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Immunization Against the Transgene but not the TetON Switch Reduces Expression From Gutless Adenoviral Vectors in the Brain

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Abstract

Immune responses against vectors or encoded transgenes can impose limitations on gene therapy. We demonstrated that tetracycline-regulated high-capacity adenoviral vectors (HC-Ads) sustain regulated transgene expression in the brain even in the presence of systemic pre-existing immune responses against adenoviruses. In this study we assessed whether systemic pre-existing immune responses against the transgene products, i.e., β -Gal or the tetracycline-dependent (TetON) regulatory transcription factors (rtTA2^{SM2} and the tTS^{Kid}), affect transgene expression levels and the safety profile of HC-Ads in the brain. We pre-immunized mice with plasmids encoding the TetON switch expressing rtTA2^{SM2} and the tTS^{Kid} or β -Gal. HC-Ads expressing β -Gal under the control of the TetON switch were then injected into the striatum. We assessed levels and distribution of β -Gal expression, and evaluated local inflammation and neuropathological changes. We found that systemic immunity against β -Gal, but not against the TetON switch, led to inflammation and reduction of transgene expression in the striatum. Therefore, the regulatory TetON switch appears to be safe to use, and capable of sustaining transgene expression in the brain even in the presence of an immune response against its components. Systemic immunity against the transgene had the effect of curtailing its expression, thereby affecting the efficacy and safety of gene delivery to the brain. This factor should be considered when developing gene therapies for neurological use.

INTRODUCTION

Gene transfer has been attempted in human patients using vectors derived from adenoviruses, adeno-associated viruses, herpes simplex virus, and retroviruses¹ for diseases ranging from cancer² to genetic defects.³ However, patients' immune response against viral vectors can lead

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to inconsistent, short-lived transgene expression,⁴⁻⁶ and even serious adverse events, including death. One of the patients in an ornithine transcarbamylase deficiency clinical trial died as a result of immune response.⁷

Immune responses associated with gene transfer and gene therapy in the brain differ from immune responses to vectors delivered to other organs. This is because of the brain's unique immune reactivity, usually referred to as "immune privilege".⁸ We showed that when first-generation adenoviral vectors (Ads) are administered intracranially in the presence of a pre-existing systemic anti-adenovirus immune response, T and B cells infiltrate the brain and transgene expression is eventually eliminated by the formation of specific immunological synapses.⁹⁻¹¹ Because the immune response against Ads targets the adenoviral gene products that are expressed, albeit at low levels, from the genome of these vectors, novel high-capacity Ads (HC-Ads) have been developed. These vectors do not encode any adenoviral genes and therefore, following uncoating, they should be invisible to a putative host adaptive immune response against Ads.^{9,10,12} We have demonstrated that HC-Ads are capable of inducing stable, long-term transgene expression in the brain even during an active systemic immune response against Ads.^{9,10,12} However, there is mounting evidence that immune responses against the transgenes encoded in gene therapy vectors (including therapeutic transgenes and regulatory transcription factors) could also affect the efficacy of therapeutic gene transfer.^{13, 14}

We have shown that tightly regulated transgene expression can be achieved using a tetracycline expression system encoded in HC-Ads, even in the presence of a systemic immune response against adenoviruses.¹² This TetON system, which encodes a trans-activator element (rtTA2^SM2) and a transcriptional silencer, tTS^{Kid} (refs. 15,16), originally derived from *Escherichia coli* and the herpes simplex virus, has been shown to be immunogenic in non-human primates.¹⁷ Immune responses against this protein negatively impact long-term transgene expression in peripheral organs,^{18,19} and it is therefore important to determine whether this immune response could affect TetON-driven transgene expression in the brain. Also, pre-existing systemic immune responses against the therapeutic transgenes encoded in HC-Ads could hamper transgene expression in the brain parenchyma. These therapeutic transgenes, that are potentially immunogenic and to which human patients could have been pre-exposed systemically (either naturally or by vaccination), include transgenes of viral or prokaryotic origin, such as thymidine kinase (HSV1-TK) from herpes simplex virus, cytosine deaminase, and purine nucleoside phosphorylase from *E. coli*, diphtheria toxin from *Corynebacterium diphtheriae*, carboxypeptidase G2 and exotoxin A from *Pseudomonas aeruginosa*. Other potentially immunogenic transgenes comprise those that replace single gene defects, such as coagulation factors in hemophilia, insulin in type I diabetes, and glial cell line—derived neurotrophic factor in Parkinson's disease. In this study, therefore, we examined the consequences of immune responses against the transactivator element, TetON and the reporter protein β -Gal, in relation to brain inflammation and transgene expression. Systemic immunity against β -Gal, but not against the TetON switch, led to inflammation and reduction of transgene expression in the striatum. We conclude that the regulatory TetON switch appears to be safe and capable of sustaining transgene expression in the brain even in the presence of an immune response against its components. Systemic immunity against the transgene curtails its expression, thereby affecting the efficacy and safety of gene delivery to the brain and should be taken into account when developing gene therapies for neurological disorders.

RESULTS

Expression of β -Gal and the TetON switch encoded within the pCI plasmid used for immunization

We have shown that intracranial transgene expression from Ads, but not from HC-Ads, is eliminated in animals that have been systemically immunized against adenovirus.⁹⁻¹² In this study, our aim was to assess whether systemic immune responses against the transgene or the components of the TetON switch, *i.e.*, rtTA2^SM2 transactivator and the tTS^{Kid} transrepressor that drive regulated expression of transgenes encoded within HC-Ads, could also inhibit transgene expression from HC-Ads when injected into the brain. In order to induce a systemic immune response against the components of the TetON switch and the reporter protein β -Gal, we pre-immunized mice systemically using the experimental paradigm illustrated in Figure 1a. For this purpose, we generated mammalian expression plasmids (Figure 1b) encoding either the regulatable TetON switch expressing rtTA2^SM2 and the tTS^{Kid} (pCI-TetON), or β -Gal (pCI- β -Gal). The efficiency of transgene expression from these plasmids was determined *in vitro* in transfected COS-7 cells by Western blot (Figure 2a, upper panel) and immunofluorescence (Figure 2a, lower panel). We found that β -Gal and the TetON switch components are expressed in COS-7 cells with similar transduction efficiency (pCI-TetON: 50% \pm 2.2; pCI- β -Gal: 54% \pm 2.8, Figure 2a, lower panel) and expression levels (transgene expression levels versus actin expression levels: pCI-TetON: 0.78; pCI- β -Gal: 0.82; Figure 2a, upper panels). In order to evaluate the ability of these plasmids to drive transgene expression *in vivo*, we injected them into the tibialis anterior muscles of mice. Transgene expression was determined by immunocytochemistry 5 days after injection. As shown in Figure 2b, we found that immunoreactive muscle fibers for both transgenes, *i.e.*, β -Gal and TetON could be detected when the expression plasmids (pCI- β -Gal and pCI-TetON) used for the immunization experiments) were delivered into the muscle. These data indicate that the levels of expression of both transgenes, *i.e.*, TetON and β -Gal, encoded in the eukaryotic expression plasmid (pCI) are very similar.

Induction of a systemic, peripheral immune response against the transgene and the TetON switch

We pre-immunized mice systemically using the experimental paradigm illustrated in Figure 1a. C57/Black6 mice were immunized using an intramuscular injection of the mammalian expression plasmids (Figure 1b) encoding either the regulatable TetON switch expressing rtTA2^SM2 and the tTS^{Kid} (pCI-TetON) or β -Gal (pCI- β -Gal) in saline containing CpG motifs that act as adjuvants to enhance the immune responses.²⁰ Non-immunized mice were used as controls, and received saline containing CpG. Two weeks later, the mice received a second intramuscular immunization using the same immunogens. In order to assess the ability of these plasmids to elicit a specific immune response, we performed an enzyme-linked immunosorbent spot (ELISPOT) assay 1 week after the second intramuscular immunization. Splenocytes isolated from each group of animals were stimulated with either the transactivator pure protein (rtTA2^S)¹⁸ or β -Gal pure protein, and an ELISPOT assay was performed to detect antigen-specific T cells producing interferon- γ (IFN- γ) (Figure 3a). The release of IFN- γ from the splenocytes prepared from pCI-TetON- and pCI- β -Gal-immunized animals was induced by stimulation with rtTA2^S and β -Gal, respectively. The number of IFN- γ producing cells was higher in the splenocytes from pCI- β -Gal-pre-immunized animals than from the mice pre-immunized with pCI-TetON (twofold higher, $P < 0.05$). Splenocytes isolated from animals immunized with saline and stimulated with rtTA2^S or β -Gal pure protein resulted in negligible base levels of IFN- γ producing splenocytes.

Pre-immunization against the transgene, but not against the TetON switch diminished the Hc-Ad-mediated transgene expression in the brain

Having demonstrated that pre-immunizations with pCI-TetON or pCI- β -Gal were successful in initiating specific systemic immune responses, we assessed their impact on HC-Ad-mediated expression in the brain. One week after the second immunization, each of the mice received a single stereotactic injection of 1×10^7 blue forming units of HC-Ad-mCMV-TetON- β -Gal (1 μ l) into the striatum. In order to activate TetON-driven transgene expression from the HC-Ad, the mice were fed chow containing the inducer doxycyclin. The mice were killed either 3 weeks or 7 weeks after intracranial delivery of HC-Ad, the brains were removed and processed, and transgene expression was assessed by evaluating the β -Gal activity (Figure 3b), distribution of β -Gal immunore-activity (Figure 4) and infiltration of inflammatory cells in the brain (Figures 5 and 6), and also by neuropathological analysis (Figure 7).

β -Gal enzymatic activity assay was performed on brain tissue isolated from pre-immunized animals at 3 and 7 weeks following intracranial stereotactic injection of HC-Ad-mCMV-TetON- β -Gal. Only the animals that had been pre-immunized with pCI- β -Gal showed a statistically significant decrease in β -Gal activity as compared to the control (non-immunized) animals. Anti- β -Gal pre-immunization induced a decrease of $\sim 80 \pm 7\%$ and $90 \pm 3\%$ in brain β -Gal activity 3 and 7 weeks after intracranial administration of HC-Ad-mCMV-TetON- β -Gal, respectively. On the other hand, β -Gal activity from pCI-TetON-immunized animals exhibited only a slight decrease in comparison with non-immunized animals ($\sim 50\%$ at both time points), which was not statistically significant when compared with values from saline-treated controls.

Using immunohistochemistry, we also assessed the impact of pre-immunization against the TetON switch components rtTA2^SM2 and the tTS^{Kid} or β -Gal on transgene expression. Striatal sections from immunized and control animals, killed at 3 or 7 weeks after intrastriatal HC-Ad delivery, were stained with a β -Gal-specific antibody (Figure 4a). Quantitative stereological analysis revealed that, at 3 weeks after injection of HC-Ad-mCMV-TetON- β -Gal, there were no changes in the distribution of β -Gal immunoreactivity in the brains of mice pre-immunized with either pCI-TetON or pCI- β -Gal (Figure 4b). However, 7 weeks after HC-Ad administration, the area occupied by β -Gal-immunoreactive cells was approximately fivefold smaller in the brains of animals pre-immunized with pCI- β -Gal than in the brains of non-immunized animals; no changes were observed in β -Gal immunoreactivity of pCI-TetON-pre-immunized mice (Figure 4b).

Pre-immunization against β -Gal, but not against the TetON switch increases infiltration of inflammatory cells into the brain

We next examined whether pre-immunization against the TetON switch or β -Gal increases the infiltration of inflammatory cells into the brain after intracranial delivery of HC-Ads. By means of immunocytochemistry, and using an antibody specific for CD3⁺T cells, we determined the number of T cells infiltrating the brains of animals pre-immunized with pCI-TetON or pCI- β -Gal (Figure 5a). Stereological quantification of CD3⁺T cells demonstrated a ~ 40 -fold increase in the number of CD3⁺ cells in the brains of animals 3 weeks after pre-immunization with pCI- β -Gal, in comparison to the brains of non-immunized mice (Figure 5b). Although CD3⁺T cell infiltration decreased 4 weeks later in the brains of pCI- β -Gal immunized mice, the number of CD3⁺T cells in their brains was still significantly higher than those in the brains of non-immunized mice (Figure 5b). Infiltration of CD3⁺T cells into the brains of pCI-TetON-immunized animals was mild, and showed an increase in comparison with the values in non-immunized animals only at 7 weeks after HC-Ad delivery (Figure 5b).

We also analyzed the infiltration of macrophages into the brains of animals immunized against TetON switch components or β -Gal before intracranial delivery of HC-Ad-mCMV-TetON-

β -Gal. Striatal sections from immunized animals were stained with the mouse macrophage marker F4/80 at 3 or 7 weeks after the striatal injection of HC-Ad (Figure 6a). Quantitative stereological analysis revealed that the area containing F4/80⁺ cells in the brains of pCI- β -Gal-immunized mice was >25-fold larger than in those of non-immunized mice, 3 and 7 weeks after HC-Ad delivery. In contrast, the F4/80⁺ immunoreactive area was small in animals pre-immunized with TetON and not significantly different from that in non-immunized mice 3 and 7 weeks after HC-Ad delivery.

Pre-immunization against β -Gal, but not against the TetON switch induces a transient decrease in the density of striatal dopaminergic terminals

Considering that immune responses in the brain can lead to severe inflammatory side effects, we performed histopathological analyses of brains from mice pre-immunized against the components of the TetON or against the transgene β -Gal. Nissl staining in striatal sections of the brains taken from these animals did not reveal gross structural changes in any of the experimental groups (data not shown). We then determined the impact of these immune responses on the density of striatal tyrosine hydroxylase (TH)-expressing dopaminergic terminals. Representative brain sections showing TH staining are depicted in Figure 7a. We estimated the optical density (OD) of TH immunoreactivity in the injected striatum and compared it with the contralateral non-injected hemisphere 3 and 7 weeks after intracranial administration of HC-Ad (Figure 7b). We found a decrease in the OD of TH in the region of the injection site at 3 weeks after HC-Ad administration, only in the animals that had been pre-immunized against β -Gal. However, TH immunoreactivity in these mice returned to normal 4 weeks later. No changes in TH immunoreactivity were detected in mice that had been pre-immunized against the TetON switch components.

Taking into account the fact that inflammatory responses against either of the transgenes could lead to demyelination or dysmyelination, we examined the integrity of the myelin sheaths in the brains of these animals. We labeled myelin by immunostaining for myelin basic protein in the brain sections from injected animals. We found that the density of myelin immunoreactivity appeared to be intact in the brains of animals from all the three groups, with none of them showing any evidence of myelin loss, thereby suggesting that the integrity of the corresponding oligodendrocytes remained intact (Figure 7a).

DISCUSSION

Ads are powerful tools for transgene delivery into the brain and can sustain high levels of transgene expression for long periods of time, as has been demonstrated in several pre-clinical animal models.^{21,22} Moreover, Ads exhibit high transduction efficiency in the brains of human patients, and are associated with a satisfactory safety profile.^{4,5,23-25} However, we (and others) have demonstrated that immune responses against first-generation Ads or their encoded transgenes and regulatory sequences may lead not only to a reduction in therapeutic efficacy^{9,10} but also to severe immune-mediated adverse side effects.^{7,10,26}

It has been reported that the vast majority of the human population exhibits circulating antibodies against Ads.⁶ This has led to the development of HC-Ads. These vectors are devoid of all viral encoding genes, are associated with very low immunogenicity, and achieve persistent transgene expression even in the presence of an anti-Ad immune response.¹⁰⁻¹² These features make them ideal vectors to deliver therapeutic transgenes in clinical trials. We have shown earlier that these HC-Ads can elicit non-leaky, regulatable transgene expression in the brain.¹² We have also recently demonstrated that transgene expression encoded by these gutless Ads can be successfully switched “on” and turned “off” even in the presence of a pre-existing systemic immune response against adenoviruses,¹² of the kind that could be encountered in humans undergoing clinical trials.⁶

Regulatory sequences and transgenes are frequently derived from microorganisms to which human patients could have been pre-exposed,²⁷ or against which they could develop an immune response after gene therapy.²⁸ For gene therapy applications, it is very desirable to include a post-transcriptional switch which should allow turning gene expression “on” and switching it “off” as and when necessary.²⁹ One of the best characterized regulatable expression systems, is the TetON system, which we have recently further engineered to encode the tTS^{Kid} element which renders this system non-leaky in the uninduced state in the context of HC-Ad.^{12,30,31} In this study we sought to elucidate the effects of the presence of a pre-existing systemic immune response against the TetON switch elements. This is particularly relevant, given that both the TetON switch and β -Gal³² are derived from *E. coli*, which is a common bacterial resident and/or pathogen in the human intestine.²⁷ The experiments were designed to assess whether HC-Ads can sustain transgene expression in the brain in the presence of systemic immune responses against the components of the TetON switch or against the reporter transgene β -Gal. β -Gal was used as a model transgene as it has been extensively used as a marker gene in both pre-clinical animal experiments^{12,33} and also in human gene marking trials.²⁵

Our results indicate that systemic pre-exposure to transgenes encoded within HC-Ads delivered into the brain parenchyma reduces their expression in the brain. Both β -Gal and the components of the TetON switch were immunogenic. However, the cellular immune response against β -Gal was stronger than the response against the components of the TetON switch. Considering that both plasmids, pCI-TetON and pCI- β -Gal, were similarly expressed in the mouse muscle, the difference in the potency of these plasmids to induce a specific immune response seems to be related to the antigenicity of each molecule rather than to the expression levels of the antigens. In fact, infiltration of inflammatory cells into the brain takes place earlier when pCI- β -Gal is used for pre-immunization than when pCI-TetON is used. This is likely to be a consequence of the higher antigenicity of β -Gal as compared to TetON. Also, the more rapid inflammation observed in the brains of pCI- β -Gal-immunized mice is probably the cause of the quickest and more drastic reduction in transgene expression levels. While pre-existing immunity against β -Gal led to a strong inhibition of transgene expression, the immune response against the components of the TetON switch induced a non-significant decrease in transgene expression, which remained stable and was detectable for up to 7 weeks after delivery of the HC-Ad into the brain parenchyma. These results demonstrate that, while systemic immunization against β -Gal elicited a stronger immune response than immunization against the components of the TetON system did in C57/Black6 mice, pre-exposure to the Tet-dependent regulatory proteins would not severely compromise transgene expression from HC-Ads delivered into the brain.

We found a difference in the transgene expression levels as determined by β -Gal activity assay and those determined by immunocytochemistry. These techniques measure essentially different parameters of transgene expression. While β -Gal immunocytochemistry measures the distribution of transduced, immunoreactive cells (the number of cells expressing the transgene), β -Gal enzymatic activity indicates the exact levels of transgene expression (amount of transgene per mass of transduced tissue). The changes in transgene expression levels are detected earlier in terms of β -Gal enzymatic activity. Differences between levels of enzymatic activity and immunostaining have also been reported for other enzymes, such as ornithine decarboxylase in epidermal and macrophage-like cells.³⁴ In addition, the polyclonal antibodies used for immunohistochemical detection may detect smaller fragments of β -Gal generated during the metabolic degradation of the enzyme, which will no longer elicit enzymatic activity. It has also been shown that β -Gal is a very stable protein when injected by direct pressure injection into neurons in culture, and that it lasts for >4 days after a single administration.³⁵ It appears, therefore, that the immune response against the transgene causes a decline in

transgene expression, which is first identified as a decrease in enzymatic activity and is later detected by immunocytochemistry.

Although our results suggest that immunity against the TetON switch components would not severely compromise transgene expression in the brain, an immune response against the components of the TetON has been shown to be involved in causing a reduction in the duration of transgene expression from HC-Ads when the vectors are administered systemically at high doses.^{18,36} This limitation has been overcome by expressing the TetON switch under the control of a cell type—specific promoter which inhibits expression of the antigen in professional antigen-presenting cells.³⁶ The immune response against the TetON switch components has been reported to be triggered only if enough monocytes or dendritic cells are transduced after intramuscular administration of adeno-associated viruses.¹⁸ Also, a reduction in the vector dose has been shown to decrease the immunogenicity of the TetON switch, leading to a less potent immune response and improvement of the longevity of transgene expression.³⁶

In peripheral tissues, immune responses against reporter or therapeutic transgenes have earlier been shown to be involved in reducing transgene expression after the administration of gene therapy vectors that express coagulation factors VIII³⁷ and IX,³⁸ α -hialuronidase,³⁹ and α -Gal.⁴⁰ Moreover, it has been reported that neutralizing antibodies against β -Gal are induced after the administration of plasmids expressing this transgene in the liver.⁴¹ Antibodies against human growth hormone have also been found in mice implanted peripherally with transgenes expressing microencapsulated allogeneic cells.⁴² In a Phase I clinical trial for mesothelioma,²⁸ 3 out of the 21 patients who received intrapleural injections of Ad expressing thymidine kinase developed antibodies against the therapeutic transgene. Our results show that systemic immune responses against the transgene would also hamper HC-Ad-mediated transgene expression in the brain, and that the development of immune responses against therapeutic transgenes, including those of prokaryotic origin and those delivered to correct protein deficiencies in genetic disorders, should be taken into account when designing and testing gene therapy approaches.^{28,37-40}

Immunity (either pre-existing, or induced after vector delivery) against Ads can also elicit very severe immune-mediated neuropathological adverse effects in the brain.^{10,26} We found that pre-immunization against the components of the TetON switch did not induce changes in the density of striatal dopaminergic terminals expressing TH in the striatum. On the other hand, mice pre-immunized against β -Gal and exhibiting decreased transgene expression levels and increased infiltration of inflammatory cells, also displayed a reduction in the density of TH immunoreactivity in the striatum 3 weeks after administration of HC-Ad. Nevertheless, these pathological changes were reversible, and reverted when inflammation subsided. Neither pre-immunization against TetON switch nor against β -Gal induced myelin loss or any structural changes in the brain after administration of HC-Ad.

In summary, our results show that pre-existing immunity against the transgene β -Gal, but not against the TetON switch components, *i.e.*, rtTA2^{SM2} and the tTS^{Kid}, leads to a local inflammatory response in the brain and rapid reduction in the levels and distribution of transgene expression after intracranial administration of HC-Ads. These findings suggest that the regulatory switch composed of rtTA2^{SM2} and the tTS^{Kid} in the context of a HC-Ad is a safe tool for regulating gene expression in the brain. However, immunity against therapeutic transgenes could affect the efficacy and safety of gene delivery to the central nervous system and should be taken into account when designing and testing novel gene therapy approaches for neurological disorders.

MATERIALS AND METHODS

Animals and surgical procedures

Female C57/Black6 mice of 15–18 g body weight (Jackson Laboratories, Bar Harbor, ME) were housed at constant temperature and humidity with a 12-hour light/dark cycle and had free access to chow and drinking water. Animal experiments were performed after receiving approval from Institutional Animal Care and Use Committee, and conformed to the policies and procedures of the Cedars-Sinai Medical Center Comparative Medicine Department. The experimental design is depicted in Figure 1a. In order to induce an immune response against the regulatable switch (TetON) or the reporter gene (β -Gal), mice (five per group) received an intramuscular injection with 100 μ g of plasmids encoding TetON (pCI-TetON) or β -Gal (pCI- β -gal) in 50 μ l saline containing 25 μ g of CpG 2216 (G* G*GGGACGATCGTCG* G*G*G*G*G*G (*: phosphothiolate modification) (Axxora LLC, San Diego, CA). The non-immunized mice that were used as controls received saline containing CpG. For determining distribution of transgene expression from these plasmids *in vivo*, a group of mice was injected with 100 μ g of pCI-TetON or pCI- β -Gal in saline without CpG and killed 5 days later. For the purpose of boosting the immune response, mice received a second intramuscular injection of pCI-TetON, pCI- β -Gal or saline 2 weeks later. One week after the second immunization, groups of animals were killed (see **Supplementary Materials and Methods**) and their spleens removed for assessing the status of immunity against TetON and β -Gal using IFN- γ ELISPOT, while others were intracranially injected with HC-Ads expressing β -Gal under the control of the regulatable switch TetON (see **Supplementary Materials and Methods**). At the end-points of the experiment (3 and 7 weeks after brain injection) the mice were killed and brain tissue was removed for transgene expression functional assay (β -Gal activity assay) or immunohistochemistry.

Plasmid construction

All restriction enzymes, enzymes, DNA ladders, and plasmids were purchased from New England Biolabs, Hercules, CA or Promega (Madison, WI) unless indicated otherwise. We constructed a plasmid expressing the TetON switch, comprising the rtTA2^SM2 and the tTS^{Kid} complementary DNAs (kindly provided by Dr. H. Bujard from ZMBH, Germany) under the control of the cytomegalovirus (CMV) promoter (pCI-rtTA2^SM2-IRES-tTS^{Kid}-pA, pCI-TetON; Figure 1b) to immunize mice against the regulatable switch, and another plasmid encoding the reporter gene β -Gal under the control of the CMV promoter (pCI- β -Gal, Figure 1b) to immunize mice against the transgene (see **Supplementary Materials and Methods** for details). Transgene expression was studied in COS-7 cells transfected with pCI-TetON or pCI- β -Gal by Western blot and immunocytochemistry (Figure 2a), using a rabbit anti-TetON antibody (1:300) newly developed by us (as described in the following text), or a rabbit anti- β -Gal antibody (1:1,000) as we have earlier reported.³⁰

Production, scale-up, and purification of HC-Ads

Our method of development of a pSTK120m plasmid harboring the TRE- β -Gal-pA expression cassette and the regulatable TetON switch under the control of the mCMV promoter has been described in detail elsewhere.¹² HC-Ads expressing β -Gal under the control of the regulatable mCMV-TetON (HC-Ad-mTetON- β -Gal) switch were scaled up from vector seed stocks, purified, and titrated as described earlier by us and also by others^{12,30,31,43} (see **Supplementary Materials and Methods**). The vector preparations were tested to ensure that they were free from contaminating lipopolysaccharide and replication competent adenovirus. The titer of HC-Ad-mTetON- β -Gal was 6.32×10^{12} viral particles/ml, 3.28×10^{11} blue forming units/ml.

Western blot detection of β -Gal and TetON expression in COS-7

1×10^6 COS-7 cells in a T75 flask were transfected with 15 μ g of plasmid pCI- β -Gal, pCI-TetON, or pCI empty vector (control). Cells were harvested in RadioImmunoPrecipitation Assay lysis buffer; 80–120 μ g of total sample protein was loaded onto a 10 or 12% sodium dodecyl sulfate-polyacrylamide gel with 5% for stacking, and run at 130 V for 90 minutes. Pure β -Gal (0.2 μ g) (Sigma-Aldrich, St. Louis, MO) or TetON (0.4 μ g) (kindly provided by Dr. Philippe Moullier, INSERM ERM, Nantes, France) (18) proteins were used as positive controls. The proteins within the gels were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with primary polyclonal rabbit anti- β -Gal (1:500), or rabbit anti-TetON (1:150), or mouse anti- β -actin (1:5,000) (Sigma, cat. no. A1978). A horseradish peroxidase—conjugated sheep anti-rabbit or rabbit anti-mouse (Bio-Rad, Hercules, CA) was used as a secondary antibody at a dilution of 1:2,000, and incubated for 1 hour to detect β -Gal, TetON, and β -actin bands. The blots were then developed using the ECL Western blotting analysis kit (Amersham Biosciences, Piscataway, NJ). The density of immunostaining in each lane was determined using Adobe Photoshop 6.0, and the amount of β -Gal and TetON present in each lane was normalized in relation to the corresponding β -actin present in each sample.

β -Gal and TetON expression in vivo in mouse tibialis anterior muscle

The distribution of transgene expression from the mammalian expression plasmids pCI-TetON and pCI- β -Gal was determined by immunocytochemistry after intramuscular injection into the tibialis anterior muscle. Mice were injected with 100 μ g pCI (empty), pCI- β -Gal, or pCI-TetON (diluted in 50 μ l saline) as described earlier. Control animals were injected with pCI plasmid without transgene (pCI-Empty). Five days after injection, animals were killed by intracardiac perfusion and fixative. The muscles were removed and cryoprotected with 20% sucrose in phosphate-buffered saline (pH 7.4) for 24 hours. The tissue block was frozen in dry ice and kept frozen at -20°C . Ten-micrometer sections were cut serially using a Microm cryostat (Leica CM3050 S; Leica, Wetzlar, Germany), and mounted on gelatin-coated slides. Transgene expression was determined by immunocytochemistry as described later.

Production of anti-TetON antibody

We generated a rabbit polyclonal antibody (anti-TetON) specific for the transactivator sequence, *i.e.*, rtTA2^SM2 (see **Supplementary Materials and Methods** for details). The coding sequence of the “M2”-mutant of TetR, fused with three minimal activation domains (F-type), was synthesized with codon usage optimized for humans. In addition, all putative splice-sites were removed. The hydrophilicity plot was constructed for the TetON protein using Hopp—Woods and Kyte—Doolittle hydrophilicity analysis. The region from amino acids 150 to 165 was considered to be a favorable site for antigen recognition, because of its hydrophilic properties. A cytosine coupling was engineered to the end of the 16-amino acid peptide EHQVAKEERETPTTDS for coupling to a protein carrier for immunization.

Assessment of antigen-specific immunity by IFN- γ ELISPOT assay

In order to determine whether TetON transactivator or β -Gal can induce a systemic immune response, female C57/Black6 mice were immunized with either pCI-TetON, pCI- β -Gal plasmids, or saline as described earlier. The mice were perfused 1 week after the second immunization, and their spleens removed aseptically. The number of IFN- γ -producing T cells was assessed using the ELISPOT kit assay (R&D Systems, Minneapolis, MN) in accordance with the manufacturer’s instructions (**Supplementary Materials and Methods**).

β -Gal enzymatic activity assay

Three and seven weeks after intracranial administration of HC-Ads, the mice were perfused, a block of brain tissue around the injection site was dissected and processed, and β -Gal enzymatic activity assay was carried out as described elsewhere¹² (see **Supplementary Materials and Methods**).

Immunohistochemistry

Transgene expression and infiltration of immune cells was determined by immunocytochemistry in 30- μ m brain sections as described in **Supplementary Materials and Methods**.^{12,30}

Quantification and stereological analysis of infiltration of immune cells

Quantitative analysis was performed in 30- μ m brain sections to determine the anatomical area occupied by cells immunoreactive with antibodies against β -Gal and immune markers. The analysis was carried out using a Zeiss AxioPlan 2 Imaging microscope (Carl Zeiss Microsystems, Thornwood, NY) controlled by a Ludl electronic MAC 5000 XY stage control (Ludl Electronics Products, Hawthorne, NY). We quantified the HC-Ad-transduced area, the number of infiltrating CD-3 ϵ cells, and the area infiltrated by macrophages in coronal sections, using Stereo Investigator software (Microbrightfield, Colchester, VT) as described elsewhere.³⁰

Striatal fiber OD measurements

TH immunostained striatal coronal sections were digitalized with Axiovision software version 3.1 (Carl Zeiss Vision, Germany) using AxioPlan 2 imaging microscope (Carl Zeiss, Germany). TH staining OD was determined in the striatum on both sides of the brain using MosaiX-AxioVision Rel 4.4 Software (Carl Zeiss Vision, Germany). Background OD was determined at the corpus callosum and subtracted. The mean OD of the injected striatum was relativized to the contralateral striatum, and expressed as a percentage of the control non-immunized group.

Statistical analysis

Data were analyzed using one-way analysis of variance followed by Newman—Keuls' test, or, when they failed the normality test or Levene equal-variance test, they were analyzed using the non-parametric Kruskal—Wallis test. Transduction efficiency in COS-7 cells was analyzed using the Chi-square test. The results were expressed as mean values \pm SEM. A *P* value <0.05 was considered the cutoff for significance. All experiments were performed at least two times.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Edelstein ML, Abedi MR, Wixon J, Edelstein RM. Gene therapy clinical trials worldwide 1989–2004—an overview. *J Gene Med* 2004;6:597–602. [PubMed: 15170730]
2. McLoughlin JM, McCarty TM, Cunningham C, Clark V, Senzer N, Nemunaitis J, et al. TNFerade, an adenovector carrying the transgene for human tumor necrosis factor α , for patients with advanced solid tumors: surgical experience and long-term follow-up. *Ann Surg Oncol* 2005;12:825–830. [PubMed: 16132372]
3. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000;288:669–672. [PubMed: 10784449]
4. Lang FF, Bruner JM, Fuller GN, Aldape K, Prados MD, Chang S, et al. Phase I trial of adenovirus-mediated p53 gene therapy for recurrent glioma: biological and clinical results. *J Clin Oncol* 2003;21:2508–2518. [PubMed: 12839017]
5. Sandmair AM, Loimas S, Puranen P, Immonen A, Kossila M, Puranen M, et al. Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther* 2000;11:2197–2205. [PubMed: 11084677]
6. Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* 1999;6:1574–1583. [PubMed: 10490767]
7. Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003;80:148–158. [PubMed: 14567964]
8. Lowenstein PR, Castro MG. Inflammation and adaptive immune responses to adenoviral vectors injected into the brain: peculiarities, mechanisms, and consequences. *Gene Ther* 2003;10:946–954. [PubMed: 12756415]
9. Barcia C, Thomas CE, Curtin JF, King GD, Wawrowsky K, Candolfi M, et al. *In vivo* mature immunological synapses forming SMACs mediate clearance of virally infected astrocytes from the brain. *J Exp Med* 2006;203:2095–2107. [PubMed: 16923851]
10. Thomas CE, Schiedner G, Kochanek S, Castro MG, Löwenstein PR. Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc Natl Acad Sci USA* 2000;97:7482–7487. [PubMed: 10840055]
11. Barcia C, Jimenez-Dalmaroni M, Kroeger KM, Puntel M, Rapaport AJ, Larocque D, et al. One-year expression from high-capacity adenoviral vectors in the brains of animals with pre-existing anti-adenoviral immunity: clinical implications. *Mol Ther* 2007;15:2154–2163. [PubMed: 17895861]
12. Xiong W, Goverdhan S, Sciascia SA, Candolfi M, Zirger JM, Barcia C, et al. Regulatable gutless adenovirus vectors sustain inducible transgene expression in the brain in the presence of an immune response against adenoviruses. *J Virol* 2006;80:27–37. [PubMed: 16352528]
13. Figueredo J, Limberis MP, Wilson JM. Prediction of cellular immune responses against CFTR in patients with cystic fibrosis after gene therapy. *Am J Respir Cell Mol Biol* 2007;36:529–533. [PubMed: 17218617]
14. Traversari C, Marktel S, Magnani Z, Mangia P, Russo V, Ciceri F, et al. The potential immunogenicity of the TK suicide gene does not prevent full clinical benefit associated with the use of TK-transduced donor lymphocytes in HSCT for hematologic malignancies. *Blood* 2007;109:4708–4715. [PubMed: 17327417]
15. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci USA* 2000;97:7963–7968. [PubMed: 10859354]
16. Witzgall R, O’Leary E, Leaf A, Onaldi D, Bonventre JV. The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc Natl Acad Sci USA* 1994;91:4514–4518. [PubMed: 8183940]

17. Latta-Mahieu M, Rolland M, Caillet C, Wang M, Kennel P, Mahfouz I, et al. Gene transfer of a chimeric trans-activator is immunogenic and results in short-lived transgene expression. *Hum Gene Ther* 2002;13:1611–1620. [PubMed: 12228016]
18. Favre D, Blouin V, Provost N, Spisek R, Porrot F, Bohl D, et al. Lack of an immune response against the tetracycline-dependent transactivator correlates with long-term doxycycline-regulated transgene expression in nonhuman primates after intramuscular injection of recombinant adeno-associated virus. *J Virol* 2002;76:11605–116011. [PubMed: 12388721]
19. Ginhoux F, Turbant S, Gross DA, Poupiot J, Marais T, Lone Y, et al. HLA-A*0201-restricted cytolytic responses to the rtTA transactivator dominant and cryptic epitopes compromise transgene expression induced by the tetracycline on system. *Mol Ther* 2004;10:279–289. [PubMed: 15294175]
20. Häcker G, Redecke V, Häcker H. Activation of the immune system by bacterial CpG-DNA. *Immunology* 2002;105:245–251. [PubMed: 11918685]
21. Brann T, Kayda D, Lyons RM, Shirley P, Roy S, Kaleko M, et al. Adenoviral vector-mediated expression of physiologic levels of human factor VIII in nonhuman primates. *Hum Gene Ther* 1999;10:2999–3011. [PubMed: 10609660]
22. Dewey RA, Morrissey G, Cowsill CM, Stone D, Bolognani F, Dodd NJ, et al. Chronic brain inflammation and persistent herpes simplex virus 1 thymidine kinase expression in survivors of syngeneic glioma treated by adenovirus-mediated gene therapy: implications for clinical trials. *Nat Med* 1999;5:1256–1263. [PubMed: 10545991]
23. Chiocca EA, Abbed KM, Tatter S, Louis DN, Hochberg FH, Barker F, et al. A phase I open-label, dose-escalation, multi-institutional trial of injection with an E1B-Attenuated adenovirus, ONYX-015, into the peritumoral region of recurrent malignant gliomas, in the adjuvant setting. *Mol Ther* 2004;10:958–966. [PubMed: 15509513]
24. Immonen A, Vapalahti M, Tyynelä K, Hurskainen H, Sandmair A, Vanninen R, et al. AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: a randomised, controlled study. *Mol Ther* 2004;10:967–972. [PubMed: 15509514]
25. Puumalainen AM, Vapalahti M, Agrawal RS, Kossila M, Laukkanen J, Lehtolainen P, et al. β -Gal gene transfer to human malignant glioma *in vivo* using replication-deficient retroviruses and adenoviruses. *Hum Gene Ther* 1998;9:1769–1774. [PubMed: 9721087]
26. Zirger JM, Barcia C, Liu C, Puntel M, Mitchell N, Campbell I, et al. Rapid upregulation of interferon-regulated and chemokine mRNAs upon injection of 10^8 international units, but not lower doses, of adenoviral vectors into the brain. *J Virol* 2006;80:5655–5659. [PubMed: 16699048]
27. Walters M, Sperandio V. Quorum sensing in *Escherichia coli* and *Salmonella*. *Int J Med Microbiol* 2006;296:125–131. [PubMed: 16487745]
28. Molnar-Kimber KL, Sterman DH, Chang M, Kang EH, ElBash M, Lanuti M, et al. Impact of preexisting and induced humoral and cellular immune responses in an adenovirus-based gene therapy phase I clinical trial for localized mesothelioma. *Hum Gene Ther* 1998;9:2121–2133. [PubMed: 9759938]
29. Goverdhan S, Puntel M, Xiong W, Zirger JM, Barcia C, Curtin JF, et al. Regulatable gene expression systems for gene therapy applications: progress and future challenges. *Mol Ther* 2005;12:189–211. [PubMed: 15946903]
30. Candolfi M, Curtin JF, Xiong WD, Kroeger KM, Liu C, Rentsendorj A, et al. Effective high-capacity gutless adenoviral vectors mediate transgene expression in human glioma cells. *Mol Ther* 2006;14:371–381. [PubMed: 16798098]
31. Candolfi M, Pluhar GE, Kroeger K, Puntel M, Curtin J, Barcia C, et al. Optimization of adenoviral vector-mediated transgene expression in the canine brain *in vivo*, and in canine glioma cells *in vitro*. *Neuro Oncol* 2007;9:245–258. [PubMed: 17522335]
32. Matthews BW. The structure of *E. coli* β -Gal. *C R Biol* 2005;328:549–556. [PubMed: 15950161]
33. Mane VP, Toietta G, McCormack WM, Conde I, Clarke C, Palmer D, et al. Modulation of TNF α , a determinant of acute toxicity associated with systemic delivery of first-generation and helper-dependent adenoviral vectors. *Gene Ther* 2006;13:1272–1280. [PubMed: 16708078]
34. Greenfield AR, Taffet SM, Haddox MK. Immunocytochemical localization of ornithine decarboxylase in cultured murine cells. *Cell Tissue Res* 1986;243:33–40. [PubMed: 2417722]

35. Smith RL, Geller AI, Escudero KW, Wilcox CL. Long-term expression in sensory neurons in tissue culture from herpes simplex virus type 1 (HSV-1) promoters in an HSV-1-derived vector. *J Virol* 1995;69:4593–4599. [PubMed: 7609023]
36. Lena AM, Giannetti P, Sporeno E, Ciliberto G, Savino R. Immune responses against tetracycline-dependent transactivators affect long-term expression of mouse erythropoietin delivered by a helper-dependent adenoviral vector. *J Gene Med* 2005;7:1086–1096. [PubMed: 15772935]
37. Shi Q, Wilcox DA, Fahs SA, Weiler H, Wells CW, Cooley BC, et al. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J Clin Invest* 2006;116:1974–1982. [PubMed: 16823491]
38. Wang L, Cao O, Swalm B, Dobrzynski E, Mingozzi F, Herzog RW. Major role of local immune responses in antibody formation to factor IX in AAV gene transfer. *Gene Ther* 2005;12:1453–1464. [PubMed: 15889137]
39. Di Domenico C, Di Napoli D, Gonzalez Y, Reyero E, Lombardo A, Naldini L, Di Natale P. Limited transgene immune response and long-term expression of human alpha-L-iduronidase in young adult mice with mucopolysaccharidosis type I by liver-directed gene therapy. *Hum Gene Ther* 2006;17:1112–1121. [PubMed: 17044753]
40. Chu Q, Joseph M, Przybylska M, Yew NS, Scheule RK. Transient siRNA-mediated attenuation of liver expression from an α -Gal A plasmid reduces subsequent humoral immune responses to the transgene product in mice. *Mol Ther* 2005;12:264–273. [PubMed: 15946902]
41. Sullivan DE, Dash S, Du H, Hiramatsu N, Aydin F, Kolls J, et al. Liver-directed gene transfer in non-human primates. *Hum Gene Ther* 1997;8:1195–1206. [PubMed: 9215737]
42. Chang PL, Shen N, Westcott AJ. Delivery of recombinant gene products with microencapsulated cells *in vivo*. *Hum Gene Ther* 1993;4:433–440. [PubMed: 8399490]
43. Palmer D, Ng P. Improved system for helper-dependent adenoviral vector production. *Mol Ther* 2003;8:846–852. [PubMed: 14599819]

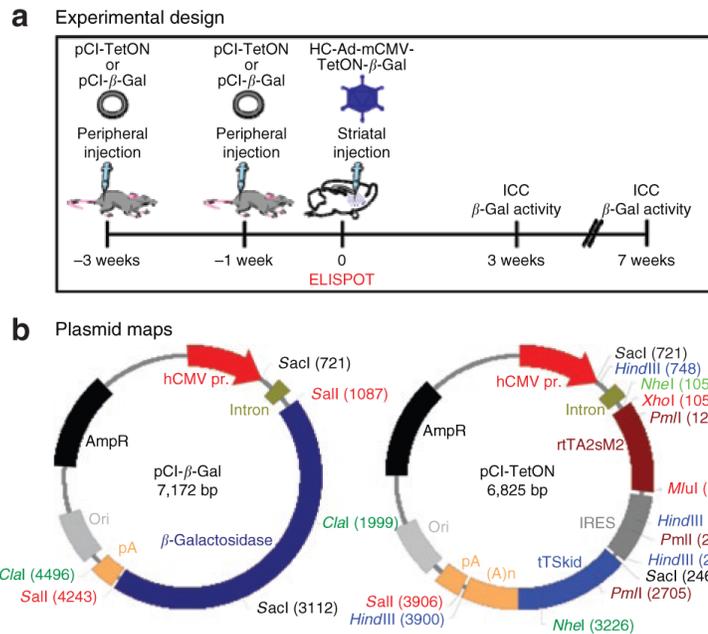


Figure 1. Experimental protocol of peripheral immunizations using plasmids encoding the TetON switch components $rtTA2^{SM2}$ and the tTS^{Kid} (pCI-TetON) and the reporter gene product β -Gal (pCI- β -Gal)

(a) Schematic representation of the experimental protocol of immunization and the experimental end-points. C57/Black6 mice were injected intramuscularly with plasmids encoding the components of the TetON switch, $rtTA2^{SM2}$ and the tTS^{Kid} (pCI-TetON) or the reporter gene β -Gal (pCI- β -Gal) in 50 μ l saline, or saline only ($n = 5$ animals per group), all containing 25 μ g of CpG. Two weeks later, the mice received a second intramuscular injection of pCI-TetON, pCI- β -Gal, or saline to boost the immune response against the TetON switch components or against β -Gal. One week after the second immunization, a group of mice received an intracranial injection of 1×10^7 blue forming units of the HC-Ad-mTetON- β -Gal, while other mice were killed and their spleens removed for performing an enzyme-linked immunosorbent spot assay to determine the effectiveness of the pre-immunization. The mice received chow containing doxycycline starting 24 hours prior to stereotactic brain surgery, so as to activate TetON-driven transgene expression. The mice were killed either 3 or 7 weeks after stereotactic surgery, and brain tissue was removed in order to perform β -Gal activity assay or β -Gal immunocytochemistry (ICC). (b) Maps of the plasmids used for immunization, indicating the constituents and orientation of the β -Gal (β -Gal) and TetON cassettes within the pCI-neo mammalian expression plasmid, both under the control of human cytomegalovirus (hCMV) promoter. IRES, internal ribosome entry site.

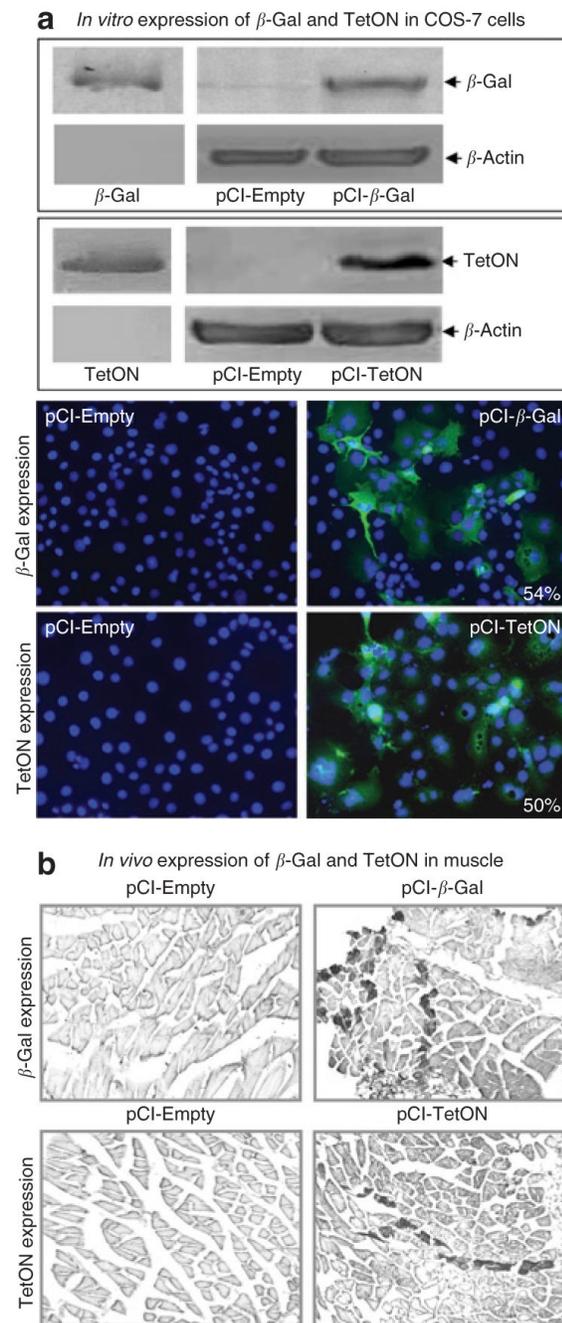


Figure 2. Expression of β -Gal and TetON protein *in vitro* and *in vivo* in the tibialis anterior muscles of mice

(a) Transgene expression from pCI-TetON and pCI- β -Gal in transfected COS-7 cells, as determined using Western blot (upper panel) and immunofluorescence (lower panel). As a negative control, COS-7 cells were transfected with a plasmid without transgene (pCI-Empty). Purified recombinant β -Gal or TetON protein were used as positive controls (left panels) for Western blot. The percentage of transduced cells, as determined by immunofluorescence, is depicted in the bottom right corner of the pictures. (b) C57/Black6 mice were injected with 100 μ g of plasmid DNA: pCI-Empty, pCI- β -Gal, or pCI-TetON (100 μ g DNA/50 μ l saline). Five days later, the animals were killed and the muscle tissue was removed for cryostat

sectioning. Transgene expression was determined in 10- μ m muscle sections, using immunocytochemistry.

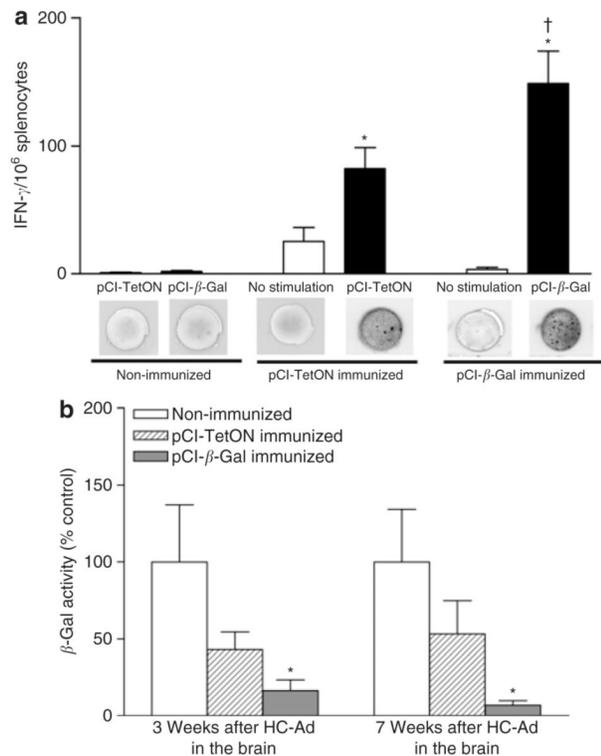


Figure 3. Assessment of the immune response against the components of the TetON switch, rtTA2^{SM2} and the tTS^{Kid}, or the reporter gene product β -Gal

Levels of high-capacity adenoviral vector (HC-Ad)-mediated β -Gal expression 3 and 7 weeks after intracranial administration. **(a)** Specific immune responses against the components of the TetON switch, rtTA2^{SM2} and the tTS^{Kid}, or the reporter protein β -Gal were determined 1 week after the second intramuscular injection, in mice pre-immunized with pCI-TetON and pCI- β -Gal. The spleens were removed and the number of interferon- γ (IFN- γ)-producing T cells was assessed using the enzyme-linked immunospot kit assay after 24 hours of stimulation with the trans-activator protein tTA2 (TetON) or the reporter protein β -Gal. The results are expressed as mean values \pm SEM of number of spots (IFN- γ producing cells) per 10⁶ splenocytes. $n = 3-4$ mice. * $P < 0.05$ versus no stimulation; † $P < 0.05$ versus pCI-TetON pre-immunized. Two-way analysis of variance followed by Newman—Keuls multiple-comparison test. **(b)** Transgene expression from HC-Ad-mTetON- β -Gal was determined in the brains of mice pre-immunized against the components of the TetON switch, rtTA2^{SM2} and the tTS^{Kid} (pCI-TetON), or the reporter protein β -Gal (pCI- β -Gal). β -Gal enzymatic activity assay was used for determining transgene expression levels 3 and 7 weeks after intracranial administration of HC-Ad-mTetON- β -Gal. Columns represent the mean values \pm SEM of β -Gal activity, expressed as *o*-nitrophenol produced (mg/ml)/sample protein content (mg/ml)/incubation time (min). $n = 4-7$ mice per group. * $P < 0.05$ versus non-immunized. Kruskal—Wallis test.

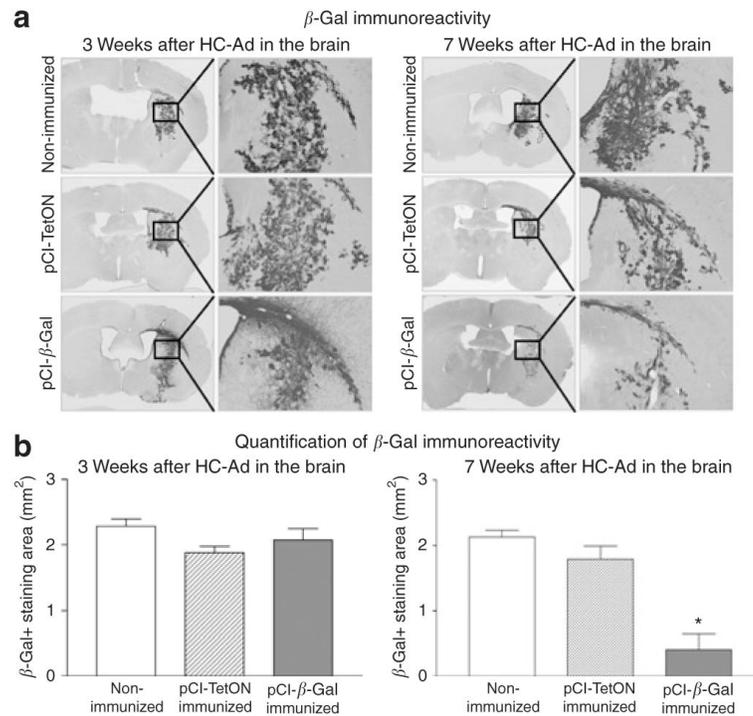


Figure 4. Distribution of β -Gal expression in the brains of mice pre-immunized against the components of the TetON switch, rtTA2^{SM2} and the tTS^{Kid}, or the reporter gene product β -Gal 3 and 7 weeks after intracranial administration of Hc-Ad-mTetON- β -Gal

The area of transgene expression from HC-Ad-mTetON- β -Gal was determined using immunocytochemistry, in the brains of mice pre-immunized against the components of the TetON switch, rtTA2^{SM2} and the tTS^{Kid} (pCI-TetON), or the reporter protein β -Gal (pCI- β -Gal). (a) Microphotographs show β -Gal staining in representative striatal sections from pre-immunized mice injected intracranially with HC-Ad- mTetON- β -Gal 3 weeks and 7 weeks earlier. (b) The β -Gal staining area was quantified using the Stereo Investigator software. Columns represent the mean values \pm SEM of β -Gal staining area (mm²). $n = 4-7$ mice per group. * $P < 0.05$ versus non-immunized. Kruskal—Wallis Test. HC-Ad, high-capacity adenoviral vector.

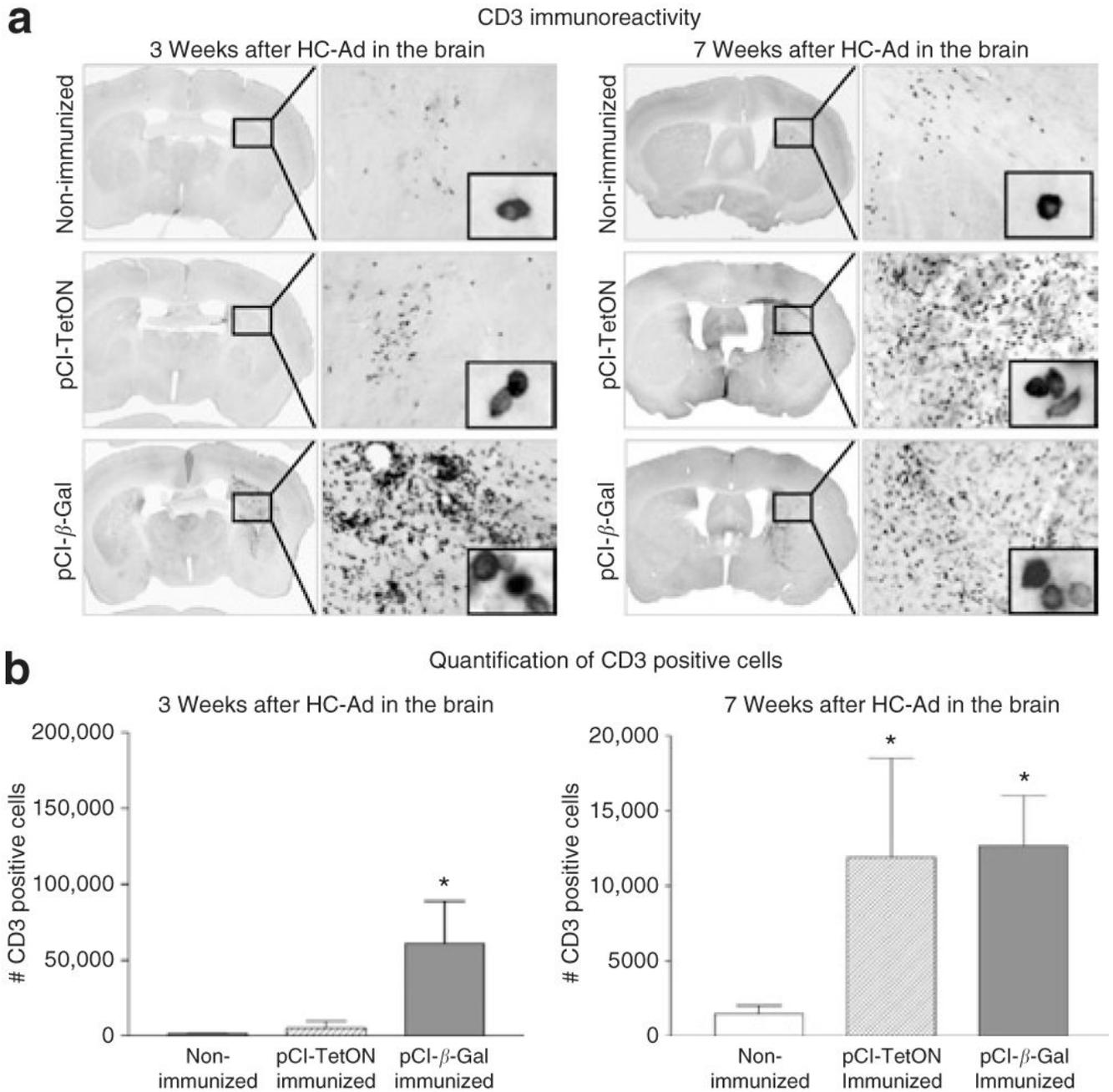


Figure 5. Infiltration of $cd3^+$ immune cells into the brains of mice pre-immunized against the components of the TetON switch, $rtTA2^{SM2}$ and the tTS^{Kid} , or the reporter gene product β -Gal 3 and 7 weeks after intracranial administration of Hc-Ad-mTetON- β -Gal

(a) The infiltration of immunoreactive $CD3^+$ cells was determined by immunocytochemistry in the brains of mice pre-immunized against the components of the TetON switch, $rtTA2^{SM2}$ and the tTS^{Kid} (pCI-TetON), or the reporter protein β -Gal (pCI- β -Gal). Microphotographs in panel a show infiltration of $CD3^+$ cells in representative striatal sections from pre-immunized mice intracranially injected with Hc-Ad-mTetON- β -Gal 3 weeks and 7 weeks earlier. (b) The number of $CD3^+$ cells was quantified using the Stereo Investigator software. Columns represent

the mean values \pm SEM of the number of CD3⁺ infiltrating cells. $n = 4-7$ mice per group. * $P < 0.05$ versus non-immunized. Kruskal—Wallis test. HC-Ad, high-capacity adenoviral vector.

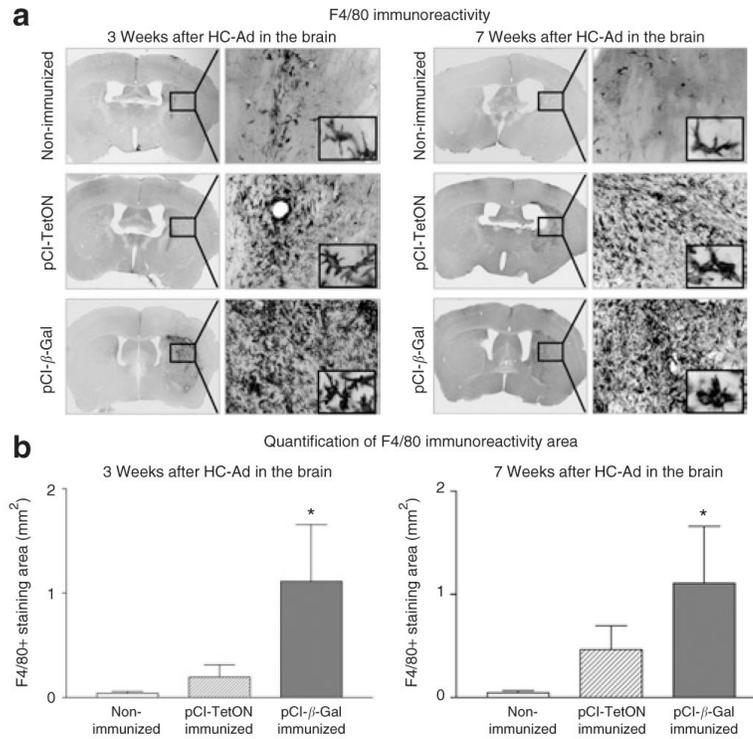


Figure 6. Infiltration of F4/80⁺ immune cells into the brains of mice pre-immunized against the components of the TetON switch, rTA2^{SM2} and the tTS^{Kid}, or the reporter gene product β-Gal 3 and 7 weeks after intracranial administration of Hc-Ad-mTetON-β-Gal

(a) The infiltration of F4/80⁺ cells was determined by immunocytochemistry in the brains of mice pre-immunized against the components of the TetON switch, rTA2^{SM2} and the tTS^{Kid} (pCI-TetON), or the reporter protein β-Gal (pCI-β-Gal). Microphotographs in panel a show infiltration of F4/80⁺ cells in representative striatal sections from pre-immunized mice intracranially injected with Hc-Ad-mTetON-β-Gal 3 and 7 weeks earlier. (b) The area infiltrated by F4/80⁺ cells was quantified using the Stereo Investigator software. Columns represent the mean values ± SEM of the area of F4/80⁺ cells (mm²). *n* = 4–7 mice per group. **P* < 0.05 versus non-immunized. Kruskal—Wallis Test. HC-Ad, high-capacity adenoviral vector.

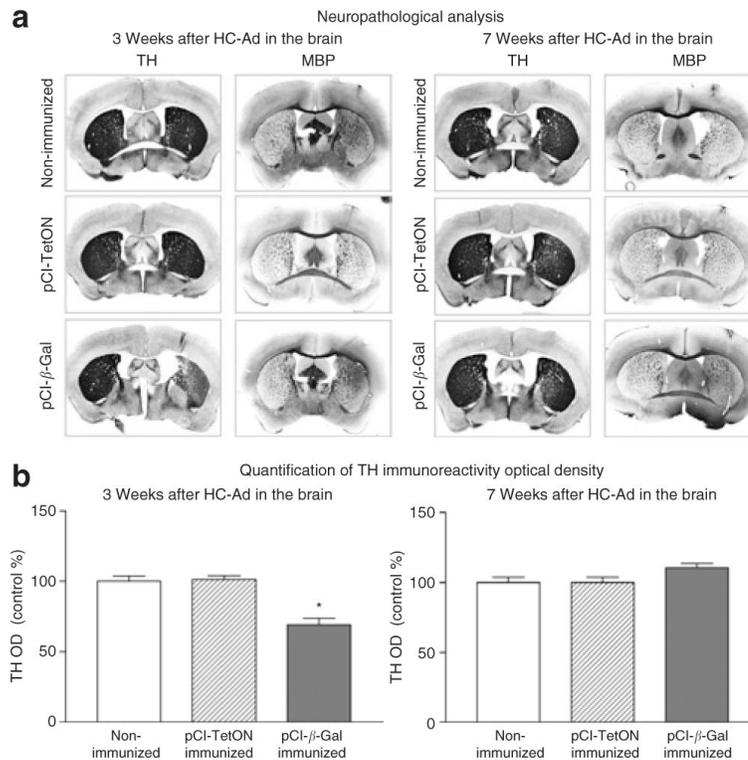


Figure 7. Tyrosine hydroxylase (TH) and myelin basic protein (MBP) immunoreactivity in the brains of mice pre-immunized against the components of the TetON switch, rTA2^SM2 and the tTS^{Kid}, or the reporter gene product β -Gal 3 and 7 weeks after intracranial administration of Hc-Ad-mTetON- β -Gal

TH and MBP were stained by immunocytochemistry in the brains of mice pre-immunized against the components of the TetON switch, rTA2^SM2 and the tTS^{Kid} (pCI-TetON), or the reporter protein β -Gal (pCI- β -Gal). **(a)** Microphotographs show TH and MBP immunoreactivity in representative striatal sections from pre-immunized mice intracranially injected with HC-Ad-mTetON- β -Gal 3 and 7 weeks earlier. **(b)** The optical density (OD) of TH staining was determined in the striatum in each side of the brain. Background staining was estimated at the corpus callosum in each side of the brain, and this value was subtracted from the OD of TH staining in either the HC-Ad-injected striatum or the contralateral striatum. Columns represent the mean values \pm SEM of the intensity of TH staining in the injected side of the brain versus the contralateral side, expressed as a percentage of the value obtained in non-immunized, control mice at 3 and 7 weeks after intracranial HC-Ad administration. * $P < 0.05$ versus non-immunized. Oneway analysis of variance followed by Newman—Keuls multiple-comparison test. HC-Ad, high-capacity adenoviral vector.