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Intensive Pharmacological Immunosuppression Allows for Repetitive Liver Gene Transfer With Recombinant Adenovirus in Nonhuman Primates

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Repeated administration of gene therapies is hampered by host immunity toward vectors and transgenes. Attempts to circumvent antivector immunity include pharmacological immunosuppression or alternating different vectors and vector serotypes with the same transgene. Our studies show that B-cell depletion with anti-CD20 monoclonal antibody and concomitant T-cell inhibition with clinically available drugs permits repeated liver gene transfer to a limited number of nonhuman primates with recombinant adenovirus. Adenoviral vector–mediated transfer of the herpes simplex virus type 1 thymidine kinase (*HSV1-tk*) reporter gene was visualized *in vivo* with a semiquantitative transgene-specific positron emission tomography (PET) technique, liver immunohistochemistry, and immunoblot for the reporter transgene in needle biopsies. Neutralizing antibody and T cell–mediated responses toward the viral capsids were sequentially monitored and found to be repressed by the drug combinations tested. Repeated liver transfer of the *HSV1-tk* reporter gene with the same recombinant adenoviral vector was achieved in macaques undergoing a clinically feasible immunosuppressive treatment that ablated humoral and cellular immune responses. This strategy allows measurable gene retransfer to the liver as late as 15 months following the first adenoviral exposure in a macaque, which has undergone a total of four treatments with the same adenoviral vector.

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INTRODUCTION

The immune system has been evolutionarily selected to fight viruses and is a serious hurdle for gene therapies based on viral vectors.[1,2] Immunity against the vector precludes readministration as reported with recombinant adenovirus[3] and adeno-associated virus (AAV)–based vectors.[4] Attempts to circumvent antivector immunity include pharmacological immunosuppression[5] or alternating different vectors with the same transgene.[6] In the case of adenoviruses, immunogenicity is very potent and has been exploited for vaccination.[7,8]

Liver tropism of adenoviruses is considered advantageous for gene therapy interventions in this organ. This is the case of helper-dependent ("gutless") adenoviruses used to correct inherited disorders with a need for sustained expression that necessarily require repeated vector administrations.[3] Tumoricidal conditionally replicating adenoviruses may need immunosuppression to allow the agent to spread sufficiently within the malignancy.[9] Adenoviruses turn on innate immune functions such as type I interferon and other proinflammatory cytokines.[10–12] Innate responses can prime for a humoral immune response generating neutralizing antibodies to adenoviral capsid antigens, which are augmented by T-cell help.[1,2]

Visualization and measurements of transgene expression are needed to refine and optimize gene therapy strategies. We have recently set up in nonhuman primates a semiquantitative transgene-specific positron emission tomography (PET) technique,[13] which allows detection and imaging of the reporter gene herpes simplex virus type 1 thymidine kinase (*HSV1-tk*). *HSV1-tk* as a reporter transgene can be traced by means of PET imaging in nonhuman primates[13,14] and in humans.[15,16] This imaging technique is based on measurements of retention of [^{18}F]9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine ([^{18}F]FHBG) in the liver parenchyma once phosphorylated by HSV1-tk. This noninvasive imaging technique is useful for assessing the possibility of repeated gene transfer. We reasoned that if first-generation recombinant adenovirus could be readministered, it would be easier to repeatedly use less immunogenic adenoviral vector generations. An additional advantage of our adenoviral system is the transient time course of transgene expression.[15] Once transgene expression is extinguished in about 2 weeks, the visualization of the efficiency of subsequent gene transfers is experimentally feasible. Transgene expression extinction is explained by immune elimination of transduced cells and gene silencing affecting the cytomegalovirus (CMV) promoter.[17]

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Many approved drugs exist[5,18–22] that thwart the immune response and might allow for viral vector readministration. A previous report[23] has shown the feasibility and safety of pharmacological immunosuppression of three macaques in an attempt to block T-cell responses to AAV encoding factor IX coagulation factor. In this study, we have demonstrated that pharmacological immunosuppression can achieve repeated liver gene transfer with the same first-generation adenoviral vector in spite of the high degree of immunogenicity attributed to this viral vector. B-cell depletion with anti-CD20 monoclonal antibody[19,24] and concomitant T-cell inhibition with clinically available drugs permits repeated liver gene transfer in macaques.

RESULTS

Rituximab and FK506 treatment reduce the antiadenoviral humoral and cellular immunity that prevents gene-transfer repetition with the same vector

To test whether immunosuppression could allow repeated administration of an adenoviral vector, a group of macaques treated i.v. with the clinical-grade first-generation recombinant adenovirus AdCMVHSV1.tk[25] (**Figure 1a**) received two courses of Rituximab to deplete B cells[26] plus a conventional daily oral regimen with FK506 (ref. 18) to repress T cells (**Figure 1a**).

Two doses of AdCMVHSV1-tk were given i.v. 4 weeks apart to three macaques. Only two of the animals received immunosuppression. PET imaging was performed 2 days after each adenovirus administration. The liver of the three animals showed evidence of transgene expression following the first administration, but only one of the immunosuppressed subjects showed a certain degree of reexpression upon the second administration (**Figure 1b,c**). To rule out residual transgene expression from the first dose of adenovirus, a PET study was performed 1 week before the second adenoviral administration. This is shown in **Supplementary Figure S1a** that summarizes the sequential PET measurements of transgene expression performed in these animals.

In the nonhuman primates, the two-drug regime reduced the titer of neutralizing antibodies by one log, but failed to abolish the humoral response (**Figure 1d**). One potential reason is that the depletion of B cells had not been complete as previously suggested by other authors.[22] In this regard, we found that in the subject who attained HSV1-tk partial reexpression, $CD20^+$ B-cells in peripheral blood samples were virtually undetectable (**Figure 1e**). In the other animals treated with the same immunosuppressive regimen, B-cell counts were lowered only about 2.5 times in comparison to the control macaque (**Figure 1e**). This difference could account for the different gene-transfer outcome. Peripheral blood T-lymphocytes (both CD4 and CD8) proliferated avidly upon *in vitro* exposure to adenoviral capsids, 6 weeks after the first administration (**Figure 1f,g**). Clearly less marked, albeit detectable, proliferation was substantiated in the CD4 T-cell compartment of the animal with partial transgene reexpression, and it is likely that these T-lymphocytes were still providing sufficient help for antibody responses (**Figure 1f,g**). Of note, all the animals tolerated the treatments well with only moderate increases of serum liver enzymes (**Supplementary Figure S1b**).

More intensive immunosuppression with Rituximab, FK506, antithymocyte immunoglobulin, MMF, and steroids permit repeated transfer of the HSV1-tk transgene

The partial success obtained in our first study (**Figure 1**) suggest that B-[22] and T-cell suppression is important for permitting adenoviral vector readministration, but that the two-drug regimen may not be sufficient.[1] Therefore, we pursued a more intensive immunosuppressive protocol. Accordingly, a more intense five-drug immunosuppression regime was tested in a new group of three animals. Antithymocyte immunoglobulin (ATG),[19,27] mycophenolate mofetil (MMF),[28] and steroids were added. The course of Rituximab[27] was intensified with weekly doses between adenoviral administrations. FK506 was given as in the first group of macaques (**Figure 2a**). The goals were to decrease T cells (ATG), to prevent B-cell rebounds (with more frequent readministrations of Rituximab) and to further inhibit residual B- and T-cell signaling with MMF. Steroids were included mainly to prevent systemic inflammatory responses elicited by ATG and in addition methylprednisolone can also diminish the early innate immune response[2] toward the adenoviral capsids.

Preexisting low titers of antiadenovirus antibodies seen in patients had not precluded gene transfer upon first intratumoral administrations of the vector.[15] Such weak reactivity probably reflects previous exposure to partially cross-reactive adenoviral serotypes. None of the animals in the first group had shown this feature (data not shown). However, one of the macaques in the second group showed a low titer of pretreatment antibodies that fell below the detection threshold in four other sequential samples (**Figure 2b**). In this subject, a weak cellular response toward adenoviral capsids was also detected immediately before AdCMVHSV1-tk exposure (**Figure 2c**). None of the other two animals showed pretreatment signs of antiadenoviral immunity (**Figure 2b,c**). To study the possible influence of this weak pretreatment antiadenoviral immunity, this animal was chosen to receive immunosuppression, whereas the other two were randomly distributed to receive or not the five-drug regimen.

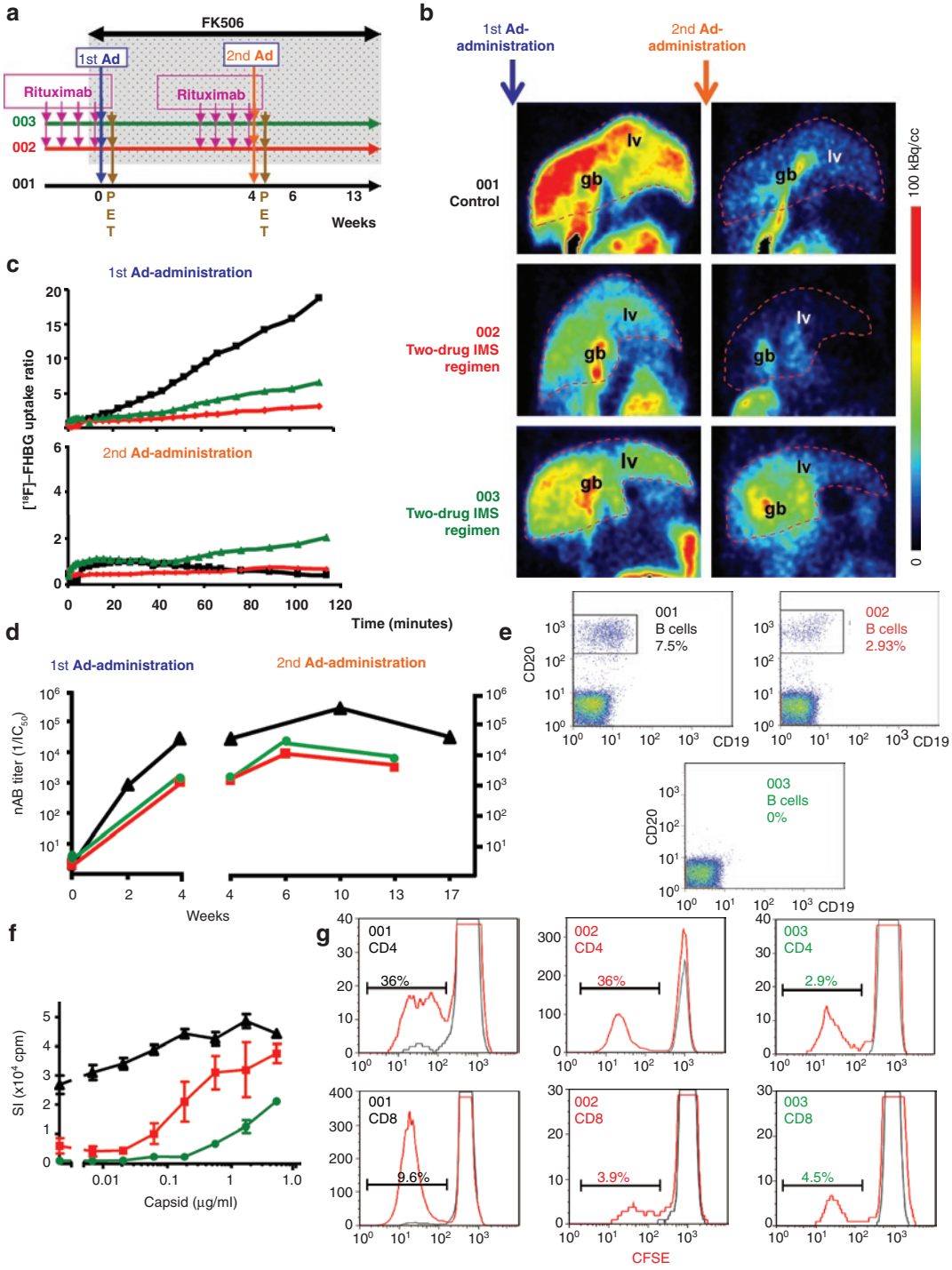
Again all the macaques showed intense PET signal following the first adenovirus exposure (**Figure 2d**). Upon second administration 1 month later (**Figure 2d**), one of the immunosuppressed macaques remarkably showed liver transgene expression of the same order of magnitude as in the previous administration. As expected, the control subject did not show reexpression of the transgene. This was also the case with the animal which had showed low levels of pretreatment antiadenoviral immunity.

In the animal with positive PET signal upon adenoviral readministration, immunohistological (**Figure 2e**) and immunoblot analyses (**Figure 2f**) on ultrasound-guided needle biopsies taken from the right and left liver lobes of the animals confirmed the expression of the *tk* transgene. There were no histological signs of liver inflammation although serum transaminases were moderately increased (**Supplementary Figure S2a,d**). Tk was present both in parenchymal cells and liver macrophages (Kupffer cells) (**Figure 2e** and **Supplementary Figures S2b,c and S4**). Kupffer cells were identified by morphological criteria and CD68 immunostaining in serial sections (**Supplementary Figure S2c**). Quantitative analyses 3 days after the adenoviral readministration

concluded that about 8% of hepatocytes and virtually all Kupffer cells were HSV1-tk$^+$ (**Figure 2e**). Two nonmutually exclusive possibilities for the tk presence in Kupffer cells can be considered: (i) direct transfection by the adenovirus or (ii) phagocytosis from expressing hepatocytes. Phagosome granular images were not observed, favoring the former option. Full transduction of liver macrophages has important implications for transgene functionality and immunogenicity.[12,29] This is also important because previous reports in mice indicate that Kupffer cell depletion increases gene transfer to liver parenchyma cells.[29]

HSV1-tk expression was confirmed by immunoblot in these biopsy samples taken from the right and left liver lobes 3 days after the second AdCMVHSV1-tk administration (**Figure 2f**). Immunoblot and immunohistochemical tk stainings were found negative in the control macaque 004 and treated macaque 005 (**Figure 2e,f**), indicating a good correlation with the PET results.

B-lymphocyte depletion by Rituximab was far more effective in the second cohort of animals and B cells remained almost undetectable for the duration of the protocol (**Figure 3a**). CD4



T-cell counts were maintained low following treatment, although CD8 T-cells rebounded faster (**Figure 3a**). The reduction of lymphocyte counts in the control animal (**Figure 3a**) could reflect sequestration in lymphoid organs as is the case in lymphocytopenia secondary to acute viral infections.[25]

Neutralizing antibodies and T-cell responses against adenovirus remained very low in the 006 macaque who reexpressed the HSV1-tk reporter transgene (**Figure 3b,c**). In contrast, the control subject (004 macaque) and the individual with preexisting low adenoviral immunity (005 macaque) produced neutralizing antibodies and anticapsid T-cell responses following the first adenoviral administration (**Figure 3b,c**). Importantly, neutralizing antibody titers eventually declined in both immunosuppressed animals but not in the control. **Figure 3d,e** shows proliferation among gated CD4 and CD8 T-cells after *in vitro* exposure to adenoviral capsids in samples obtained 6 weeks after the second adenoviral administration. In the animal that reexpressed the transgene, proliferation of both CD4 and CD8 was ablated, but only partially reduced in the CD4 compartment of the animal that did not reexpress in spite of immunosuppression.

The antibody immune response toward the foreign tk protein was undetectable after three administrations of AdCMVHSV1-tk by western blot on purified recombinant tk, although the sera from the same macaques were readily reactive to adenoviral capsid α-fiber (**Supplementary Figure S3**).

Under a discontinuous intensive immunosuppressive regimen liver gene retransfer was attained 8 months after the first administration of the same adenoviral vector

To study whether a third readministration of the same adenoviral vector was feasible, the color-coded macaques of the second cohort (**Figure 2**) were kept off immunosuppressive treatment for 4.5 months without signs of disease. Then the immunosuppressive regimen was started again in the macaques that had previously received the five-drug regimen (**Figure 4a**). A third i.v. administration of AdCMVHSV1-tk was given to the three animals (**Figure 4a**). Upon this third administration of the same vector both immunosuppressed animals showed [^{18}F]-FHBG retention in the liver that was more intense in the animal that had successfully reexpressed the *tk* transgene upon the first readministration (**Figure 4b**). Although reexpression was more modest in the animal that had shown preexisting adenoviral immunity, transgene-dependent retention of the PET tracer was also clearly detected. tk expression by both immunosuppressed macaques was confirmed by immunohistochemistry and immunoblots (**Figure 4c,d**) in the corresponding needle liver biopsies.

The humoral and cellular response against adenoviral capsids had remained suppressed before the third adenoviral readministration in the immunosuppressed animals but not in the control macaque (**Figure 4e,f**). It is of note that following the third AdCMVHSV1-tk administration even the immunosuppressed animals showed a rise in neutralizing antibodies against adenovirus, albeit one or two logs less concentrated in the immunosuppressed animals than in the control macaque. In this regard, the monkey which had had signs of low adenoviral immunity before the protocol showed higher titers at this stage of the experiment. The cellular response remained low in the immunosuppressed monkeys but was readily detected in the peripheral blood mononuclear cells from the control animal in which it peaked on day 5 after adenoviral exposure (**Figure 4f**). In **Figure 4g**, the numbers of T- and B-lymphocytes are recorded during the time around the third adenoviral administration.

Figure 5a shows the PET measurements of the second cohort color-coded macaques (**Figures 2** and **4**) to provide a summary of the results. **Figure 5b** shows the time course [^{18}F]-FHBG liver uptake in the PET imaging experiments performed 2 days after each adenoviral administration.

Moreover, 7 months after this third readministration a fourth infusion of the adenoviral vector was given to macaques 006, which had been weaned from immunosuppression during 4 months, and the control macaque 004. Importantly, macaque 006 was free of neutralizing antibodies during the previous month before the fourth adenovirus injection, while 004 still maintained titers above 1/50,000. As expected, there was expression upon immunohistochemical staining for tk in the liver biopsies from 006 taken 2 days after the fourth AdCMVHSV1-tk injection but not in case of macaque 004 (**Supplementary Figure S4a–c**). At the time of the fourth readministration there was a transient and mild peak of alanine aminotransferase and aspartate aminotransferase, whereas alkaline phosphotase remained normal (**Supplementary Figure S4d–f**).

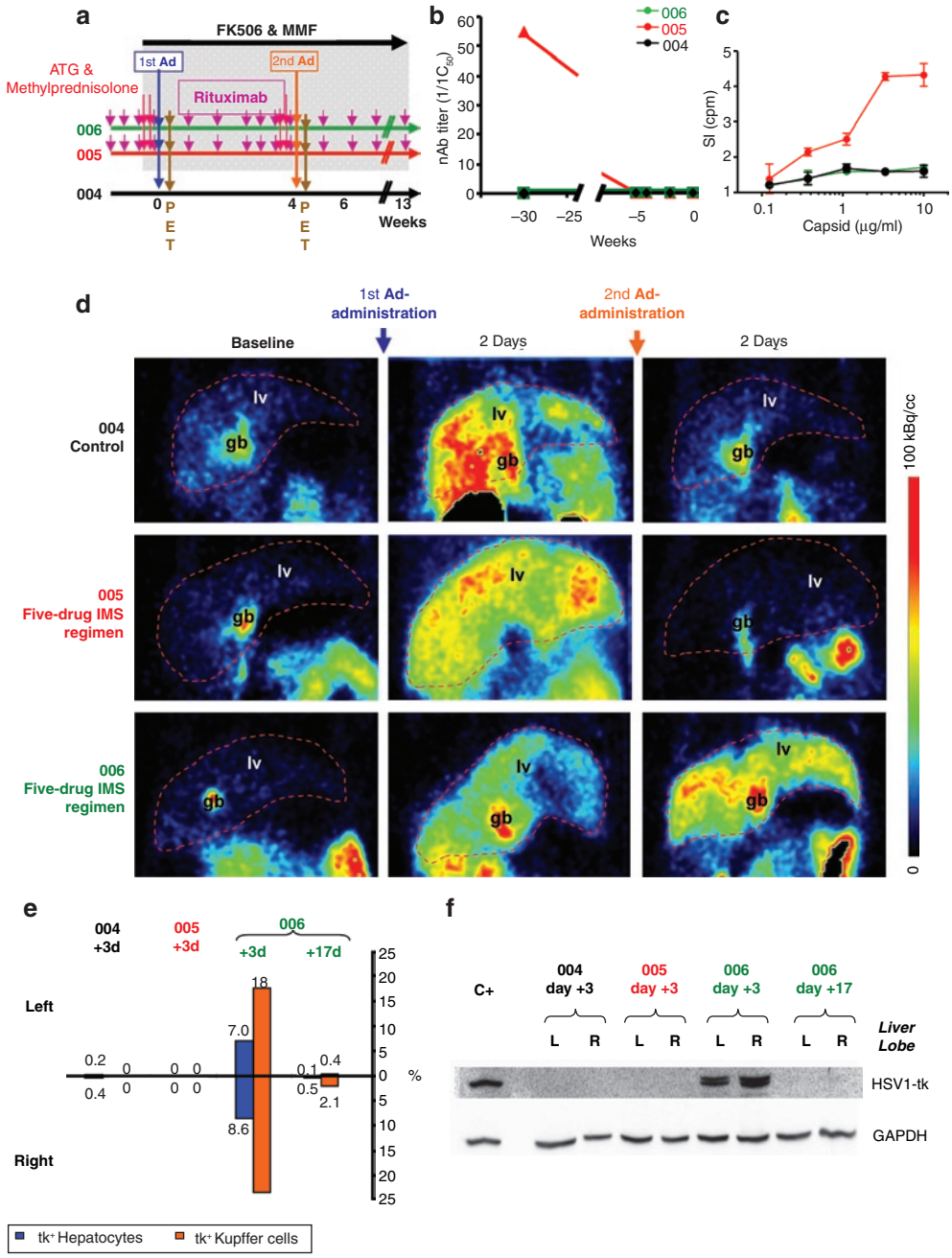
Figure 1 Immunosuppression of macaques with Rituximab and FK506 partially fails in an attempt to transfer twice a transgene to the liver with the same first-generation adenoviral vector. **(a)** Schematic time line representation of two administrations of a first-generation adenovirus encoding HSV1-tk to three female macaques (individually color-coded) and the immunosuppressive treatments given to two of the animals with Rituximab (20 mg/kg, i.v.) or daily doses of FK506 during the shaded time (1 mg/day). **(b)** Positron emission tomography (PET) images from the three macaques 2 days after each i.v. administration of AdCMVHSV1-tk performed on the dates marked in **a** (green arrows) to nonimmunosuppressed 001 control (black), and those animals receiving the immunosuppressive drugs (002-red, 003-green). PET images monitor [^{18}F]FHBG tracer, which becomes phosphorylated and intracellularly retained inside tk-expressing cells. **(c)** Time course of quantitative analyses of tracer retention in the hepatic region during PET analyses in the animals after the first and second dose of the recombinant AdCMVHS1-tk. Values are normalized by subtraction of the intensity of baseline positron emission in each macaque as measured 1 week before the administration of adenovirus. **(d)** Follow-up of the titers of neutralizing antiadenoviral antibodies after the first and second administration of AdCMVHSV1-tk to the macaques (color-coded as in **a**). **(e)** Double immunostainings of CD20$^+$ and CD19$^+$ cells in peripheral blood mononuclear cell (PBMC) from the indicated color-coded macaques in blood samples drawn upon termination of the second Rituximab course (13 weeks after the first adenoviral administration). **(f)** *In vitro* mitogenic responses to adenoviral capsids measured by ^{3}H-Thy incorporation (mean ± SD) of PBMC taken from the indicated macaques (color-coded) 13 weeks after the second adenovirus administration. **(g)** Fluorescence-activated cell-sorting histograms showing proliferation as estimated on gated CD4$^+$ and CD8$^+$ lymphocytes by CFSE dilution in response to adenoviral capsids at the same time point as in **f**. Analyses performed in the indicated color-coded macaques. Ad, adenovirus; CFSE, carboxyfluorescein succinimidyl ester; [^{18}F]FHBG, [^{18}F]9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine; HSV1-tk, herpes simplex virus type 1 thymidine kinase; gb, gall bladder; i.v., intravenous; lv, liver; nAb, neutralizing antibody; SI, stimulation index.

Of note, there was an transient peak of interleukin-6, tumor necrosis factor-α, and interleukin-1β in the serum of these animals within the 24 hours immediately following a readministration of AdCMVHSV1-tk (**Supplementary Figure S5**) that indicated activation of an innate immune response. There was a decrease of peripheral blood lymphocytes upon adenoviral administration, although platelets remained in the normal range. Transient lymphopenias (**Supplementary Figures S5 and Figures 3a and 4g**) could be related to lymphocyte sequestration in secondary lymphoid organs.

This second cohort of macaques offers a proof-of-the concept that pharmacological immunosuppression may permit gene liver retransfer with adenoviral vectors at least for four times.

DISCUSSION

This study explores in nonhuman primates the potential of immunosuppression to allow repetitive gene transfer with viral vectors. The small number of nonhuman primates available precludes taking the data as definitive evidence, but clearly offers a proof-of-the concept to support that strategies combining immunosuppression and gene therapy can be feasible and successful in at least in some cases. The limits to the size of macaque cohorts were imposed by ethical approval and logistics. A question that we face now is how much animal experimentation must be implemented for optimization of immunosuppression protocols before testing such approach in humans. More of that, taking into account that the predictability of the macaque model remains



largely undefined in this type of experimentation, macaque safety and efficacy results should be taken with caution.

The liver is a preferred target organ for gene therapy not only for liver-specific diseases but also for disorders that require systemic delivery of a protein. The pharmacology of immunosuppression is fast advancing and gene therapy may benefit from it, as has been the case in organ transplantation. Rituximab is a B-cell-depleting anti-CD20 fully human monoclonal antibody that is widely used for the treatment of Non-Hodgkin B-cell lymphomas.[24] Additionally, it has demonstrated efficacy in various autoimmune conditions[30,31] and a promising role for allogenic tissue grafting.[19] At the beginning of this project, we reasoned that if repeated liver gene transfer with adenovirus vectors was impossible mainly because of neutralizing antibodies, the depletion of B lymphocytes should be helpful to repeat gene transfer. The side effects of Rituximab in humans[24] are quite tolerable and the ensuing humoral immunodeficiency, if serious, should be reversible by immunoglobulin infusions.

High avidity neutralizing antibodies need T–B cell cooperation[32] and therefore a daily course of the calcineurine inhibitor FK506 was considered adequate to prevent T-cell activation in this regard. The results in the first cohort of macaques indicated that this two-drug regimen reduced humoral and cellular immunity directed to the viral capsids, albeit the overall pharmacological effect was not intense enough as to both completely suppress the immune response and allow successful subsequent gene transfer.

The obvious next step was to enhance the intensity of the immunosuppressant cocktail. With these ideas in mind, a much stronger pharmacological regimen was given to a second cohort of macaques including drugs that drastically, but transiently diminish T-cell counts and reactivity. ATG would decrease numbers of circulating T-cells,[33] and MMF would repress signaling in B- and T-lymphocytes.[34] Steroids were given immediately before each administration of ATG and adenovirus to mitigate possible systemic inflammation. This regimen controlled both the cellular and humoral response more efficaciously. In an animal without previous signs of immunoreactivity to viral capsids, this resulted in complete success upon readministration up to four times over a 15-month period. Interestingly, in this animal each successful gene retransfer coincided in time with transient increases in transaminases (~2 days after adenoviral administration) that may indicate some hepatocyte cytolysis also seen at lower levels in the nonimmunosuppressed controls.

Preexisting immunity might be less amenable to be restrained by pharmacological immunosuppression. Pretreatment plasmapheresis to remove antibodies could be considered. Lack of effect of Rituximab on plasma cells[35] is a hurdle because of their role as long-lasting sources of antibody that remain in the anti-CD20 monoclonal antibody treated subjects.[36]

It is intriguing that primary gene transfer under immunosuppressive treatments tended to attain less efficiency in transgene expression as assessed by PET. We do not understand the reasons, but intriguingly the group of Katherine High has observed a similar phenomenon in animals undergoing different immunosuppressive regimens and transduced in the liver with AAV vectors (ref. 37 and K. High, Children's Hospital, University of Pennsilvania, personal communication, 24 November 2009). We are currently experimentally addressing this phenomenon in murine models.

Repeated liver gene therapy can be highly efficacious for conditions such as hemophilia,[37] familiar hypercholesterinemia,[38] glycogen storage diseases,[39] phenylketonuria,[40] hepatic porphyrias,[41] etc. Likewise it is possible that tumor-selective oncolytic adenovirus could become more efficacious and be successfully readministered under this kind of pharmacological immunosuppression protocols.[42] Ongoing experiments in our institution will address the role of transient pharmacological immunosuppression to permit readministration of gutless adenoviral vectors that are being developed to correct liver metabolic diseases.

In the case of AAV, it has been seen that clearance of transduced hepatocytes is mediated by a CD8 T-cell response that recognizes

Figure 2 An uninterrupted five-drug immunosuppressive regimen (Rituximab+FK506+MMF+ATG+methyl prednisolone) permits efficient liver gene-transduction upon a second administration of an adenoviral vector given 1 month later to an adenovirus naive macaque. This was not feasible in a macaque with signs of previous weak immunity to the viral vector. **(a)** Schematic representation of the time course of the experiment with the i.v. administrations of AdCMVHSV1.tk that were received by three color-coded macaques and the immunosuppressive regimen given to two of the animals when indicated by the corresponding arrows at the following doses: Rituximab (20 mg/kg), antithymocyte γ-globulin (3 mg/kg), methyl-prednisolone (100 and 50 mg/animal in two daily doses before each adenoviral administration), as well as daily mycophenolate mofetil (30 mg/kg) and FK506 (0.25 mg/kg) during the time indicated by the shaded area. **(b)** Representation of the pretreatment baseline responsiveness of the color-coded animals to adenoviral capsids as detected by the presence of neutralizing antibodies and **(c)** PBMC proliferation to serial dilutions of adenoviral capsids performed 2 weeks before the first infusion of AdCMVHSV1-tk. **(b)** One of the animals (color-coded red) showed low titer antibodies 30 weeks before first adenoviral vector treatment, which became negative in sequential determinations thereafter and **(c)** low level of antiadenoviral lymphocyte proliferation 10 days before the first administration of adenovirus. **(d)** Coronal 1 mm-thick positron emission tomography (PET) images from the three macaques 2 days after each intravenous administration of AdCMVHSV1-tk performed on the dates marked in **a** to nonimmunosuppressed [004 control (black)], and those animals receiving the immunosuppressive drugs [005-red, 006-green]. PET images monitor [^{18}F]FHBG tracer which becomes phosphorylated and intracellularly retained inside tk-expressing cells. Gall bladder (gb) accumulation shows hepatobiliary clearance of the tracer. Dotted lines outline an approximate contour of the liver (lv) based on 3D stacked PET images. **(e)** Percentage of tk$^+$ cells in ultrasound-guided needle biopsies taken from the right and left liver lobes of the color-coded macaques upon immunohistochemistry stainings. Quantitative data were generated by computer assisted image analyses of 8 non-serial slides counting >1,000 cells. Liver macrophages were distinguished by morphology confirmed with CD68 immunostaining (**Supplementary Figure S2c**). In the positive macaque a second biopsy performed on day 17 after the second adenoviral administration and showed transgene expression extinction (day +17). **(f)** Immunoblot analysis of tk and GAPDH (house keeping control) on liver tissue homogenates from the liver biopsies. As a positive control COS7 cells transduced with AdCMVHSV1-tk were used. L and R indicate the hepatic lobe (left and right, respectively) from which the punch biopsy were taken. An additional biopsy was performed 2 weeks after the first set of biopsies (day +17) to assess persistence of expression in the animal successfully retransduced with tk (color-coded green). To corroborate transgene extinction an extra PET study performed one day before that biopsy was negative (images not shown are summarized in **Figure 5**). Ad, adenovirus; ATG, antithymocyte immunoglobulin; [^{18}F]FHBG, [^{18}F]9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine; i.v., intravenous; MMF, mycophenolate mofetil; nAb, neutralizing antibody; PBMC, peripheral blood mononuclear cell; SI, stimulation index.

the internalized capsid antigens.[4,23,43] The situation with adenoviral vectors may be different and highly modulated by the fact that liver macrophages are readily infected by adenovirus to the point that, according to our immunohistochemistry data, most Kupffer cells express the transgene.[29,44] We have examined the humoral immune response to HSV-tk without finding specific antibodies in serum but the cellular immune response toward the product of this transgene could not be measured for technical limitations.

Strikingly, despite intensive immunosuppression, a macaque with weak signs of preexisting adenoviral immunity was not successfully retransferred 4 weeks following the first exposure to the viral vector and only attained partial reexpression following

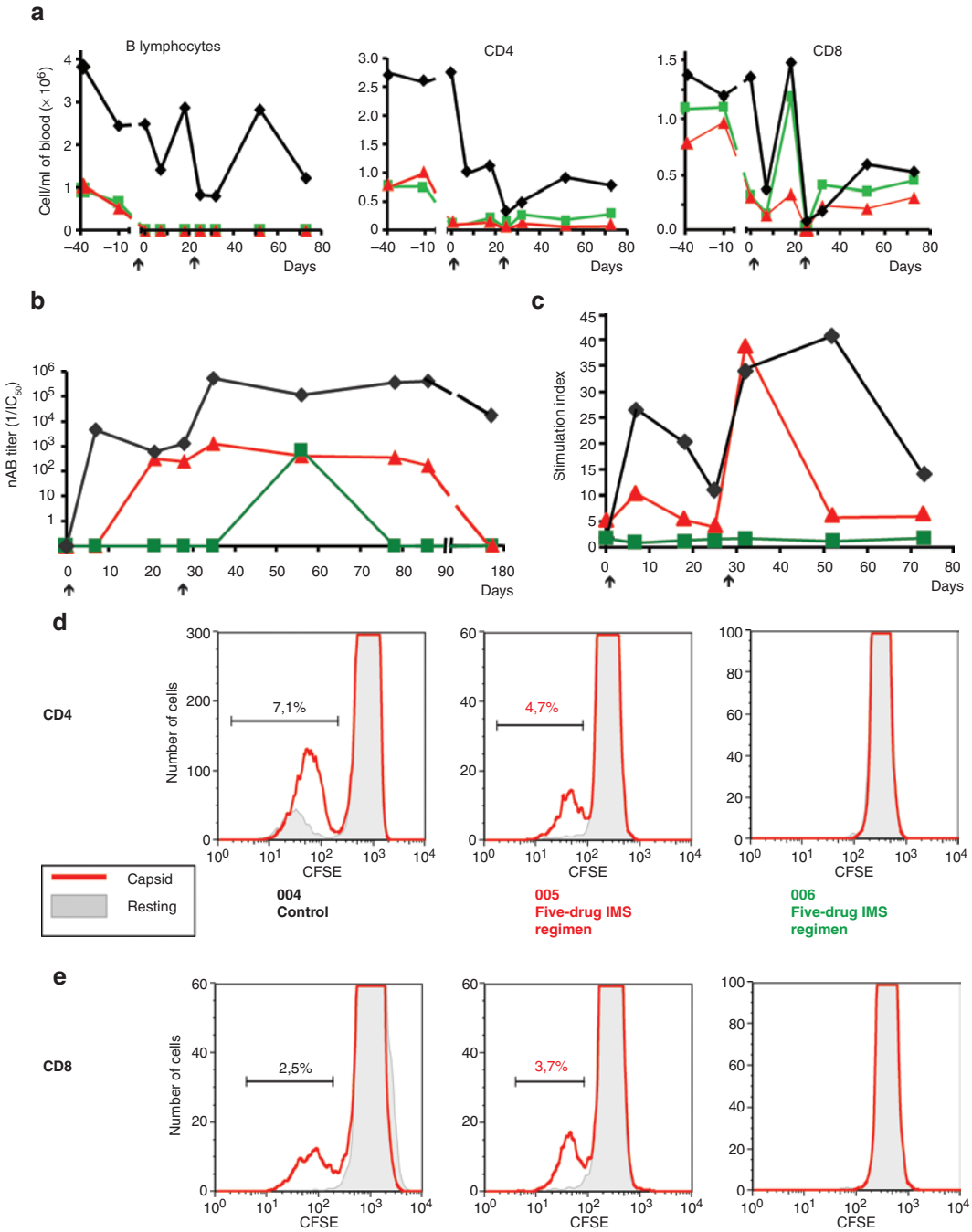


Figure 3 The five drug immunosuppressive regimen lessens humoral and cellular immunity against adenoviral capsid antigens. **(a)** Follow-up by flow cytometry assessments of the absolute numbers of CD19$^+$ B-lymphocytes, CD4$^+$ T-cells, and CD8$^+$ T-cells in the peripheral blood of the indicated macaques. Arrows point to the dates of AdCMVHSV1-tk injections. **(b)** Sequential follow-up of serum antiadenoviral neutralizing antibodies in the color-coded animals as described in **Figure 2a**. **(c)** Sequential follow-up of the proliferative response of PBMC from the indicated color-coded macaques to adenoviral capsids. **(d,e)** Proliferation assessed by CFSE dilution in gated **(d)** CD4$^+$ T-cells and **(e)** CD8$^+$ T-cells drawn from the peripheral blood of the indicated color-coded macaques 6 weeks after the second adenoviral infusion. CFSE, carboxyfluorescein succinimidyl ester; nAb, neutralizing antibody.

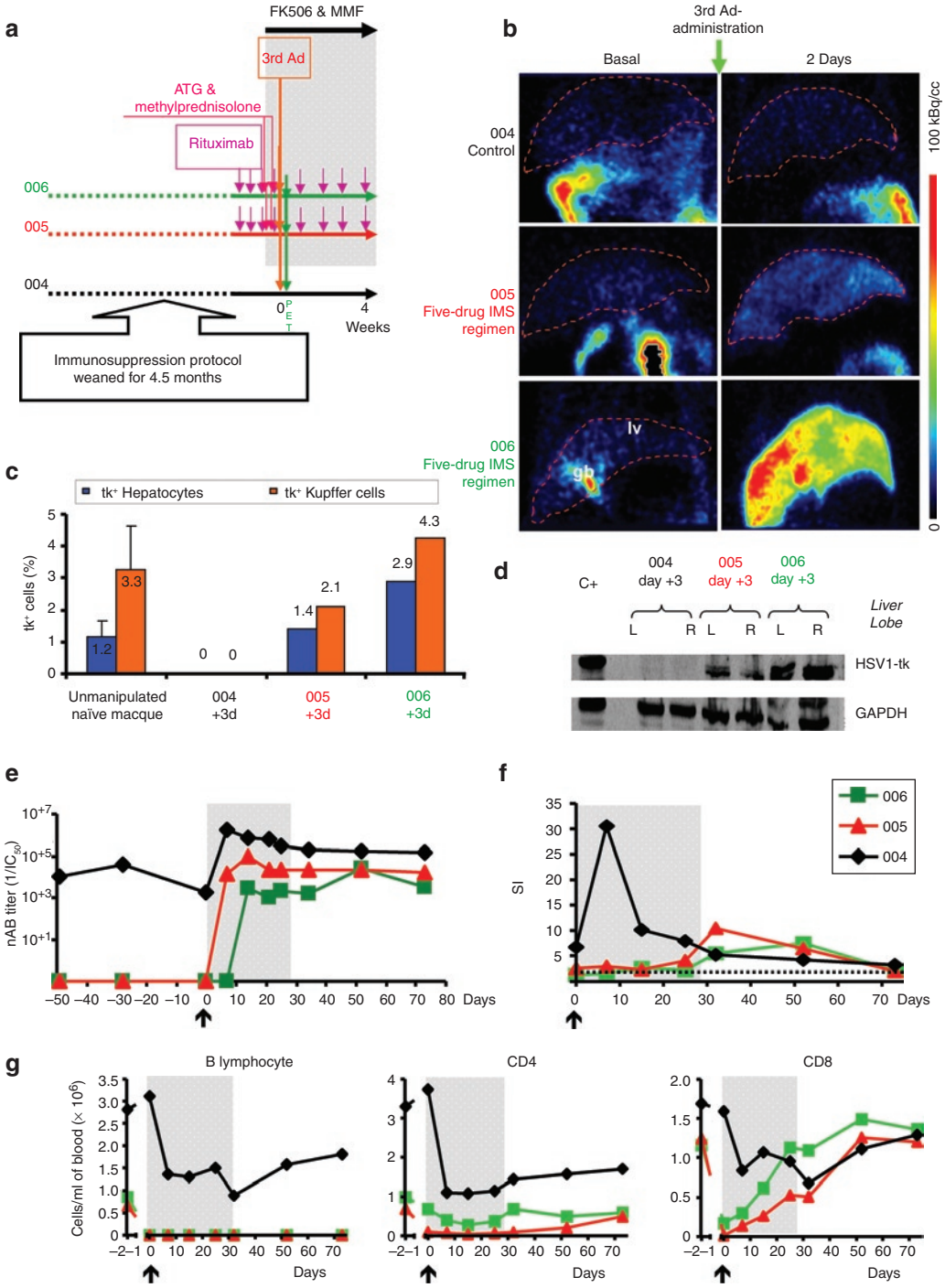


Figure 4 The five-drug regimen permits gene retransfer upon a third readministration of AdCMV-tk given 8 months after the first intravenous adenoviral vector administration. The macaques in the second cohort (005 and 006) were weaned from immunosuppressive treatment 9 weeks after the second administration of AdCMVHSV1-tk and remained 18 weeks off immunosuppressants. **(a)** Schematic timeline representation of a third administration of AdCMVHSV1-tk to the second cohort of macaques on day 0 and the indicated immunosuppressive regimen given to the color-coded macaques 005 and 006, which had undergone immunosuppression in the previous treatment. **(b)** Positron emission tomography (PET) images showing liver expression of the transgene before and 2 days after adenoviral administration in the indicated macaques. Of note, the 005 macaque achieved objective gene transfer. **(c**,**d)** Immunohistochemical and immunoblot assessment of tk expression in ultrasound-guided liver biopsies from the indicated animals. A biopsy from an additional macaque which had not received adenovirus previously was included as a control in **c**. Experiments were performed as in **Figure 2e,f** and biopsies were from the left (L) and right (R) liver lobe as indicated: **(e)** Follow-up of antiadenovirus neutralizing antibody (nAb) titers in the indicated macaques. **(f)** PBMC proliferation to adenoviral capsids from the indicated macaques assessed in blood samples drawn at the indicated days following the third AdCMVHSV1-tk administration. **(g)** Follow-up of the B- and T-cell counts in the peripheral blood of the color-coded macaques in the period of time surrounding the third adenoviral administration. PBMC, peripheral blood mononuclear cell.

a third administration given 4 months later. Interestingly, in this macaque neutralizing antibodies were below the detection threshold at the time of first exposure to the vector, but T-cell reactivity was still measurable in its peripheral blood, pointing once again to the key role played by T-lymphocytes. This macaque with low preexisting adenoviral immunity provides a clue to a situation of low titer antiadenoviral antibodies that is commonly found in human beings.[15] Intratumoral readministration of the same adenoviral vector to patients with liver cancer has been reported as being fruitless in terms of transgene reexpression because of neutralizing antibodies.[15] But in these patients low preexisting immunity did not prevent gene transduction following the first vector dose.

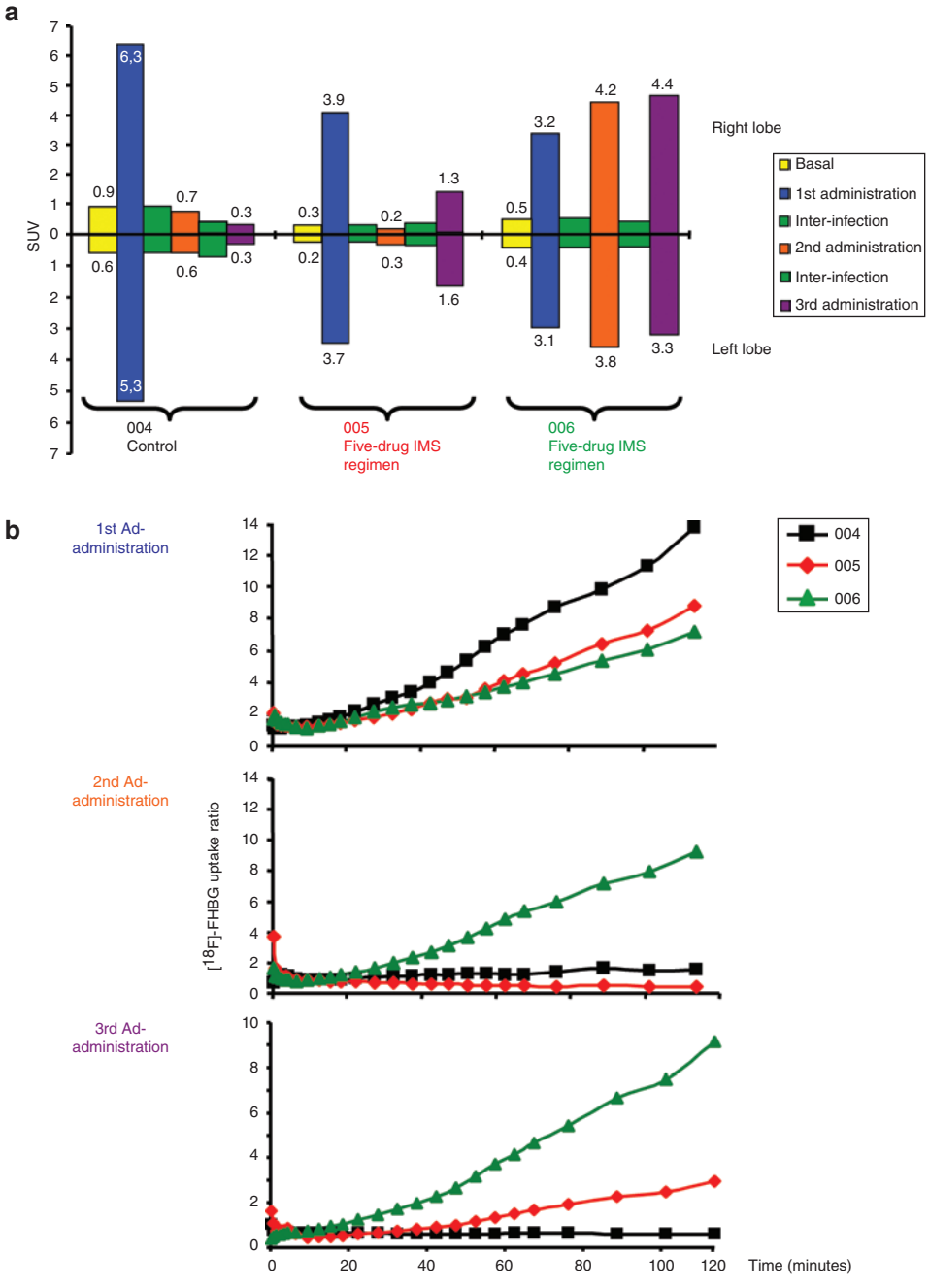


Figure 5 Summary of positron emission tomography (PET) imaging data upon follow-up of the second cohort of macaques undergoing AdCMVHSV1-tk readministrations. **(a)** Quantitative data from the sequential PET experiments shown in **Figure 2d** and **4b** carried out as indicated again in the graph legend. In these graphs, numeric data represent [^{18}F]FHBG tracer retention measured from 95 to 120 minutes (SUV25) after tracer infusion given separately for the right and left lobe liver areas (upper and lower bars). Results include baseline studies and those performed following the first, second and third AdCMNHSV1-tk administrations. Data include PET studies performed 1 week before the second and third administrations of adenovirus to verify extinction of transgene expression. **(b)** Time course of quantitative analyses of tracer retention in the hepatic region during the indicated PET analyses in the color-coded animals after each dose of the recombinant AdCMVHS1-tk. Values are normalized by the intensity of baseline positron emission in each macaque as measured 1 week before the administration of adenovirus. [^{18}F]FHBG, [^{18}F]9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine.

In agreement with these observations, low levels of preexisting immunity did not preclude transgene expression upon the first dose of AdCMVHSV1-tk to the macaque.

It is worth noting that a similar immunosuppressive regimen is under clinical trial (NCT 00782821). Importantly, no overt infectious complications were observed and all our animals were alive 15 months after the first adenoviral dose and lymphocyte counts returned to normal when the treatment was discontinued. This indicates that strong combined T- and B-cell immunosuppression if maintained for a 3–4 month period is relatively safe.[19] Longer immunosuppressive maintenances are likely to be problematic as is the case in transplantation patients and in a gene therapy patient treated with an AAV encoding a tumor necrosis factor-α antagonist transgene.[45] An aspect to be taken into account is that the functional thymus in macaques at this age facilitates the repopulation of the T-cell compartment.

Considering the risks, serious opportunistic infections could be eventually palliated in this therapeutic setting with exogenous infusions of immunoglobulins or cryopreserved self T-lymphocytes frozen and stored before treatment. On the other side, pharmacological immunosuppression could have affected the early cytokine response that is detected following adenovirus injections.[10,12,46] If exacerbated, such innate response is a well-known mediator of severe acute toxicity.[47] However, our data on interleukin-6 and tumor necrosis factor-α serum concentrations fortunately indicate that there are no signs of such an augment, at least in the tested conditions.

Risk must be balanced in terms of the expected benefit and the available alternatives for the patients. In this sense, the concept of acceptable risk in gene therapy is to be refined and depends on the outcome of the disease, existence of alternative treatments, and the expected benefit.[48] What is clear is that immunological end points are to be included is the clinical trials to optimize immunosuppression regimens. This is because immune parameters correlate with successful repeated gene transfer according to our results.

Research on the persistence of adenoviral antigens under immunosuppressive regimens will determine just how long immunosuppressive therapy will need to be continued. It is conceivable that if antibodies are a major driving force for clearing the capsid proteins, such proteins may persist much longer under B-cell suppression.

It is likely that immunosuppression requirements will be less demanding for less immunogenic helper-dependent adenoviruses. Noninvasive PET imaging can be a useful tool to evaluate the feasibility of readministrations in the clinical arena when combining a reporter and a therapeutic gene.

All in all, our results in a limited number of nonhuman primates indicate that comprehensive T- and B-cell transient pharmacological suppression can overcome the obstacles to readministration of adenoviral vectors used in gene therapy and have clear potential for clinical applications.

MATERIALS AND METHODS

Animals. Three-year-old captive bred female nonhuman primates (*Macaca fascicularis*) were purchased from R.C. Hartelust (Tilburg, The Netherlands). Animal experiments were performed following a protocol previously approved by the Ethics and Biosafety Committee according to guidelines from the University of Navarra and government of Navarra with emphasis in the reduce, replace and refine standards which prevented enlargement in the number animals involved in this protocol. Before the studies, macaques underwent complete physical and biochemical examinations, evaluation of clinical pathology parameters and were screened for tuberculosis. Biochemical parameters in serum were measured in a Cobas Integra 400 (Roche Diagnostics, Barcelona, Spain). Platelet and blood cell counts were performed in an automated Sysmex XT-1800i (Sysmex America, Mundelein, IL) with software set-up for macaque analysis.

Vector infusion procedure. A first-generation clinical-grade recombinant adenovirus encoding tk (AdCMVHSV1-tk) has been described.[25] Adenovirus was diluted in phosphate-buffered saline to a final volume of 10 ml and slowly infused to the anesthetized animal via a peripheral vein at 1.4×10^{12} viral particles per dose.

Immunomodulation therapy. Immunosuppressants were administered as described in **Figures 1a** and **2a**. Two-drug regimens consisted of (i) 20 mg Rituximab/kg/dose intravenously (i.v.) (Mabthera, ROCHE, Basel, Switzerland) at days −9, −6, −3 and immediately before adenovirus injections; and (ii) Tacrolimus (FK506; Astellas Pharma, Madrid, Spain) at a dose of 0.25 mg/kg administered orally from day −2 and daily to the end of the study (**Figure 1a**). The five-drug regime included (i) Rituximab (20 mg/kg/dose i.v.) at days −9, −6, −3, immediately before adenovirus injections and weekly after the viral administration, (ii) two doses of 3 mg/kg of rabbit ATG (Genzyme Polyclonals, Marcy l'Etoile, France) at days −2 and −1 before the adenovirus injection. (iii) Methyl-prednisolone (Solu-moderin, Pfizer SA, Spain) was applied intramuscularly 10 minutes before the ATG infusion at a dose of 100 mg on day −2 and 50 mg on day −1. (iv) MMF (CellCept; Roche Pharma, Madrid, Spain) at a dose of 25–30 mg/kg/day, and (v) FK506 0.25 mg/kg/day. MMF and FK506 were orally given from day −2 daily during the indicated periods (**Figure 2a**).

PET analysis. Transgene expression in the liver parenchyma was visualized and quantified by PET[13] 1 week before and 48 hours after the AdCMVHS1-tk administration ("PET analyses of HSV1-tk expression" in **Supplementary Materials and Methods**).

Neutralizing antibody assays[49] to AdCMVHSV1-tk. Serial dilutions of macaque serum starting from 1/25 were mixed with 1×10^5 plaque-forming units of Ad5CMV-luc encoding firefly luciferase (a similar recombinant adenovirus also based on serotype 5 backbone) and incubated at 37 °C for 1 hour then the mixture was added to PCL-PRF5 cells (1×10^4 cells/well) in a 96-well plate. Forty-eight hours later, cells were washed in phosphate-buffered saline and D-luciferin substrate (Xenogen, Alameda, CA) was added at a final concentration of 150 µg/ml and placed in a light-tight chamber. The intensity of light emission from individualized wells was detected using the IVIS cooled charge-coupled device camera (Xenogen) and Living Image 2.20 software package (Xenogen). Sera were scored as positive if the light intensity was 50% when compared to negative control sera. A curve was adjusted to extrapolate the serum dilution for 50% inhibition (IC_{50}).

Fluorescence-activated cell-sorting analysis of the lymphocyte populations and cytokine serum concentrations. The percentage of B, CD4, and CD8 lymphocytes in peripheral blood was determined by flow cytometry as detailed in "FACS analysis" in **Supplementary Materials and Methods.** Serum concentrations of cytokines were measured with a BD Cytometric Bead Array (Inflammatory Cytokine Kit, Ref.: 551811) analyzed in a FACSCalibur (Brussels, Belgium). Culture supernatants of macaque lymphocytes stimulated with Concanavalin-A at 1 µg/ml (Sigma, St Louis, MO) were used as a positive control.

In vitro *evaluation of the cellular immune response against the adenoviral vector.* To functionally evaluate the cellular immune response induced against the vector a number of techniques were set up as detailed

in "Measurement of cellular immune responses against adenoviral vector" in **Supplementary Materials and Methods**.

Immunoblot for HSV1-tk expression in liver biopsies, liver histology, and immunohistochemical analysis. Western blot analyses were carried out to detect HSV1-tk expression in liver tissue biopsies using GAPDH as a housekeeping internal control as detailed in "Western blot analysis of liver biopsies" in **Supplementary Materials and Methods** and ref. 13. Histological analysis of the liver was carried out on 3-µm paraffin-embedded sections. Hematoxylin–eosin stain was used to determine structure. Immunohistochemical analyses were carried out as detailed in "Immunohistochemistry of liver biopsies"in **Supplementary Materials and Methods**. HSV-tk production in eukaryotic cells with a Semliki forest virus-based expression system[50] to screen by immunoblot for anti-tk antibodies was performed as described in "Construction of SFV-histk vector" and "Transfection of cells for TK production" in **Supplementary Materials and Methods**.

SUPPLEMENTARY MATERIAL

Figure S1. Summary of tk expression data and well-being from macaques in the three-drug cohort.
Figure S2. Histological images from the biopsies presented in figure 2.
Figure S3. Anti-tk antibodies in the treated macaques.
Figure S4. Ultrasound-guided liver biopsies were performed from the left and right liver lobes of macaques 004 and 006 two days after the fourth adenovirus administration.
Figure S5. Platelets and serum inflammatory cytokines following adenoviral administration.
Materials and Methods.

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