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Locoregional intravenous delivery of AAV vector in macaque results in persistent transgene expression despite a dedicated humoral and cellular immune response

Gwladys Gernoux¹, Marie Devaux¹, Laurence Dubreil², Nicolas Jaulin¹, Mickael Guilbaud¹, Jack-Yves Deschamps², Thibaut Larcher², Lydie Guigand², Maeva Dutilleul², Caroline Le Guiner¹, Yan Cherel², Philippe Moullier^{1,3} and Oumeya Adjali¹.

¹ Inserm UMR 1089/Atlantic Gene Therapies, Institut de Recherche Thérapeutique, Nantes, France

² INRA UMR 703/Atlantic Gene Therapies, ONIRIS, Nantes, France

³ Department of Molecular Genetics and Microbiology department, University of Florida, Gainesville, United States

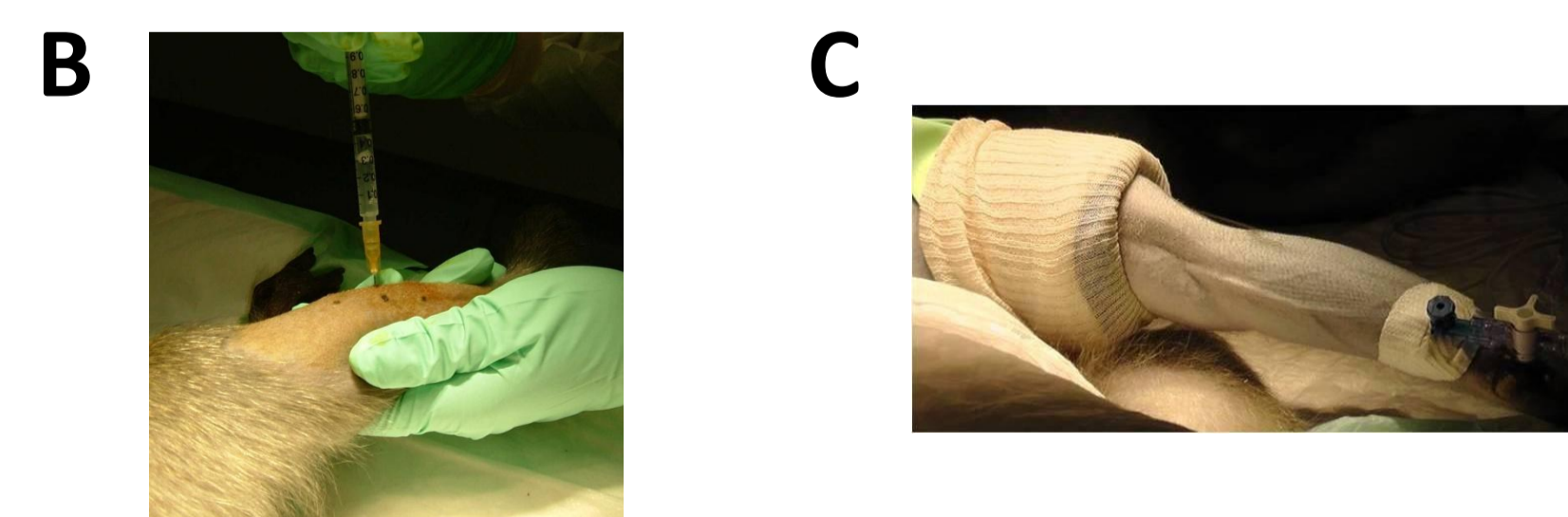


Introduction

Locoregional intravenous delivery (LR) is a promising method to deliver recombinant adeno-associated virus (AAV) vector to large skeletal muscle territories. In addition, LR as opposed to intramuscular (IM) route of AAV administration was previously reported to allow sustained expression of rtTA, an immunogenic transgene product in primates¹ and also promote a tolerogenic profile in factor IX-deficient dogs². To further explore these observations, non-human primates (NHP) were injected by IM or LR routes with a self-complementary AAV8 (scAAV8) vector encoding for the green-fluorescent protein (GFP), in the absence of an immunosuppressive regimen. We studied for 8 months post scAAV8.GFP administration, the relationship between the AAV vector and the host immune system by analyzing vector biodistribution and transgene expression in the skeletal muscles and the lymphoid organs. The host humoral and cellular immune responses against the GFP transgene and the AAV8 viral capsid were also monitored at different time points after vector injection.

Methods

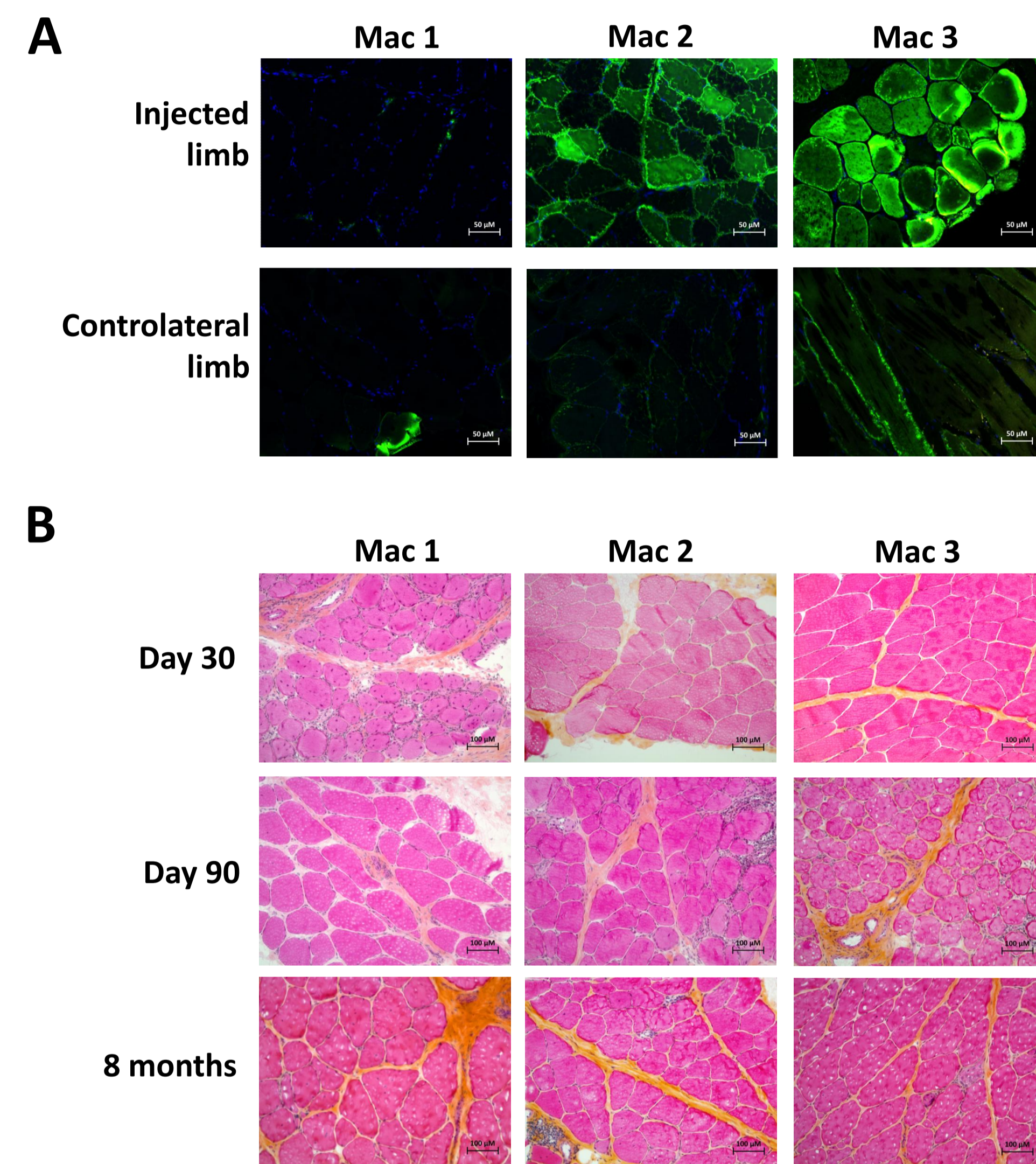
NHP ID	Serotype	Promotor	Route	Dose (vg/kg)	Follow-up
Mac 1	scAAV8	CMV	IM	7 ^e 12	8 months
Mac 2	scAAV8	CMV	LR	7e12	8 months
Mac 3	scAAV8	CMV	LR	7e12	8 months



(A) Cynomolgus macaques were injected in the posterior limb with a self-complementary serotype 8 adeno-associated virus-derived vector (scAAV8). The vector genome consisted in cytomegalovirus (CMV) promoter, the coding sequence of green fluorescent protein (GFP) and the SV40 polyadenylation signal (SV40 pA). Non-human primates were injected either by intramuscular (IM, Mac1) or intravenous locoregional (LR, Mac 2 and 3) routes with 7^e12 viral genomes/kg. (B) Mac 1 was injected intramuscularly in the tibialis anterior over 5 pretattooed injection sites. (C) Mac 2 and 3 were injected by LR route consisting in the injection of 20% of the limb volume³ through the saphenous vein under pressure, while the blood flow in and out of the limb is blocked by a tourniquet placed above the knee.

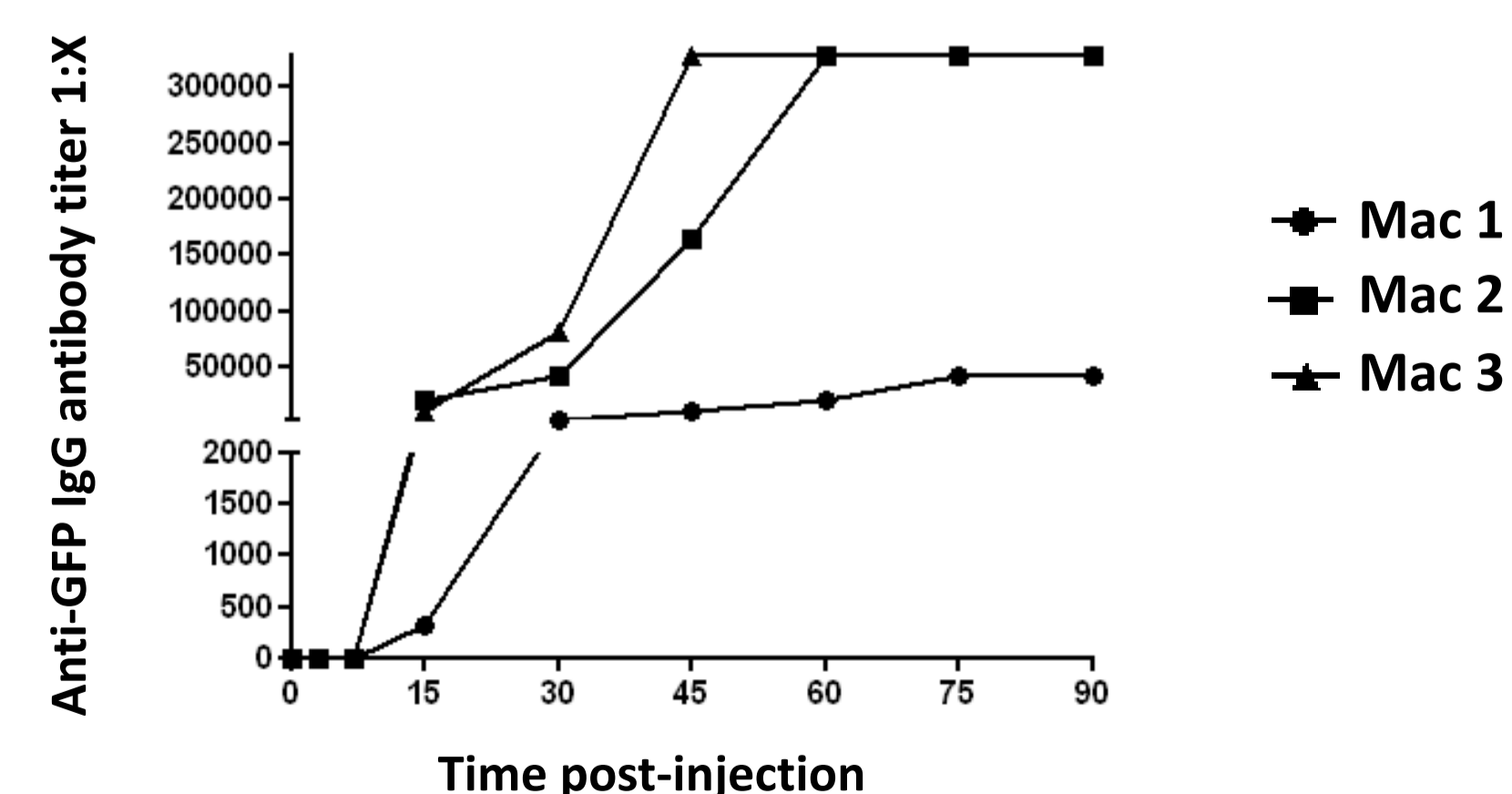
Results

GFP transgene expression is maintained until at least 8 months post-injection after LR delivery, with delayed and mild muscle cell infiltration, as compared to IM route



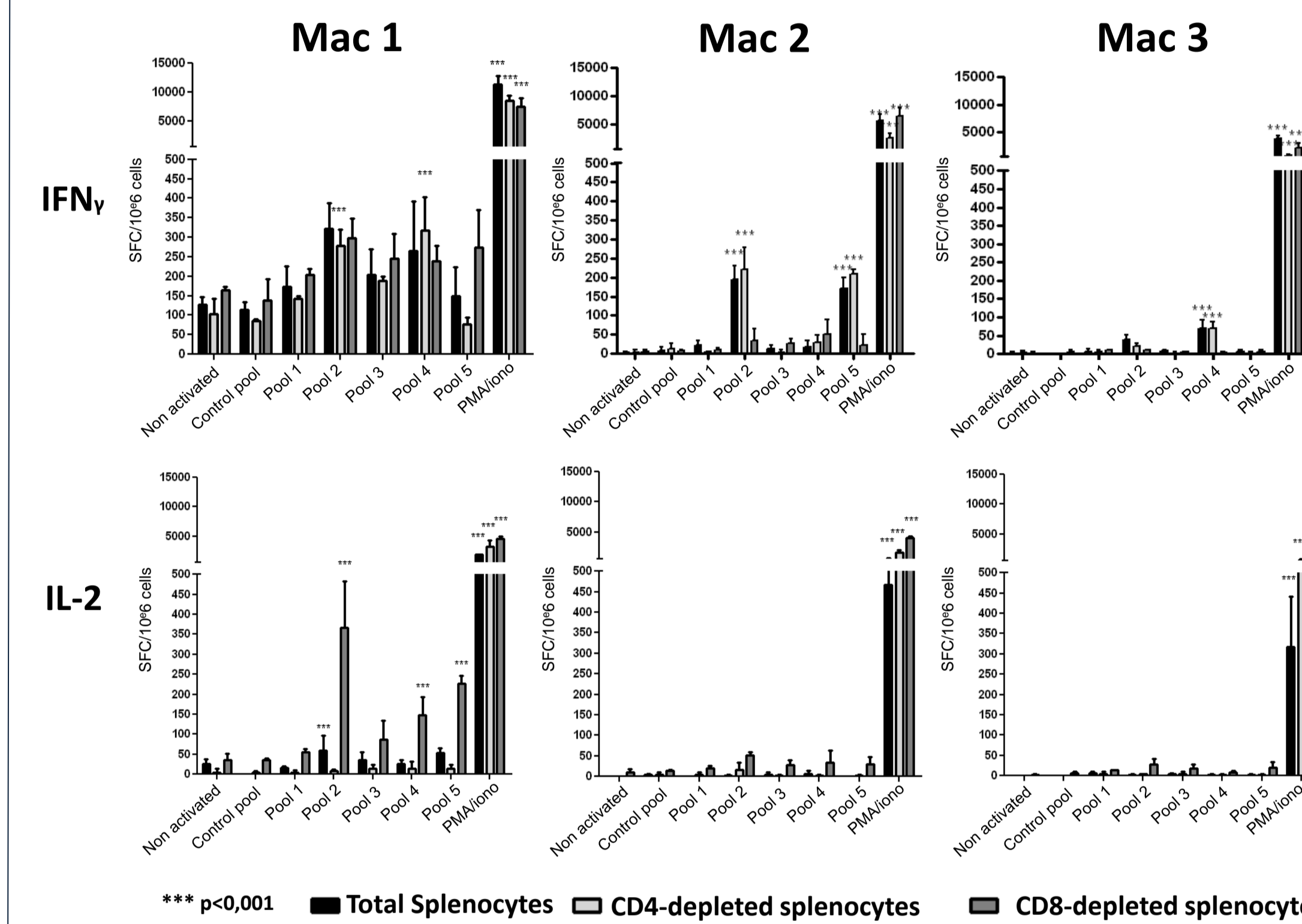
Cynomolgus macaques were injected in the posterior limb with scAAV8-CMV-GFP vector (7^e12vg/kg) either by IM (Mac1) or LR (Mac 2 and 3) routes. Muscle biopsies were realised at day 30, day 90 and 8 months post-injection in the tibialis anterior of injected and controlateral limbs. (A) GFP protein detection was analysed by direct immunofluorescence in muscle biopsies performed at 8 months post-injection. (B) Cellular infiltrates were analysed on muscle biopsies obtained at different time points, using hematoxylin erythrosine saffron (HES) staining. Our results show the persistence of GFP expression until at least 8 months post-injection after LR administration, not after IM delivery. Moreover, GFP protein was detected in both injected and controlateral limbs. HES staining showed a mild and delayed inflammation after LR (day 90) as compared to IM (day 30).

Transgene persistence after LR was observed despite the detection of anti-GFP IgG antibodies



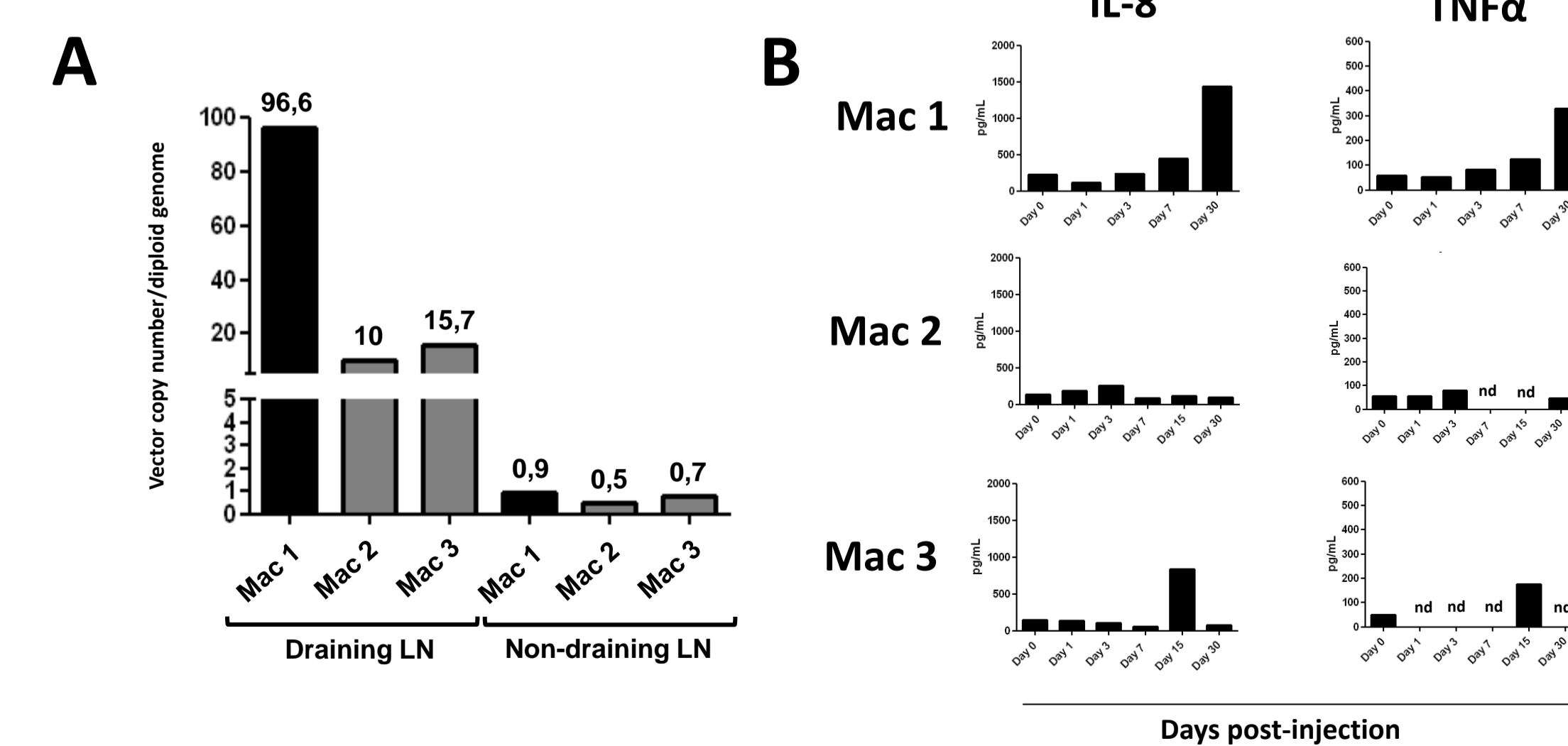
Anti-GFP IgG antibodies were analysed in primate sera by ELISA at days 0, 3, 7, 15, 30, 45, 60, 75 and 90 post-injection. Each sample was tested in duplicates. Anti-IgG antibody titers were assigned at the highest dilution where the average of the duplicates was higher than the average of serum before vector injection \pm 2SD. Antibodies were detected from day 15 to at least day 90 post-injection after both IM and LR delivery. Anti-GFP antibody titer was even higher after LR than IM delivery. For instance, at day 60 post-injection, titers >1:327680 versus 1:20480 observed for Mac 2 (LR) and Mac 1 (IM) respectively

Persisting transgene expression after LR was associated to IFN γ but not IL2 secretion



Secretion of IFN γ and IL2 from GFP-restimulated splenocytes was measured by ELISpot. Splenocytes were isolated at day 60 post-injection and stimulated *in vitro* with overlapping peptides spanning the GFP sequence, and divided in 5 pools. Negative controls consisted in non activated cells and an unrelated peptide pool not corresponding to any known antigen in the macaque, whereas phorbol 12-myristate 13-acetate and ionomycin (PMA/iono) stimulation was used as a positive control for cytokine secretion. Responses were considered positive when the number of spot-forming colony (SFC) per 10⁶ cells were >50 and at least 3-fold higher than the unrelated peptide pool (control pool). Cytokine secretion was measured by stimulation of total splenocytes (black), CD4-depleted splenocytes (light-gray) or CD8-depleted splenocytes (dark-gray). After LR delivery, GFP stimulated T cells secreted IFN γ but not IL-2 and the IFN γ secretion was mainly mediated by CD8-enriched T cell fraction. In contrast, after IM injection, stimulated T cells secreted both IFN γ and IL-2. IFN γ secretion was mediated by both CD4 and CD8 T-cells whereas IL-2 secretion seemed to be mainly mediated by CD4-enriched T-cell fraction.

Decreased lymph node vector draining after LR was associated to less secretion of inflammatory TNF α and IL8 cytokines, as compared to IM



(A) Viral genomes were detected by quantitative PCR targeting the GFP transgene in inguinal draining and non-draining lymph nodes surgically obtained at day 7 post-injection. The results were expressed as vector copy numbers per diploid genome and the value for each sample is indicated above the corresponding bar chart. (B) Circulating IL-8 and TNF α cytokines were quantified in plasmas by Luminex technology at days 0, 1, 3, 7, 15 and 30 post-injection. Each sample was tested in duplicates. Viral genomes were detected in draining and non-draining lymph nodes for both IM and LR routes. Although GFP copy number per diploid genome was similar after IM or LR in non-draining lymph node, a difference of 10-fold copy number was observed in the draining lymph node after IM as compared to LR. IL-8 and TNF α increased between day 7 and day 30 post-injection after IM administration, not after LR.

Conclusion

After LR delivery of an AAV8 vector expressing GFP in non-human primates, we showed detection of transgene expression until at least 8 months post-injection in contrast to IM where GFP was lost at 3 months post-injection. Interestingly, GFP persistence after LR was observed despite the detection of anti-GFP IgG antibodies, even at higher titers than IM route, and the presence of T cells responding to GFP peptides in the spleen. Surprisingly, GFP-restimulated T cells secreted IFN γ but not IL2 after LR as compared to IM route where both cytokines were detected.

Histological analysis showed a delayed and mild inflammation in the perfused limb whereas IM injection resulted in earlier and robust muscle infiltrates at the site of injection. The characterization of these cellular infiltrates still needs to be performed. Finally, inflammatory IL8 and TNF α cytokines and chemokines did not increase in plasma after LR delivery in opposition to IM.

All these observations suggest that immune signals induced after LR administration are not sufficient to allow fully destructive immune responses. One observation consistent with this hypothesis is the fact that transgene copy number in the draining lymph node was found 10-fold lower in draining lymph node after LR as compared to IM.

In conclusion, this study in the nonhuman primate model further confirms the promising clinical potential of LR mode of delivery for AAV-based gene transfer to the skeletal muscle.

¹ Toromanoff et al, Mol Ther, 2010

² Haurigot et al, Mol Ther, 2010

³ Fan et al, Mol Ther, 2012