

ORIGINAL ARTICLE

The pros and cons of immunomodulatory IL-10 gene therapy with recombinant AAV in a *Cftr*^{-/-}-dependent allergy mouse model

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Cystic fibrosis (CF) patients have decreased levels of lung epithelial interleukin (IL)-10 and increased levels of proinflammatory cytokines (tumor necrosis factor- α , IL-4, IL-8 and IL-6). This has also been documented in *Cftr* (cystic fibrosis transmembrane conductance regulator)-deficient mice (*Cftr* 489X^{-/-}, *FABP-hCFTR*^{+/+}). Our laboratory has recently characterized a peculiar hyper-IgE phenotype in these mice, in response to *Aspergillus fumigatus* crude protein extract (Af-cpe). Thus, we hypothesized that sustained systemic circulating IL-10 levels achieved through skeletal muscle transduction with recombinant adeno-associated vectors expressing IL-10 (rAAV1-IL-10) would serve to downregulate Th1 and Th2 cytokine production. This in turn would dampen the allergic response in the *Cftr*^{-/-}-dependent mouse model of allergic bronchopulmonary aspergillosis. After Af-cpe

sensitization and airway challenge, mice treated with rAAV1-IL-10 had markedly lower IgE levels when compared to the control-treated rAAV1-GFP group. This was accompanied by a significant reduction in the levels of IL-5, IL-4 and IL-13 in the lung compartment. The lower lung cytokine profiles resulted in a near absence of eosinophil recruitment in the lung and a lower inflammatory response in the lung tissue of mice receiving rAAV1-IL-10. Unfortunately, sustained secretion of IL-10 from transduced muscle did lead to thrombocytopenia and splenomegaly in mice injected with rAAV1-IL-10. These results highlight that while IL-10 gene therapy is very effective for treating allergic responses caution must be taken with the prolonged secretion of IL-10. Gene Therapy advance online publication, 25 September 2008; doi:10.1038/gt.2008.156

Keywords: allergy; *Aspergillus*; Cystic fibrosis; IL-10; AAV; thrombocytopenia

Introduction

Cystic fibrosis is the most common autosomal recessive genetic disease in the United States, affecting up to 1 in 3000 live births. Patients with cystic fibrosis (CF) suffer a wide range of pathological consequences of the disease including pancreatic insufficiency, with subsequent malabsorption and impaired nutrition, and chronic lung infection with *Pseudomonas aeruginosa* and other pathogens.^{1–4} The connection between cystic fibrosis transmembrane conductance regulator (CFTR) mutation and the propensity to chronic lung infection and inflammation is not clearly defined, but it appears to result from a combination of effects, including altered ion and water transport in the airway epithelium,^{5–7} increased binding of *P. aeruginosa*^{8,9} and increased proinflammatory cytokine production in the CF airway.^{10–14} The overproduction of inflammatory cytokines is also accompanied by lower levels of the anti-inflammatory interleukin (IL)-10.

Some studies have shown that IL-10 secretion in the bronchoalveolar lavage (BAL) of CF patients is reduced; still others have reported that T-lymphocyte clones from CF patients also have decreased production of IL-10.^{15–18} These results along with other studies suggest that IL-10 deficiency in CF plays a major role in the dysregulation of inflammatory responses observed in cystic fibrosis.^{19–21} For these reasons much interest has been focused on the potential role that IL-10 plays in the altered inflammatory and infectious lung processes in CF. Data from murine models have consistently supported the view that IL-10 is an important regulator of the response to endobronchial infection with *P. aeruginosa* and T-cell-mediated responses to *A. fumigatus*.^{22–24} Skewed, aberrant or ineffective immune responses towards these two particular pathogens are deleterious to CF patients. In fact, CF patients were recently characterized to have excessive IL-4 and IL-13 production, which may have a direct influence in the prevalence of allergic asthma in this population.^{25–28} Approximately 5.5% of American children are afflicted with asthma. Although this represents a very high prevalence, asthma is much more common in children with CF, where the rate of bronchial hyper-responsiveness approaches 50%, and up to 10–15% may experience a hyper-IgE asthma variant

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known as allergic bronchopulmonary aspergillosis (ABPA).^{29–40} ABPA is characterized by a predominantly Th2-mediated immune response to inhaled *Aspergillus* antigens in the airways, leading to reversible airway obstruction and bronchial hyper-reactivity. This inflammatory response correlates with the local infiltration of effector cells including mast cells, activated neutrophils, eosinophils, basophils and macrophages into the airway mucosa and the airway lumen.^{41,42} Although ABPA is a multifactorial disease, there is a crucial role for lymphocytes producing mainly type-2 cytokines. The recruitment and/or activation of effector cells appear to be orchestrated by the secretion of certain cytokines including IL-4, IL-5, IL-9 and IL-13. Thus, it is conceivable that aberrant cytokine regulation^{43–46} and lower IL-10 activity, which is a recently characterized feature of CF airway epithelial cells, could promote the development of Th2 allergic type responses to Af antigens.

The interest in IL-10 stems from its broad immunoregulatory activity as it has been shown to be produced by several cell types, including macrophages, monocytes, T lymphocytes, B cells, dendritic cells, mast cells and in certain cases also tumor cell lines.^{47–51} Specifically, IL-10 will inhibit the synthesis of the proinflammatory cytokines IL-1 β , tumor necrosis factor- α , IL-6 along with the Th2-derived cytokines IL-4 and IL-5. The latter two cytokines have been shown to play an important role in the development of the allergic response and are involved in IgE production, eosinophil recruitment and maturation. More importantly related to lung inflammation in CF, IL-10 downregulates IL-8 and eotaxin. IL-8 (or its mouse analog KC) is upregulated in CF⁵² and increases cell trafficking to the lung. Eotaxin is also involved in eosinophil recruitment to the lungs during allergic exacerbations such as those seen in ABPA, which commonly complicates CF. As IL-10 is constitutively produced in bronchial epithelial cells¹⁶ and is found in smaller amounts in BAL of CF patients,¹⁹ it is of considerable interest in regulating the pulmonary immune response in the Cftr-dependent ABPA model developed in our laboratory.⁵³ In previous studies, IL-10 has been shown to effectively prevent inflammation and injury in several animal studies.^{54–57} Intratracheal delivery of adenoviral vectors expressing IL-10 have been previously shown to decrease lung inflammation but not IgE levels in an ovalbumin asthma mouse model.⁵⁸ However, to this date clinical studies with IL-10 remain difficult due to its short half-life (1.1–2.6 h) in serum and the requirement for subcutaneous or intravenous routes of administration.⁵⁹

The studies described here were performed in a previously described Cftr^{-/-} mouse model that is characterized by a peculiar hyper-IgE phenotype, in response to *A. fumigatus* crude protein extract (Af-cpe).⁵³ We hypothesized that sustained systemic circulating IL-10 levels achieved through skeletal muscle transduction with recombinant adeno-associated vectors expressing IL-10 (rAAV1-IL-10) would circumvent the problems associated with short half-life of IL-10 protein therapy and would serve to dampen the allergic response in the Cftr^{-/-} ABPA model. The systemic circulation of IL-10 would also lower IgE levels, which was not achieved in previous studies using local delivery of AAV1-IL-10.⁵⁸ Although our results indicate that IL-10 acted to inhibit pulmonary eosinophilia and inflamma-

tion and was successful at lowering both IgE levels and proinflammatory cytokine production, they also uncovered serious side effects associated with sustained IL-10 serum levels. Serum IL-10 levels correlated with thrombocytopenia, splenomegaly and liver inflammation.

Results

rAAV1-IL-10 gene dose-response study

Recombinant AAV vectors were used to transduce muscle to serve as site of secreted protein production with the aim of reaching systemic circulation.⁶⁰ To determine the appropriate dose of vector needed to reach systemic IL-10 levels between 200 and 500 pg ml⁻¹, (previously demonstrated to be therapeutic in a NOD diabetes model^{56,57,61}) three dose groups of B6 mice (low, medium and high, that is, 2.8×10^{10} , 1.4×10^{10} and 7.4×10^9 vector genomes per mouse) and one group of Cftr^{-/-} mice (medium dose that is, 1.4×10^{10}) were injected with pseudotyped rAAV1-IL-10. To assess transgene function, serum cytokine levels were determined throughout the length of the study (Figure 1). This study identified elevated IL-10 levels in the serum of rAAV1-IL-10-transduced animals that were not detectable in the control animals (rAAV1-GFP) or were below the limit of detection of our assay (16 pg ml⁻¹). From the dose-response study it was determined that mice receiving 1.4×10^{10} particles of virus (medium dose) achieved levels within the desired range. The mice were followed for 1 year and IL-10 levels were present throughout the duration of the study and reached a plateau through day 120 after which they dropped back down.

The drop in systemic levels of IL-10 was significant across all dose groups. Despite the negligible detection of IL-10 in the later months of the study, local staining of the rAAV1-IL-10 injected muscle 1-year post-vector administration still showed presence of IL-10 production (Figures 2a and c). Along with the local production of IL-10 in the injected muscle, the hematoxylin and eosin staining revealed a significant amount of lymphocyte accumulation at the site of injection (Figures 2b and d). This inflammation was not observed in the control animals injected with rAAV1-GFP.

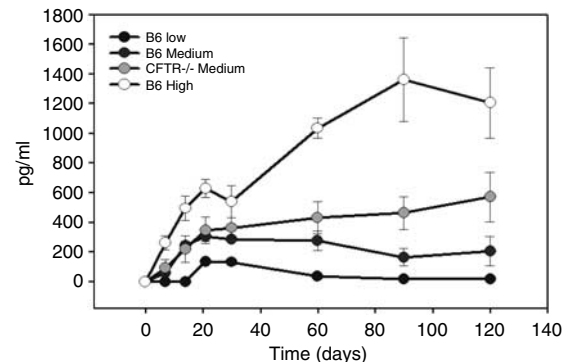


Figure 1 Recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV1-IL-10) dose-response in B6 and Cftr^{-/-} mice. Three different groups of B6 mice and one group of Cftr^{-/-} mice were utilized for a dose-response with low, medium and high dose (2.8×10^{10} , 1.4×10^{10} and 7.4×10^9 vector genomes per mouse) of rAAV1-IL-10. The dose-response measured systemic IL-10 over a 120 day time period. Results are reported as group means $\pm$ s.e.m. (N = 4).

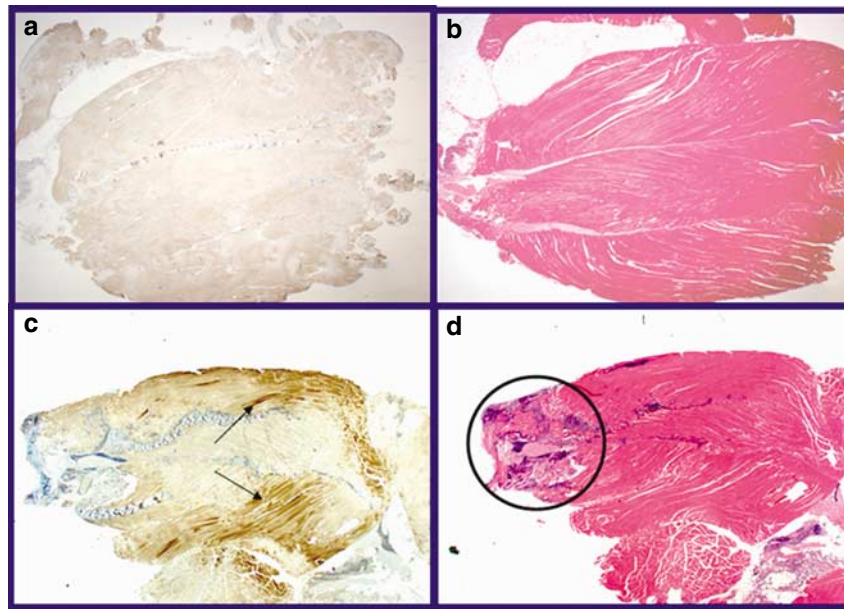


Figure 2 Representative sections of interleukin (IL)-10 and hematoxylin and eosin (H&E) staining of recombinant adeno-associated vectors expressing IL-10 (rAAV-IL-10) injected and non-injected muscle. *Cftr*^{-/-} gastrocnemius muscle stains (a) (non-injected) and (c) (rAAV1-IL-10) are IL-10-specific stains. (b) (non-injected) and (d) (rAAV1-IL-10) are H&E stains circled area indicates extensive lymphocyte infiltration as determined by dark blue staining.

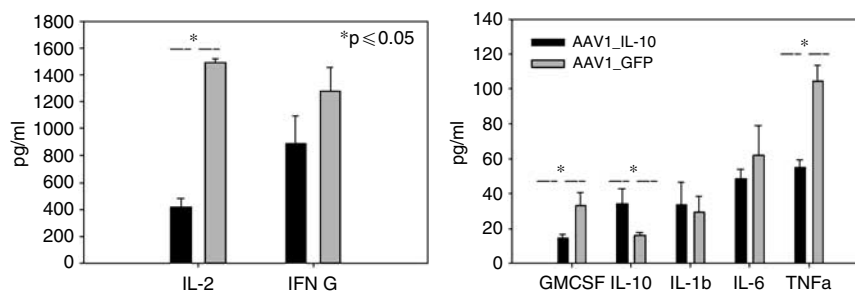


Figure 3 ConA cytokine profile of splenocytes from recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV-IL-10) and rAAV-GFP injected *Cftr*^{-/-} mice. Splenocytes from *Cftr*^{-/-} mice were harvested and stimulated with ConA at concentration of 0.5 μg per well. Supernatants were collected 48 h after activation and were analyzed for several cytokines using a Luminex assay. Results are reported as group means ± s.e.m. (*N* = 4).

Splenocytes from the *Cftr*^{-/-} mice in the mid-dose injected rAAV1-IL-10 group were harvested at the time of sacrifice and stimulated with ConA, to compare their cytokine production with the rAAV1-GFP control group. In the rAAV1-IL-10 group (Figure 3), the secretion level of IL-10 was high, whereas secretion of many of the proinflammatory cytokines decreased 48 h post-ConA stimulation. Importantly, IL-2, interferon-γ, tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor were all secreted in lesser quantities in mice expressing IL-10.

Immunomodulatory effects of rAAV1-IL-10 on circulating antibody levels

With the optimal vector delivery dose established, two groups of mice were used to test whether systemic IL-10 would be able to attenuate the allergic ABPA response in *Cftr*^{-/-} mice. We hypothesized that the immunosuppressive effect of IL-10 would downregulate cytokines such as IL-4, IL-13 and IL-5 and prevent eosinophilic inflammation and isotype switching in B cells. To test

this, a control group of mice was injected with 1.4×10^{10} vector genomes (vg) of rAAV1-GFP to control for any possible vector or nonspecific transgene effects. The treatment group received 1.4×10^{10} vector genomes (vg) of rAAV1-IL-10.

The mice were then exposed to Af-cpe to reproduce our previously described ABPA model.⁵³ As in previous studies, mice were allowed 2 weeks between vector dosing and the administration of the first intraperitoneal (i.p.) injection with Af-cpe. Serum IgE levels were measured before the first i.p. injection, before the second i.p. injection and 48 h after the last challenge (Figure 4a).

Both groups demonstrated negligible IgE levels at baseline with the IgE levels being marginally higher at the time of the second i.p. injection in the rAAV1-IL-10 group. However, by the end of the study the rAAV1-IL-10 group had a significant reduction (40%) in the total serum IgE levels. To further characterize the effects of IL-10 on B-cell function, we analyzed the end point serums for the levels of other antibody isotypes. This revealed that IL-10 had a significant inhibitory effect on all the antibody isotypes (Figure 4b). The levels of both

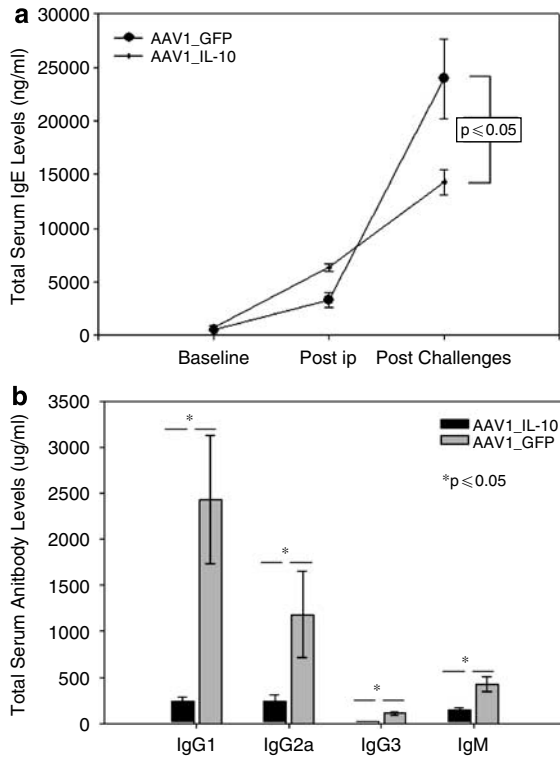


Figure 4 (a) Total serum IgE levels 48 h after *A. fumigatus* crude protein extract (Af-cpe) challenge. Total serum IgE levels in Af-sensitized Cfr^{-/-} mice injected with recombinant adeno-associated vectors expressing IL-10 (rAAV1-IL-10), Cfr^{-/-} mice injected with rAAV1-GFP mice. Cfr^{-/-} mice were sensitized with 200 µg of *A. fumigatus* crude extract dissolved in 200 µl of phosphate-buffered saline on days 1 and 14. The blood samples were collected 48 h after the third and last Af challenge. Total serum IgE in the *A. fumigatus*-sensitized mice were measured by enzyme-linked immunosorbent assay. Results are reported as group means ± s.e.m. (N = 4). (b) Total levels of serum circulating antibody isotypes. Total serum IgG subclass pattern in Cfr^{-/-} mice sensitized with *A. fumigatus*. Total serum IgG1, IgG2b and IgM were measured in the *A. fumigatus* sensitized receiving rAAV1-IL-10 and rAAV1-GFP-injected mice using a multiplex bead assay on day 32, 48 h after the last challenge. Mean ± s.e.m. (N = 4).

IgG1 and IgG2a were significantly lower in mice treated with IL-10, which suggests that IL-10 was responsible for a general suppression of B-cell antibody production. This is further demonstrated by the significantly lower levels of IgG3 and IgM in the rAAV1-IL-10 group compared with the rAAV-GFP control group.

rAAV1-IL-10 cytokine suppression

We then analyzed the cytokine profiles within the lung compartment, as reflected in the BAL. It was observed that systemic IL-10 had potent effects within the local immune environment of the lung. The most important cytokines involved in the allergic ABPA response, IL-4, IL-13 and IL-5, were all lower in the lungs of the mice that had been treated with rAAV1-IL-10 (Figure 5). Additionally, the level of KC, which is usually elevated in the Cfr^{-/-} mice, was also lowered by the rAAV1-IL-10 treatment. These results further strengthen the notion that IL-10 was responsible for a general suppression of cytokine production shutdown as opposed to a Th2 → Th1 shift.

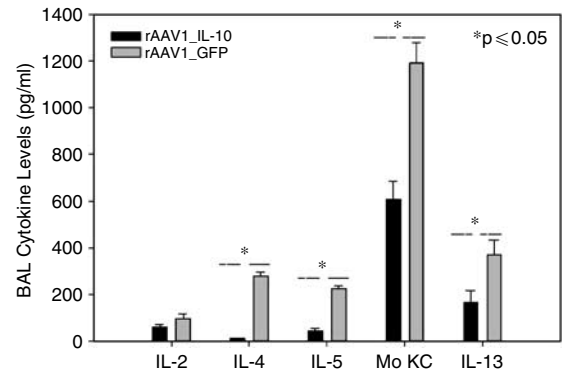


Figure 5 Cytokine levels in the bronchoalveolar lavage (BAL) of recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV1-IL-10)- and rAAV-GFP-treated Cfr^{-/-} mice. The effects of *A. fumigatus* crude extract aerosol challenge on the cytokine levels in the BALs of rAAV1-IL-10- and rAAV1-GFP-injected mice. Results are expressed as means ± s.e.m (N = 4).

Amelioration of ABPA-related pathology in IL-10-treated mice

The reduced production of cytokines in the lung compartment had significant effects on the overall immune responses in the lungs. The reduction in inflammatory cytokines in the lungs was accompanied by a difference in the amount and composition of cellular infiltrates in the BAL. As can be seen in Figure 6a, IL-10 treatment attenuated lymphocyte, polymorphonuclear and eosinophil recruitment to the lungs, with BAL cytopins from the IL-10 group mainly composed of macrophages with very few eosinophils (less than 5%) (Figure 6b).

The histological findings reflect this lack of eosinophilic infiltrate in the lungs and are consistent with ability of IL-10 to downregulate IL-5 and eotaxin production.

The decrease in eosinophil recruitment is evident within the periodic acid-Schiff-stained lung tissue. As shown in Figure 7, the lung sections from the rAAV1-GFP control mice demonstrate the usual degree of airway inflammation and goblet cell hyperplasia, accompanied by lymphocytic and eosinophilic infiltration as seen in previous reports.⁵³ This is nearly absent in the lung tissue from the rAAV1-IL-10-injected mice. Table 1 shows the overall inflammatory scores on the lung sections as determined by a pathologist blinded as to the assignment of specific mice. All the rAAV1-GFP-treated mice demonstrated inflammatory scores of 2 (4 being the most severe, 3-multifocal or severe, 2-multifocal or moderate, 1-focal or mild and 0 being normal), while the majority of the samples in the rAAV1-IL-10 group had a score of 1.0.

Adverse effects from rAAV1-IL-10 therapy

The effects of IL-10 are widespread. Treatment not only lowers IgE levels but antibody secretion across all isotypes and it also prevents inflammation in the lung as evidenced by the histology and the BAL cell composition and infiltration. However, these studies also show some of the side effects that can be associated with the sustained expression of IL-10. From the dose-response study it was observed that rAAV1-IL-10-treated

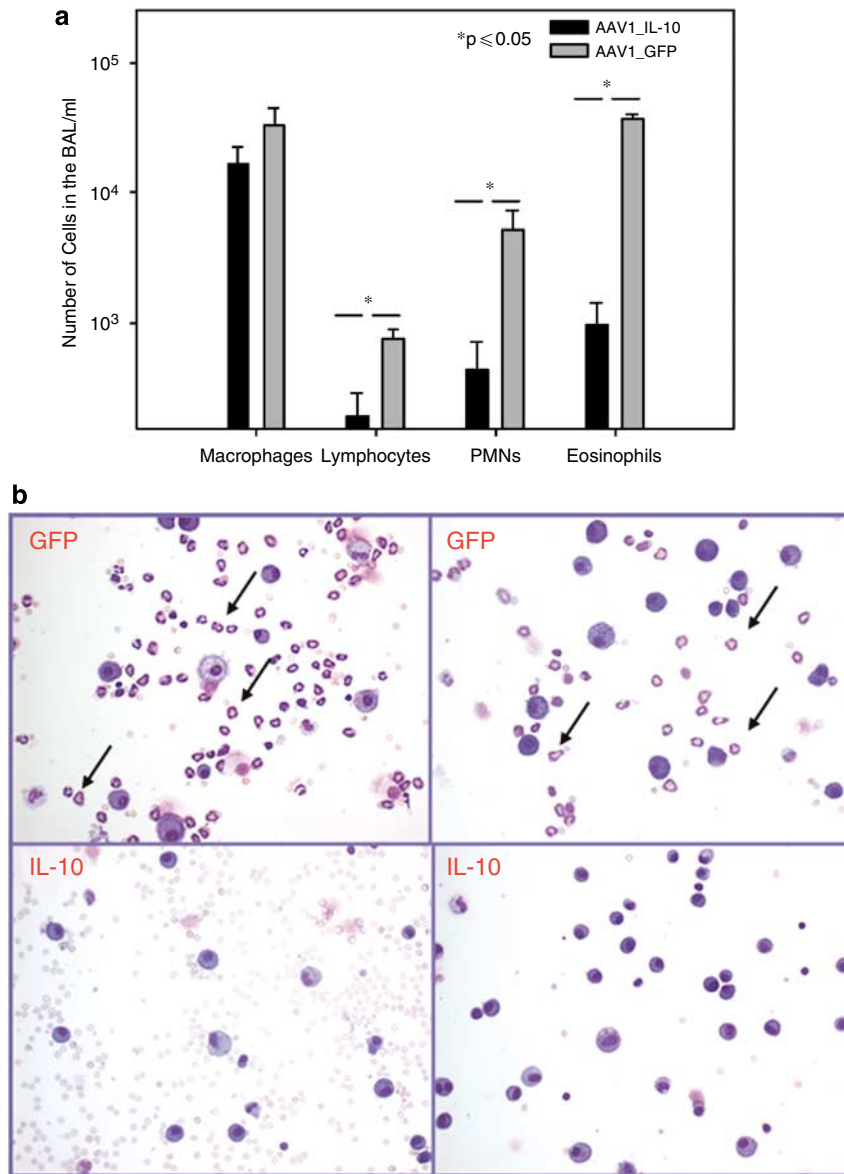


Figure 6 (a) Bronchoalveolar lavage (BAL) cell counts after *A. fumigatus* crude protein extract (Af-cpe) exposure in interleukin (IL-10) and green fluorescent protein (GFP) mice. Results are expressed as means ± s.e.m. (N = 4). (b) Wright–Giemsa stain of the BAL cytospin samples. The upper panels are representative images from the rAAV1-GFP control group. The arrows indicate characteristic eosinophils. The bottom panels are representative images from the recombinant adeno-associated vectors expressing IL-10 (rAAV1-IL-10)-injected group.

mice developed anemia accompanied by thrombocytopenia. As shown in Figure 8 there is a clear correlation between the IL-10 dose and platelet levels. rAAV1-IL-10-injected mice also had splenomegaly as indicated by markedly greater spleen weights when compared with rAAV1-GFP controls (Figure 8).

Histological analysis of spleen sections from the AAV1-IL-10-injected mice showed morphological changes that suggested disruption in the follicular structure of the organ. The Prussian blue stain also revealed abnormally low amounts of hemosiderin deposition in these spleens (Figure 9). The GFP (green fluorescent protein) controls had normal spleen morphology and hemosiderin deposition. The overall morphology of the spleens can be appreciated in the low magnification panels b and d, while the panels a and c show clear difference in hemosiderin (iron) staining.

In addition to the striking morphologic defects in the spleen of the IL-10-injected mice, further evaluation of organs collected during the necropsies showed an IL-10-associated inflammation in the liver. The liver infiltrates were comprised of more polymorphonuclear cells in the rAAV1-IL-10-injected mice than the control mice and included hepatocyte degeneration. In the IL-10-treated mice, infiltrates along portal tracts were widespread with moderate-to-severe extensions into surrounding areas. Focal hepatocyte degeneration in the parenchyma was also widespread and focally severe (Figure 10).

Further evaluation of the liver was performed by using a Prussian blue stain to determine iron deposition levels in the liver. In contrast with the results from the spleens in the rAAV1-IL-10 group, the livers from these animals had significant amount of hemosiderin deposits along the inflamed tracts of the liver. This can be seen

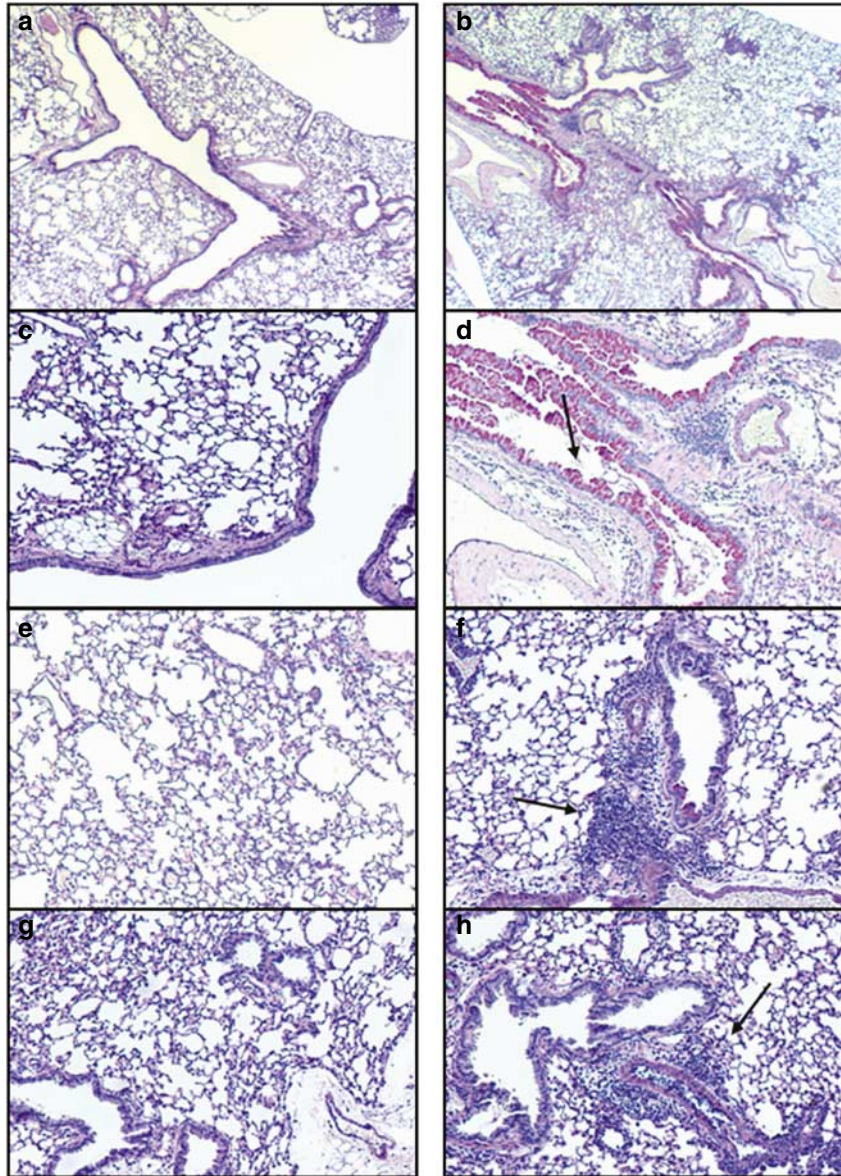


Figure 7 Representative periodic acid-Schiff lung sections from $Cftr^{-/-}$ mice 48 h after challenges. $Cftr^{-/-}$ mice were sensitized with 200 μ g of *A. fumigatus* crude extract and were treated with recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV-IL-10) (a, c, e, g) or rAAV-GFP (b, d, f, h). (a and b) Are $\times 5$ and all other panels are $\times 10$ images. Arrows indicate airway inflammation and goblet cell hyperplasia accompanied by lymphocytic and eosinophilic infiltration.

Table 1 Relative inflammatory scores on lung hematoxylin and eosin sections

	0.5	1	1.5	2
rAAV1-IL-10	20%	60%	20%	—
rAAV1-GFP	—	—	—	100%

when comparing panels a and b from IL-10-injected mice with panels c and d from the GFP-control group in Figure 11.

Discussion

In CF patients, the massive influx of proinflammatory cytokines in the lung is accompanied by very low

levels of the anti-inflammatory cytokine, IL-10.^{19,37,62,63} Although the typical inflammatory lung disease elicited by *pseudomonas* infection has been modeled in $Cftr^{-/-}$ mice,⁵² our group was the first to describe a somewhat simpler experimental method to elicit a difference in the lung inflammation in CF mice. ABPA represents a common allergic complication in CF patient's airways. We hypothesized that the exaggerated IgE inflammatory response seen in the murine CF-ABPA model can be downregulated with rAAV vectors expressing the anti-inflammatory cytokine IL-10. The main features of this ABPA model include high IgE levels, elevated peripheral and lung eosinophils, pulmonary inflammation, elevated levels of IL-4, IL-5, IL-13 and interferon- γ , and goblet cell hyperplasia. As shown in the previous studies, what set the $Cftr^{-/-}$ phenotype apart from $Cftr^{+/+}$ mice in this model is the

higher IgE levels attained in the allergic response toward Af-cpe.⁵³

In our first studies, a single intramuscular injection of AAV1-IL-10 vectors led to a systemic transgene expression for more than 4 months. The levels of IL-10 were observed to drop after this time; however, as confirmed by the muscle histology local production of IL-10 was still present more than 1 year after injection. Lymphocyte accumulation could provide an explanation for the decline in IL-10 production in the later months. As this was observed 1 year after injection, it suggests a persistent and chronic reaction elicited by the transgene

product. It is not clear whether this represented the local biological activity of IL-10 or an immune response to IL-10. Although this was not determined, it is possible that the transduced myofibers were damaged or killed as a result of the cellular infiltration. It can be speculated that this chronic inflammation is more likely to be an effect of IL-10 expression itself rather than immune response to the AAV1 vectors or the transgene itself, as this was not observed in the rAAV1-GFP group. This would be consistent with the notion that immunostimulatory effects of IL-10 include the increase in cytotoxic T-lymphocyte activation.

Having determined that the medium dose group produced IL-10 levels that were consistent and within the desired range, two cohorts of *Cftr*^{-/-} mice were intramuscularly injected with either rAAV1-IL-10 or rAAV1-GFP 2 weeks before the first i.p. sensitization of Af-cpe. After the Af-cpe challenges, serum from three time points was examined for IgE levels. It was observed that before Af-cpe sensitization all mice had negligible levels of IgE antibodies. After the Af-cpe lung challenges, IgE levels peaked and were 40% lower in the IL-10-treated group. In addition, IL-10 treatment was also effective at decreasing the secretion of IgG1, IgG2, IgG3 and IgM, suggesting that general B-cell activation was greatly attenuated.

The BALs of the two cohorts in these studies demonstrated findings consistent with the ability of IL-10 to downregulate nuclear factor- κ B-dependent cytokines. The mice treated with rAAV1-IL-10 exhibited markedly lower levels of KC (IL-8 mouse analog), IL-4 and IL-13 in the BAL. Similarly the non-nuclear factor κ B-dependent cytokine IL-5 was also inhibited by IL-10 treatment. This reduction in IL-5 although not directly involved in the production of IgE plays a significant role in the therapeutic benefits of IL-10 for allergic responses. It is this cytokine along with eotaxin that are responsible for the second arm of the allergic response, which involves the maturation and recruitment of eosinophils to the site of inflammation. Clearly IL-10 served to prevent this recruitment as evidenced by the significantly decreased influx of eosinophils in the lungs of the treated mice. It is evident that the circulating levels of IL-10 not only affect systemic antibody secretion but also exerts a direct effect on the local environment of the lung. The overall anti-inflammatory properties of IL-10 also reduced the inflammatory state of the lung, as

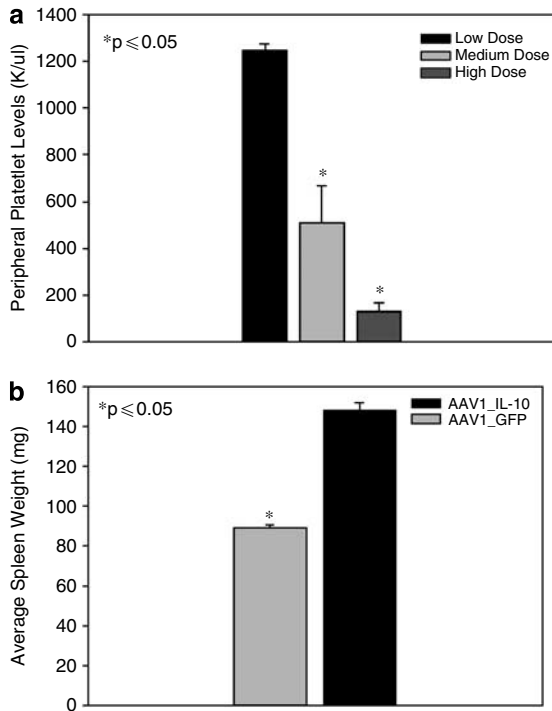


Figure 8 Side effects associated with prolonged and sustained interleukin (IL)-10 gene therapy. (a) Illustrates the levels of peripheral platelet cells in dose-response group's low, medium and high dose (2.8×10^{10} , 1.4×10^{10} and 7.4×10^9 particles per mouse) of recombinant adeno-associated vectors expressing IL-10 (rAAV-IL-10). (b) Indicates the difference in spleen weight between rAAV-IL-10-injected mice and rAAV-GFP control. Results are reported as group means $\pm$ s.e.m. ($N = 4$).

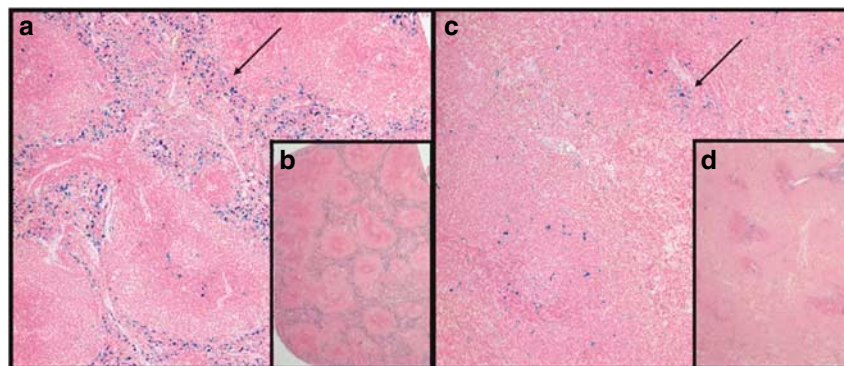


Figure 9 Prussian Blue stain of spleen section. (a) ($\times 10$) and (b) ($\times 2.5$) are sections of the spleen from rAAV-GFP-injected mice. (c) ($\times 10$) and (d) ($\times 2.5$) are sections of the spleen from recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV-IL-10)-injected mice. Arrows indicate iron depositions.

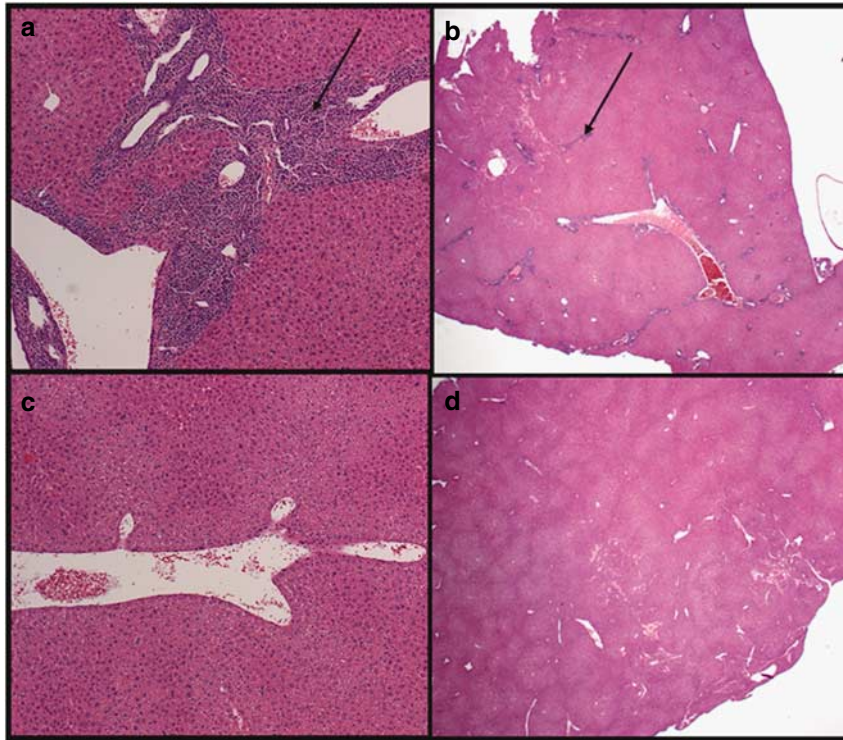


Figure 10 Hematoxylin and eosin stained liver sections. (a) ($\times 10$) and (b) ($\times 2.5$) are recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV-IL-10)-injected mice. (c) ($\times 10$) and (d) ($\times 2.5$) are rAAV-GFP-injected mice. Arrows indicate liver infiltrates.

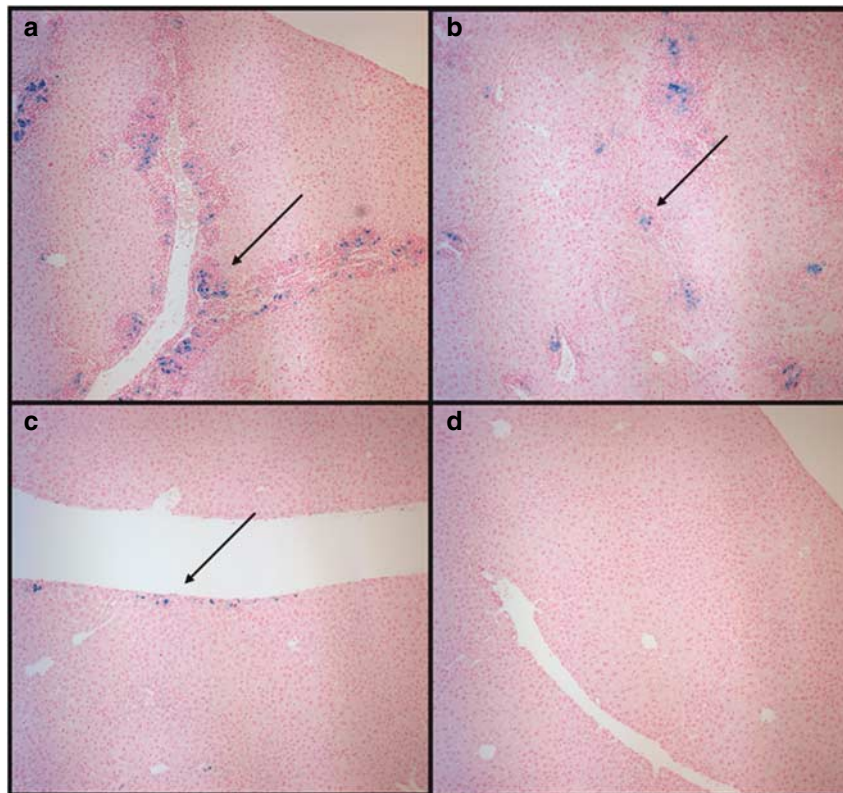


Figure 11 Prussian Blue stain of liver sections. (a and b) ($\times 10$) are recombinant adeno-associated vectors expressing interleukin (IL)-10 (rAAV-IL-10)-injected mice. (c and d) ($\times 10$) rAAV-GFP-injected mice. Arrows indicate iron depositions in the liver.

can be seen from the histological findings inflammation is nearly absent in the lung tissue of the IL-10-treated mice.

Unfortunately, the sustained systemic levels of IL-10 had multiple side effects in treated mice. From the early IL-10 dose-response study, it was found that there was a

negative correlation between serum IL-10 levels and platelet counts in the blood. All of the mice in the high-dose group were thrombocytopenic and those mice that were grouped in the middle dose on average were at the low end of normal platelet counts. Only mice from the low-dose group maintained normal platelet levels. These results suggest an effect of IL-10 on platelets that may be derived from its ability to block or suppress the production of cytokines from monocytes, macrophages and progenitor cells that play role in the hemopoiesis development. The granulocyte-macrophage colony-stimulating factor, which was reduced (see Figure 3), and other thrombopoietic factors such as IL-3 could fall within this category. In fact, this IL-10-associated thrombocytopenia has been reported recently by Sosman *et al.*,⁶⁴ in human patients receiving a recombinant human IL-10 protein subcutaneously for 10 days. This study also unmasked a twofold reduction in the platelet counts in patients receiving the IL-10 protein when compared to the placebo cohort; it was also noted that the thrombocytopenia resolved once the patients stopped receiving protein with no observable lasting toxic effects.

Our studies, however, point to a more complex toxicity to sustained gene therapy with IL-10. Along with the thrombocytopenia, mice also suffered from splenomegaly. Although this may be tied to the fact that there could be an increased sequestration of platelets in the spleen, the histological findings reveal a more complex situation. Spleens from mice receiving IL-10 not only were larger, they also had extensive follicular degeneration. When the spleens were stained for hemosiderin deposition to determine if their enlargement could in part be due to RBC sequestration, it was surprising to find an almost complete absence of iron deposition. Hyperfunction of the spleen as a result of clearing RBC in autoimmune anemias results in its enlargement, which would also show a high amount of iron deposition; however, this was not the case in our study. (Instead it appears that the spleen is not sequestering RBC or platelets but that the development of these is being inhibited by IL-10, further studies are needed to determine this.) The toxicity of IL-10 was not isolated to hematopoiesis and the spleen but also the liver. Hematoxylin and eosin sections of the liver revealed consistent and severe accumulation of lymphocytes along the portal vein tract. This is only evident in mice injected with AAV1-IL-10 and is absent in the GFP group. Focal hepatocyte degeneration in the parenchyma is also widespread and focally severe. On the contrary to the spleens the iron deposits are evident in the liver of IL-10 and not the GFP mice. Although there were no overt signs of liver failure in these mice, iron deposition in the liver can lead to hemochromatosis and eventually liver failure.

In summary, our studies demonstrate that intramuscular rAAV1-IL-10 treatment inhibits the synthesis of a number of cytokines including IL-4, IL-13, IL-5, IL-2 and tumor necrosis factor- α both in the lung and from activated splenocytes. This along with the other known functions of IL-10 involving the downregulation of antigen presentation and costimulatory molecules lead to the reduction in the IgE antibody levels as well as to a very effective shutdown of the inflammatory process in the lung of the Cftr^{-/-}-dependent ABPA model. The exact mechanism of IL-10 action in this model warrants

further investigation. However, intramuscular rAAV1-IL-10 gene therapy appears to mediate high enough levels of IL-10 to impart a state of anergy in B and T cells probably potentiated by the delay of suppressed maturation of antigen-presenting cells. Unfortunately, the subdued immune system and the decreased release of cytokines, such as granulocyte-macrophage colony-stimulating factor, could also have played a part in the altered platelet counts in treated animals. It is important to note that sustained IL-10 secretion in addition to splenomegaly caused liver inflammation, which eventually could lead to liver degeneration and fibrosis. One aspect of IL-10 overexpression that was not addressed by these studies is whether in the presence of live *A. fumigatus*, this type of intervention could actually lead to invasive aspergillosis by subduing macrophage function and fungal clearance. This is particularly of concern as certain studies have revealed possible associations between alleles in the IL-10 promoter region responsible for higher IL-10 secretion and *A. fumigatus* colonization of lung.⁶⁵ These potential toxicities indicate that caution must be exercised in considering clinical applications of the potent immunomodulatory effects of rAAV1-IL-10, which might otherwise be therapeutic in a number of human diseases.

Materials and methods

Mouse strains

The primary Cftr knockout strain (Whitsett mouse) used for these studies was the Cftr S489X^{-/-} neo insertion in C57BL/6 mice developed initially at the University of North Carolina⁶⁶ and then modified with the transgenic gut-specific expression of the human CFTR gene driven by the fatty acid-binding protein (FABP) promoter to prevent the intestinal obstruction and improve viability.

Muscle injection

For muscle administration of the rAAV1 vector expressing GFP or IL-10 under control of the chicken β -actin promoter, 5- to 6-week-old Cftr S489X^{-/-}; FABP-hCFTR mice were anesthetized with 3.5% isoflurane inhalation. 50 μ l of viral vector suspension was administered percutaneously by intramuscular injection into the right or left gastocnemius muscle 2 weeks before (day -14) Af-cpe sensitization.

Aspergillus sensitization and challenge

Five- to six-week-old Cftr S489X^{-/-}; FABP-hCFTR were housed in the SPF mouse colony of the University of Florida according to the NIH guidelines and were allowed access to food and water *ad libitum*. All experimental procedures were approved by the IACUC of the University of Florida. Animals were sensitized to Af-cpe (Greer Laboratories, Lenoir, NC, USA). Briefly, animals were administered with i.p. injections of 200 μ g of Af-cpe dissolved in 100 μ l of phosphate-buffered saline on days 0 and 14. Aerosol challenge was performed with 0.25% Af-cpe for 20 min in a 30 $\times$ 30 $\times$ 20 cm acrylic chamber using a jet nebulizer Pari model LC-D with an air flow of 6 l min⁻¹ on days 28, 29 and 30.

Dose-response of rAAV1-IL-10

A dose-response of murine IL-10 was preformed with nine C57BL/6 and four Whitsett mice. The intramuscular injections were preformed as previously described. Three doses of rAAV1/2-IL-10 were used (2.8×10^{10} , 1.4×10^{10} and 7.4×10^9 particles per mouse) with three C57BL/6 mice for each dose and four Whitsett mice for the intermediate dose. Blood was collected through retro-orbital bleeds on the day of intramuscular injection day 0 and subsequently on days 7, 14, 21, 28, 60, 90 and day 120 post-injection.

Cytokine detection for serum IL-10

BioPlex cytokine assays for IL-10 (BioRad Laboratories, Hercules, CA, USA) were carried out according to the protocol for serum samples collected preinjection of virus vector and before killing. The standard was made with seven serial dilutions within the range of 500–7.8 pg ml⁻¹. Both assays were carried out on a Luminex 100 instrument BioPlex System (BioRad Laboratories). IL-10 concentrations were determined utilizing SOFT-max PRO software (Molecular Devices, Sunnyvale, CA, USA) with four-parameter data analysis.

Serum IgE ELISA

The blood was collected on day 32 after killing the mice, centrifuged in a serum separator and stored at -80 °C until analysis. Total IgE levels were measured with the BD OptEIA ELISA (BD Biosciences, San Jose, CA, USA), triplicate sera samples were used for each mouse. Tetramethyl-benzidine substrate was used to develop the assay and was read at 450 nm with correction at 570 nm on the VersaMax Micro Plate Reader (Molecular Devices, Sunnyvale, CA, USA).

Serum IgG isotyping

The blood was collected on day 32 after killing the mice, centrifuged in a serum separator and stored at -80 °C until analysis. Samples were assayed on the Bioplex Suspension Array system (BioRad Laboratories) using the Beadlyte Mouse Immunoglobulin Isotyping Kit (Millipore, Billerica, MA, USA) as per manufacturer's instructions. Samples were diluted 1:25 000.

BAL for cell differentials and cytokine profiles

The BAL fluid was retrieved from each animal through cannulation of the exposed trachea and gentle flushing of the lungs with two separate 1 ml aliquots of phosphate-buffered saline. Aliquots were pooled for individual animals preceding centrifugation and separation of pelleted cells and supernatant. Cytospin cell preparations made with 200 µl of BAL were stained using Hema 3 (Biochemical Sciences, Swedesboro, NJ, USA) differential stain, and relative cell populations were determined using the standard morphological criteria. Total cell numbers were calculated by counting the cells on the cytospin slide obtained from the 200 µl BAL aliquot. Assessments of cytokine profiles from the BAL were performed using a commercially available multiplexed kit (Biorad Mouse Multi-Cytokine Detection System; BioRad Laboratories) and the Bioplex Suspension Array System. Simultaneous measurement of several cytokines was performed. All assays were performed according to the manufacturer's protocols. Cytokine concentrations

were determined utilizing Bioplex software with four-parameter data analysis. The sensitivity of the assay is less than 10 pg ml⁻¹ and has a range from 0.2–32 000 pg ml⁻¹ with an inter- and intra-assay coefficient of variation of less than 10%.

Histology

Immediately after collection of lavage fluid, the left lobes were tied off and, ice-cold freshly prepared 4% paraformaldehyde in 1 × phosphate-buffered saline (pH 7.4) was instilled through the tracheal cannula into the right two lobes to inflation-fix the lung. Specimens were immersed in 4% paraformaldehyde at 4 °C overnight. Tissue was treated with standard methods for fixation, dehydration and paraffin embedding and stained with hematoxylin and eosin and periodic acid-Schiff for determination of inflammatory cells and goblet cell hyperplasia, periodic acid-Schiff-stained slides were semiquantitatively scored in a blinded manner. The left lobes were snap frozen in liquid nitrogen. The spleen and livers were fixed and stained with standard methods for Mallory Iron staining.

Immunohistochemistry

Tissue from injected and non-injected muscle where treated with standard methods of fixation, dehydration and paraffin embedding. The 4 µm sections were dewaxed and rehydrated. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide in methanol. Sections were subjected to heat-based antigen retrieval using a citrate buffer (Citra, BioGenex, San Ramon, CA, USA) for 10 min by a steamer. Slides were subsequently rinsed in buffer and incubated with a universal protein blocker, Sniper (Biocare Medical, Walnut Creek, CA, USA), for 15 min. Slides were incubated in primary antibody, rabbit anti-mouse IL-10 (1:1000, Biosource, Carmarillo, CA, USA) overnight at 4 °C. Slides were rinsed followed by application of conjugated secondary polymers, goat anti-rabbit-HRP (Mach-2, Biocare Medical) for 30 min at room temperature. Detection of IL-10 was achieved by incubating slides in Cardassian diaminobenzidine (Biocare Medical) for 5 min at room temperature. Slides were rinsed for 5 min in water then counterstained with hematoxylin (Vector Laboratories Inc., Burlingame, CA, USA). Slides were mounted and coverslipped with cytoaseal XYL (Richard-Allen Scientific, Kalamazoo, MI, USA). Negative controls included incubation of the non-injected muscles with anti-IL-10 and substitution of the primary antibody with rabbit IgG in IL-10-injected muscle sections.

Splenocyte studies

Splenocytes were harvested from the mice on the day of killing. The spleens were meshed into a nylon screen to create a single-cell suspension into a 50 ml conical tube. This suspension of cells was washed with 30 ml of Hank's buffer saline, spun for 8 min at 1000 g and the Hank's buffer saline was aspirated. Then the splenocyte pellet was treated with ice-cold ammonium chloride and incubated on ice for 2 min to lyse red blood cells. The pellet was then washed two more times with Hank's buffer saline. Splenocytes were then resuspended in 5 ml of RPMI supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (pH 7.2). The spleno-

cytes were counted under a hemocytometer and cultured at 1×10^5 per well in 200 μ l of RPMI medium in a 96-well round bottom plate. Splenocytes were activated with ConA (0.5 μ g per well). Supernatants were harvested at 24 and 48 h for cytokine analysis in response to ConA.

Statistical analysis

All data were compared using an unequal variance two-tail student's *t*-test, unless stated otherwise. Data were considered statistically significant when $P \leq 0.05$.

Abbreviations

ABPA, allergic bronchopulmonary aspergillosis; Af-cpe, *Aspergillus fumigatus* crude protein extract; CFTR, cystic fibrosis transmembrane conductance regulator; rAAV, recombinant adeno-associated virus.

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