Modulation of T_{reg} function improves adenovirus vector-mediated gene expression in the airway

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Virus vector-mediated gene transfer has been developed as a treatment for cystic fibrosis (CF) airway disease, a lethal inherited disorder caused by somatic mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in humans, affecting about 1 in 2500 live births.¹ Pathobiological symptoms of CF include mucosal obstruction of exocrine glands and pulmonary inflammation.² Chronic neutrophilic inflammation and pulmonary infections are among the chief contributors to morbidity and mortality in CF.³ Patients with CF have increased levels of proinflammatory cytokines in the airways that include tumor necrosis factor-α, interleukin (IL)-1, IL-6, and IL-8, and reduced levels of anti-inflammatory cytokines such as IL-10.⁴

The success of gene therapy for CF airway disease is dependent on the efficient delivery and expression of the CFTR gene in the cells of the respiratory epithelium.⁵⁻⁶ Virus (that is, adenovirus (Ad), lentivirus and adeno-associated virus) vector-based gene transfer has been developed to correct the underlying Cl-secretion defect in the CF airway epithelium.⁹,¹⁰ Cellular and humoral immune responses to viral antigens, and the epitopes on the expressed proteins have been shown to be barriers to efficient airway gene transfer.¹¹,¹² Successful gene therapy regimens using virus-based vectors may also require the elimination of the anti-vector capsid immune responses.¹³⁻¹⁵ It is likely that two sets of antigens induce a host immune response, the viral antigens associated with the virus vector and the non-tolerated antigens of the newly expressed proteins.

Regulatory T cells (T_{reg}) reduce pulmonary inflammation and lung injury in animal models of Pneumocystis pneumonia.¹⁶ Activated CD4⁺ CD25⁺ FOXP3⁺ T_{reg} cells also suppress allergic airway inflammation.¹⁷ We are particularly interested in the post-translational modification of FOXP3 as a mechanism to regulate the activity of T_{reg} cells.¹⁸,¹⁹ Acetylation of FOXP3 has been shown to increase the stability of the FOXP3 proteins.¹⁹⁻²¹ Previously valproic acid (VPA), an HDAC inhibitor and a clinically safe compound, was shown to enhance the function of T_{reg} cells to suppress effector cells.²² Here we report a strategy that utilizes VPA to improve virus vector-based gene transfer in the CF mouse lung.

VPA treatment increased T_{reg} activity and reduced inflammation in the CF mouse lung, as demonstrated by the reduction of the number of neutrophils. Furthermore, following VPA treatment, we observed an increase in Ad vector-mediated gene expression. This study suggests that HDAC inhibitors may be developed and used as immune modulators to improve lung gene therapy.

RESULTS

Increased number of T_{reg} cells in the bronchoalveolar lavage (BAL) fluid of CF mice

A CFTR knockout mouse model²² has been used extensively to study CF-related disease and explore therapies. In our experiments to examine the role of T_{reg} cells in the CF mouse lung, we compared the number of T_{reg} cells in both CF and wild-type (WT) age-matched mice. Splenocytes as well as cells isolated from the BAL fluid were harvested from CF and WT mice and analyzed for the presence of T_{reg} cells by fluorescence-activated cell sorting (FACS). No difference was observed in the frequency of spleen-derived T_{reg} cells between CF and WT mice. However, we did observe a higher percentage of T_{reg} in the BAL fluid of CF mice when compared with the WT mice (7.7% ± 2.6 vs 0% ± 0, Figure 1b), a likely consequence of the preexisting inflammatory status of the CF mouse lung. Compared with WT mice, CF mice had an increased number of Ly6G⁺ neutrophils (Figure 1c), which are myeloid cells shown to contribute to the innate immune defense against microbial pathogens.²⁴

HDACi enhances Ad-mediated transgene expression in lung

We first investigated if VPA could enhance Ad vector-mediated LacZ expression in mouse lung. CF mice were treated...
injured pulmonary neutrophil number by indirectly limiting inflammation and thereby affecting the frequency of neutrophils and Treg frequency in spleen each experimental group. VPA treatment did not significantly affect the frequency of neutrophils, we also examined the population of splenocytes from each experimental group. To rule out a possible toxic effect of VPA on neutrophils, we isolated neutrophils from the spleen and studied for their ability to suppress the proliferation of carboxyfluorescein diacetate succinimidyl ester-labeled primary CD4⁺ CD25⁰ T cells (representing effector T cells (Teff)). Treatment with VPA dose-dependently increased Treg activity in the BAL fluid of CF mice that received the Ad.CFTR vector and less Teff cells underwent proliferation (Figure 2f). We then studied the effect of VPA on CFTR gene transfer. Independent of the Ad.CFTR vector, VPA treatment only slightly increased the percentage of Treg cells in the BAL fluid of CF mice (Figure 3a). The difference, however, was not statistically significant due to the significant variation between samples. Treatment with the Ad.CFTR vector alone did not appear to have an effect on the frequency of Treg cells in the BAL fluid. In contrast, VPA treatment or Ad.CFTR vector delivery led to a reduction in the frequency of neutrophils (Ly6G⁺) in the BAL fluid, suggesting less inflammation in these mice. VPA treatment combined with the Ad.CFTR vector delivery had an additive significant effect on the reduction of neutrophils compared to the naive group (P < 0.05) (Figure 3b). To rule out a possible toxic effect of VPA on neutrophils, we also examined the population of splenocytes from each experimental group. VPA treatment did not significantly affect the frequency of neutrophils and Treg frequency in spleen (Figure 3). Our data suggest that VPA treatment reduced BAL fluid neutrophil number by indirectly limiting inflammation and not by directly inducing cytotoxicity towards this population.

Although the frequency of Treg cells was not changed (Figure 3a), we investigated whether the Treg activity was affected by either the VPA or the Ad.CFTR vector treatment. We examined Treg cells for their ability to suppress Teff cells as described above. Treg and Teff cells were mixed at a ratio of 2:1. The percentage of proliferative Teff cells was reduced from 75.4 (in the absence of Treg cells) to 57 by the addition of Treg cells from naive CF mice (Figure 4). In the presence of VPA-treated Treg cells, the population of proliferating Teff cells was further reduced to 48.1%, indicating an enhanced Treg activity in CF mice following VPA treatment (Figure 4). Interestingly, Ad.CFTR vector treatment had little effect on Treg activity, and the effect of the VPA and Ad.CFTR combination treatment was similar to the VPA-only treatment (Figure 4). These data are consistent with the previously reported pro-Treg activity of VPA. We expect that the CFTR expression in lung epithelial cells by the Ad.CFTR vector would limit inflammation by restoring CFTR activity and not by increasing Treg activity. We speculate that by invoking two mechanisms the Ad.CFTR and VPA combination treatment may lead to lower inflammation, as seen by the lower frequency of neutrophils in BAL fluid (Figure 3).

DISCUSSION

VPA has been shown to inhibit HDAC activity and affect the acetylation of histones and as such is expected to modulate gene transcription by promoting DNA decondensation. Previous studies have demonstrated that HDAC inhibitors alone are insufficient to broadly modulate gene expression. In a microarray study, the pan-HDAC inhibitor trichostatin was shown to influence (equal distribution of up- or downregulation) the transcription of ~2% genes in T cells. In another study using the pan-HDAC inhibitor LAQ824, the Toll-like receptor 4-dependent activation of macrophages was examined and only 5% of genes were found to be either up- or downregulated.

Fan et al. reported that treatment with VPA resulted in increased expression of exogenous genes in cells transduced with various viral-based gene transfer vectors, including Ad, adeno-associated virus and herpesvirus vectors. Recently, the effect of VPA on Ad vector-mediated transduction was reported. VPA concentrations as low as 1 mm increased the Ad-vector transduction of glioma cells by seven-fold. Although the pleiotropic effects of VPA may also contribute to the enhanced gene transfer in the airway observed in our study, the focus of our study was on the HDAC inhibitory activity of VPA.
studies was the immunosuppressive effect of VPA through the regulation of Treg activity (Figure 2).

Inflammatory responses that arise following virus-mediated gene therapy in CF airway may involve cytotoxic T cells, 3 T helper cells, 30 and dendritic cells. 31 CD4+ CD25+ FOXP3+ Treg cells suppress the function of all these immune cells. 32 Although Treg abnormalities have been reported in CF patients, 33 the role of Treg cells in CF disease pathogenesis remains unclear.

We examined CD4+ CD25+ FOXP3+ Treg cells isolated from CF mice and observed an increase in the number of both Treg cells and neutrophils in BAL fluid. Our observations are consistent with previous reports of higher frequency of Treg cells at sites of ongoing chronic inflammation in lung 34 and the pathological influx of neutrophils in the airways of CF due to the loss of the CFTR function. 35 In some CF patients, signs of inflammation, such as neutrophil accumulation, high concentration of IL-8 and abundance of free protease, are often observed in the airways even in the absence of an infection. 36

We hypothesize that the HDAC inhibitor VPA functions as an immune suppressor by promoting FOXP3 acetylation as previously shown 18 and enhancing the function of Treg cells, as demonstrated in Figures 2f and 4.

FOXP3 has an essential role in the development and function of natural and induced Treg cells and as such represents a key target to modulate Treg functions. 18,37 Acetylation of FOXP3 is linked to the stability of FOXP3 21,38 that can be regulated by acetytransferases (that is, p300 and TIP60) and deacetylases (that is, HDAC7, HDAC9 and SIRT1). 20,37,39 Recent studies suggest that in vivo treatment with VPA increases the number and function of CD4+ CD25+ FOXP3+ cells and reduces disease severity in the collagen-induced arthritis-animal model. 22 In our study CF mice were characterized by highly elevated levels of Treg cells in the BAL fluid compared with WT mice (Figure 1b). Although VPA may further increase the frequency of Treg cells in CF mice, we propose that the ability of VPA to enhance Treg suppressive function is more critical and may instantly induce the pre-localized Treg cells.

Unexpectedly, we found that treatment with Ad vector reduced Treg activity in CF mice. As shown in Figure 2f, mice treated with only Ad.LacZ had much lower Treg activity than naive mice. It is unclear whether the induced inactivation of Treg function by Ad.LacZ vector is related to the influence of the virus vector on the host immune system. Treatment with VPA prior to Ad.LacZ gene transfer restored Treg activity. We found that VPA treatment decreases neutrophil infiltration in the lung of CF mice (Figure 3b). Co-treatment of VPA with the Ad.CFTR vector further reduced the number of neutrophils in the BAL fluid of CF mice.

The capability of CD4+ CD25+ Foxp3+ Treg cells to reduce neutrophil survival and limit inflammatory response has been reported in several models. 40,41 In a lipopolysaccharide-induced lung injury model, alveolar infiltration of both Treg cells and neutrophils was observed after acute lung injury, and the transfer of Treg cells to injured mice enhanced the clearance of neutrophils from BAL fluid. 42 Our data suggest that by inducing the activity of Treg cells, VPA appears to reduce neutrophils in the lung.

The clinical utility of Ad-based vectors for lung-gene therapy is severely limited by their immunogenicity and the low transduction efficiency of airway epithelial cells. 43 Compared with Ad-based vectors, adenov-associated virus-based vectors are less immunoinflammatory and thus favorable for repeat administration and long-term transgene expression. Furthermore, several adenov-associated virus serotypes exist with improved targeting and transduction of airway epithelial cells. 43,44 Here, we demonstrate the use of the immune-suppressive HDAC inhibitor VPA to enhance transgene expression.

Figure 2. Adenovirus-mediated LacZ gene expression in CF mouse lung. CF mice were subjected to VPA treatment and dosed with 5 x 10^10 of the Ad.LacZ vector intranasally. Representative images from (a) Naïve mice, (b) Ad.LacZ vector-treated mice, (c) low-dose VPA (1.33 mg daily) and Ad.LacZ vector-treated mice and (d) high-dose VPA (2.67 mg daily) and Ad.LacZ vector-treated mice. (e) Quantification of LacZ-positive cells in the lung of the Ad.LacZ vector-treated mice. Values are presented as the average of three mice. Error bar denotes s.d. *P<0.05, Dunnett’s multiple comparison test. (f) Treg function in CF mice treated with the Ad.LacZ vector and VPA. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled Treg cells were isolated from the spleen and incubated with Treg cells at the indicated ratios. The proliferative fraction of T eff cells is shown. CF, cystic fibrosis; WT, wild type.
In summary, VPA may complement the effectiveness of CFTR gene transfer by inducing T_{reg} activity in vivo. Our studies support further evaluation of VPA as a potential complimentary therapeutic to diminish inflammation in CF airway. Other HDAC inhibitors (for example, vorinostat and romidepsin) that have been approved for clinical use can also be explored for their activity to facilitate virus vector-based gene transfer.

Figure 3. In vivo effect of VPA. BAL fluid or spleen cells from mice subjected to different treatments as noted were collected for FACS analysis. Frequency of (a) T_{reg} cells and (b) neutrophils was analyzed using FACS. *P<0.05, t-test.

Figure 4. VPA enhances T_{reg} function. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T_{eff} cells were co-cultured with T_{reg} cells from CF mice that were treated with VPA, the Ad.CFTR vector or with the combination of VPA and the Ad.CFTR vector. Cells were analyzed by FACS; the proliferative fraction of T_{eff} cells as calculated is presented.

MATERIALS AND METHODS

Animal studies

Studies utilizing mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Ad-based vector at a dose of 5 x 10^{10} particles per mouse was delivered in 50μl of phosphate-buffered saline (PBS) intranasally as described previously. For the VPA plus the Ad.LacZ vector...
group, mice were treated daily with either 1.33 mg (low-dose group) or 2.67 mg (high-dose group) delivered intraperitoneally for 4 consecutive days. The AdLacZ vector was used on the second day after VPA injection. On the fifth day, BAL fluid and lung tissue were collected. For the VPA plus the Ad.CFTR vector group, mice were first treated with 2.67 mg VPA or PBS by intraperitoneal injection on the day before as well as on the day of virus vector administration. Mice were further treated with 8 mg VPA three times a week over the period of 1 week after receiving the Ad.CFTR vector. Spleen cells and BAL fluid were collected for the analysis. PBS was used as the control treatment. Lungs from mice were inflated with 1:1 PBS/CT (optimum cutting temperature) compound, prepared as 8 μm tissue sections and stained for LacZ expression. The slides were counterstained with Safranin O.

Flow cytometry

Spleen cells and BAL fluid cells were collected and a single suspension of cells was incubated with 5% FCS containing PBS to block the Fc receptor. Cells were stained with anti CD4-FITC, CD25-PE (BD Pharmingen, San Diego, CA, USA) and anti-Ly6G (Biolegend, San Diego, CA, USA). After washing, cells were fixed and stained with anti Foxp3-APC (eBioscience) using Foxp3 staining buffer set (eBioscience, San Diego, CA, USA). Flow cytometry was performed by LSRII (BD) at the University of Pennsylvania Flow Cytometry Core Facility.

In vitro Treg suppression assay

CD4+ T cells were isolated from the spleen of mice using the MACS CD4+ T cell isolation kit II (Milteny), CA, USA), CD4+ CD25+CD45RBhigh Treg cells and CD4+ CD25−CD45RBlow Treg cells were isolated by FACS Aria II, yielding a purity of ~97% for both type of cells. Treg cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Invitrogen, Carlsbad, CA, USA) and 2 mM sodium pyruvate (Invitrogen) and 50 μM non-essential amino acids (Invitrogen), 2 mM sodium pyruvate (Invitrogen) and 50 μM β-mercaptoethanol (Sigma, St Louis, MO, USA), were harvested and in vitro proliferation of lymphocytes was analyzed by the FACSCount Flow Cytometry.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Massie J, Cumow L, Gaffney L, Carlin J, Francis I. Declining prevalence of cystic fibrosis since the introduction of newborn screening. Arch Dis Child 2010; 95: 531–533.
2 Koehler DR, Downey GP, Sweezy NB, Tanswell AK, Hu J. Lung inflammation as a therapeutic target in cystic fibrosis. Am J Respir Cell Mol Biol 2004; 31: 377–381.
3 Rowe SM, Miller S, Sorscher EJ. Cystic fibrosis. N Engl J Med 2005; 352: 1992–2001.
4 Bonfield TL, Panuska JR, Konstan MW, Hilliard KA, Hilliard JB, Ghnaim H et al. Histone deacetylase inhibitors improve gene transfer and expression of CFTR in CF lung epithelial cells. J Cyst Fibrosis 2006; 5: 371–380.
5 Conese M, Copreni E, Di Gioia S, De Rinaldis P, Fumarulo R. Neutrophil recruitment into the airway epithelium in cystic fibrosis. J Immunol 2009; 182: 114–126.
6 Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. Immunity 2008; 29: 114–126.
7 Cohen TS, Prince A. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med 2012; 18: 509–519.
87 Zipf et al. Current prospects for gene therapy of cystic fibrosis. Gene Therapy (2014) 219 – 224
37 Xiao Y, Li B, Zhou Z, Hancock WW, Zhang H, Greene MI. Histone acetyltransferase-mediated regulation of FOXP3 acetylation and Treg function. Curr Opin Immunol 2010; 22: 583–591.

38 Li B, Saouaf SJ, Samanta A, Shen Y, Hancock WW, Greene MI. Biochemistry and therapeutic implications of mechanisms involved in FOXP3 activity in immune suppression. Curr Opin Immunol 2007; 19: 583–588.

39 Li B, Greene MI. FOXP3 actively represses transcription by recruiting the HAT/HDAC complex. Cell Cycle 2007; 6: 1432–1436.

40 Richards H, Williams A, Jones E, Hindley J, Godkin A, Simon AK et al. Novel role of regulatory T cells in limiting early neutrophil responses in skin. Immunology 2010; 131: 583–592.

41 D’Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF et al. CD4+ CD25+ Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. J Clin Invest 2009; 119: 2898–2913.

42 Conese M, Ascenziioni F, Boyd AC, Coutelle C, De Fino I, De Smedt S et al. Gene and cell therapy for cystic fibrosis: from bench to bedside. J Cyst Fibrosis 2011; 10(Suppl 2): S114–S128.

43 Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. Proc Natl Acad Sci USA 2006; 103: 12993–12998.

44 Limberis MP, Vandenberghe LH, Zhang L, Pickles RJ, Wilson JM. Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro. Mol Ther 2009; 17: 294–301.

45 New M, Olzicha H, La Thangue NB. HDAC inhibitor-based therapies: can we interpret the code? Mol Oncol 2012; 6: 637–656.

46 Price A, Limberis M, Gruneich JA, Wilson JM, Diamond SL. Targeting viral-mediated transduction to the lung airway epithelium with the anti-inflammatory cationic lipid dexamethasone-spermine. Mol Ther 2005; 12: 502–509.