Modulation of Exaggerated-IgE Allergic Responses by Gene Transfer-mediated Antagonism of IL-13 and IL-17e

Christian Mueller[1,2], Allison Keeler[1], Sofia Braag[1], Timothy Menz[1], Qiushi Tang[1,2] and Terence R Flotte[1,2]

[1]Gene Therapy Center, University of Massachusetts Medical School, Worcester, Massachusetts, USA; [2]Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts, USA

Asthma and allergic rhinitis are almost invariable accompanied by elevated levels of immunoglobin E (IgE), and more importantly a genetic link between IgE levels and airway hyper-responsiveness has been established. We hypothesized that expression of soluble receptors directed against interleukin (IL)-13 and IL-17e would prevent the cytokines from engaging the cell-bound receptors and therefore help to attenuate allergic responses in a $Cftr^{-/-}$-dependent mouse model of exaggerated-IgE responses. $Cftr^{-/-}$ mice were injected with recombinant adeno-associated virus 1 (rAAV1) intramuscularly expressing soluble receptors to IL-17e (IL-17Rh1fc) or IL-13 (IL-13Rα2Fc). Total IgE levels, in mice receiving the IL-17Rh1fc and IL-13Rα2Fc therapy, were lower than in the control group. Interestingly *Aspergillus fumigatus* (Af)–specific IgE levels were undetectable in both the mice receiving the IL-17Rh1fc and IL-13Rα2Fc therapies. Further flow cytometry analysis of intracellular gene expression suggests that blocking IL-17e may be interfering with signaling upstream of $CD4^+$ and $CD11b^+$ cells and reducing IgE levels by affecting signaling on these cell populations. In contrast it appears that IL-13 blockade acts downstream to reduce IgE levels probably by directly affecting B-cell maturation. These studies demonstrate the feasibility of targeting T helper 2 (Th2) cytokines with rAAV-delivered fusion proteins as a means to treat aberrant immune responses.

Received 13 July 2009; accepted 17 October 2009; published online 24 November 2009. doi:10.1038/mt.2009.264

INTRODUCTION

Asthma, a chronic disease involving inflammation of the airways, affects ~5% of the population in the United States. This includes ~10–12% of children under age 18, according to 2003 data from the Centers for Disease Control. Although two general types of asthma medications are available, anti-inflammatory and bronchodilators, they only help to control asthma as a life-long affliction; and long-term, daily medications are required over extended periods to achieve and maintain control of persistent asthma. Thus, alternative therapeutic approaches are clearly warranted. Delivery of therapeutic proteins to the systemic circulation using gene therapy has the ability to provide durable expression after a single administration. It offers the convenience of replacing frequent injections of recombinant proteins by intravenous treatment. Asthma and allergic rhinitis are almost invariable accompanied by elevated levels of immunoglobin E (IgE), and more importantly a genetic link between IgE levels and airway hyper-responsiveness has been established.[1] It is known that the interaction of mast cell-bound IgE with antigen leads to the release of vasoactive mediators, increased synthesis of T helper 2 (Th2) cytokines and the production of leukotrienes and prostaglandins. This acute response leads to immediate-hypersensitivity in the lungs followed by mucous production, smooth muscle constriction, and the eventual inflammatory infiltration. Thus, here we evaluate the possibility of using recombinant adeno-associated virus (rAAV) to express soluble cytokine receptors as a treatment to allergic IgE driven responses in a previously characterized mouse model. This model presents with an exaggerated-IgE phenotype in cystic fibrosis (CF) mice where the cystic fibrosis transmembrane conductance regulator (CFTR) defect causes an increased expression of interleukin (IL)-4 and IL-13. This immune aberration may lead to the increased sensitivity of CF patients to develop allergic disease, because ~50% of all CF patients have elevated levels of serum IgE.[2] Allergic reactions in CF patients complicate diagnosis and more importantly accelerate pulmonary deterioration. Interestingly 10–12% CF patients develop a unique allergic induced asthmatic response to common house mold *Aspergillus fumigatus* (Af), known as allergic bronchopulmonary aspergillosis (ABPA) which is characterized by an unusually high-IgE response. Moreover, a recent study by Hartl *et al.* revealed the prevalence of a pulmonary Th2 immune response in *Pseudomonas aeruginosa*–infected lungs of CF.[3] Thus, the modulation of the pulmonary Th2 response may not only beneficial for treating *P. aeruginosa* infections but may also lead to decrease the prevalence of allergic diseases associated with CF. As stated above, clinical asthma and ABPA are accompanied by elevated levels of Th2 cytokines such as IL-4 and IL-13.[4] IL-13 is a pleitropic cytokine that is secreted from wide array of cells including T cells,[5] B cells,[6] mast cells,[7] basophils, natural

Correspondence: *Christian Mueller, Department of Pediatrics, University of Massachusetts Medical School, 381 Plantation Street, GTC Suite 250, Worcester, Massachusetts 01605, USA. E-mail: chris.mueller@umassmed.edu*

killer and dendritic cells[8–10] with activities that partially overlap those of IL-4. Over expression of IL-13 in the mouse lung results in an intricate phenotype that recapitulates the classical symptoms of asthma. This includes goblet cell hyperplasia, mononuclear and eosinophil inflammation, subepithelial fibrosis, and airway hyperresponsiveness.[11] In addition it has been shown that IL-13 is able to initiate B-cell isotype switching for the production of IgE.[12–14] These features of IL-13, along with its major role in the Cftr-dependent ABPA model make it an interesting target for immune deviation for exaggerated-IgE responses. As an alternative to targeting IL-13, we have also designed a soluble receptor antagonist for the recently discovered IL-17e (a.k.a. IL-25) cytokine. IL-17e is a member of the newly described IL-17 family, which is compromised of five members that share between 20 and 50% homology. Within this family IL-17e is the only member to date that has been shown to promote the development of Th2 responses.[15] In fact IL-17e treatment of mice resulted in the production of the cytokines IL-4, IL-13, and IL-5, extensive eosinophilia, increased serum IgE, and striking histologic changes in the lung and gastrointestinal tract.[15] In addition, IL-17e was shown to activate nuclear factor-κβ and induce the production of IL-8 human cell lines.[16] Targeting IL-17e with a soluble receptor is possible due to the recent identification of the IL-17 receptor homologue 1 (IL-17Rh1) which has shown to preferentially bind with IL-17e and somewhat with a lower affinity to IL-17B.[16] In this study, we investigate targeting IL-13 and IL-17e for neutralization, by designing soluble receptor antagonists that will circulate systemically after being expressed by rAAV vectors targeting the muscle.

RESULTS

Upregulation of IL-13 in Af-induced allergic inflammation

As previously described, we have recapitulated a peculiar exaggerated-IgE phenotype in Cftr$^{-/-}$ mice in response to Af-crude protein extract (Af-cpe) sensitization and challenge. Although this model results in an increase in lung eosinophil recruitment and goblet cell hyperplasia in both wild type and CF mice, it uniquely leads to a more vigorous IgE response in Cftr$^{-/-}$ mice. In the original studies, we demonstrated that IL-13 mRNA levels in the lungs of sensitized mice were more than twofold higher when compared to the control mice.[17] The present study extends the initial findings and demonstrates that both wild type and Cftr$^{-/-}$ mice have substantial increase in the production of IL-13 in NK1.1^{+} (natural killer-cell marker) and CD14^{+} (macrophage marker) cells. **Figure 1** depicts the change in number and type of cells expressing IL-13 intracellularly; where unsensitized Cftr$^{-/-}$ or wild-type mice served as a baseline controls for IL-13 expression. Curiously, although IL-13 levels are detected in CD4^{+} cells in wild-type mice after Af-cpe sensitization and challenge, we only observed an increase in this cell population after Af-cpe challenge in Cftr$^{-/-}$ mice. The prominent increase of IL-13^{+} cells in this model, in conjunction with the newly described role for IL-17e in regulating IL-13 prompted us to attempt immunomodulating the exaggerated-IgE phenotype targeting IL-13 and IL-17e with soluble receptors.

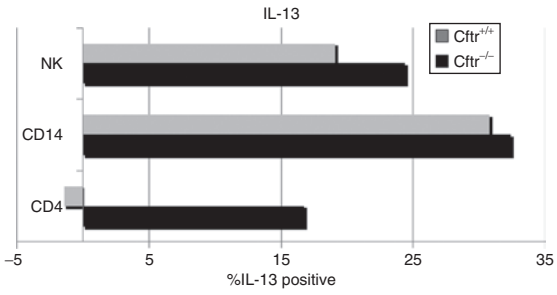

Figure 1 Overexpression of IL-13 in Cftr-deficient mice after ABPA. Intracellular staining for the number of cells expressing IL-13 intracellularly after ABPA, graphed as a percent change over unsensitized animals. Gray bars depict wild-type animals whereas black bars are Cftr$^{-/-}$ mice -deficient animals. The number of positively gated cells from NK1.1, CD14, and CD4 divided by the lymphocyte gate determined by FSC and SSC and multiplied by the percent IL-13^{+} of that population. $N = 5$ except CFTR wild-type ABPA which has an $N = 6$. ABPA, allergic bronchopulmonary aspergillosis; CFTR, cystic fibrosis transmembrane conductance regulator; FSC, forward scatter; NK, natural killer; SSC, side scatter.

Functional studies with IL-17Rh1Fc

To create the soluble receptor fusion proteins, the extracellular domains for the IL-13Rα2 (amino acid 1–332) and for 17Rh1

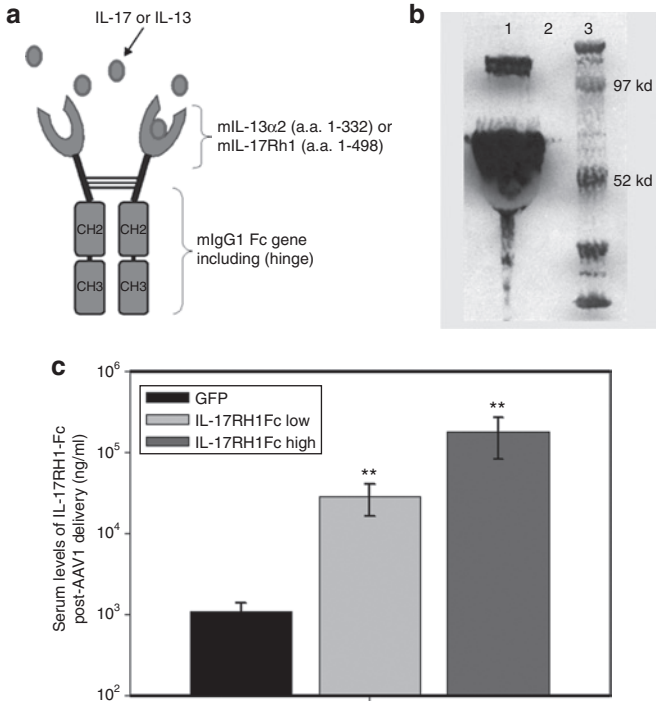

Figure 2 IL-17Rh1Fc fusion protein is stable and efficiently secreted. (**a**) Illustration of soluble receptor fusion protein. The extracellular domains of IL-17e or IL-13 are fused to the CHCH3 domains of a mouse IgG1κ antibody. (**b**) Western blot of IL-17RH1fc protein. Supernatants from transfected HEK-293 cells were blotted with an anti-IL-17Rh1 antibody. Lane 1: IL-17Rh1Fc supernatants; lane 2: IL-13Rα2Fc supernatants; and lane 3: protein ladder. (**c**) Total serum IL-17RhFc levels after rAAV1 administration. Total serum IL-17RhFc levels in CFTR$^{-/-}$ mice analyzed by ELISA from mice receiving either AAV1-GFP or AAV1-IL-17Rh1Fc (low and high dose). Results are reported as group mean + SEM ($N = 5$) $^{**}P < 0.05$. a.a., amino acid; CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; IgG, immunoglobin G; IL, interleukin.

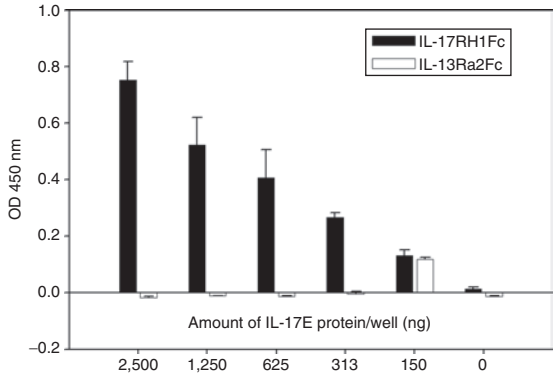

Figure 3 Binding capacity of IL-17RhFc. Ninety-six well plated were coated with different amount of IL-17e protein. Cell supernatants from IL-17Rh1Fc and IL-13Rα2Fc cell transfections were incubated in the IL-17e coated wells, after washing an HRP-conjugated mouse anti-IgG (able to bind the Fc portion of the fusion proteins) was used as to analyze the binding capacity of the supernatants to IL-17e. HRP, horseradish peroxidase; IgG, immunoglobin G; IL, interleukin; OD, optical density .

(amino acid 1-489) were cloned in frame using a linker with the mouse *IgG1 Fc* gene containing the CH2, CH3 domains as well as the hinge region to facilitate the formation of dimers (**Figure 2a**). Although there has not been a published delivery of the IL-13Rα2Fc soluble receptor with rAAV vectors, the characterization and function of the protein have been previously reported by Willis-Karp *et al.* in a mouse model of ovalbumin-induced asthma.[18] To the best of our knowledge the use of a soluble receptor for IL-17e has never before been tested, thus it was imperative that its function be evaluated *in vitro* before administration *in vivo*. To determine whether the fusion construct was expressed and secreted, human embryonic kidney-293 cells were transfected with CB-IL-17RH1Fc, CB-IL-13Rα2Fc or CB-GFP; the latter two constructs serving as controls. Forty-eight hours after the transfection, the supernatants were collected and run on a western blot assay.

As shown in lane 1 of **Figure 2b**, detectable protein was secreted into the media of the transfected human embryonic kidney-293 cells. Two prominent bands were observed when probed with the antibody against IL-17Rh1 receptor. One of the bands is the expected size of the fusion protein (~58 kd) and the second band is above 100 kd. This second band may be representative of dimerization through the hinge region in the Fc portion of the soluble receptor (**Figure 2a**). Supernatants from cells transfected with CB-IL-13Rα2Fc do not contain a soluble receptor detected by antibody raised against the mouse IL-17Rh1 (lane 2 on **Figure 2b**).

To further determine whether the IL-17Rh1 receptor Fc-fusion protein would be secreted *in vivo*, we packaged rAAV1 vectors expressing IL-17RH1Fc and injected mice at two different doses. The first cohort of mice received either rAAV1CB-IL-17Rh1Fc or rAAV1CB-GFP at a dose of 1.0×10^{11} rAAV vector genomes per mouse and the second cohort at a dose of 3.0×10^{11} vector genomes. Dose-dependent detection of IL-17Rh1Fc was observed in the serum of these mice 3 weeks after injection. There was a 200-fold increase in the serum levels of IL-17Rh1 in mice injected with high dose of AAV1CB-IL-17Rh1Fc as compared to the green fluorescent protein (GFP) group (**Figure 2c**). The serum from mice injected with CB-GFP also showed basal levels of soluble

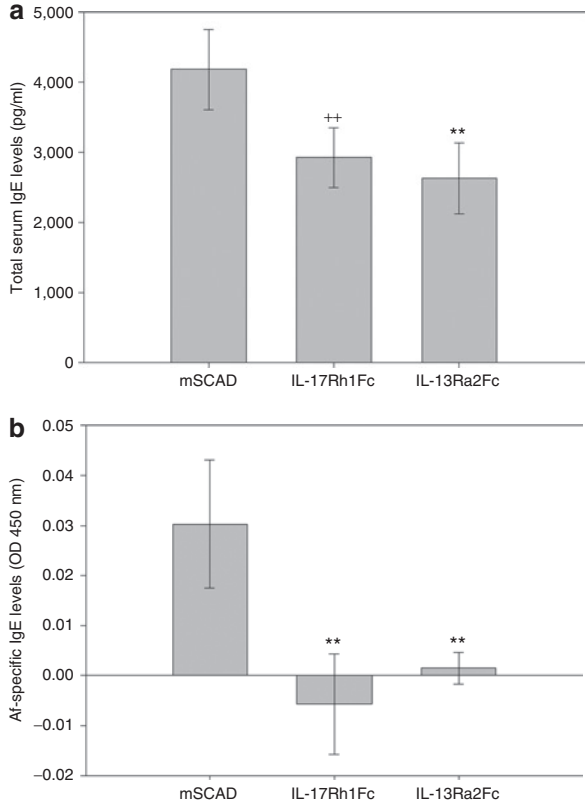

Figure 4 Reduction in circulating IgE levels after soluble receptor therapy. (**a**) Total serum IgE levels after intramuscular rAAV delivery and Af-cpe sensitisation and challenge. (**b**) $Cftr^{-/-}$ mice were injected with rAAV1-IL-17Rh1Fc, rAAV1-IL-13Rα2Fc, rAAV1mSCAD, and rAAV1-GFP. Total Serum IgE in the Af-sensitized mice were measured by ELISA. Serum IgE levels specific for Af-cpe. $N = 5$ Results expressed as a mean + SEM $^{**}P < 0.05$, $^{++}P < 0.1$. Af-cpe, *Aspergillus fumigatus*-crude protein extract; CFTR, cystic fibrosis transmembrane conductance regulator; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobin E; IL, interleukin; rAAV, recombinant adeno-associated virus; OD, optical density.

IL-17Rh1, which can be interpreted as the endogenous level of IL-17Rh1 protein. Endogenous protein present in the serum is supported by the evidence of an alternative splice variant of the protein that is secreted as a decoy receptor.

A modified indirect enzyme-linked immunosorbent assay (ELISA) was used to determine if the secreted IL-17Rh1Fc soluble receptor would be effective at binding IL-17e. A flat bottom 96-well plate was coated with different concentrations of IL-17e protein and subsequently the supernatants from the human embryonic kidney-293 cell transfections of either the IL-17Rh1Fc or IL-13Rα2Fc plasmids were incubated on the plate with IL-17e protein coated wells. An horseradish peroxidase -conjugated secondary antibody directed against the Fc portion of the soluble receptors was used to detect whether the soluble receptors bound to the wells. Thus, the secondary antibody would detect only supernatants that contained Fc-fusion proteins able to bind IL-17e. As shown in **Figure 3** there was a significant correlation between the amounts of IL-17e coated on the plate and the signal absorbance from the supernatant of the IL-17RH1Fc transfection only, whereas supernatants containing the IL-13Rα2Fc protein did produce an appreciable signal.

Intramuscular gene therapy for ABPA with rAAV1-IL-17R1Fc and IL-13Rα2Fc

Subsequent to the *in vitro* analysis, the IL-17Rh1Fc and the previously characterized IL-13Rα2Fc constructs were packaged into rAAV1 vectors for intramuscular administration in Cftr$^{-/-}$ mice. The mice received IL-13Rα2Fc, IL-17Rh1Fc, or CB-mSCAD expressing vectors at a dose of 1.0×10^{11} rAAV particles per mouse. Mouse short-chain acetyl CoA dehydrogenase (mSCAD), was used as a control instead of GFP. Since GFP is a foreign protein it could potentially skew the Th2 response in favor of a Th1 cytotoxic T-lymphocyte response in order to clear cells expressing it, thus inadvertently lowering IgE levels. Recombinant AAV was administered 2 weeks before Af sensitization and challenge, to allow for vector uncoating, second strand synthesis and gene expression. Two weeks after intramuscular delivery of the therapeutic soluble receptors, the mice were subjected to the previously described exaggerated-IgE allergy model, which consists of a series of intraperitoneal injections with Af-cpe followed by airway challenges with nebulized Af-cpe.[17,19]

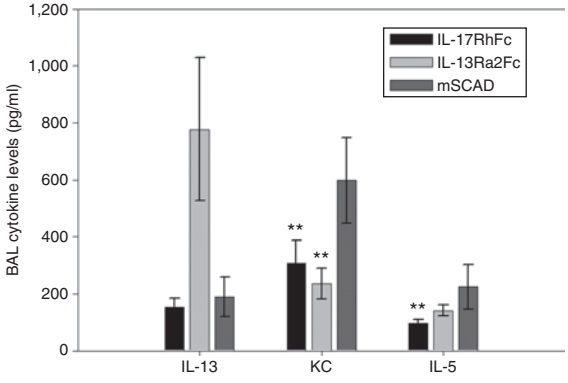

Figure 5 Cytokine levels in the BAL of mice treated with rAAV1 expressing; IL-13Rα2Fc, IL-17Rh1Fc, or mSCAD. *N* = 5. Results expressed as a mean + SEM $^{**}P < 0.05$. BAL, bronchoalveolar lavages; IL, interleukin; mSCAD, mouse short-chain acetyl CoA dehydrogenase; rAAV1, recombinant adeno-associated virus 1.

To determine whether the therapies offered protection from the B-cell isotype switching into an IgE-mediated allergic response, the serum samples were evaluated for total IgE levels. As seen on **Figure 4a** the total circulating serum IgE levels were ~30–40% lower in mice receiving either of the two soluble receptors; this is comparable to the decrease we have seen in other studies using IL-10 as means to reduce IgE responses by immunomodulation.[20] Further analysis of Af-specific IgE responses revealed that both soluble receptors were able to completely abrogate this response as detected by our assay (**Figure 4b**). This is in striking contrast to the Af-specific IgE levels observed in the mSCAD control injected mice.

In order to determine whether the systemically circulating soluble receptors were having an effect locally in the lung, bronchoalveolar lavages (BALs) from the three vector-treated groups were analyzed for various cytokines. As shown in **Figure 5**, the BALs of mice receiving the IL-13 soluble receptor had very high levels of IL-13 in the lungs, while there was a slight but not significant decrease in the IL-13 levels between the mSCAD group

Table 1 Proportions of inflammatory cells in the BAL

	Macrophages	Lymphocytes	Neutrophils	Eosinophils
IL-17Rh1Fc	42.9 ± 4.7	10.7 ± 0.6	5.2 ± 1.2	41.3 ± 4.2**
IL-13Rα2Fc	32.2 ± 5.2	9.8 ± 2.1	7.2 ± 1.3	50.8 ± 3.8
mSCAD	33.9 ± 2.0	9.2 ± 1.4	2.7 ± 0.7	54.2 ± 3.1**

Abbreviations: BAL, bronchoalveolar lavages; IL, interleukin; mSCAD, mouse short-chain acetyl CoA dehydrogenase.
Mean ± SEM.
$^{**}P \leq 0.03$.

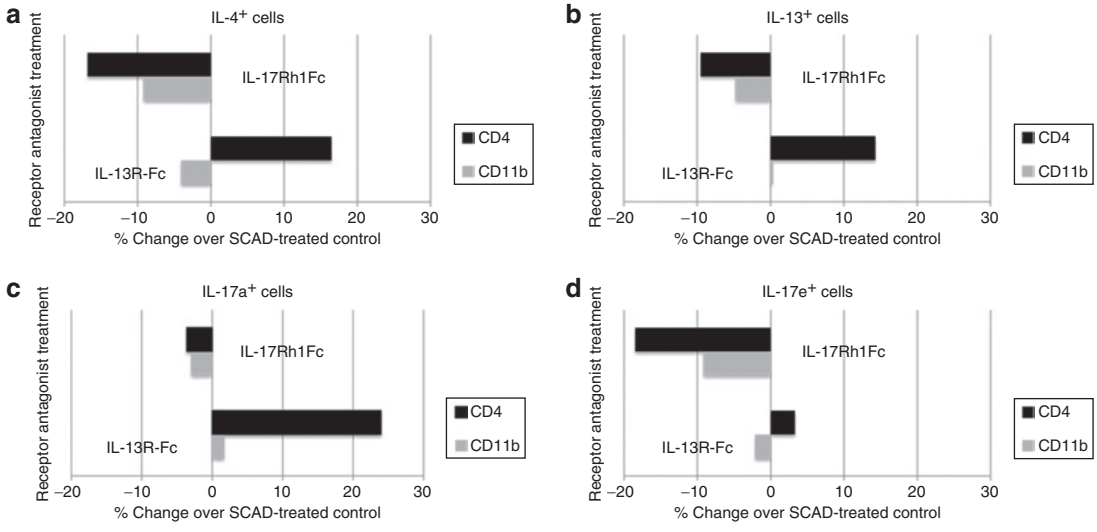

Figure 6 Intracellular cytokines produced after receptor agonist treatment. Cells were stained for surface markers and intracellular cytokines using fluorescently conjugated antibodies that were detected by FACS analysis. CD4$^+$ staining cells were gated and are depicted by the black bars; CD11b$^+$ staining cells were gated and are depicted by the gray bars. IL-17Rh1Fc and IL-13Rh1Fc receptor agonist-treated mice cytokine expression after ABPA treatment are graphed as a percent change over the SCAD-treated control. (**a**) IL-4 staining, (**b**) IL-13 (**c**) IL-17a, and (**d**) IL-17e. *n* = 6 per group. ABPA, allergic bronchopulmonary aspergillosis; FACS, fluorescence activated cell sorting; IL, interleukin; SCAD, short-chain acetyl CoA dehydrogenase.

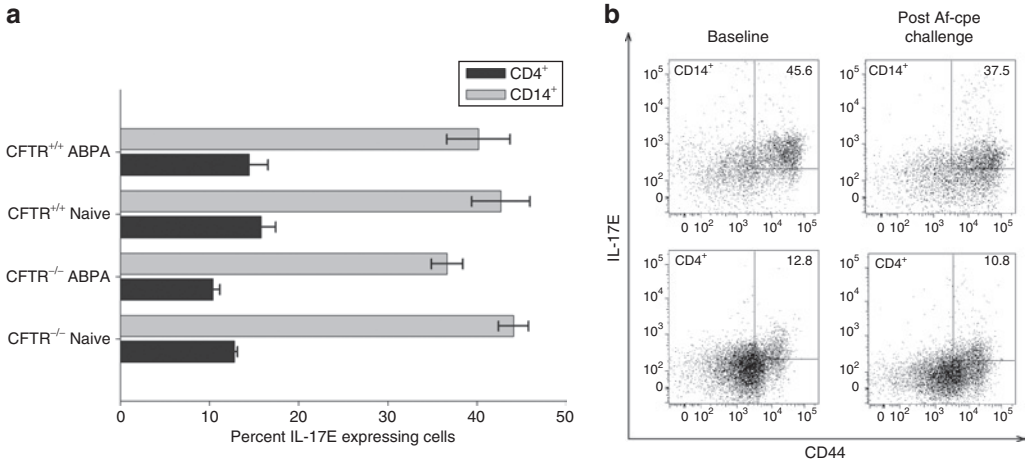

Figure 7 Expression of IL-17e in CD14$^+$ and CD4$^+$ cells. Intracellular staining for IL-17e with and without ABPA. (**a**) Graph of the mean percent IL-17e$^+$ cells. $n = 5$ except Cftr$^{+/+}$ ABPA which has a $n = 6$. Gray bars are CD4$^+$ cells expressing IL-17e; black bars are CD14$^+$ cells expressing IL-17e. Error bars are SE. (**b**) Representative dot blot of data depicted in **a**. Activation marker CD44 staining is on the x axis, IL-17e is on the y axis. CD14$^+$ gated cells are on the top; CD4$^+$ cells are on the bottom. Cells without ABPA are on the left and ABPA cells are on the right. ABPA, allergic bronchopulmonary aspergillosis; Af-cpe, *Aspergillus fumigatus*-crude protein extract; IL, interleukin.

and the IL-17Rh1Fc-treated group. Furthermore, the BAL analysis revealed that both of the soluble receptor treatments were significantly effective at lowering the keratinocyte-derived cytokine response to Af-cpe in the lung (**Figure 5**). Also as shown in **Figure 5**, IL-17Rh1Fc therapy resulted in a successful downregulation of IL-5, whereas IL-13Rα2Fc therapy trended lower but was not statistically significant (**Figure 5**). IL-5 is an important cytokine involved in the maturation and recruitment of eosinophils. Consistent with this role, there was a decrease in the eosinophil infiltration found in the BALs from the lungs of the IL-17Rh1Fc-treated mice (**Table 1**).

The broader effect on cytokine responses from mice receiving IL-17Rh1Fc was also observed from studying the splenocytes by flow cytometry after intracellular cytokine staining (**Figure 6**). The Th2 cytokines (IL-4, IL-13) and IL-17 family members (IL-17a, IL-17e) were measured from soluble receptor-treated mice and compared to mSCAD-treated controls. Th2 cytokines and IL-17 cytokines were reduced in both CD4$^+$ cells and CD11b$^+$ cells treated with the IL-17e antagonist (**Figure 6**). The IL-13 antagonist had minimal effect on the levels of the Th2 cytokines as well as the IL-17 cytokines in the CD11b$^+$ population of cells. Curiously, the CD4$^+$ population of mice treated with the IL-13 antagonist showed an increase of IL-4, IL-13, and IL-17a, with little to no change in IL-17e (**Figure 6**). In contrast to IL-13Rα2Fc treatment the IL-17Rh1Fc soluble receptor seems to be inhibiting IL-17e signaling which may be acting upstream of both CD4$^+$ and CD11b$^+$ cells as seen by the decrease in cytokines in these cells (**Figure 6**) To determine what cell types could be the source of IL-17e in this ABPA model, we compared splenocytes from Cftr$^{-/-}$ and Cftr$^{+/+}$ mice before and after Af-cpe sensitization and challenge. As seen in **Figure 7**, we detected only slight differences in the levels of IL-17e between Cftr$^{-/-}$ and Cftr$^{+/+}$ mice with a modest reduction in the percent of IL-17e$^+$ cells Cftr$^{-/-}$ mice after Af-cpe challenge. More importantly, we identified that CD14$^+$ macrophages are a significantly more abundant source of IL-17e than CD4$^+$ T cells (**Figure 7**).

DISCUSSION

These studies extend the potential utility of rAAV vectors for gene therapy to target allergic disease and it offers another example of the use of these vectors for the expression of therapeutic proteins designed to deviate allergic immune responses. The experiments described here compared the efficacy of the neutralization of IL-17e and IL-13 with a single-intramuscular injection of rAAV vectors expressing soluble receptors targeting these cytokines. Intramuscular injection of the vectors led to a significant production of protein as determined by the ELISA for serum levels of IL-17Rh1Fc. Although the IL-13 soluble receptor was previously described to bind IL-13 with high affinity, the IL-17Rh1 receptor has not been as well characterized. We demonstrated that fusing the extracellular domain of the IL-17e receptor to the Fc portion of a mouse IgG1 antibody resulted in stable, secreted soluble receptors able to bind IL-17e protein.

Intramuscular delivery of rAAV vectors resulted in the reduction of total IgE levels by both IL-13Rα2Fc and IL-17Rh1Fc soluble receptor therapy. When total IgE levels were analyzed the 13Rα2Fc-treated mice had a more significant reduction; however, when Af-specific IgE was analyzed it was evident that both soluble receptors were equally effective at completely abrogating the antigen-specific IgE response.

Further characterization of the effects of the soluble receptors was performed by looking at the cytokine levels in the lung compartment. Analysis of the BALs unexpectedly revealed that neutralization of IL-13 with IL-13Rα2Fc led to higher levels of IL-13 in the lung. It is possible that IL-13 may act as a negative regulator of it own production, so interrupting this negative feedback loop may be causing cells in the lungs to secrete more IL-13. It is also possible, that the soluble receptor may be acting as depot or reservoir for IL-13, which would likely extend the half life of IL-13 and thus artificially increase its concentration. In this scenario, although there is a higher concentration of IL-13, the cytokine would be mostly bound to the decoy receptor and thus would have little biologic activity. This scenario is far more

likely taking into account that there was no increase in goblet cell hyperplasia accompanying the increase the IL-13 levels in the lung (data not shown). In fact the increase in the IL-13 has also been documented before in a model of helminth infection that employed IL-13Rα2 protein as a therapy.[21] Although IL-17Rh1Fc treatment did not seem to reduce the levels of IL-13 in the lung compartment, both treatments reduced levels of keratinocyte-derived cytokine, a chemokine that has been previously shown to be upregulated in CF and has been implicated in pulmonary neutrophil-mediated inflammation. However, more importantly for allergy and asthma-associated inflammation, IL-5 was significantly reduced in mice treated with rAAV-CB-IL-17Rh1Fc. Because IL-5 is responsible for the recruitment and maturation of eosinophils, the reduction of this response is also important for control of allergy driven inflammation. As shown in **Table 1**, this reduction in IL-5 was accompanied by a reduction in BAL infiltration of eosinophils in the IL-17Rh1Fc-treated group. The reduction in eosinophil recruitment by dampening IL-5 and the prevention of Af-specific IgE responses with IL-17Rh1Fc hint that a broader effect is achieved by targeting IL-17e rather than IL-13. In fact when comparing intracellular cytokine staining of $CD11b^+$ monocytes and $CD4^+$ T cells to control-treated mice, it was determined that IL-17Rh1Fc-treated mice had a significant reduction in IL-4, IL-13, and IL-17e in both cell populations. In contrast, macrophages in mice treated with IL-13Rα2Fc did not any significant changes in the production of IL-13, IL-17a, or IL-17e and only a slight decrease in production of IL-4. Even more compelling are differences seen in $CD4^+$ T cells, where IL-13Rα2Fc-treated mice have increased production of IL-13 and IL-4, again hinting at a feedback inhibition loop, which surprisingly led to drastic increase in the production of IL-17a.

This phenomenon may partially explained by the recent discovery that a functional IL-13 receptor is present on $CD4^+$ Th17 cells and that in the presence of IL-13 signaling, IL-17 production is attenuated.[22] In this case the absence of IL-13 signaling may be causing an increase production of IL-17; which lends further proof that IL-13 may also be involved in the regulation of Th17 responses. The broader effect of IL-17Rh1Fc therapy achieved in this allergy model suggests that IL-17e is acting upstream of $CD4^+$ T and B cells; and interferes with the signaling mediated by IL-17e dampening the cytokine production of Th2 $CD4^+$ T cells thereby curbing downstream B-cell isotype switching to IgE and Th2 cytokine-mediated and eosinophil-mediated inflammation. This data also seems to suggest that targeting IL-13 signaling, which is mainly an effector cytokine, may be useful at inhibiting downstream processes such as B-cell isotype switching but may not be helpful at curbing responses at the $CD4^+$ T cell level. In fact it seems that reduced bioavailability of IL-13 seems to make $CD4^+$ T cells increase the production of both IL-4 and IL-13. Having established that interfering with IL-17e signaling with a soluble IL-17Rh1Fc receptor may offer broader benefits for allergic driven pulmonary inflammation we tried to determine what cell type may be the primary source of this cytokine. To begin to elucidate what cells may be possible sources of the IL-17e in the ABPA model, we stained splenocytes for the production of IL-17e in both $Cftr^{-/-}$ and $Cftr^{+/+}$ mice before and after *Aspergillus* challenge. As shown in the **Figure 7**, it seems that the more abundant source of this cytokine is present in $CD14^+$ macrophages as opposed to $CD4^+$ T cells.

In conclusion these studies demonstrate a marked therapeutic effect of IL-13 and IL-17e immunoneutralization with the use of soluble receptors fusion proteins delivered with rAAV. The striking abrogation of Af-specific IgE response from the two different therapies has important implications for the treatment of ABPA and other allergic diseases. This data in combination with the IL-17e staining data in the ABPA model suggest that IL-17e is an innate cytokine that may help to bridge and regulate the production of Th2 cytokines. IL-17e is substantially produced by $CD14^+$ macrophage cells, and the receptor antagonist reduced production of IL-17a, IL-17e, IL-13, and IL-4 when compared to the SCAD control both in $CD4^+$ T cells and $CD14^+$macrophages. As discussed above, the IL-13 antagonist appears to have more downstream affects as illustrated by the BAL cytokine data and flow cytometry. Although, IL-13 antagonists did not reduce Th2 or IL-17 cytokines, it did greatly reduce total and Af-specific IgE responses. Presumably the IL-13 antagonist blocks the IL-13 from signaling the B cells to isotype switch to make IgE, whereas the IL-17e antagonist acts further upstream in preventing Th2 and IL-17 signaling to T cells as well as B cells. Future experiments should look at the possibility that interfering with the signaling of both cytokines in combination may produce a synergistic effect in decreasing allergic response to Af as well as to other antigens. Finally, although these experiments are proof-of-concept animal studies, in light of recent data from intramuscular delivery of rAAV1 vectors in humans, it is conceivable that a single administration with rAAV1 could provide sustained serum levels of the recombinant soluble receptors for up to 1 year.[23]

MATERIALS AND METHODS

CB-mIL-13Rα2-Fc. This construct consists of the extracellular domain (amino acid 1–332) of the mouse IL-13Rα2 receptor fused with the CH2 and CH3 including the hinge region (Fc) domains of a mouse IgG1κ antibody. The IL-13Rα2 receptor was synthesized by reverse transcription (RT)–PCR from RNA of mouse thymus. The RT-PCR was performed with the one-step RT-PCR kit (Qiagen, Valencia, CA) using the following primers: IL-13_sense 5′AGAATAAATGGCCTCGTG3′ and IL-13_antisense 5′AATAACAGAAACACGGAAG3′. The resulting 1 kb fragment was the gel purified (Qiagen gel extraction kit). Subsequently, the CH2 and CH3 domains of a mouse IgG1κ antibody were amplified by RT-PCR from RNA extracted from a mouse hybridoma cell line. A small Gly-Ser-Gly linker was added to the 5′ end of the PCR product. This product was also gel purified. The two fragments were then subjected to a blunt end ligation (T4 DNA ligase) at an equimolar ratio this ligation was then subjected to 30 rounds of PCR using the forward IL-13 primer and the reverse Fc primer to yield a 1,770-bp fragment. The fragment was then cloned into a TA vector backbone (Stratagene PCR 2.0) which was used to transform Top 10 cell for subsequent screening. Once the sequence of the fusion gene was verified by sequencing, it was cloned into a chicken β-actin (CBA) vector backbone for packaging. This was done by digesting the TA plasmid with the IL-13Rα2Fc fusion with Not1. The linearized plasmid was then treated with Klenow to fill in the 3′ overhang. The plasmid was then cut with *Spe*1 and the resulting 1,777 bp fragment was gel purified. To create a CBA backbone to clone the insert into the pTR2-CBA-rIL-10 plasmid was cut with *Hind*III to linearize it and also treated with Klenow to fill in the 3′ overhang. Then it was cut with *Nhe*1 and the large ~5,300 bp fragment was gel purified to serve as the pTR2-CBA-backbone for the IL-13Rα2Fc fusion insert. This two fragment were then

ligated (T4 DNA ligase) at 5:1 molar ratio (insert:backbone) to yield the final plasmid of 7,061 bp.

CB-mIL-17Rh1Fc. This vector is composed of the extracellular domain of the IL-17Rh1 (a.k.a. IL-17BR) fused with the with CH2 and CH3 including the hinge region (Fc) domains of a mouse IgG1κ antibody. The methods used for the cloning of this fusion protein were the same as the ones describe above for the pTR-CBA-mIL-13Rα2Fc vector with the exception of the primer used for the RT-PCR. The following primers were use to amplify the extracellular domain of the *IL-17Rh1* gene; IL-17Rh1-for 5′GGGCCATGTTGCTAGTGTTG3′ and IL-17Rh1-REV 5′TCCCAAATGTAGGTCCCACTC3′.

In vitro *expression and analysis of IL-17Rh1Fc.* Expression of IL-17Rh1Fc was first investigated in cell cultures of 293 human kidney epithelial cells. The cells were cultured in T-75 plates with 10 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 1% penicillin/streptavidin (Cellgro, Manassas, VA) in a 37 °C humidified CO_2 incubator until cells were 90% confluent expression of IL-17Rh1Fc was first investigated in cell cultures of 293 human kidney epithelial cells. Cells were transfected at 90% confluency with lipofectamine 2000. Forty-eight hours after transfection GFP-transfected cells were evaluated for expression and supernatants were collected for further western blot analysis.

Serum IL-17Rh1Fc ELISA. Blood was collected upon sacrificing the mice, centrifuged in a serum separator and stored at −80 °C until analysis. Total IL-17Rh1Fc levels were measured with the BD IL-17Rh1 (a.k.a. IL-17BR) antibody pairs. The antibodies were used at the suggested concentration for coating and detection (BD Biosciences, San Jose, CA) . 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 microplate reader (Molecular Devices, Sunnyvale, CA).

Mouse strains. The primary Cftr knockout strain used for these studies was the CFTR S489X$^{-/-}$ neo insertion in C57BL/6 mice developed initially at the University of North Carolina[24] and then modified with the transgenic overexpression of gut-specific expression of human CFTR from the fatty acid–binding protein promoter in order to prevent intestinal obstruction and improve viability.[25] These mice have then been backcrossed 10 generations onto a C57BL/6 mouse.

Muscle injection. For muscle administration of the rAAV1 vectors expressing 5–6-week mice were anesthetized with 3.5% isofluorane inhalation. Fifty micro liters of viral-vector suspension was administered percutaneously by intramuscular injection into the right or left gastrocnemius muscle 2 weeks before (day −14) Af-cpe sensitization.

Aspergillus sensitization and challenge. Six to eight-week-old Cftr S489X$^{-/-}$; fatty acid–binding protein human CFTR$^{+/+}$, and wild-type littermate mice were housed in the specific pathogen-free mouse colony of the University of Massachusetts Medical School (Worcester, MA) according to National Institutes of Health guidelines and were allowed food and water *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Animals were sensitized to Af-cpe (Greer Laboratories, Lenoir, NC). Briefly, animals were administered with intraperitoneal injections of 200 µg of Af-cpe extract dissolved in 100 µl of phosphate-buffered saline on days 0 and 14. Aerosol challenge was performed with 0.25% Af-cpe for 20 minutes in a 30 × 30 × 20 cm acrylic chamber using a jet nebulizer Pari model LC-D (PARI Respiratory Equipment, Midlothian, VA) with an air flow of 6 l/min on days 28, 29, and 30.

Serum IgE ELISA. Blood was collected on day 32 after sacrificing 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) 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).

Af-specific ELISA was performed by coating a 96-well plates with 10 µg of Af-cpe (Greer Laboratories) and detected using BD OptEIA IgE ELISA (BD Biosciences) detection antibody.

Cytokine determination for BALs. Assessments of cytokine profiles from the BAL were performed using a commercially available multiplexed kit (Biorad mouse multi-cytokine detection system; Bio-Rad Laboratories, Hercules, CA) and the Bioplex suspension array system (Bio-Rad Laboratories). Simultaneous measurement of five cytokines was performed: specifically, IL-2, IL-5, INF-G, IL-4, and IL-13. All assays were performed according to the manufacturer's protocols. Cytokine concentrations were determined utilizing Bioplex software (Bio-Rad Laboratories) with four-parameter data analysis. The sensitivity of the assay is <10 pg/ml and has a range from 0.2 to 32,000 pg/ml with an inter and intra-assay coefficient of variation of <10%.

Intracellular staining of cytokines for flow cytometry. Splenocytes were incubated for 3 hours with GolgiStop (BD Biosciences) in RPMI + 10% fetal calf serum at 37 °C to block intracellular transport of cytokines. The cells were then Fc blocked by CD16/CD32 antibodies for 5 minutes at 4 °C. Cells were then stained for surface antigens (including CD4, CD44, CD11b, CD14, NK1.1) for 20 minutes at 4 °C. Cells were fixed and permeabilized for 20 minutes at 4 °C with CytoFix/CytoPerm buffer (BD Biosciences). Anticytokine antibodies (IL-17a, IL-17e, IL-13, and IL-4) were stained for 25 minutes at 4 °C. Cells were washed and then analyzed on BD LSR II (BD Biosciences) using FACSDIVA software . Analysis was done using FlowJo (Treestar, Ashland, OR).

Statistical analysis. All data was compared using an unequal variance two-tail Student *t*-test, unless stated otherwise. Data was considered statistically significant when $P \leq 0.05$.

ACKNOWLEDGMENTS

We thank Mark Atkinson and Clive Wasserfall for their helpful comments and insights. This work was supported by grants from NHLBI (HL69877) the Cystic Fibrosis Foundation, the Alpha-1 Antitrypsin Foundation, a fellowship from the Parker B. Francis Foundation and the Diabetes and Endocrinology Research Center of the University of Massachusetts Medical School (supported by Grant P30 DK32520).

REFERENCES

1. Postma, DS, Bleecker, ER, Amelung, PJ, Holroyd, KJ, Xu, J, Panhuysen, CI *et al.* (1995). Genetic susceptibility to asthma–bronchial hyperresponsiveness coinherited with a major gene for atopy. *N Engl J Med* **333**: 894–900.
2. Hauber, HP, Gholami, D, Koppermann, G, Heuer, HE, Meyer, A and Pforte, A (2003). Increased expression of Interleukin-13 but not Interleukin-4 in cystic fibrosis patients. *J Cyst Fibros* **2**: 189–194.
3. Hartl, D, Griese, M, Kappler, M, Zissel, G, Reinhardt, D, Rebhan, C *et al.* (2006). Pulmonary T(H)2 response in *Pseudomonas aeruginosa*-infected patients with cystic fibrosis. *J Allergy Clin Immunol* **117**: 204–211.
4. Kotsimbos, TC, Ernst, P and Hamid, QA (1996). Interleukin-13 and interleukin-4 are coexpressed in atopic asthma. *Proc Assoc Am Physicians* **108**: 368–373.
5. Brown, KD, Zurawski, SM, Mosmann, TR and Zurawski, G (1989). A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes. *J Immunol* **142**: 679–687.
6. Kindler, V, Matthes, T, Jeannin, P and Zubler, RH (1995). Interleukin-2 secretion by human B lymphocytes occurs as a late event and requires additional stimulation after CD40 cross-linking. *Eur J Immunol* **25**: 1239–1243.
7. Burd, PR, Thompson, WC, Max, EE and Mills, FC (1995). Activated mast cells produce interleukin 13. *J Exp Med* **181**: 1373–1380.
8. Gibbs, BF, Haas, H, Falcone, FH, Albrecht, C, Vollrath, IB, Noll, T *et al.* (1996). Purified human peripheral blood basophils release interleukin-13 and preformed interleukin-4 following immunological activation. *Eur J Immunol* **26**: 2493–2498.
9. de Saint-Vis, B, Fugier-Vivier, I, Massacrier, C, Gaillard, C, Vanbervliet, B, Aït-Yahia, S *et al.* (1998). The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J Immunol* **160**: 1666–1676.
10. Hoshino, T, Winkler-Pickett, RT, Mason, AT, Ortaldo, JR and Young, HA (1999). IL-13 production by NK cells: IL-13-producing NK and T cells are present *in vivo* in the absence of IFN-gamma. *J Immunol* **162**: 51–59.

11. Zhu, Z, Homer, RJ, Wang, Z, Chen, Q, Geba, GP, Wang, J *et al.* (1999). Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* **103**: 779–788.
12. Brière, F, Bridon, JM, Servet, C, Rousset, F, Zurawski, G and Banchereau, J (1993). IL-10 and IL-13 as B cell growth and differentiation factors. *Nouv Rev Fr Hematol* **35**: 233–235.
13. Cocks, BG, de Waal Malefyt, R, Galizzi, JP, de Vries, JE andAversa G (1993). IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *Int Immunol* **5**: 657–663.
14. Carballido, JM, Schols, D, Namikawa, R, Zurawski, S, Zurawski, G, Roncarolo, MG *et al.* (1995). IL-4 induces human B cell maturation and IgE synthesis in SCID-hu mice. Inhibition of ongoing IgE production by *in vivo* treatment with an IL-4/IL-13 receptor antagonist. *J Immunol* **155**: 4162–4170.
15. Fort, MM, Cheung, J, Yen, D, Li, J, Zurawski, SM, Lo, S *et al.* (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* **15**: 985–995.
16. Lee, J, Ho, WH, Maruoka, M, Corpuz, RT, Baldwin, DT, Foster, JS *et al.* (2001). IL-17e, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *J Biol Chem* **276**: 1660–1664.
17. Müller, C, Braag, SA, Herlihy, JD, Wasserfall, CH, Chesrown, SE, Nick, HS *et al.* (2006). Enhanced IgE allergic response to *Aspergillus fumigatus* in CFTR-/- mice. *Lab Invest* **86**: 130–140.
18. Wills-Karp, M, Luyimbazi, J, Xu, X, Schofield, B, Neben, TY, Karp, CL *et al.* (1998). Interleukin-13: central mediator of allergic asthma. *Science* **282**: 2258–2261.
19. Mueller, C, Torrez, D, Braag, S, Martino, A, Clarke, T, Campbell-Thompson, M *et al.* (2008). Partial correction of the CFTR-dependent ABPA mouse model with recombinant adeno-associated virus gene transfer of truncated CFTR gene. *J Gene Med* **10**: 51–60.
20. Mueller, C, Braag, SA, Martino, AT, Tang, Q, Campbell-Thompson, M, Flotte, TR *et al.* (2008). The pros and cons of immunomodulatory IL-10 gene therapy with recombinant AAV in a Cftr(–/–)-dependent allergy mouse model. *Gene Ther* **16**: 172–183.
21. Chiaramonte, MG, Mentink-Kane, M, Jacobson, BA, Cheever, AW, Whitters, MJ, Goad, ME *et al.* (2003). Regulation and function of the interleukin 13 receptor alpha 2 during a T helper cell type 2-dominant immune response. *J Exp Med* **197**: 687–701.
22. Newcomb, DC, Zhou, W, Moore, ML, Goleniewska, K, Hershey, GK, Kolls, JK *et al.* (2009). A functional IL-13 receptor is expressed on polarized murine CD4+ Th17 cells and IL-13 signaling attenuates Th17 cytokine production. *J Immunol* **182**: 5317–5321.
23. Brantly, ML, Chulay, JD, Wang, L, Mueller, C, Humphries, M, Spencer, LT *et al.* (2009). Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proc Natl Acad Sci USA* **106**: 16363–16368.
24. Grubb, BR, Pickles, RJ, Ye, H, Yankaskas, JR, Vick, RN, Engelhardt, JF *et al.* (1994). Inefficient gene transfer by adenovirus vector to cystic fibrosis airway epithelia of mice and humans. *Nature* **371**: 802–806.
25. Zhou, L, Dey, CR, Wert, SE, DuVall, MD, Frizzell, RA and Whitsett, JA (1994). Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human CFTR. *Science* **266**: 1705–1708.