyvinylidene difluoridemembrane (PVDF) MultiScreen®HTS filter plates (Millipore). Positive and negative controls were obtained using Concanavalin A (Sigma) or medium alone, respectively. After incubation with a biotinylated anti-IFN $\gamma$  antibody (clone 7-B6- 1, MabTech, France) and

ExtrAvidin® Alkaline Phosphatase (Sigma-Aldrich, France), enzymatic reaction was revealed using NBT/BCIP (Thermo). Spot number was determined using an ELISpot reader ELR07 (AID, Germany) and analyzed with AID ELISpot Reader Software V7.0. Responses were considered positive when the number of spot-forming colonies (SFC) *per* million cells were >50 and at least 3-fold higher than the negative control (C-). The rtTA-immunized macaque was obtained by the administration of the same vector rAAV1-rtTA/Epo combined with 2 intradermal injections of rtTA-pulsed dendritic cells as published in Moreau *et al.*<sup>28</sup>.

### ***Follow-up of anti-AAV2/1 humoral immune responses***

Detection of serum anti-AAV2/1 antibodies was conducted using an enzyme-linked immunosorbent assay (ELISA). Nunc MaxiSorp P96 plates (Sigma) were coated with 1E9 vg/well of recombinant AAV2/1 rtTA-Epo. Following saturation, sera were added in wells at various dilutions (twofold dilutions from 1/10 to 1/40960) and incubated 2 hours. A biotinylated anti human IgG (Jackson ImmunoResearch, USA) was incubated for 1 hour at 37°C followed by a horseradish peroxidase (HRP)-conjugated Streptavidin (Vector, USA). Finally, revelation was performed using 2,2',3,3',5,5'-Tetramethylbenzidine (TMB, BD OptEIA, BD Biosciences) and absorbance of duplicate samples were read at 450 nm with a correction at 570 nm on a Multiskan Go reader (Thermo Scientific). As for rtTA, threshold of positivity was determined using 14 negative sera obtained from naïve NHPs as mean of optic density for each dilution + 2\*SD. IgG titers for experimental animals were defined as the last sera dilution whose optic density remained above the threshold curve.

### **Follow-up of anti-AAV1 Neutralizing Factors**

Two hours prior to rAAV transduction, a permissive cell line was infected with wild-type Adenovirus serotype 5. During this incubation time, a rAAV2/1 expressing the reporter gene LacZ (encoding beta-galactosidase) was incubated with diluted serum and the mix was added to the cell line. 24 hours later, the cells were fixed with 0.5% of Glutaraldéhyde (Sigma) and stained with X-gal solution (Promega). The transduction was determined by light microscopy. The titer of neutralizing factors was defined as the last dilution that inhibits the transduction as compared to the transduction control (without serum).

### ***Histopathological analysis***

Muscles were sampled at euthanasia more than 5 years post-injection. Hematoxylin-phloxine-saffron staining (HPS) was performed as *per* standard histological protocols using formol-fixed and paraffine-embedded muscle sections. The slides were observed using a NanoZoomer Slide Scanner (Hamamatsu).

## **RESULTS**

### ***Persisting expression of an inducible transgene 5 years after locoregional delivery of rAAV2/1.***

Three NHPs (Mac 19, 20 and 21) were LR-perfused with 1E11 vg/kg of rAAV2/1 rtTA/Epo. Doxycycline was then administered periodically to induce the rtTA-mediated transactivation of the Tet-O-CMV promoter, and subsequently the expression of the Erythropoietin (Epo) protein<sup>8,10,24,25</sup>. Epo protein secretion in the serum was measured by ELISA during the 21 days after every doxycycline administration cycle. Our previous results showed a persisting Epo transgene expression, with a secretion peak of recombinant Epo protein in the serum following every doxycycline administration, during one year after LR delivery of rAAV2/1, without any immune suppression<sup>10</sup>. Moreover, although the rtTA transactivator was permanently expressed (under the control of the Desmin promoter), we did not detect any immune response against this immunogenic foreign protein in any of the three LR-injected NHP, as opposed to IM-injected NHP<sup>10</sup>.

Here, we extended the follow-up of the three LR-perfused animals over a period of 5 years (Table 1 and Figure 1). Interestingly, all three animals still exhibited a peak of Epo protein secretion in the serum after each doxycycline induction, which was systematically correlated with an increase of the levels of circulating reticulocytes (Supplementary Fig.1). However, we noticed a slight decrease of Epo protein levels in the serum after the 7<sup>th</sup> month post-perfusion: Epo peak expression was 1.3 to 2.6 fold lower than after the first 6 inductions. This level then stabilized until the end of the protocol. Nonetheless, despite this decrease, each Epo protein peak was higher than physiologic variations of intrinsic Epo protein (Figure 1). These results demonstrate for the first time a persistent inducible transgene expression up to 5 years after LR delivery of AAV in non-human primates.

***Most of vector genomes and transgene transcripts are localized in perfused muscles 5 years after locoregional delivery.***

Vector biodistribution was evaluated by measuring viral vector genomes and constitutively-expressed rtTA transcripts through quantitative PCR and Reverse Transcriptase quantitative PCR, respectively. Quantifications were performed in 4 muscles of the perfused limb (biceps femoris, tibialis anterior, gastrocnemius medialis and gastrocnemius lateralis), in the same muscles of the contralateral limb, in distant muscles (biceps brachialis and brachioradialis), and in various organs (diaphragm, heart, liver and spleen). At 5 years post-infusion, most of the vector genomes (up to 0.08 vg/dg) were detected in perfused muscles and in the liver (Figure 2A). Only Mac 21 had detectable, though rare (less than 0.007 vg/dg), copies of vector genomes in contralateral or distant muscles.

Related to these results, rtTA transcripts were mostly detected in perfused muscles (most of RQ comprises between 0.0264 and 0.3843) and not or few transcripts were detected in contralateral/distant muscles as well as in others organs as heart, diaphragm or spleen with RQ values always below the limit of PCR quantification (Figure 2B). We also detected rtTA transcripts in liver of the three NHP, though to a lesser degree than in perfused muscles (RQ = 0.0058, 0.0076 and 0.0340 for Mac 19, 20 and 21 respectively). Overall, these data indicate that the transgene is essentially found and expressed in the muscles of the perfused limb, with low to no expression at distant sites, except in the liver.

***Monitoring humoral and cellular immune responses***

We have observed a slight decrease in Epo protein secretion 7 months post-LR infusion of rAAV2/1 vectors. To investigate whether this could be attributed to a transient immune response targeting transduced cells, we monitored anti-transgene humoral and cellular responses in the periphery. We first investigated the kinetics of serum anti-rtTA humoral immune using an ELISA assay at 15 days, 3 months, 10 months, 2.5 years and 5 years post-gene transfer. As shown in Table 1 and Table 2, we couldn't detect any anti-rtTA IgG antibodies at any time during the protocol. In the same manner, anti-rtTA T-cell responses were assessed at 5 year post-injection on PBMCs stimulated with rtTA peptide pools

through an IFN- $\gamma$  ELISpot assay (Table 1 and Figure 3). We did not detect any IFN- $\gamma$  secretion at 5 year post-perfusion in contrast to an rtTA-immunized macaque. Overall, these results indicate that LR administration did not induce any detectable anti-transgene immune response at least at the time points analyzed in this study.

To complete vector immunogenicity assessment, we evaluated AAV2/1 humoral response by determining anti-AAV2/1 IgG antibody titers in the serum using an ELISA assay at various time-points over 5 years (Table 1 and Table 2). For all three NHPs, serum anti-AAV2/1 antibodies occurred from day 15 post-perfusion onward, peaking between 3 and 10 months, with titers ranging from 1/5120 to 1/10240. Afterwards, anti-AAV2/1 IgG antibody amounts tended to decrease slowly over time but remained relatively high for two NHPs at 5 years post-perfusion (1/5120 and 1/1280 for Mac 19 and Mac 21 respectively).

Anti-AAV2/1 IgG antibodies were associated with the induction of anti-AAV2/1 neutralizing factors in the serum. Neutralizing factors were detected from month 3 post-perfusion onward, with titers ranging from 1/1000 to 1/5000. As for anti-AAV2/1 IgG antibodies, neutralizing factor titers decreased over time but remained relatively high at 5 years post-perfusion (1/100 and 1/1000) (Table 1 and Table 2). These results indicate that LR administration induced a long-term anti-AAV2/1 humoral response, which tends to gradually decrease over time.

#### ***Absence of infiltrating immune cells in muscles 5 years after locoregional delivery***

To deepen our analysis of anti-transgene cellular response after LR delivery, we investigated the potential infiltration of immune cells in perfused muscles. Therefore, we performed Hematoxylin-phloxine-saffron (HPS) staining on four muscle sections from the injected limb (gastrocnemius lateralis, tibialis anterior, gastrocnemius medialis and biceps femoris) and one muscle section from the contralateral limb (tibialis anterior) obtained at sacrifice, 5 years after vector delivery (Table 1 and Figure 4 for representative sections). No infiltrated immune cells were detected in any of the muscle sections analyzed at 5 years post-LR infusion.

## DISCUSSION

Gene therapy using rAAV has grown by leaps and bounds among the past decade, culminating with the first EMA-approved product Glybera delivered in 2012 to treat lipoprotein lipase deficiency<sup>1,3</sup>. Since then, another rAAV gene therapy product has been approved at the end of 2017 by the FDA for a genetic retinal dystrophy<sup>3,4</sup>. Pre-clinical studies in large animal models have largely contributed to these successful developments, although few of them provide insights on the long-term efficiency and stability of rAAV-mediated gene transfer. The highest number of long term reports (between 2.5 and 6 years post-injection) have been provided for gene transfer studies targeting retinal immune privileged site and the central nervous system in NHP and canine models<sup>29-31</sup>. When it concerns systemic, intramuscular or intrahepatic routes, there are now also numerous promising long term reports (between 2 and 8 years post-injection) in NHP<sup>32</sup> and canine models for diabetes<sup>33</sup>, metabolic diseases<sup>34</sup>, hemophilia<sup>35,36</sup> or muscular dystrophies<sup>37,38</sup>. Moreover, the field has now the benefit of up to 10 years hindsight in humans with persisting gene transfer following 1 single injection<sup>39-42</sup>. All these studies highlight the tremendous achievements of rAAV-based gene therapy.

When it concerns rAAV-mediated gene transfer to the skeletal muscle, IM-based strategies were first described and have shown long term efficient gene transfer when host effector immunity is not triggered. This was achieved in large animal models<sup>8,10,33</sup>, and even up to 5 and 10 years, in phase I/II clinical trials for alpha-anti-trypsin deficiency<sup>43</sup>, and hemophilia B<sup>40</sup>, respectively. However, IM route generally requires multiple sites of injection to achieve sufficient amounts of gene expression with a wider distribution, which in turns could increase the risk of gene transfer immunogenicity as described in multiple studies<sup>8,10-13,15,25,45,46</sup>. There is clear evidence that the vector dose *per* IM site is a major determinant of unwanted immune responses to the transgene as shown for instance for FIX in hemophilia B dogs<sup>47</sup>.

LR mode of delivery was described as a promising alternative to overcome limited gene transfer efficiency following IM vector delivery and associated potential immunogenicity towards the transgene product. We and others have indeed shown that LR-based gene

transfer allows persisting gene transfer in larger muscle territories of the perfused limb using NHP or canine models<sup>10,22–24,38,48</sup>.

In the present study, we followed-up three NHPs over, to our knowledge the longest follow-up (a 5-year time course), following a LR perfusion of an AAV vector. Inducible Epo transgene expression over time was measured in serum by ELISA and revealed a systematic peak of expression after each doxycycline induction phase, which evidenced for the first time the ability of rAAV2/1-LR administration to sustain long-term TetON-mediated inducible transgene expression in the NHP model, not only for one year, but also for at least 5 years, in the absence of any immunosuppressive treatment.

Biodistribution analyses at 5 years post-perfusion showed that most of vector genomes are confined to perfused muscles and to the liver, confirming our previous results wherein most of transgene copies were found in the injected limb after LR delivery of rAAV2/1 or rAAV2/8 in NHPs<sup>24</sup> and rAAV8 in Duchenne GRMD dogs<sup>23,38</sup>. When, an important biodistribution of the vector to the liver was shown initially to be a particular feature of serotype 8<sup>49</sup>, it is now admitted that other AAV serotypes including AAV1 are able to partially home to the liver even following IM injection<sup>24,50</sup>.

In parallel, rtTA transcript quantification validated that vector copies in perfused muscles are the main source of transgene expression. Whereas we detected transgene mRNA in perfused muscles, we also detected rtTA transcripts in the liver of the 3 NHPs. This event parallels the leak of the desmin promoter in the liver initially described by Toromanoff and colleagues in this model<sup>10</sup>, and could be explained by the transduction of Hepatic Stellate Cells by rAAV2/1 where desmin protein is expressed<sup>10,51</sup>.

The long term follow-up of our animals highlighted that at 7 months post-injection, the level of Epo protein secretion in the serum decreased to approximatively half of the initial level for each NHP, and remained stable until the end of the protocol, a phenomenon already observed in previous studies<sup>8,24,52–54</sup>. A moderate or partial silencing of the transgene, as described by Suzuki and colleagues for adenoviral vectors<sup>55</sup>, might account for this partial loss. Nonetheless, the decrease of Epo inducible expression in our model

didn't affect the subsequent rate of reticulocytes in blood that remained constant after each doxycycline induction step (Supplementary Fig. 1).

Otherwise, another possibility to explain Epo expression decrease is a partial and transitory immune-mediated destruction of the genetically modified myofibers. To further understand this event, we explored capsid and transgene-directed immune responses. Concerning anti-transgene immune responses, we were not able to detect transgene-directed antibodies at any time during the protocol, even at early time points. Nonetheless, these results are not in line with previous LR studies in Duchenne and hemophilia canine models where transgene-directed IgG antibodies were detected<sup>22,38,48</sup>. However, in these studies, rAAV2 or rAAV8 serotypes were used instead of rAAV1, and vectors were administered at higher doses ranging from 1E12 to 1E13 vg/kg, instead of 1E11 in the present study. Interestingly, persisting  $\mu$ dystrophin or FIX transgenes expression was anyways observed suggesting a particular humoral immunity. Concerning anti-transgene cellular response, we did not detect rTA-directed IFN- $\gamma$  secreting cells at 5 years post-perfusion. Unfortunately, except at the end of the protocol, we did not collect PBMC at intermediate time points and a transitory anti-transgene cellular immune response cannot be excluded. On the other hand, this result is consistent with the previous LR studies in dogs<sup>22,23,48</sup>.

Regarding anti-capsid immunity and as expected, we were able to detect circulating IgG antibodies as well as neutralizing factors in all NHPs during the whole protocol. Neutralizing factors and anti-capsid antibodies titers reached high levels during the first year and, despite an important decrease, remained high even at 5 years post-injection, indicating a long term capsid immunization after rAAV2/1 delivery similar to the reports by others after IV administration of rAAV8 in dogs<sup>32</sup>. Importantly, these events seem to have no effect on the persistence of transgene expression, consistent with previous observations in preclinical and clinical trials<sup>13</sup>. We didn't check the anti-capsid cellular response but the long term persistence of transgene expression indicates that even if a cellular anti-capsid response did occur, it did not completely abrogate transgene expression.

In addition, we did not detect any infiltrated cells in muscle sections at 5 year post-infusion and, what's more, muscles didn't show any evidence of past immune cells infiltration (observation of an anatomy pathology EMA-certified expert). In opposition to those findings, *in situ* infiltrated cells have been detected at 4 weeks and 6 months in muscles of dogs from Arruda *et al.* and Haurigot *et al.* LR-studies respectively<sup>22,48</sup>. The presence of cell infiltrates does not systematically mean an effector immune response. Indeed, regulatory T cells (Tregs) and/or exhausted/anegetic T cells were shown to potentially play a role in the modulation of anti-transgene or capsid immune responses following AAV-mediated gene transfer<sup>43,56-60</sup>. The involvement of Tregs in muscle local tolerance was reported in two phase I/II clinical trials for alpha anti-trypsin and lipoprotein lipase deficiencies and associated to persisting gene expression<sup>43,57,60</sup>. In addition and regarding the transgene product, liver-directed gene transfer was shown to be associated to tolerance<sup>61-63</sup> *via* the induction of specific regulatory T cells<sup>64</sup>. Although, in our study, our observations argue in favor of no cellular infiltration after LR injection or rAAV2/1 at least in this particular model of rAAV-mediated inducible gene transfer.

Overall, our model suggests that LR delivery doesn't elicit anti-transgene immune rejection with neither humoral, nor cellular immunity. Even at relatively low doses of vectors (1E11 vg/kg), in our hands, IM injection of the same vector from various serotypes induced in the majority of animals transgene directed immune-response<sup>8,10,24,25,28</sup>. This study confirms that, when the immune system is not mobilized against the rtTA transactivator, the tetR-regulatable system is able to support long-term inducible transgene expression following either IM or LR vector administration.

Following LR delivery, we didn't detect any adverse event both at clinical and biochemical levels (supplementary Table 1), whether at early<sup>10,24</sup> or late time points. Our results, along with previous studies in large animal models<sup>21-23,38</sup>, confirm the safety of this procedure of injection which has already been validated for the perfusion of leg muscles in dystrophic patients<sup>65</sup>.

Our past and present studies involving LR administration of rAAV2/1 without immunosuppression demonstrated that the LR mode of delivery allowed a diffused

transduction of large muscle territories within the perfused limb, with minor diffusion to other organs (except for the liver). The expression of an inducible transgene product was found stable at long term, over-ranging 5 years, without any detectable deleterious effect of immune responses or toxicity associated to the expression of a foreign and highly immunogenic protein (rtTA transactivator). Whether these observations remain true across various AAV serotypes, transgene products and high vector doses remain to be investigated. Interestingly and despite the induction of a humoral response against the transgene product, data from other previous studies has also shown safe and long term gene transfer following LR delivery<sup>22,38,48</sup>. Altogether, this study along with previous reports, strongly support that LR route, in the same way as the conventional intravenous route currently used in clinics, is safe and efficient and promotes its potential use as an alternative to IM injection for muscle delivery, in particular in the context of metabolic diseases needing the secretion of a therapeutic factor. Moreover, this study further confirms the critical impact of vector mode of delivery on host immunity.

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#### **AUTHOR DISCLOSURE**

No competing financial interests exist

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**Table 1. Summary table of long-term transgene expression and host immunity at 5 years post-perfusion**

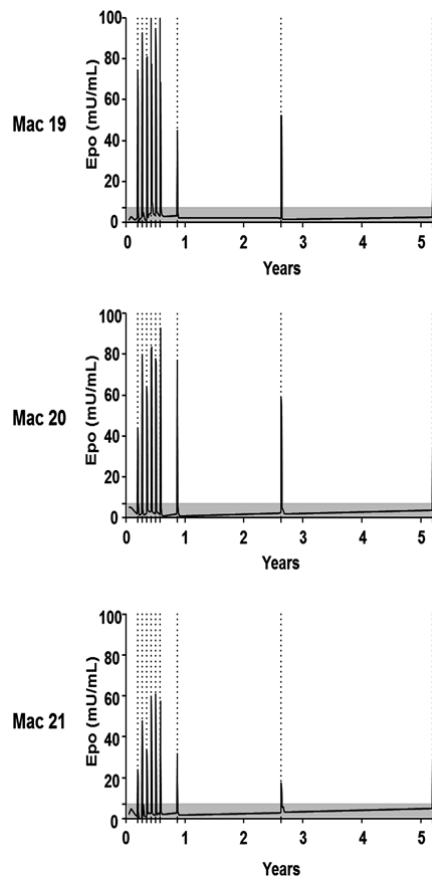
<b>NHP</b>	<b>Mode of vector delivery</b>	<b>Transgene expression</b>	<b>Anti-AAV1 Ab</b>	<b>Anti-AAV1 NF</b>	<b>Anti-rtTA Ab</b>	<b>rtTA directed IFN<math>\gamma</math> Cellular response</b>	<b>Muscle infiltrates</b>
<b>Mac 19</b>	LR	Persisting	+	+	-	-	-
<b>Mac 20</b>	LR	Persisting	+	+	-	-	-
<b>Mac 21</b>	LR	Persisting	+	+	-	-	-

**Table 2. Kinetic of humoral Response (anti-rtTA, anti-AAV1 responses and anti-AAV1 Neutralizing Factors)**

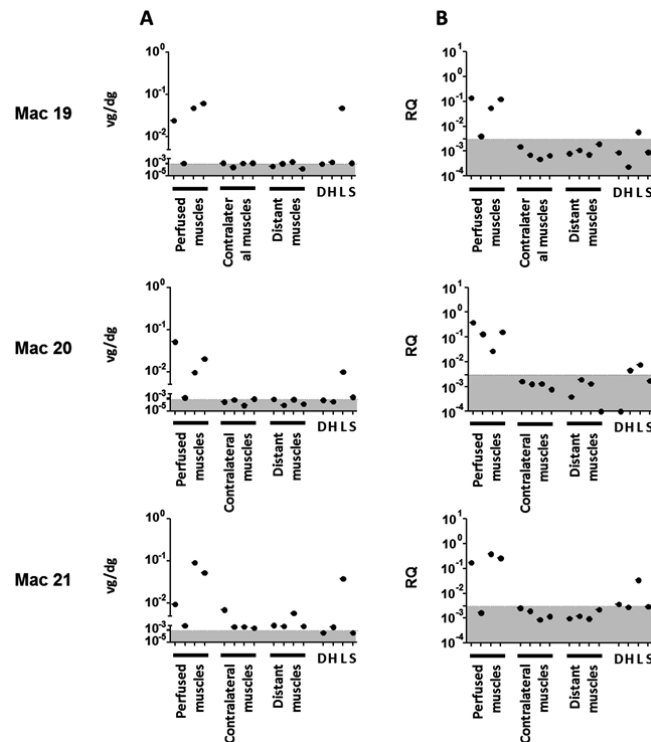
	Before inj	15 days pi	3 months pi	10 months pi	2,5 years pi	5 years pi	
Mac 19	<b>Anti-AAV1 Ab</b>	Negative	1/40	1/10240	1/10240	1/5120	1/5120
	<b>Anti-AAV1 NF</b>	Negative	ND	1/5000	ND	ND	1/1000
	<b>Anti-rtTA Ab</b>	Negative	Negative	Negative	Negative	Negative	Negative
	<b>Anti-AAV1 Ab</b>	Negative	1/80	1/5120	1/1280	1/320	1/80
Mac 20	<b>Anti-AAV1 NF</b>	Negative	ND	1/1000	ND	ND	1/100
	<b>Anti-rtTA Ab</b>	Negative	Negative	Negative	Negative	Negative	Negative

Mac 21	<b>Anti- AAV1 Ab</b>	Negative		1/80	1/10240	1/10240	1/5120	1/1280
	<b>Anti- AAV1 NF</b>	Negative		ND	1/5000	ND	ND	1/1000
	<b>Anti- rtTA Ab</b>	Negative	Negative	Negative	Negative	Negative	Negative	Negative

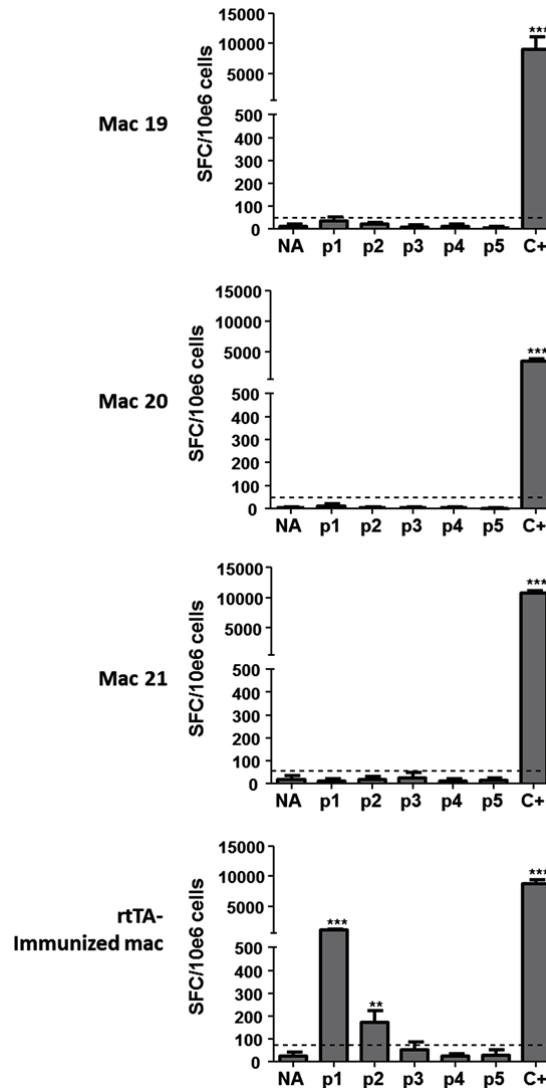
ND = Not Determined, Ab = Antibodies, NF = Neutralizing Factors

Guilbaud *et al.*, FIGURE LEGENDS

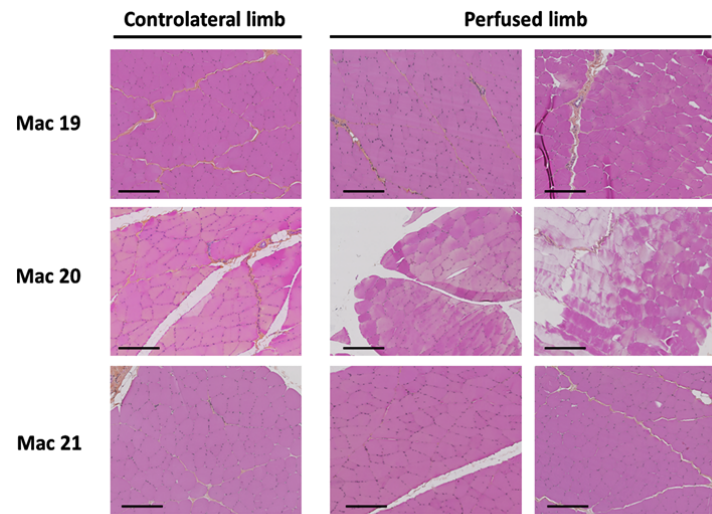
**Fig1. Long-term dox-mediated transgene regulation.** Long term follow-up (5 years) of serum Epo level in 3 LR injected macaques (Mac 19, Mac 20 and Mac 21)<sup>10</sup>. Dox administrations are indicated by dotted lines. The grey area illustrates the physiologic basal levels of Epo (established as mean  $\pm$  2SD of 182 measures of Epo in serum samples obtained from 32 cynomolgus macaques)



**Fig2. Detection of viral genomes and transgene transcripts.** Tissue samples were collected during necropsy. For each panel, perfused muscles correspond to Biceps femoris, tibialis anterior, gastrocnemius medialis and gastrocnemius lateralis of the injected limb. Contralateral muscles correspond to the same muscles of the non-injected limb and distant muscles correspond to biceps brachialis and brachioradialis of the 2 anterior limbs. D, H, L and S correspond to Diaphragm, Heart, Liver and Spleen respectively. **(A)** Viral genomes were determined by quantitative PCR (qPCR) and expressed as viral genome per diploid genome (vg/dg). **(B)** Transcripts were detected by reverse-transcription and qPCR (RT-qPCR) and expressed as relative quantity (RQ) to an endogenous gene. The grey areas corresponds to the limit of quantification of PCR detection (0.000809 vg/dg for panel A and RQ of 0.00304 for panel B)



**Fig3. No detection of anti-rtTA IFN $\gamma$  T cell response at 5 years post-injection.** PBMCs were collected at euthanasia (5 years post-injection) and secretion of IFN $\gamma$  by rtTA-stimulated PBMCs was evaluated by ELISpot. Cells were stimulated using an overlapping 15 per 10 amino-acids peptide library covering the rtTA protein and divided in 5 pools (p1 to p5). Negative controls (C-) consisted in PBMCs cultured with medium alone and positive control (C+) consisted in mitogenic Concanavalin A (Con A) stimulation. IFN $\gamma$  was measured as Spot Forming Cells (SFC). Threshold of positivity (dotted line) consists in SFC > 50 spots and 3 times the number of SFC obtained with negative control (C-). For positive responses, statistical analysis was performed using a DFR test, \*\*\* for P value  $\leq$  0,001, \*\* for P value  $\leq$  0,01, \* for P value  $\leq$  0,05.



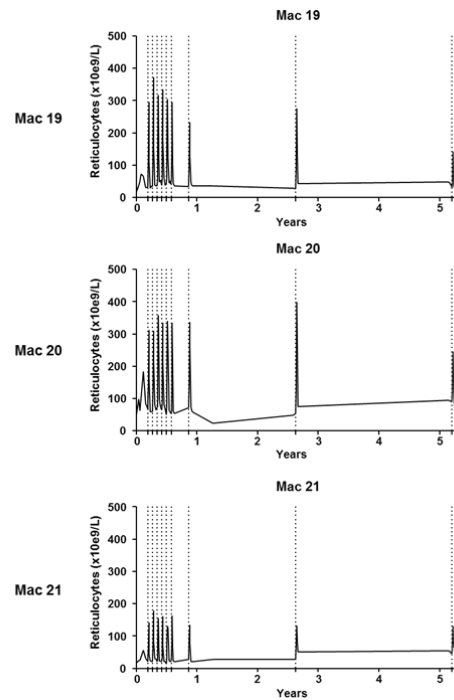
**Fig4. No detection of muscle lesions at 5 years post-injection.** Perfused (tibialis anterior and gastrocnemius lateralis) and contralateral (tibialis anterior) muscles were collected at euthanasia (5 years post-injection). HPS stainings were performed on formol-fixed and paraffin-treated muscles sections. Pictures were representative of whole section and indicate no infiltration of immune cells or lesions in perfused muscles. Scale bar = 200  $\mu$ m.

Supplementary Table 1. Follow up of biochemical parameters

Biochemical parameter	Time-point	Mac 19	Mac 20	Mac 21
<b>C-Reactive Protein</b> (mg/mL)	D-30	< 4	< 4	< 4
	D4	< 4	< 4	< 4
	D14	25.2	8.9	< 3
	D446	<3	<3	<3
<b>Alkaline Phosphatase</b> (U/L)	D-30	581	837	681
	D4	461	869	765
	D14	443	791	722
	D446	583	558	519
<b>ALAT</b> (U/L)	D-30	33	53	23
	D4	58	67	35
	D14	43	58	36
	D446	32	60	32
<b>γ-GT</b> (U/L)	D-30	146	140	169
	D4	102	127	157
	D14	106	111	138
	D446	110	101	114
<b>Creatin Kinase</b> (U/L)	D-30	367	600	888
	D4	308	878	329
	D14	637	421	447
	D446	196	3928	869
<b>Creatinin</b> (mg/L)	D-30	6.3	6.1	6.6
	D4	6	5.6	5.8
	D14	5.4	6.2	6.4
	D446	9.2	8.7	8.7

Human Gene Therapy  
 5 years of successful inducible transgene expression following locoregional AAV delivery in nonhuman primates with no detectable immunity (DOI: 10.1089/hum.2018.234)  
 This paper has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

	D-30	0.4	0.4	0.4
<b>Urea</b> (g/L)	D4	0.3	0.4	0.4
	D14	0.3	0.3	0.4
	D446	0.3	0.34	0.37

Guilbaud *et al.*, Supplementary file

**Supplementary Fig.1. Long-term induction of Reticulocytes in blood.** Reticulocytes (Red blood cells precursors) were measured throughout the duration of the protocol. Monitoring of Blood parameters were performed by technical core facility of University Hospital of Nantes (CHU of Nantes). Doxycyclin administrations are indicated by dotted lines. Reticulocyte peaks are correlated with those of Epo.