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ORIGINAL ARTICLE

Helper-dependent adenovirus achieve more efficient and persistent liver transgene expression in non-human primates under immunosuppression

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Helper-dependent adenoviral (HDA) vectors constitute excellent gene therapy tools for metabolic liver diseases. We have previously shown that an HDA vector encoding human porphobilinogen deaminase (PBGD) corrects acute intermittent porphyria mice. Now, six non-human primates were injected in the left hepatic lobe with the PBGD-encoding HDA vector to study levels and persistence of transgene expression. Intrahepatic administration of 5×10^{12} viral particles kg^{-1} (10^{10} infective units kg^{-1}) of HDA only resulted in transient (≈14 weeks) transgene expression in one out of three individuals. In contrast, a more prolonged 90-day immunosuppressive regimen (tacrolimus, mycophenolate, rituximab and steroids) extended meaningful transgene expression for over 76 weeks in two out of two cases. Transgene expression under immunosuppression (IS) reached maximum levels 6 weeks after HDA administration and gradually declined reaching a stable plateau within the therapeutic range for acute porphyria. The non-injected liver lobes also expressed the transgene because of vector circulation. IS controlled anticapsid T-cell responses and decreased the induction of neutralizing antibodies. Re-administration of HDA-*hPBGD* at week +78 achieved therapeutically meaningful transgene expression only in those animals receiving IS again at the time of this second vector exposure. Overall, immunity against adenoviral capsids poses serious hurdles for long-term HDA-mediated liver transduction, which can be partially circumvented by pharmacological IS.

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INTRODUCTION

Immunity against viral vectors is of utmost importance in the development of gene therapies for monogenic diseases, such as acute intermittent porphyria (AIP, OMIN 176000). This is a rare metabolic disorder resulting from a haploinsufficiency of porphobilinogen deaminase (PBGD, EC 4.3.1.8), which encodes for the third enzyme involved in the production of heme. AIP is characterized by acute neurological manifestations associated with high serum accumulation of heme precursors produced by the liver.[1–4] Previous studies in a murine model of the disease indicate that high PBGD expression in a small number of corrected hepatocytes protects against induced attacks of AIP and improves neuromotor function.[5–8]

Persistence of the therapeutic levels of the transgene throughout the life of the patient is the goal of gene therapy for most metabolic diseases, including AIP.[9,10] Helper-dependent adenoviral (HDA) vectors are devoid of all coding sequences for viral antigens, and hence, they are less immunogenic than first-generation adenoviruses.[11,12] Moreover, it has been demonstrated that they can provide long-term expression without chronic toxicity in large animal models.[9,11,13–18] Nevertheless, the host innate immune response and preexisting immunity against adenoviral capsid proteins are serious obstacles for efficient transduction and persistent expression of the transgenes. Thereby immunity precludes more favorable results in liver-targeted gene therapy.[19–23]

Given the fact that liver transplantation requiring lifelong immunosuppression (IS) is the only curative alternative for patients who develop repeated and severe porphyria attacks,[24–27] the use of intensive pharmacological IS can be considered to support translational approaches based on gene therapy. Furthermore, previous reports suggest that the use of IS at the same time of adenoviral vector administration allowed for successful re-administration of the vector.[28–32]

In the present study, we evaluated the therapeutic potential of a HDA vector encoding the human PBGD protein using ultrasound-guided percutaneous intrahepatic administration in non-human primates (NHPs) to maximize bioavailability. Direct intrahepatic injections in rodents not only reduce dose requirements for gene transfer[6,33,34] but also limit the dissemination of HDA into multiple organs, including the spleen, which is likely to contribute to an increased inflammatory/immune response.[33] In previous studies performed in NHPs, direct injection of an HDA vector into the surgically isolated liver[35] or via the hepatic artery[18] provided high levels and persistence of transgene expression.[15]

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The first aim of our study was to evaluate hepatic transduction efficiency by HDA and to explore whether clinically available immunosuppressive drugs would improve persistence of transgene expression within the relevant therapeutic range for AIP. We also evaluated whether the IS regimen permits re-administration of the very same HDA vector.

RESULTS

Experimental design to evaluate IS as a strategy to improve HDA transduction and transgene persistence in the liver of NHPs

The performance of the HDA-*hPBGD* vector to achieve long-term transgene expression in primates was explored in six juvenile NHPs (designated M#01–M#06), which received 1×10^{10} infective units (iu) kg^{-1} (5×10^{12} viral particles (vp) kg^{-1}) of the vector in the left liver lobe by ultrasound-guided intrahepatic injection. In an attempt to control the immune response against the adenoviral capsid, one animal (M#03) received a 1-month course of IS (Figure 1a) and two NHPs (M#05 and M#06) received a 3-month course of IS (Figure 1b) circa the date of vector injection in both settings. Immunosuppressive agents included methylprednisolone, tacrolimus, methyl-mycophenolate and rituximab. Although rituximab partially depletes B cells, the other agents are known to decrease T-cell activation and inflammation.

The persistence of PBGD overexpression was closely monitored by serial liver biopsies. In the control group, ultrasound-guided percutaneous intrahepatic administration of HDA-*hPBGD* attained weak and transient liver transduction only in one out of three non-immunosuppressed animals (Figure 2a). PBGD activity in the non-immunosuppressed positive case (M#02) showed a 69% gain with respect to its endogenous pretreatment enzymatic activity. No evidence for any PBGD activity (Figure 2a) or HDA vector content (Figure 2b) was detected in the other two control NHPs despite the fact that they were injected with the same vector batch and dose.

In the M#03 macaque receiving the 1 month course of the IS regimen, a weak PBGD overexpression was observed (57% gain) compared with its pretreatment liver enzymatic activity 10 days after vector administration. This PBGD overexpression declined over time to disappear within 14 weeks after the HDA injection both in the control M#02 and the M#03 1-month immunosuppressed macaque (Figure 2a). Concomitantly, vector DNA content also declined in these two animals (Figure 2b).

In striking contrast, the two animals receiving the 3-month IS regimen showed robust liver transduction as established by both elevated hepatic PBGD enzymatic activity (Figure 2a) and vector DNA content (Figure 2b). The highest hepatic PBGD expression was achieved 6 weeks after the HDA injection. Gain in PBGD activity in M#05 and M#06 NHPs treated with 3-month IS represented 628% and 1681% of the endogenous baseline activity measured in each animal. Serial liver biopsies revealed a progressive decrease for up to 33 weeks (Figure 2) and thereafter a plateau was reached lasting at least for the following 36 weeks of observation. In Supplementary Figure S1, representative immunoblot and immunoshistochemical staining for PBGD are shown in liver samples from M#04 and the immunosuppressed M#06 NHPs taken 14 weeks after HDA administration.

At the plateau (+72 weeks), the gain of PBGD activity in the liver over pretreatment levels of these two NHPs remained 20% (M#05) and 60% (M#06) over endogenous pretreatment PBGD expression. These data are statistically significant if compared with liver samples from eight necropsied untreated macaques (subjected to

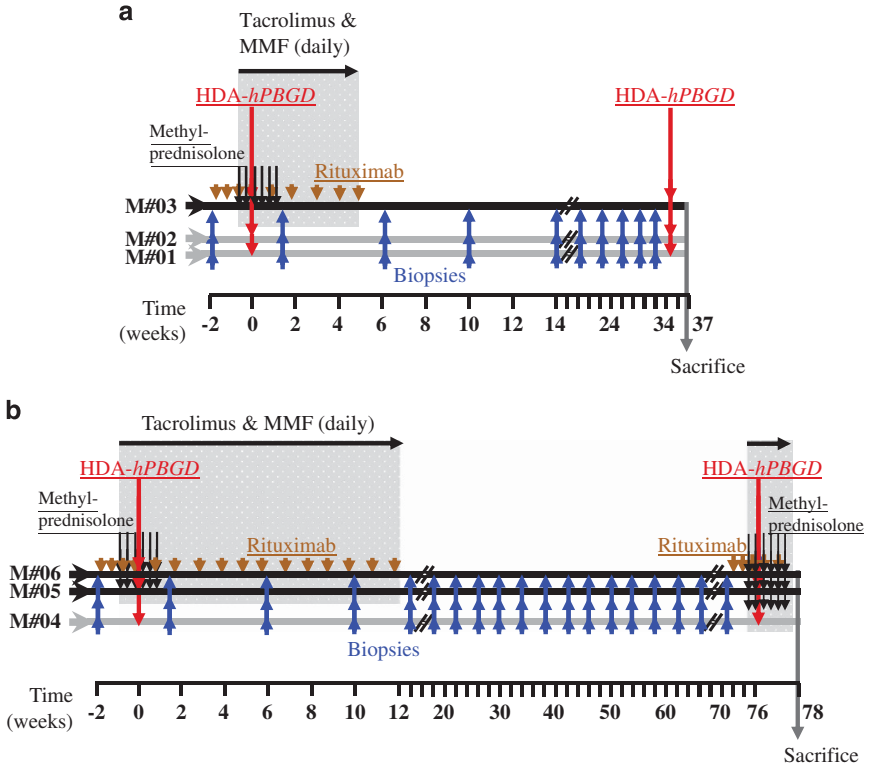

Figure 1. Schematic timeline representation of two administrations of HDA-*hPBGD* vector to NHPs under combined pharmacological IS. Animals are identified by the digit code (M#) and the timeline is represented by the horizontal axes. Shaded areas represent the period of immunosuppressive treatment that lasted for 1 month in panel (**a**) or 3 months in panel (**b**). Arrows indicate the different treatments (rituximab in brown and methylprednisolone in black) given and the dates of ultrasound-guided liver biopsies are represented by blue arrow heads. Ultrasound-guided percutaneous intrahepatic administrations of HDA-*hPBGD* are indicated by red arrows. For each administration, 5×10^{12} viral particles kg^{-1} in a total volume of 1.5 ml were distributed in up to 10 different sites of the target liver lobe. All animals were killed at the end of the experiment and autopsies were performed 11 days after the second HDA-*hPBGD* injection.

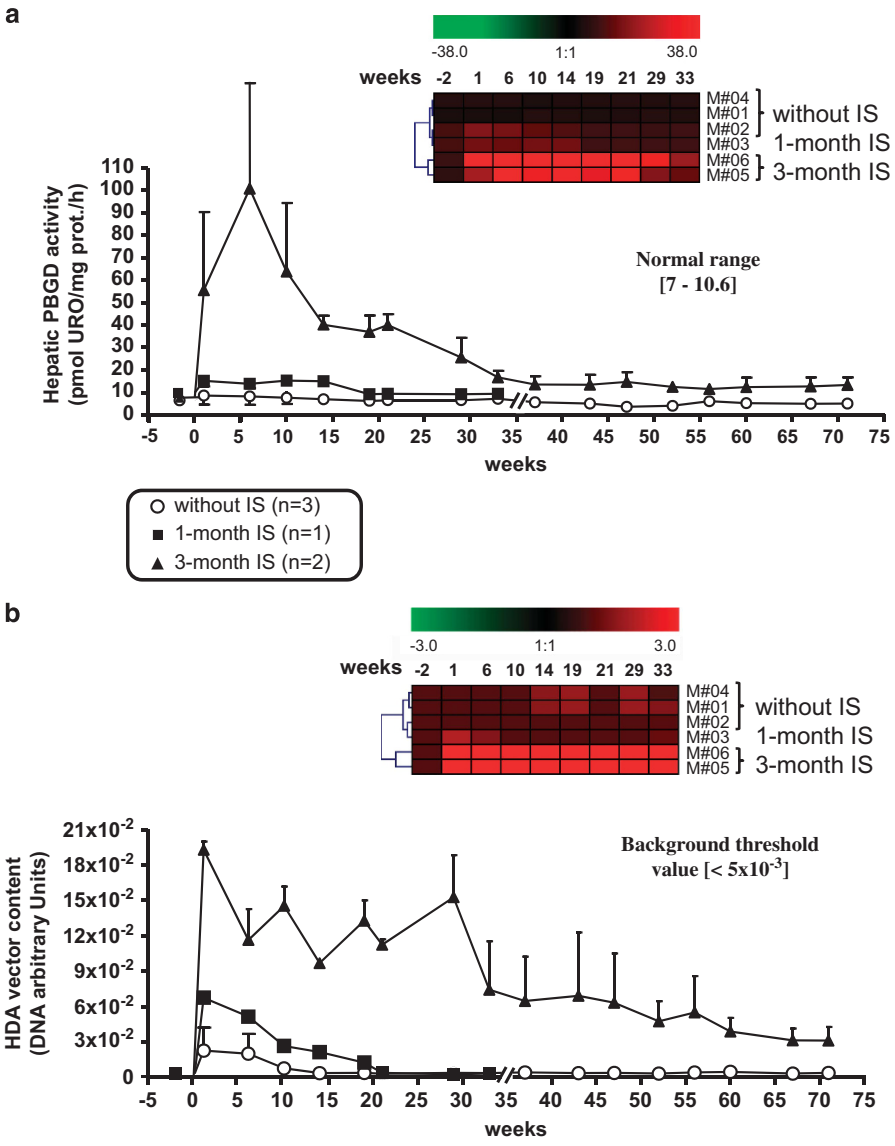

Figure 2. A 3-month IS regimen enhanced HDA-mediated liver transduction in NHPs. Follow-up of PBGD enzymatic activity (**a**) and HDA vector DNA content (**b**) in serial liver biopsies of the injected liver region taken at the indicated time points. The normal range of PBGD activity (defined as mean ± 2 × s.ds.) was estimated in liver samples taken at necropsy in eight non-injected age-matched NHPs. Samples were run in duplicate or triplicate. The proviral DNA content was measured by real-time QPCR in these liver biopsies. The amount of human PBGD transcript was expressed according to the formula $2^{Ct(GAPDH)-Ct(gene)}$, where Ct is the cycle at which the fluorescence rises appreciably above background fluorescence. Background threshold value was estimated in liver samples from eight non-injected NHPs. An unsupervised cluster analysis was performed in an attempt to group animals with similar liver transduction. In both analyses (PBGD activity and vector DNA content), two clusters are observed: one cluster grouping the two animal receiving 3-month IS and the other groups without IS and with 1-month IS (as described in Materials and methods section). This type of statistical analysis was performed to optimize the information from the necessarily limited numbers of animal in the experiments. Closed symbols represent NHPs receiving the IS regimen and open symbol animals without IS.

experimental surgical techniques for auditive impairment) of the same age (8.8 ± 0.9 pmol uroporphyrinogen III per mg protein per h) and with the pretreatment baseline values of the six macaques used in this study (7.3 ± 2.4 pmol uroporphyrinogen III per mg protein per h). Importantly, overexpression of PBGD was observed both in the injected (Figure 2) and non-injected liver regions (Supplementary Figure S2), albeit at lower levels than in the directly injected lobe thus indicating systemic circulation of viable vector.

Immune responses to HDA capsids are downmodulated by pharmacological IS

Following HDA-*hPBGD* administration to control non-immunosuppressed NHPs, interleukin-6 (IL-6) concentrations rose 8 h after injection (Figure 3a). This increase was almost abolished in animals undergoing the IS regimen, indicating an attenuation of the innate inflammatory response to the viral vector. Upon intrahepatic HDA vector release, there were slight and transient increases in serum transaminases, alanine transaminase and aspartate transaminase, which were similar in both immunosuppressed and non-immunosuppressed NHPs (Figures 3b and c). All these parameters rapidly returned to pretreatment normal values. Transaminases levels were measured every 15 days between day +30 and +90 and monthly later. None of the measurements revealed any significant elevation in serum transaminases (data not shown). However, raises in transaminases in the intervals between blood extractions cannot be completely ruled out.

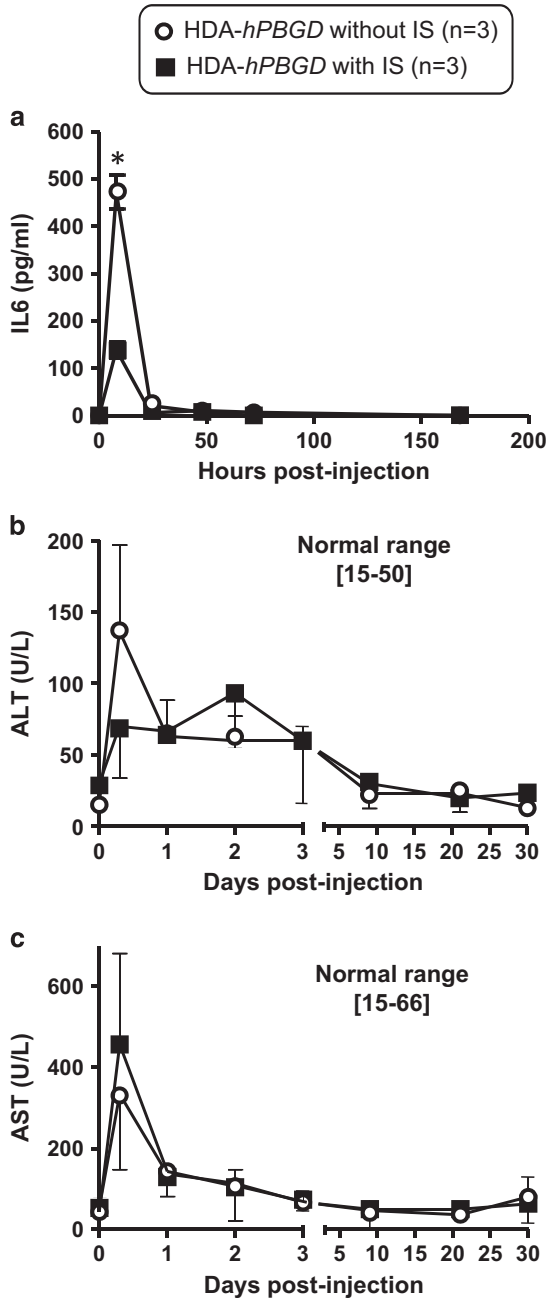

Figure 3. Transient acute response and liver function tests monitored by sequential measurements of serum IL-6 and aspartate and alanine aminotransferase before and after the ultrasound-guided percutaneous intrahepatic injections of the HDA vector. (**a**) Sequential ELISA follow-up of serum IL-6 concentrations, which is considered a key biomarker of acute systemic inflammatory response. There were no major differences between the peak values of the three immunosuppressed animals (M#03: 120 pg ml^{-1}; M#05: 173 pg ml^{-1} and M#06: 125 pg ml^{-1}). All three IS animals showed that at 8 h post-HDA administration grouped value increases, which were clearly lower as compared with the control non-immunosuppressed group (535, 411 and 473 pg ml^{-1}). Liver damage was monitored by sequential follow-up of alanine aminotransferase (**b**) and aspartate aminotransferase (**c**) in the sera from NHPs after the first HDA-*hPBGD* administration. AST, plasma aspartate aminotransferase; ALT, alanine aminotransferase. Normal ranges (defined as mean $\pm 2\times$s.ds.) were estimated in eight non-injected NHPs of the same range of age. Data are showed as mean $\pm$ s.d. The non-parametric Mann–Whitney *U*-test was used for comparison of two groups (two-tailed *P*-values; $^*P < 0.05$).

None of the NHPs exhibited preexisting immunity against the adenoviral capsid. As expected, HDA injection rapidly induced both specific antibodies and T-cell responses in control non-immunosuppressed animals (Figures 4a and b). In the animal that received the 1-month IS course, the immune response became apparent with rising neutralizing antibodies titers immediately following withdrawal of the immunosuppressants (Figure 4a) and T-cell proliferation levels as high as those observed in the control non-immunosuppressed NHPs (Figure 4b). The extension of the IS protocol for a period of 3 months reduced neutralizing antibodies and T-cell reactivity against HDA capsids in the two animals that received this longer IS regimen (Figures 4a and b). Following withdrawal of the 3-month immunosuppressive regimen, the immune response did not rebound. Reactivity against capsid antigens in peripheral blood mononuclear cells remained undetectable by a sensitive T-cell proliferation-based assay following a transient elevation after exposure to the vector (Figure 4b).

Immunoglobulin M (IgM) has been reported to hamper liver gene transfer owing to its innate ability to bind to adenoviral hexon proteins. This has been shown in first-generation[36–38] and HDA adenoviral vectors.[39] B-cell depleting rituximab was a component in our immunosuppressive regimen, and hence we reasoned that this agent could reduce the baseline concentration of IgM in serum, potentially facilitating HDA gene transfer. To explore this point, plasma concentrations of IgM were monitored in the three NHPs over the 10 days previous to the HDA-*hPBGD* administration. In the two NHPs that received three rituximab doses, serum IgM levels measured by enzyme-linked immunosorbent assay (ELISA) clearly fell (Figure 4c, left). Importantly, rituximab also reduced the ability of serum IgM to bind to HDA capsids absorbed onto ELISA plastic plates[36,39] to 40% and 30% of their baseline values (Figure 4c, right). It is of note that M#06, which attained the highest expression in the 3-month IS regimen, was the animal that showed a more marked decrease in serum IgM following rituximab.

Re-administration of HDA-hPBGD under IS achieves relevant transgene expression in NHPs

To determine whether higher neutralizing antibody titers (Figure 4a) correlated with the *in vivo* non-viability of subsequent repetition of gene transfer procedures with the same vector, we administered a second dose of HDA-*hPBGD* vector (1×10^{10} iu kg^{-1}) to M#01–M#03 NHPs. No increase in PBGD activity or vector DNA content was observed in the liver of these three animals (Figure 5). Even if the vector was delivered intrahepatically, our data strongly support that high neutralizing antibody titers preclude HDA liver transduction. However, when an IS regimen is applied at the time of the second administration (Figure 1b), it allows for repeated liver transduction at measurable levels as determined by PBGD activity (Figure 5a), vector DNA content (Figure 5b), immunohistochemistry (Figure 5c) and immunoblotting (Figure 5d). Samples were analyzed in the necropsy performed 11 days after vector re-administration. One of the immunosuppressed (M#06) NHPs had circa re-administration a very low titer of neutralizing antibodies, but the two others (M#04 and M#05) showed a $1/IC_{50}$ titer of about 3500 (Figure 6a). These data suggested that providing a secondary IS course was able to partially circumvent preexisting immunity against adenoviral capsids serotype 5 to achieve at least some gene transduction.

Cellular immune responses against HDA capsids (Figure 6b) were controlled upon re-administration in animals under the IS regimen, even in the M#04 animal, which had not received IS during the first exposure to the viral vector. It is worth noting that, 11 days after the re-administration, there was no evidence of necrosis or mononuclear cell infiltration in any of the animals, regardless of whether they had or had not received IS (data not shown).

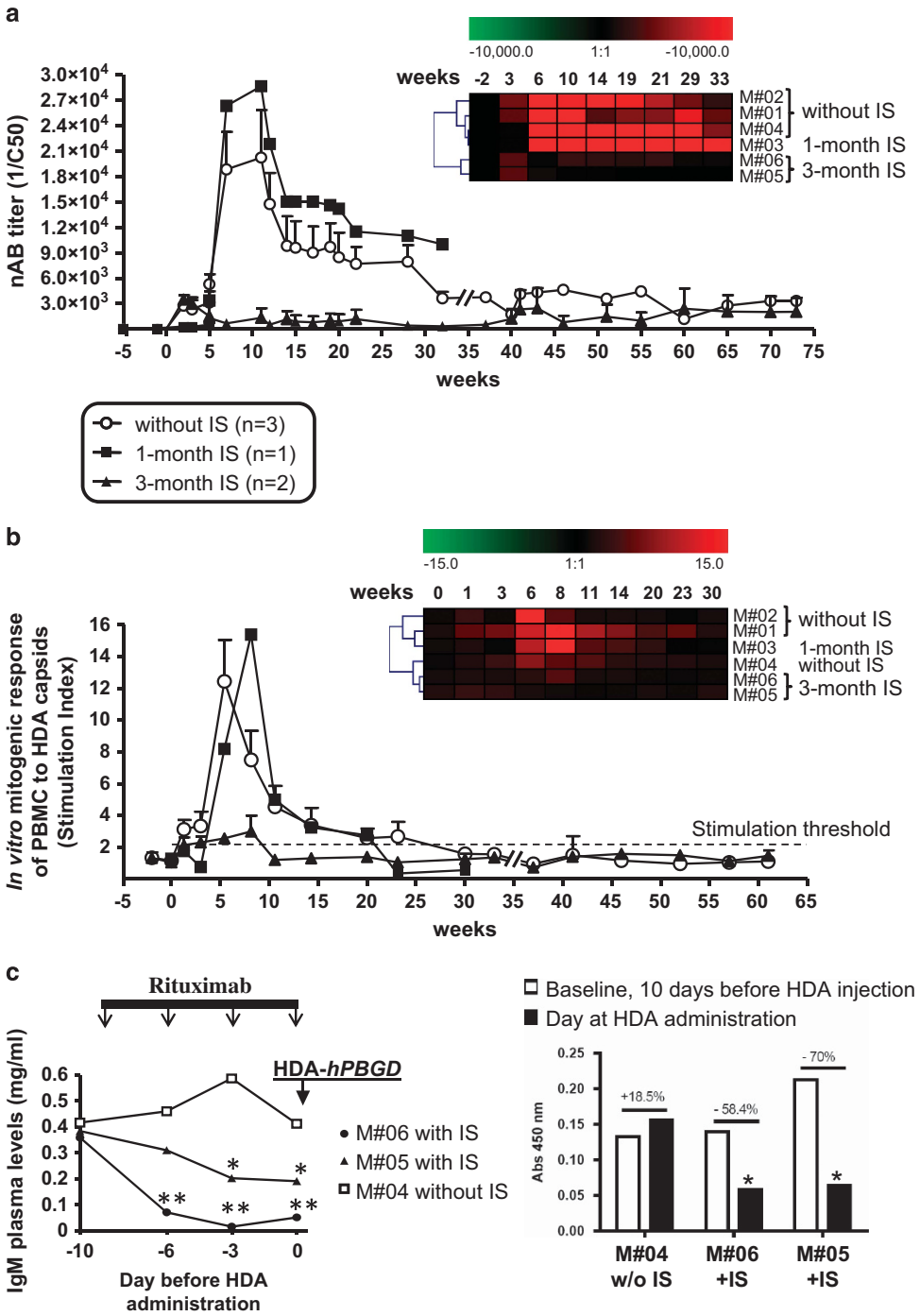

Figure 4. A 3-month IS regimen controls immunity against HDA capsid antigens, whereas a 1-month IS regimen only delays antivector immune responses. (**a**) Sequential follow-up of serum-neutralizing antibodies against HDA capsids in control NHPs and animals under 1- or 3-month IS regimens. (**b**) Peripheral blood mononuclear cell (PBMC) mitogenic responses to HDA capsids in peripheral blood mononuclear cultures at the indicated time points. Responses were measured by ^{3}H-Thymidine incorporation and referred to cultures without capsids (stimulation indexes) in the experiments testing the 1- and 3-month IS protocols. The unsupervised cluster analysis concluded that the NHPs receiving 3-month IS are grouped together with respect to the remaining macaques (without IS and with 1-month IS). Data are showed as mean ± s.d. (**c**, left) Follow-up of plasma IgM concentrations before the first HDA-*hPBGD* administration in the two NHPs receiving 3-month IS protocol (closed symbols) and one control animal without IS (open symbol). The background signal with irrelevant secondary antibodies was virtually non-existent. (**c**, right) ELISA experiments, as described in Materials and methods section, were performed to test the ability of serum IgM from the three NHPs to bind HDA capsids. Open bars represent values in animals pretreated without IS and closed bars represent values immediately before the treatment with HDA following or not the IS regimen as indicated. Experiments were repeated twice in replicates. Results are presented as mean of absorbance at 450 nm ± s.d. The non-parametric Wilcoxon matched pairs test was used for comparison of data at the indicated time points versus baseline values for each animal (two-tailed *P*-values; $*P < 0.05$; $**P < 0.01$).

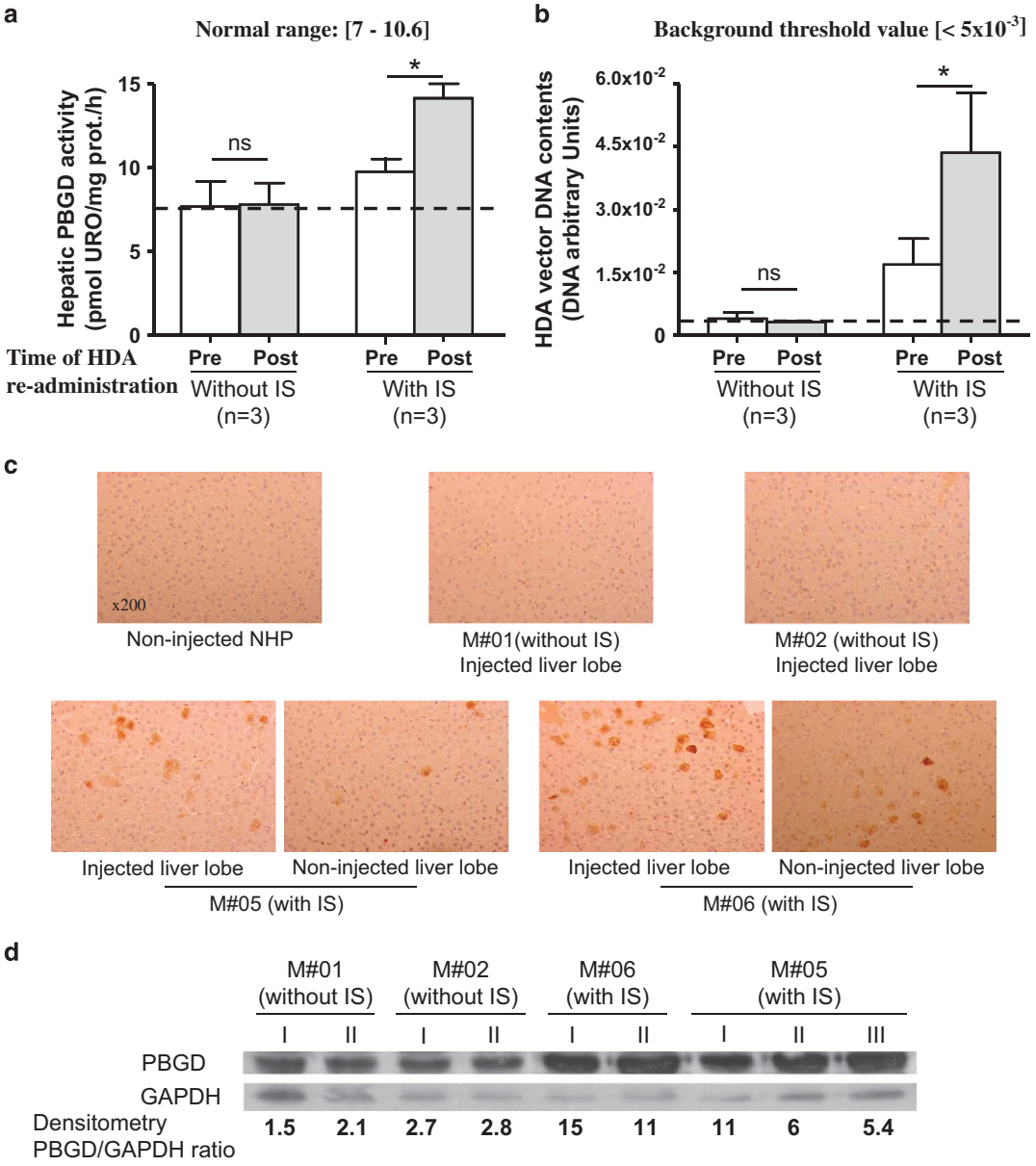

Figure 5. Successful repeated liver transfer with the same HDA-*hPBGD* vector was achieved in NHPs undergoing IS. Liver PBGD overexpression attained at killing 11 days upon re-administration of intrahepatic HDA-*hPBGD* at the same doses as in the first injection. (**a**) PBGD enzyme activity measurements. Normal range was estimated in liver samples taken at necropsy in eight non-injected age-matched NHPs. (**b**) The proviral DNA content that was analyzed by real-time QPCR specific for transgenic *hPBGD* excluding the endogenous allele. The amount of human PBGD transcript was expressed according to the formula $2^{Ct(GAPDH)-Ct(gene)}$, where Ct is the cycle at which the fluorescence rises appreciably above background fluorescence. Results were statistically compared with averaged biopsy material obtained in the last three biopsies before HDA re-administration. Background threshold value was estimated in liver samples from eight non-injected NHPs. Samples were run in duplicate or triplicate. The discontinuous line marks the averaged baseline levels of enzymatic activity and HDA vector DNA content. Data are showed as mean ± s.d. The non-parametric Mann–Whitney *U*-test was used for comparison of two groups (two-tailed *P*-values; NS, non-significant; $*P < 0.05$). (**c**) Immunohistochemistry and (**d**) western blotting assays performed on the necropsy liver samples upon immunostaining (**c**) or immunoblotting (**d**) with an anti-PBGD polyclonal antibody. Densitometry of western blots referred to internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loading controls was determined using the ImageJ software. I, II and III are independent samples from the injected liver lobe.

DISCUSSION

As a case study for liver gene transfer, AIP has several pros and cons. On the bright side, the transgenic protein is only required to repair a quantitative deficiency. Moreover, it is a self-protein from an immunological point of view, in such a way that immunity to the transgene is not a problem as the patient has been exposed for life to the PBGD protein.[40,41] Because of the lack of toxicity of the transgene, transcription does not need to be regulated and gene therapeutic approaches are considered safe.[41] However, overexpressing and maintaining high intracellular levels of PBGD protein in at least 10% of hepatocytes constitutes a formidable delivery problem from a technical point of view that has only been solved in mice so far.[5,6]

Effective gene transfer by adenoviral vectors is constrained by the innate immune system that limits the amount of transduced hepatocytes[42,43] and may cause severe side effects as a result of

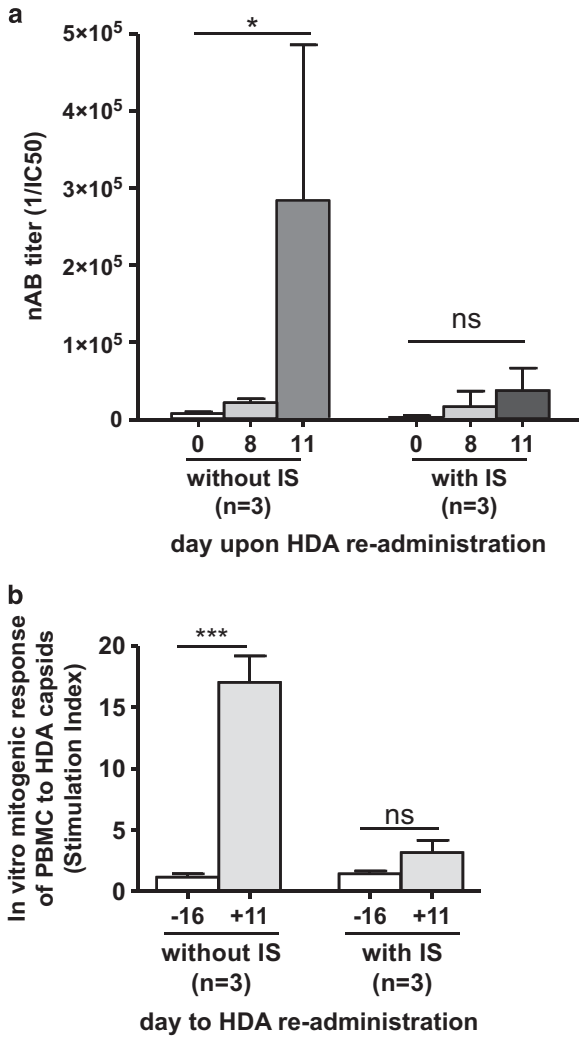

Figure 6. The IS protocol administered to preexposed animals ablates cellular immune responses to capsid antigens. (**a**) Sequential follow-up of neutralizing antibodies in serum against HDA capsids after re-administration. (**b**) Mitogenic responses of peripheral blood T cells to HDA capsids measured by ^{3}H-Thymidine incorporation. The results were obtained 11 days upon HDA-*hPBGD* re-administration and are statistically compared with the anti-capsid cellular response detected 1 week before the start of the secondary IS protocol. The non-parametric Mann–Whitney *U*-test was used for comparison of two groups (two-tailed *P*-values; NS, non-significant; $^{*}P < 0.05$; $^{***}P < 0.001$). PBMC, peripheral blood mononuclear cells.

systemic inflammation syndromes.[44] In our study, efficient liver transduction was only detected in one out of three control *Macaca fascicularis* injected with 5×10^{12} vp of HDA per kg. Other reports on experiments carried out in NHPs have described sustained expression of α1-antitrypsin[16] or coagulation factor IX,[18] following the administration of HDA vectors. A recent report described transgene α-fetoprotein expression for up to 7 years in baboons[15] injected with doses from 1×10^{11} to 1×10^{12} vp of HDA per kg and described a 80-fold improvement in transgene expression when using a balloon occlusion catheter–based delivery method for liver gene transfer through the hepatic artery.[45] The route of administration and a more infective HDA vector (lower vp-to-iu ratio) are likely to explain these efficient results even without IS. Genetic differences in the immune response depending on the age of the animals[46] and NHP species[47–50] are also to be taken into account to elucidate such differences in transduction.

As previously reported with a first-generation adenovirus,[32] the IS regimen, either 1 or 3 months long, was well tolerated by the animals (veterinary appraisal), and it allowed the cellular and humoral immune responses to be controlled. Moreover, the immunosuppressive regimen critically enhanced the transduction efficiency of HDA and reduced pro-inflammatory cytokines such as IL-6 upon administration. Many contributing factors could have resulted in more efficient transduction in our case, including modulation of Kupffer macrophages[38,51,52] by steroid therapy and a partial reduction in serum IgM concentrations. Previous reports have revealed that adenovirus capsids can interact with natural IgM antibodies, factor IX and complement without need for any immunization[36,37,53] and thereby decrease gene transfer.[39] Flow cytometry analyses of peripheral blood mononuclear cells at the time of vector administration confirmed that rituximab depleted circulating B cells in our NHPs (Figure 4c and Fontanellas *et al.*[32] and Unzu *et al.*[40]), and as a result, there was a partial reduction in serum IgM concentrations. However, the small number of animals under treatment precludes drawing definitive conclusions regarding decreases in IgM levels.

Nevertheless, the 30-day IS course was insufficient and upon IS withdrawal both cellular and humoral responses rebounded in the M#03 macaque, suggesting that the capsid antigens somehow persisted in the animal for >1 month, preserving their immunogenicity. In our hands, the cellular immune response to capsid proteins seems to be associated with a more rapid decline in transgene expression in the M#03 macaque and the control animal that exhibited a similar level of gene transduction. It has even been postulated that the proteins of incoming vector particles might be processed and presented at the surface of infected cells as immunogenic major histocompatibility complex–peptide complexes.[16] In line with this, we observed viral genomes in the sera of the macaques during the first month following vector delivery (Supplementary Figure S3), but we cannot conclude a cause–effect relationship.

Consequently, extending IS for 3 months paid off in terms of transgene persistence. T-lymphocyte responses were almost undetectable from week 12. Indeed, M#05 and #06 exhibited a more robust hepatic PBGD overexpression, which slowly declined over the following 7.5 months and then remained remarkably stable during the following 9 months of the study, stabilizing over the therapeutic threshold estimated in AIP mice. A possible explanation for the drop in transgene expression is the increased weight of these fast growing juvenile NHPs during the 80 weeks of the study (Supplementary Figure S4). Despite this fall, PBGD gains in these two NHPs remained around 20% and 61% over endogenous baseline expression at least until 18 months after the HDA administration. Such levels of expression would be protective with conversion of a severe AIP phenotype to a moderate or asymptomatic one. This statement would be true if data from the AIP mice results could be extrapolated to large animals.[6]

Previous studies also reported an initial high expression of the transgene after HDA administration, followed by a slow decay and stabilization of transgene levels over time.[15,16,18] The mechanisms of transgene expression decay remain undefined. We have to emphasize that there are no signs of cellular immune responses to human PBGD in the NHPs (Supplementary Figure S5). To a certain extent, the physiological turnover of liver cells[54] or the growth of the organ in these juvenile NHPs[16,42] or a combination of both factors might have diluted out gene-transduced cells.

Re-administration with the same HDA vector was attempted in the NHPs in which overexpression had been successfully attained. Our experiment concludes that intrahepatic delivery is possible again only if IS is given along with this second vector administration. Even though transduction was quantitatively poorer upon re-administration, IS treatment is an option to be considered for re-administration of an HDA vector. An interesting

finding is that while in the first intrahepatic delivery the non-injected liver lobes became transduced due to vector re-circulation, this dissemination was lost in the second administration (Supplementary Figure S6), most likely because of neutralizing antibodies. Given our results[41] with an AAV vector carrying the same transgene NHPs, sequential use of different viral vectors with the same transgene could be considered as an approach to prolong the efficacy. However, according to our data HDA vectors should be preferred and used first, because transgene persistence is more efficaciously sustained by the immunosuppressive regimens.

Overall, this macaque study has demonstrated the feasibility of controlling host immune responses against HDA vectors to allow increased and prolonged PBGD transgene expression. These results confirm the key role of the immune system in adenoviral liver transduction even when the vector has been administered directly into the liver. Doses and agents of the immunosuppressive regimens must be optimized in terms of risk versus benefit, considering that liver transplantation, involving lifelong IS, is currently the only curative option for AIP patients.

MATERIALS AND METHODS

Plasmids and production of HDA-*hPBGD*

The vector gene expression cassette encodes the human cDNA of the codon optimized *PBGD*.[5] The transgene is under the transcriptional control of the liver-specific human α-1-antitrypsin promoter with regulatory sequences from the human albumin enhancer (EalbAAT promoter) and includes the human PBGD polyadenylation sequence.[5] This cassette of 1.9 kb was inserted into the shuttle plasmid pLPBL1-Zeo (courtesy of Dr Brendan Lee, Baylor College of Medicine, Houston, TX, USA) and subcloned by AscI sites into pDelta28E4 (courtesy of Dr Philip Ng) to obtain the pHDA-*hPBGD* (with 33.1 kb length). HDA vector production and purification was performed as detailed elsewhere.[55]

Viral particle concentration of the pool from nine different batches was determined by spectrophotometry. Helper virus contamination was tested and levels were under 0.0001%. In addition, quantitative PCR (QPCR) for E4 and E1 adenoviral genes corresponding to helper wild-type adenovirus at 9, 40, 70, 100, 140 and 300 days after the HDA-*hPBGD* administration were always under the detection threshold in liver biopsies (data not shown). The iu/vp ratio in HDA-*hPBGD* preparations was determined as described.[6] Briefly, Huh7 cells infected with serial dilutions of the vector were harvested 24 h postinfection. Cells were washed five times with phosphate-buffered saline and lysed by freezing/thawing. DNA was extracted and quantification of iu was performed by real-time QPCR of vector DNA. Specific primers for the untranslated region 3′ region of the vector were used (pAPBGDfw 5′-GCTAGCCTTTGAATGTAACCA-3′, pAPBGDrv 5′-CCTTCAGAACTGGTTTATTAGTAGG-3′). The ratio of iu/vp was 1/500.

Macaque experiments and IS treatment

NHP experiments were approved by the ethics committee of the University of Navarra in accordance with Spanish regulations (study approval CEEA038-09). Ethical and regulatory constraints brought the numbers of animals available for this experimentation down to a minimum. Six juvenile male captive-bred NHPs (*M. fascicularis*, 2–3 years of age) showing anti-Ad capsid antibody titers of 1/100 units were included in the study. Animals were injected with 1×10^{10} iu kg^{-1} (5×10^{12} vp kg^{-1}) of HDA-*hPBGD* in the left lobe of the liver using a ultrasound-guided intrahepatic injection method. Percutaneous injections were performed in one side of the liver (on the left for the first administration and the right side for the second administration). Administration procedures were performed by a trained radiologist using 22-G needles under ultrasound guidance. For each HDA-*hPBGD* administration procedure, a total volume of 1.5 ml was distributed as homogeneously as possible in up to 10 different sites of the liver parenchyma as visualized by ultrasonography. The dose chosen in this study (5×10^{12} vp kg^{-1}) is considered under the threshold affected by innate immunity, although it is considered safe.[42,56]

As indicated in Figure 1, one of the animals received an immunosuppressive treatment for 1 month (Figure 1a) and two for 3 months (Figure 1b). The IS regimen was administered as described: (i) B-cell-depleting rituximab (20 mg kg^{-1} dose^{-1} intravenously, Mabthera, ROCHE, Switzerland) at days −9, −6 and −3 immediately before HDA injection and weekly after the vector administration, (ii) methylprednisolone (Solu-moderin, Pfizer SA, Spain) applied intramuscularly at a dose of 100 mg on day −2, 50 mg on day −1, 40 mg on days 0, +1 and +2, 20 mg on days +3 and +4 and 10 mg on day +5, (iii) mycophenolate mofetil (MMF) (CellCept, Roche Pharma AG, Demark) at a dose of 25–30 mg kg^{-1} day^{-1}, and (iv) 0.25 mg kg^{-1} day^{-1} of tacrolimus (or FK506) (Astellas Pharma, Madrid, Spain). MMF and tacrolimus were given orally from day −2 daily to the end of IS protocol. IS treatment was aimed at controlling the release of early cytokines with steroids upon adenovirus exposure, depleting B cells with rituximab and avoiding activation and proliferation of T cells with tacrolimus and MMF. As the immunosuppressed regimen included daily oral administration of MMF and tacrolimus, the animals which quickly accepted the food offered by caregivers were included in the immunosuppressed groups. Animals were identified by a permanent tattoo in the internal gastrocnemius muscle. To blind the study, only the principal investigator and caregivers who gave the drugs could identify the code for the immunosuppressed animals but not the technicians who performed the laboratory analysis.

Transgene expression

Transgene expression was detected by PBGD activity in liver biopsies and by enzymatic activity, immunoblot and immunohistochemistry in eight different regions of the liver at necropsy.[40] Liver biopsies were obtained from left and right liver regions 10 days before HDA-*hPBGD* injection and then monthly until complete loss of transgene expression. Vector copy number and PBGD activity were measured from a mix of two samples of each region of the liver corresponding to each animal at each time point. For biopsies and blood extractions, animals were anesthetized by intramuscular injection of ketamine and midazolam 10 and 0.8 mg kg^{-1}, respectively. The analgesic administered after biopsies was one dose of 2 mg kg^{-1} ketoprofen delivered intramuscularly. Hepatotoxicity was assessed by measuring serum aspartate transaminase and alanine transaminase, which remained within the normal range for the entire observation period with the exception of a moderate rise after HDA injection. Before each biopsy, ultrasound examination of the liver revealed no abnormalities, and weight gain showed that the animal was in good clinical condition.

Proviral DNA quantification in liver samples and serum vector detection

Genomic DNA of harvested liver specimens or serum samples was isolated using the QIAmp Blood&Tissue DNA Mini-Kit (Qiagen, Madrid, Spain). Real-time QPCR for detection of the 3′ untranslated region poly A PBGD region of the vector were performed using specific primers detailed above (pAPBGDfw and pAPBGDrv). For normalization of the amount of genomic DNA, specific primers for simian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) sequence were used (sGAPDHfw 5′-GTCAGTGGTGGACCTG ACCT-3′; sGAPDHrv, 5′-TGCTGTAGCCAAATTCGTTG-3′). Real-time QPCR was performed in an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using iQ SYBR green supermix. PCR amplification conditions were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 25 s and 80 °C for 10 s; and a final extension for 10 min at 72 °C). A melting curve was generated by raising the incubation temperature from 65 °C to 95 °C to confirm amplification specificity. The amount of each transcript was expressed according to the formula $2^{Ct(GAPDH)-Ct(gene)}$, where Ct is the cycle at which the fluorescence rises appreciably above background fluorescence.

Measurement of humoral and cellular anti-HDA responses

Sera were obtained by tail vein puncture and stored at −80 °C. IL-6 serum levels were quantified by enzyme-linked immunosorbent assay ELISA (BD Biosciences, San Diego, CA, USA). Transaminase serum levels were determined in a Cobas Integra 400 Analyzer (Roche Diagnostics SL, Barcelona, Spain).

Neutralizing antibody assays against serotype 5 vector were performed with serial dilutions of serum that were mixed with 1×10^5 plaque-forming units of HDA luciferase. The mix was incubated at 37 °C for 1 h, and then the mixture was added to 1×10^4 cells per well of hepatic cell line PLC/PRF/5 (ATCC CRL-8024, Barcelona, Spain) in a 96-well plate. Cells were harvested 48 h later and assayed for luciferase activity (D-luciferin Luciferase Kit, Promega, Madison, WI, USA) using the Living Image 2.20 software package (Xenogen, Lincolnshire, UK). Sera were scored as positive

for neutralizing anti-HDA antibodies if the light intensity was $< 50\%$ of that observed when AdCMV-*luciferase* was preincubated with negative control sera. Data are reported using a curve adjusted to extrapolate the serum dilution for 50% inhibition of AdCMV-*luciferase* transduction (IC_{50}).

To evaluate the cellular immune response induced against the vector and the exogenous hPBGD, monkey leukocytes were purified from peripheral blood by centrifugation through Ficoll-Hypaque (GE Healthcare, Piscataway, NJ, USA) and counted in a Z2 Coulter Counter (Beckman Coulter, Barcelona, Spain). Mitogenic responses to HDA capsids or hPBGD were measured by [methyl-^{3}H] Thymidine incorporation of isolated leukocytes (5×10^5 cells ml^{-1}) cultured for 72 h in medium alone X-vivo medium (BioWhittaker, LONZA, Basel, Switzerland) supplemented with 2 mM glutamax (Invitrogen, Life technologies, Madrid, Spain) and 1% penicillin/streptomycin (Invitrogen, Life technologies) or containing serial dilutions of HDA vector from 10 to 0.01 μg protein per ml or of PBGD from 5 to 0.1 μg protein per ml. To assess the ability of cells to respond to mitogens, a mix of phorbol ester 12-myristate-13-acetate (0.01 μg ml^{-1}) and ionomycin (1 μg ml^{-1}) was used in each experiment (Sigma-Aldrich, St Louis, MO, USA) as a positive control. [^{3}H]-Thymidine uptake was assessed by filtration on an automatic cell harvester and by measuring nuclear radioactivity (filters) on a scintillation plate reader Packard (Topcount, Meriden, CT, USA). The stimulation index was defined as the mean counts per minute (c.p.m.) of the response of the antigen-stimulated cells divided by the mean c.p.m. of the cell cultures without antigen.

ELISA test for detection of IgM antibodies

The follow-up of plasma IgM concentrations before the first HDA-*hPBGD* administration was studied by ELISA in two NHPs receiving the IS protocol and one control animal without IS. Ninety-six-well plates were coated at 4 °C overnight with 3×10^9 vp of HDA-luciferase vector in bicarbonate buffer, pH 9.5. After being washed five times with 2% bovine serum albumin in phosphate-buffered saline, plates were blocked with 2% bovine serum albumin in phosphate-buffered saline for 3 h and a half at room temperature. Plates were washed 10 times in washing buffer (phosphate-buffered saline+0.05% Tween20) and incubated for 2 h and a half at 4 °C with 10 μg ml^{-1} of each monoclonal or polyclonal purified Igs (Ig added to reaction 1 μg) diluted in assay diluent (phosphate-buffered saline+2% bovine serum albumin+0.05% Tween20). In all, 10 μl of serum were diluted 1/10 in assay buffer. Plates were washed 10× in washing buffer and then incubated for 2 h at 4 °C with horseradish peroxidase-conjugated goat anti-mouse IgM (1:4000; Pierce, Rockford, IL, USA) or anti-mouse IgG (1:4000; Pierce) antibodies at 0.2 μg ml^{-1} diluted in assay diluent. Horseradish peroxidase was detected with tetramethylbenzidine. The same ELISA setting without HDA coating was used to detect and subtract the background signal. ELISA was also used to test the ability of serum IgM from the three NHPs to bind HDA capsids.

Statistics

An unsupervised classifier approach was used to identify the sample groups in the data set. Briefly, a hierarchical clustering analysis using Pearson correlation as the measure of distance between samples was performed for each measured parameter (PBGD activity, vector DNA content, neutralizing antibodies and *in vitro* mitogenic response to HDA capsids). All the data analysis was performed using the statistical environment R. R is a freely available language and environment for statistical computing and graphics that provides a wide variety of statistical and graphical techniques (http://cran.r-project.org/).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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