

SUPPLEMENTARY FIGURES

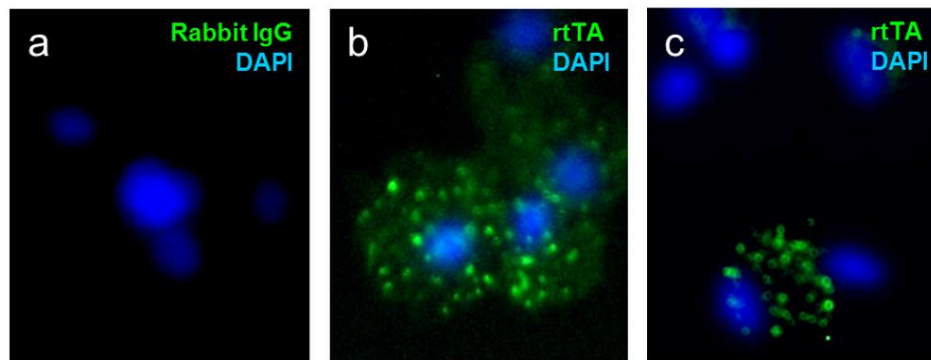


Fig. S1: iBMDC10 cells were efficiently pulsed with rtTA protein. iBMDC10 were loaded overnight with rtTA protein in the presence of IL-10. Cells were then stained using anti-rtTA antibody (green staining) and DAPI (blue staining). (b) and (c) panels are representative of six experiments using iBMDC10 from different animals. Panel (a): negative control after cell incubation with the anti-rabbit biotinylated secondary antibody alone.

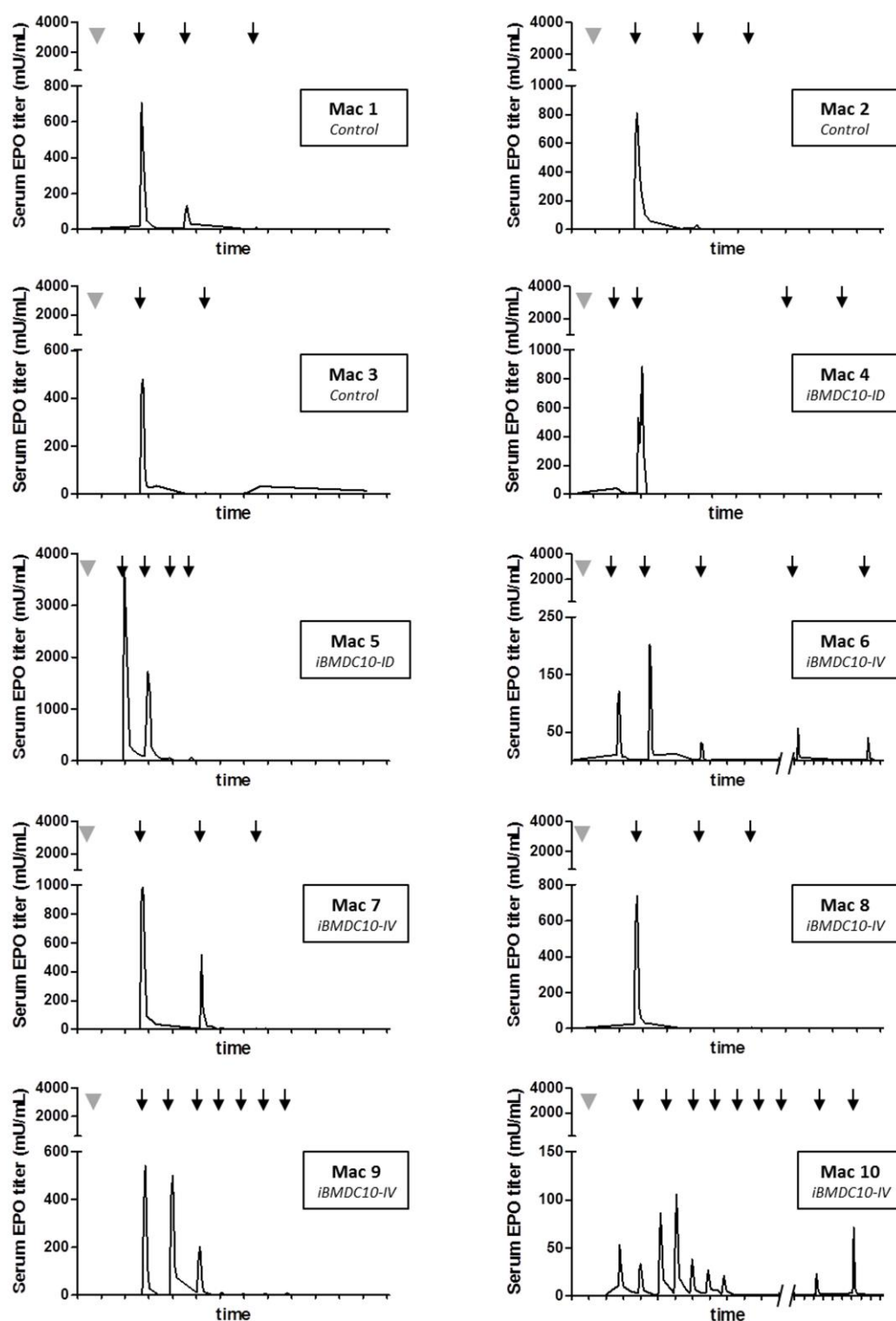


Fig. S2: Epo Serum levels in animals from control (Mac 1 to Mac 3), iBMDC10-ID (Mac 4 and Mac 5) and iBMDC10-IV (Mac 6 to Mac 10) groups. The grey arrow indicates the IM injection of rAAV1 that expresses the cynomolgus erythropoietin (cmEpo) under the control of the doxycyclin (Dox)-sensitive transactivator rtTA. Black arrows indicate induction cycles with Doxycycline. Mac 6

and Mac 10 were followed-up during a longer time in order to confirm their persisting Epo expression under dox induction.

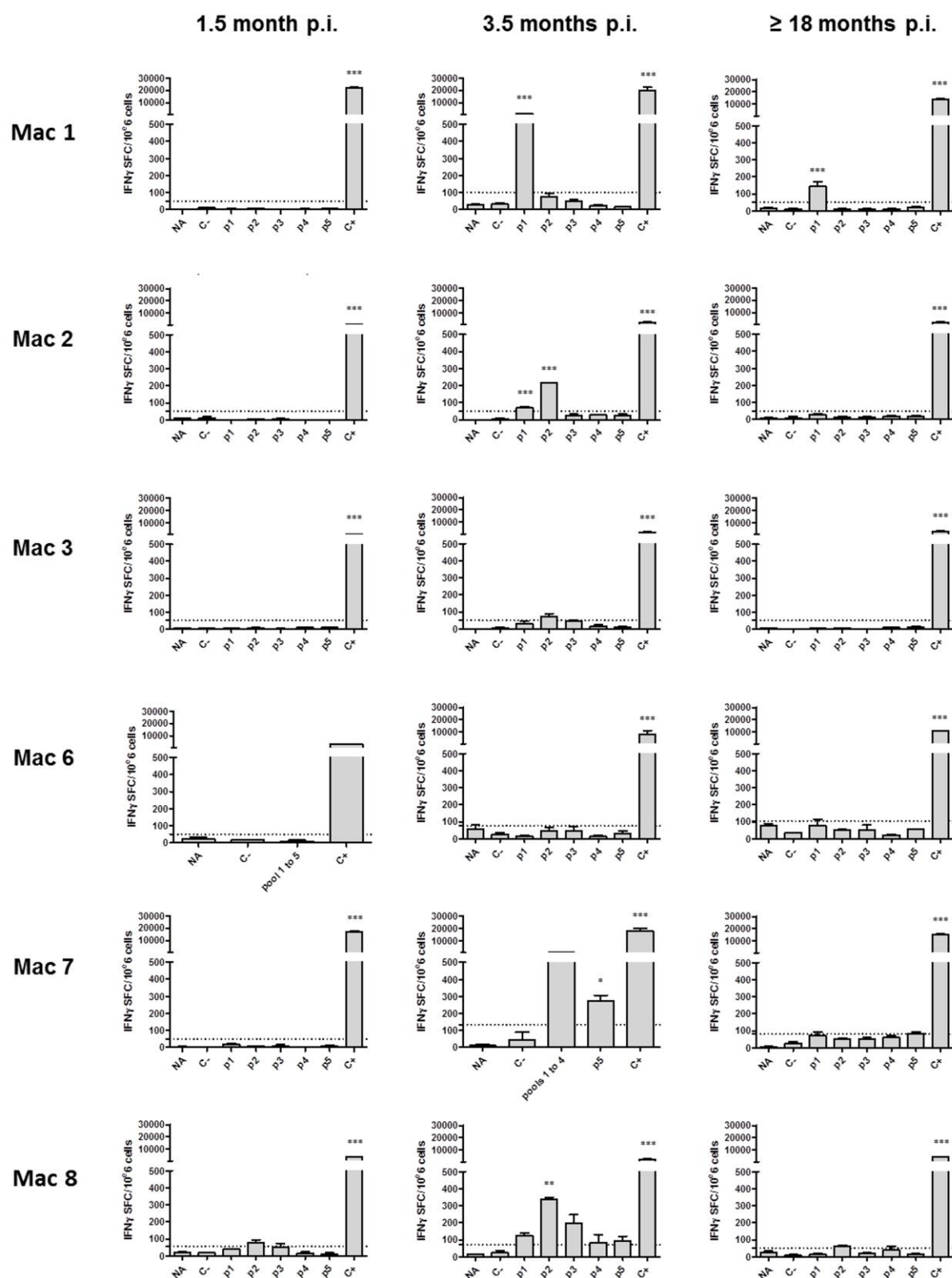


Fig. S3: ELISPOT IFN γ kinetic for Mac 1, 2 and 3 (control group) and Mac 6, 7 and 8 (iBMDC10-IV group). PBMC obtained at 1.5, 3.5 and ≥ 18 months post-injection were stimulated using an overlapping 15 per 10 amino acid peptide library covering the rtTA protein and divided in 5 pools (p1 to p5). When PBMC number was limiting, all or some pools were tested together (Mac 6 at

1.5 months pi and Mac 7 at 3.5 months pi). Negative controls consisted in PBMC cultured with either medium alone (non activated cells, NA) or an unrelated peptide pool (C-). Positive control (C+) consisted in PMA/ionomycin activation. IFN γ secretion was measured as spot forming cells (SFC) per 10⁶ PBMC. Threshold of positivity (dotted line) consists in SFC>50 spots and 3 times the number of SFC obtained with negative peptide pool control (C-). For positive responses (except those performed in duplicates when cell number was limiting), 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.

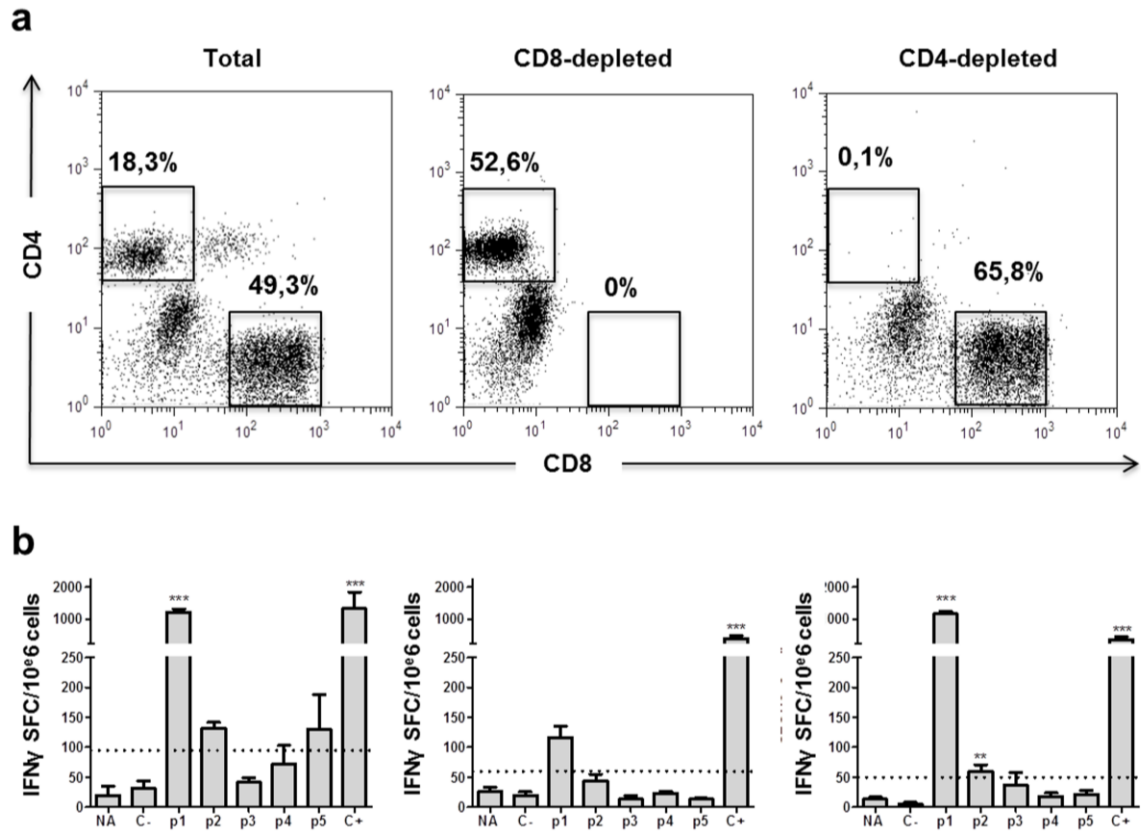


Fig. S4: Anti-rtTA IFN γ T cell response is predominantly mediated by CD8 T cells in Mac 4 Secretion of IFN γ by rtTA-stimulated PBMCs (Mac 4, iBMDC10-ID group) was evaluated by ELISpot. The analysis was realized using the same PBMC sample after either CD4⁺ or CD8⁺ magnetic cell depletions.

(a) The efficiency of CD4⁺ and CD8⁺ T cell depletions were assessed using flow cytometry after CD3⁺ CD8⁺ and CD4⁺ stainings. The percentages of CD4⁺ and CD8⁺ cells among CD3⁺ T cells are shown for total, CD8-depleted and CD4-depleted PBMC populations.

(b) An IFN γ ELISpot assay was realized for total, CD8-depleted and CD4-depleted PBMC populations. Cells were stimulated using an overlapping 15 per 10 amino acid peptide library covering the rtTA protein and divided in 5 pools (p1 to p5). Negative controls consisted in PBMC cultured either with medium alone (non activated cells, NA) or with an unrelated peptide pool (C-). Positive control (C+) consisted in PMA/ionomycin activation. IFN γ secretion was measured as spot forming cells (SFC) per 10⁶ PBMC. Threshold of positivity (dotted line) consisted in SFC > 50 spots and 3 times the number of SFC obtained with negative peptide pool 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 $\leq 0,05$. Total, CD8-depleted and CD4-depleted PBMCs from a non-injected mock macaque were assessed in parallel and responses for rtTA were all negative (data not shown).

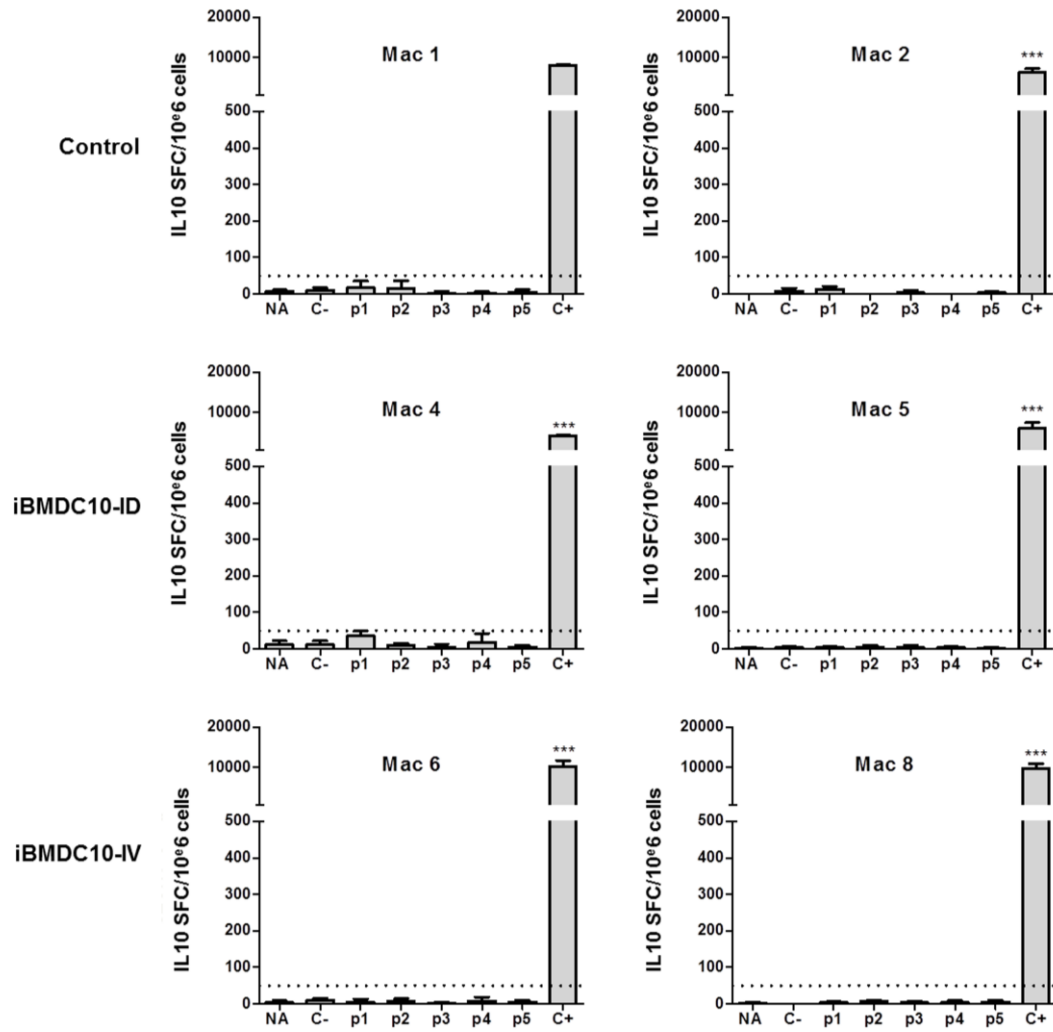


Fig. S5: Assessment of IL10 rtTA-specific T cell responses in control, iBMDC10-ID and iBMDC10-IV macaques using an ELISpot assay. Representative results of 2 macaques from each group are shown. Macaque PBMC obtained at ≥ 18 months post-gene transfer were stimulated using an overlapping 15 per 10 amino acid peptide library covering the rtTA protein and divided in 5 pools (p1 to p5). Negative controls consisted in PBMC cultured either with medium alone (non activated cells, NA) or with an unrelated peptide pool (C-). Positive control (C+) consisted in mitogenic PMA/ionomycin activation. IL10 secretion was measured as spot forming cells (SFC) per 10^6 PBMC. Threshold of positivity (dotted line) consists in $SFC > 50$ spots and 3 times the number of SFC obtained with negative peptide pool control (C-).

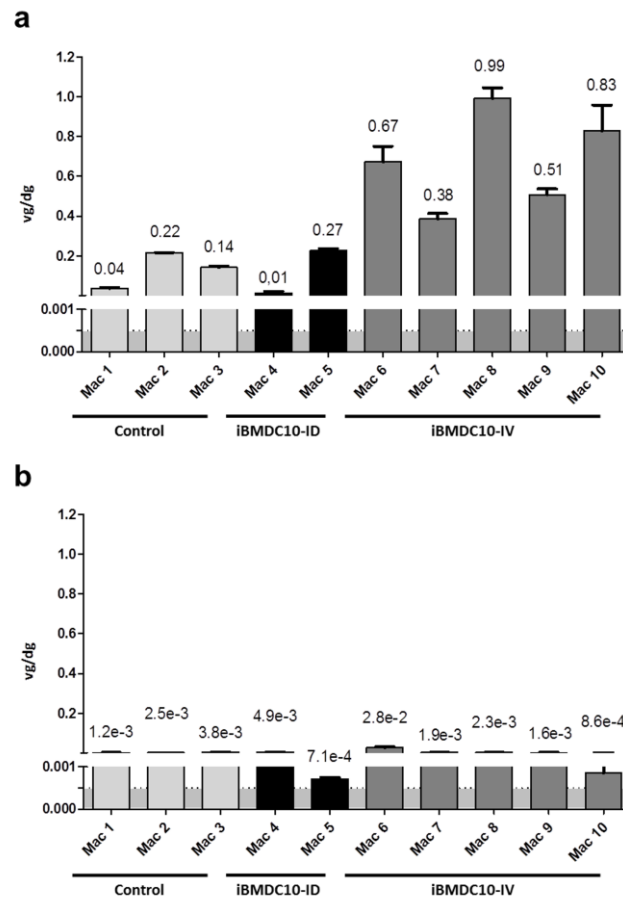


Fig. S6: Detection of viral genome copy numbers in the muscle. Viral genome copy numbers were determined by quantitative PCR in (a) the injected tibialis (one IM site) and (b) non-injected controlateral tibialis, for macaques from control, iBMDC10-ID and iBMDC10-IV groups. Results are expressed as viral genome (vg) per diploid genome (dg). The threshold of PCR sensitivity is represented in grey in the graph and determined at 5.10^{-4} . Control and iBMDC10-IV groups are statistically different: P value= 0.03 (*). Statistics were not performed for iBMDC10-ID group (only 2 individuals).

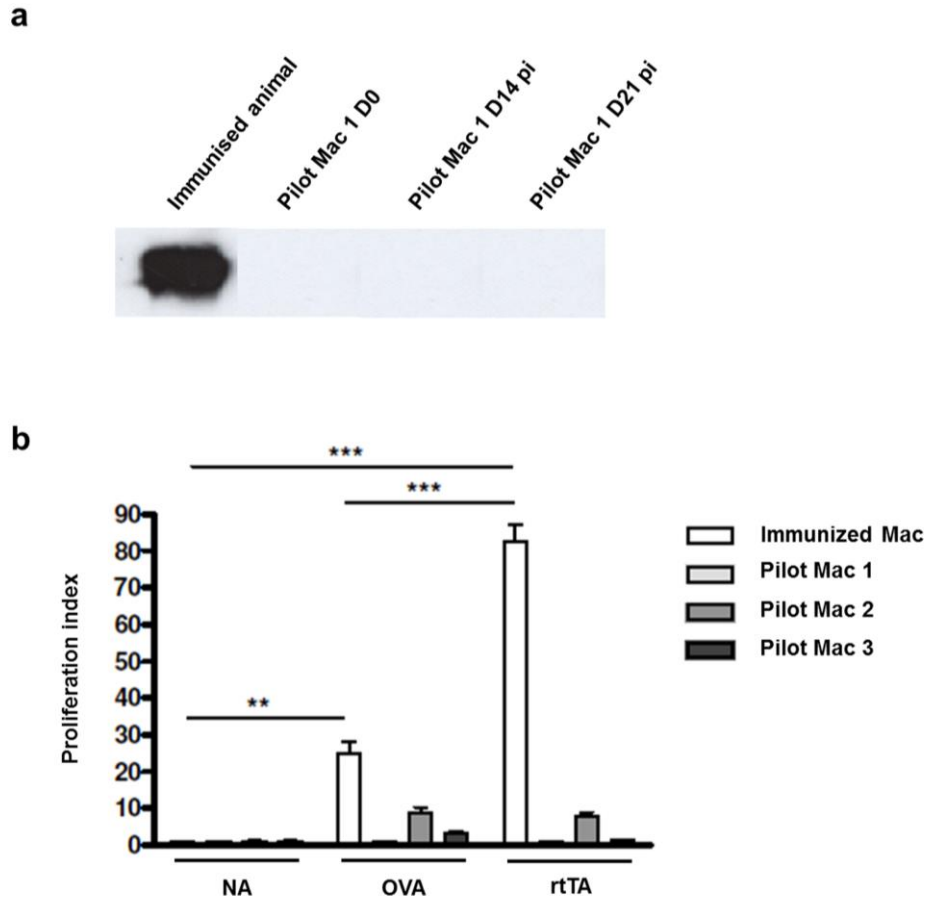


Fig. S7: Monitoring of anti-rtTA humoral and cellular responses in 3 pilot macaques (pilot Mac 1, pilot Mac 2 and pilot Mac 3) injected intradermally with autologous iBMDC10 pulsed with rtTA in the absence of gene transfer.

(a) IgG anti-rtTA antibody detection in Pilot Mac 1 sera obtained at days 0, 14 and 21 post-iBMDC10 intradermal administration using a western-blot-based assay. A macaque immunized against rtTA is shown as a positive control.

(b) T cell proliferation after rtTA protein stimulation in pilot macaques 1, 2 and 3 using PBMC obtained at day 30 post-iBMDC10 intradermal delivery. PBMC from a macaque immunized against rtTA were used as a positive control. PBMC were either non-activated (NA) or stimulated with rtTA or Ovalbumin (OVA) recombinant proteins. T cell proliferation was measured by ^3H thymidine uptake and normalized with non-activated cells for each individual (proliferation index). *** for P value $\leq 0,001$, ** for P value $\leq 0,01$.

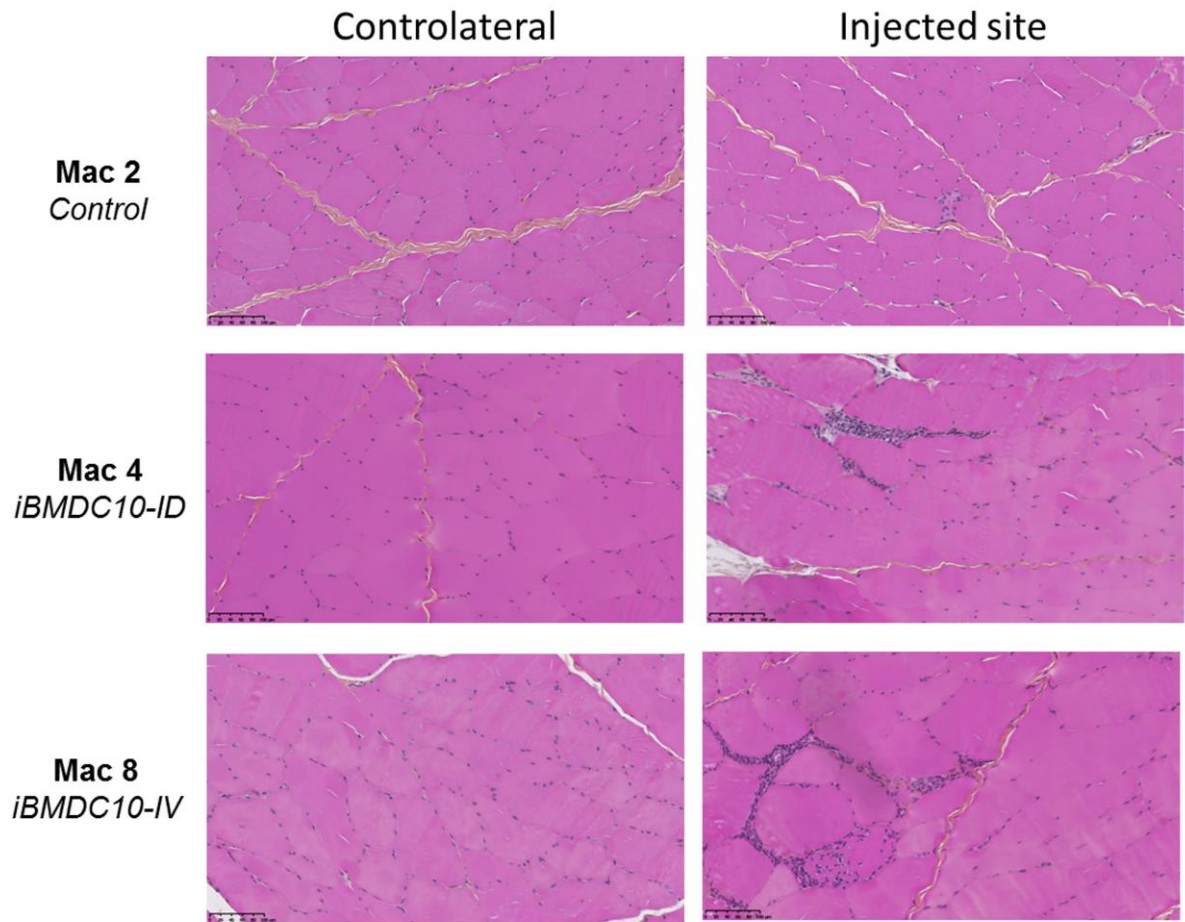


Fig. S8: Muscle infiltrates in injected muscles. Hematoxylin phloxin saffron (HPS) stainings were performed for injected and non-injected tibialis muscles obtained at ≥ 18 months post-gene transfer. For the injected muscle, pictures are representative of two IM sites of vector injection except for control animals where only one IM injection site, obtained by surgical biopsy, was analyzed. One representative macaque is shown from control (Mac 2), iBMDC10-ID (Mac 4) and iBMDC10-IV (Mac 8) groups. Scale bar: 100 μ m.

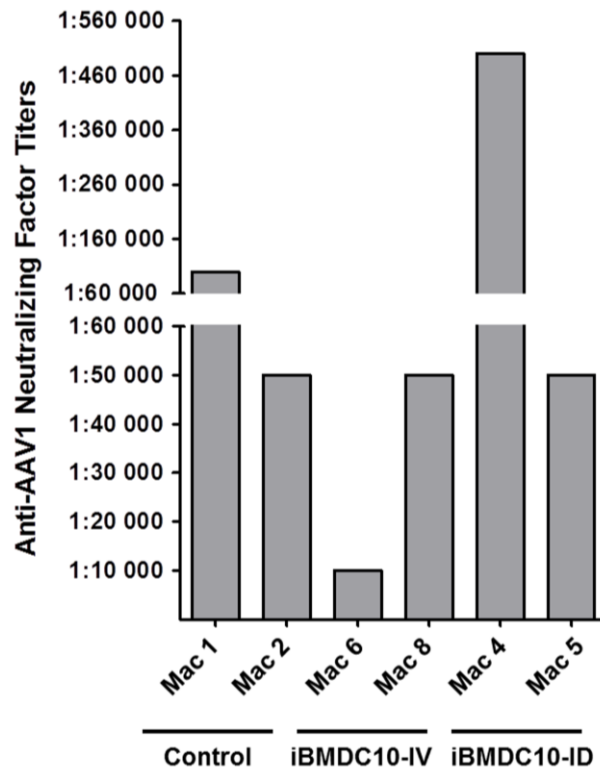


Fig. S9: Titers of anti-AAV1 neutralizing factors in NHP sera at 3 months post-injection. Two representative macaques are shown for control group (Mac 1 and Mac 2), iBMDC10-IV (Mac 6 and Mac 8) and iBMDC10-ID (Mac 4 and Mac 5). Titers of neutralizing factors were defined as the last dilution allowing the same cell transduction with rAAV1- CMV-LacZ vector as the control condition without serum.

Table S1: SFC/10⁶ values of anti-rtTA IFN γ ELISpot assay.

Group	Macaque	Non activated	Control Peptide pool	rtTA Pool 1	rtTA Pool 2	rtTA Pool 3	rtTA Pool 4	rtTA Pool 5	PMA/iono
Control	Mac 1	67.50	97.50	393.33	90.0	60.0	52.5	25.0	13750.0
	Mac 2	17.5	28.33	40.0	61.67	61.67	28.33	80.00	17000.0
	Mac 3	23.33	26.67	56.67	35.0	66.67	25.0	40.0	17366.67
iBMDC10-ID	Mac 4	40.0	41.67	1051.67	158.33	96.67	60.0	73.33	11300.0
	Mac 5	8.33	18.33	163.33	88.33	1.67	10.00	13.33	30050.0
iBMDC10-IV	Mac 6	37.5	28.33	30.0	36.67	58.33	22.5	32.5	17316.67
	Mac 7	12.50	12.50	37.50	30.0	27.50	20.0	52.20	14033.33
	Mac 8	5.0	13.33	30.0	75.0	63.33	38.33	30.0	14375.00
	Mac 9	90	53.33	73.33	86.67	46.67	73.33	200	4400
	Mac 10	0	3.33	10	6.67	5	3.33	3.33	10016.67

The number of SFC normalized to 10⁶ PBMC in non activated (NA), negative control using an unrelated peptide pool (C-), positive control (PMA/ionomycin stimulation, C+) and rtTA-peptide stimulations (pools 1 to 5) are indicated for all the macaques tested in control, iBMDC10-ID and iBMDC10-IV groups. Positive responses are highlighted in grey.

SUPPLEMENTARY MATERIALS AND METHODS

Anti-rtTA immunostaining. The detection of rtTA protein in iBMDC10 (**Fig. S1**) was realized by immunohistochemistry using polyclonal anti rtTA antibody (TET01, MoBiTec, final concentration of 2.5 µg/mL) overnight at 4°C, donkey anti-rabbit biotinylated secondary antibody (Jackson Immuno Research) for 1 hour at room temperature and FITC-conjugated streptavidin (Beckman Coulter) during 1 hour at room temperature. Negative control consisted in rabbit serum (2.5 µg/mL). Cell nucleus were stained with DAPI and slides mounted with Prolong GOLD medium (Invitrogen, Life Technologies). Cells were observed with a confocal microscope (Nikon, Champigny, France).

Follow-up of Epo serum levels. Epo levels (**Fig. S2**) were measured in serum samples by Enzyme-Linked Immunosorbent Assay (ELISA) according to manufacturer instructions (Quantikine IVD, R&D Systems).

Follow-up of TetR-specific immune responses in NHP. For Mac 4 (group iBMDC10-ID), an IFN γ ELISpot assay was performed with either CD4 or CD8-depleted PBMC fractions (**Fig. S4**) using Special StemSep Rhesus CD4+ or CD8+ Tetramer kits (Stem cell technologies, France). The efficacy of T cell subpopulation depletions was assessed by flow cytometry after CD3, CD4 and CD8 staining. The kinetic in **Fig. S3** was performed using total PBMC. IFN γ ELISpot assays were performed as described in the Materials and Methods section.

For all macaques, IL10 secretion was assessed with frozen PBMC isolated at >18 months post-injection (**Fig. S5**). Cells were stimulated with an overlapping peptide library covering the rtTA sequence (15 per 10 mers, Pepscreen, Sigma) split in 5 peptide pools and used at a final concentration of 10µg/mL for each peptide. Briefly, the method consisted in plating 2×10^5 cells on monkey anti-IL10 antibody (Ucytech, Netherlands) pre-coated MultiScreen®HTS filter plates with polyvinylidene difluoride membrane (PVDF, Millipore, France). Medium alone and an irrelevant pool of peptides served as negative controls whereas PBMC stimulated with Phorbol-12-myristate-13-acetate (PMA, 10ng/mL, Sigma) and Ionomycin, (250ng/mL, Sigma) served as a positive control. After incubation with a biotinylated anti-IL10 antibody (Ucytech, Netherlands), assay revelation was performed as described for the IFN γ ELISpot assay in the Materials and Methods section. Responses were considered positive if the number of spot-forming colonies (SFC) per million cells were >50 and at least 3-fold higher than the negative control.

For the group of pilot macaques (**Fig. S7, panel a**), anti-rtTA antibodies were detected by western-blot as previously described {Favre, 2002 #12; Toromanoff, 2009 #124}. Anti-rtTA cellular response (**Fig.**

S7, panel b) was monitored by ^3H thymidine uptake after PBMC restimulation with rtTA recombinant protein. Briefly, PBMC (10^5 cells) were cultured with medium only, or with different amounts of ovalbumin or rtTA protein (5 to 50 $\mu\text{g/mL}$) in triplicate using 96-well U-bottom plates. After 3 days, PBMC proliferation was measured by ^3H thymidine uptake and expressed as counts per minute (cpm). As a positive control of the experiment, one animal was immunized against the rtTA protein. After IM injection of an AAV1-rtTA-Epo vector, the immune response was challenged with an emulsion of Complete Freund Adjuvant and the purified rtTA protein injected by the intradermal route.

Detection of vector copy numbers by qPCR. Muscle genomic DNA was extracted using Gentra Puregene kit (Qiagen) according to manufacturer instructions, after tissue dissociation using Tissue Lyser II (Qiagen). Quantification of viral genome (**Fig. S6**) was obtained targeting the polyA\BGH sequence with the following primers: *forward primer* 5'-TCTAGTTGCCAGCCATCTGTTGT-3'; *reverse primer* 5'-TGGGAGTGGCACCTTCCA-3' and polyA\BGH probe 5' (6 FAM)-TCCCCCGTGCCTTCCTTGACC-3' TAMRA. ϵ -globin sequence was quantified as endogen control with the following primers: *forward primer* 5'-ACATAGCTTGCTTCAGAACGGT-3'; *reverse primer* 5'-AGTGTCTTCATCCTGCCCTAAA-3' and ϵ -globin probe 5' (6 FAM)-TGCAGGCTGCCTGGCAGAAGC-3' TAMRA. Quantification was conducted using AB StepOne Plus (Applied Biosystems) machine with Taqman chemistry for polyA\BGH and ϵ -globin targeting. The polyA\BGH PCR was done using the following program: initial denaturation 20s at 95°C followed by 45 cycles of 1sec at 95°C and 20sec at 60°C. The ϵ -globin PCR was done using the following program: initial denaturation 20s at 95°C followed by 45 cycles of 3sec at 90°C and 30sec at 60°C. For each sample, Ct values were compared with those obtained with plasmid standard dilutions (containing either the polyA\BGH sequence or the ϵ -globin sequence).

Muscle infiltrates. Hematoxyllin phloxin saffron staining (HPS) was performed as per standard histological protocols using formol-fixed and paraffin-embedded muscle sections obtained either at animal necropsy (iBMDC10-ID and iBMDC10-IV groups) or via a surgical biopsy (control group). Pictures of the sections (**Fig. S8**) were acquired using NanoZoomer (HAMAMATSU, Japan). Acquisitions were analyzed by three independent investigators in a blind manner.

Neutralization Assay. Circulating neutralizing factors against rAAV vector were detected in animal sera (**Fig. S9**) obtained 3 months post-injection and decomplexed 30 minutes at 56°C, using a transduction inhibition assay. HeLa cells were infected with wt Adenovirus serotype 5 (multiplicity of infection MOI= 8) during two hours at 37°C. Nine sera dilutions were prepared in DMEM – 2% FCS

(1:10 to $1:5 \times 10^6$), and incubated 30 minutes at room temperature with rAAV1-CMV-LacZ vector. HeLa cells media was removed and *sera* containing rAAV vector was added (at MOI corresponding to 4,000 vg/cell). After one day of incubation at 37°C, cells were fixed and β -galactosidase expression was revealed by incubating the cells overnight at 37°C with an X-gal substrate (Promega, France). Control conditions consisted in cells left uninfected, cells transduced with rAAV without serum (negative neutralization control) and cells transduced with rAAV incubated with previously validated positive macaque *sera* (positive neutralization control). Titer of neutralizing factors was determined as the last dilution allowing Hela cell transduction as the negative neutralization control condition (without serum).