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

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Abstract

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 CFTR gene. The pathological pro-inflammatory environment of CF as well as the naïve and adaptive immunity induced by the virus vector itself limit the effectiveness of gene therapy for CF airway. Here, we report the use of an HDAC inhibitor, valproic acid (VPA), to enhance the activity of the regulatory T cells (T_{reg}) and improve the expression of virus vector-mediated gene transfer to the respiratory epithelium. Our study demonstrates the potential utility of VPA, a drug used for over 50 years in humans as an anticonvulsant and mood-stabilizer, in controlling inflammation and improving the efficacy of gene transfer in CF airway.

Keywords

cystic fibrosis; gene therapy; VPA; T_{reg}; airway; mouse

Introduction

Cystic fibrosis (CF) is a lethal inherited disorder caused by somatic mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in humans, affecting about 1 in 2,500 live births [1]. Pathobiological symptoms of CF include muscosal obstruction of exocrine glands and pulmonary inflammation [2]. Chronic neutrophilic inflammation and pulmonary infections are among the chief contributors to morbidity and mortality in patients with CF [3]. Patients with CF have increased levels of proinflammatory cytokines in the airways that include TNF-α, IL-1, IL-6 and IL-8, and reduced levels of anti-inflammatory cytokines such as IL-10 [4].

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The success of gene therapy for CF airway disease is dependent on the efficient delivery and expression of the CFTR gene [5] in the cells of the respiratory epithelium [6–8]. Virus (i.e. adenovirus, 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]. However, 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} also suppress allergic airway inflammation [17]. We are particularly interested in the post-translational modification of FOXP3 as mechanisms to regulate the activity of T_{reg} [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} 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 adenovirus vector-mediated gene expression. This study suggests that HDAC inhibitors may be developed and used as immune-modulators to improve gene therapy.

Results

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

A CFTR knockout mouse model [23] has been used extensively to study CF-related disease and explore therapies. In our experiments to examine the role of T_{reg} in the CF mouse lung, we compared the number of T_{reg} 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} by FACS. No difference was observed in the frequency of spleen-derived T_{reg} between CF and WT mice. However, we did observe a higher percentage of T_{reg} in the BALF of CF mice when compared to the WT mice (7.7% ± 2.6 v.s. 0% ± 0, Fig 1B), a likely consequence of the preexisting inflammatory status of the CF mouse. Compared to WT mice, CF mice had an increased number of Ly6G+ neutrophils (Fig 1C), which are myeloid cells shown to contribute to the innate immune defense against microbial pathogens [24].

HDACi enhances adenovirus-mediated transgene expression in lung

We first investigated if VPA could enhance adenovirus vector-mediated LacZ expression in mouse lung. CF mice were treated intraperitoneally with either low dose (1.33 mg) or high dose (2.67 mg) of VPA once daily for 4 days. On day 2 the mice were given intranasally (IN) $5x10^{10}$ particles of Ad.LacZ vector in 50μl. Expression of LacZ was inspected on day 5.

As expected, no transgene expression was observed in the lung of naïve mice. LacZ expressing cells were present in the lung of animals given the Ad.LacZ vector IN. Treatment with the high dose of VPA resulted in a significant increase of LacZ expression in mouse airway when compared to the Ad.LacZ vector only treated group (Figure 2E). No improvement was observed in the level of LacZ expression in the lung of mice treated with the low dose of VPA (Figure 2E).

VPA reduces inflammation in the lung of CF mice

In CF mice treated with either the low or high dose of VPA, there was a considerable reduction in the neutrophil population but not in T_{reg} numbers (data not shown). To understand if the T_{reg} activity was changed by the VPA treatment, $CD4^+CD25^{high}$ T_{reg} cells were isolated from the spleen and studied for their ability to suppress the proliferation of carboxyfluorescein diacetate succinimidyl ester (CFSE) –labeled primary $CD4^+CD25^-CD45RB^{high}$ T cells (representing effector T cells, T_{eff}). Treatment with VPA dose-dependently increased T_{reg} activity in the BAL fluid of CF mice that received the Ad.LacZ vector and less T_{eff} underwent proliferation (Fig 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 T_{reg} in the BAL fluid of CF mice (Figure 3A). The difference, however, was not statistically significant due to the significant variations between samples. Treatment with the Ad.CFTR vector alone did not appear to have an effect on the frequency of T_{reg} 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 naïve group ($p < 0.05$) (Fig 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 T_{reg} frequency in spleen (Fig 3). Our data suggested that VPA treatment reduced BAL fluid neutrophil number by indirectly limiting inflammation and not by directly inducing cytotoxicity towards this population.

While the frequency of T_{reg} was not changed (Fig 3A), we investigated whether the T_{reg} activity was affected by either the VPA or the Ad.CFTR vector treatment. We examined T_{reg} for their ability to suppress T_{eff} cells as described above. T_{eff} and T_{reg} were mixed at a ratio of 2:1. The percentage of proliferative T_{eff} cells was reduced from 75.4% (in the absence of T_{reg}) to 57% by the addition of T_{reg} from naïve CF mice (Fig. 4). In the presence of VPA-treated T_{reg}, the population of proliferating T_{eff} cells was further reduced to 48.1%, indicating an enhanced T_{reg} activity in CF mice following VPA treatment (Fig. 4). Interestingly, Ad.CFTR vector treatment had little effect on T_{reg} activity, and the effect of the combination of VPA and Ad.CFTR treatment was similar to the VPA only treatment (Fig. 4). These data are consistent with previous reported pro-T_{reg} activity of VPA [22]. We expect that that CFTR expression in lung epithelial cells by the Ad.CFTR vector would limit inflammation by restoring CFTR activity and not by increasing T_{reg} activity. We speculate that by invoking two mechanisms the combination of the Ad.CFTR and VPA treatment may

lead to lower inflammation as seen by the lower frequency of neutrophils in BAL fluid (Fig 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 [18, 25]. However, previous studies have demonstrated that HDAC inhibitors alone are insufficient to broadly modify gene expression. In a microarray study, the pan-HDAC inhibitor Trichostatin (TsA) was shown to influence (equal distribution of up- or down-regulation) the transcription of ~2% genes in T cells[26]. 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 down-regulated [27].

Fan and colleagues reported that treatment with VPA resulted in increased expression of exogenous genes in cells transduced with various viral-based gene transfer vectors, including adenovirus, adeno-associated virus and herpesvirus vectors [28]. Recently, the effect of VPA on adenovirus vector-mediated transduction was reported. VPA concentrations as low as 1 mM increased adenoviral transduction of glioma cells by 7 fold [29]. 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 studies was the immunosuppressive effect of VPA through the regulation of T_{reg} activity (Fig 2).

Inflammatory responses that arise following virus vector-mediated gene therapy in CF airway may involve cytotoxic T cells [13], T helper cells [30], and dendritic cells [31]. $CD4^+CD25^+FOXP3^+$ T_{reg} suppress the function of all these immune cells [32]. While T_{reg} abnormalities have been reported in CF patients [33], the role of T_{reg} in CF disease pathogenesis remains unclear.

We examined $CD4^+CD25^+FOXP3^+$ T_{reg} isolated from CF mice and observed an increase in the number of both T_{reg} and neutrophils in BAL fluid. Our observations are consistent with previous reports of higher frequency of T_{reg} 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, is often observed in the airways even in the absence of an infection [36].

Despite the increased number of cells, the T_{regs} in BAL fluid were not sufficient to control the pulmonary inflammation in CF due to airway infection. 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 T_{reg}, as demonstrated in Figures 2F and 4.

FOXP3 has an essential role in the development and function of natural and induced T_{reg} and as such represents a key target to modulate T_{reg} functions[18, 37]. Acetylation of FOXP3 is linked to stability of FOXP3 [21, 38] that can be regulated by acetyltransferases (i.e. p300 and TIP60) and deacetylases (i.e. 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 T_{reg} cells in the BAL fluid compared to WT mice (Fig 1B). Although VPA may further increase the frequency of T_{reg} in CF mice, we propose that the ability of VPA to enhance T_{reg} suppressive function is more critical and may instantly induce the pre-localized $T_{reg.}$

Unexpectedly, we found that treatment with Adenovirus vector reduced T_{reg} activity in CF mice. As shown in Fig. 2F, mice treated with only Ad.LacZ had much lower T_{reg} activity than the control. It is unclear whether the induced inactivation of T_{reg} function by Ad.LacZ vector is related to the influence of the virus vector on host immune system. Treatment with VPA prior to Ad.LacZ gene transfer restored T_{reg} 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 numbers of neutrophils in the BAL fluid of CF mice.

The capability of $CD4^+CD25^+Foxp3^+$ T_{reg} to reduce neutrophil survival and limit inflammatory response was reported in several models [43, 44]. In a LPS induced lung injury model, alveolar infiltration of both T_{reg} and neutrophils were observed after acute lung injury, and the transfer of T_{reg} to injured mice enhanced the clearance of neutrophils from BAL fluid [44]. Our data suggest that by inducing the activity of T_{reg}, 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 low transduction efficiency of airway epithelial cells [40]. Compared with Ad-based vectors, adeno-associated virus (AAV)-based vectors are less inflammatory and thus favorable for repeat administration and long-term transgene expression. Furthermore, several AAV serotypes exist with improved targeting and transduction of airway epithelial cells [41, 42]. Here, we demonstrate the use of the immune-suppressive HDAC inhibitor VPA to enhance transgene expression.

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 (e.g. vorinostat and romidepsin [45]) that have been approved for clinical use can also be explored for their activity to facilitate virus vector-based gene transfer.

Materials and Methods

Animals studies

Studies utilizing mice were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Adenovirus-based vector at a dose of $5x10^{10}$ particles /mouse were delivered in 50μl of PBS IN as described previously [46]. For the VPA plus the Ad.LacZ vector group, mice were treated daily with either 1.33 mg (low dose group) or 2.67mg (high dose group) delivered intraperitoneally (IP) for 4 consecutive days. The Ad.LacZ vector was dosed 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 mg VPA or PBS by i.p. injection before and on the day of virus vector administration. Mice were further treated with 8 mg VPA three times over the period of one 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/OCT, prepared as 8μm tissue sections and stained for LacZ expression. The slides were counterstained with Safranin O for LacZ expression [46].

Flow cytometry

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

In vitro T_{reg} suppression assay

$CD4^+$ T cells were isolated from the spleen of mice using the MACS CD4+ T cell isolation kit II (Miltenyi). $CD4^+CD25^-CD45RB^{high}$ T_{eff} cells and $CD4^+CD25^{high}$ T_{reg} were isolated by FACS Aria II, yielding a purity of ~ 97% for both type of cells. T_{eff} were labeled with CFSE (Invitrogen), stimulated with anti-CD3/CD28 beads (Invitrogen), and co-cultured with different ratio of T_{reg}. After 3 days of co-culture in RPMI supplemented with 10% FBS, 1X non-essential amino acids (Invitrogen), 2mM sodium pyruvate (Invitrogen) and 50μM β-mercaptoethanol (Sigma), cells were harvested and in vitro proliferation of lymphocytes was analyzed by the FACSCanto Flow Cytometry.

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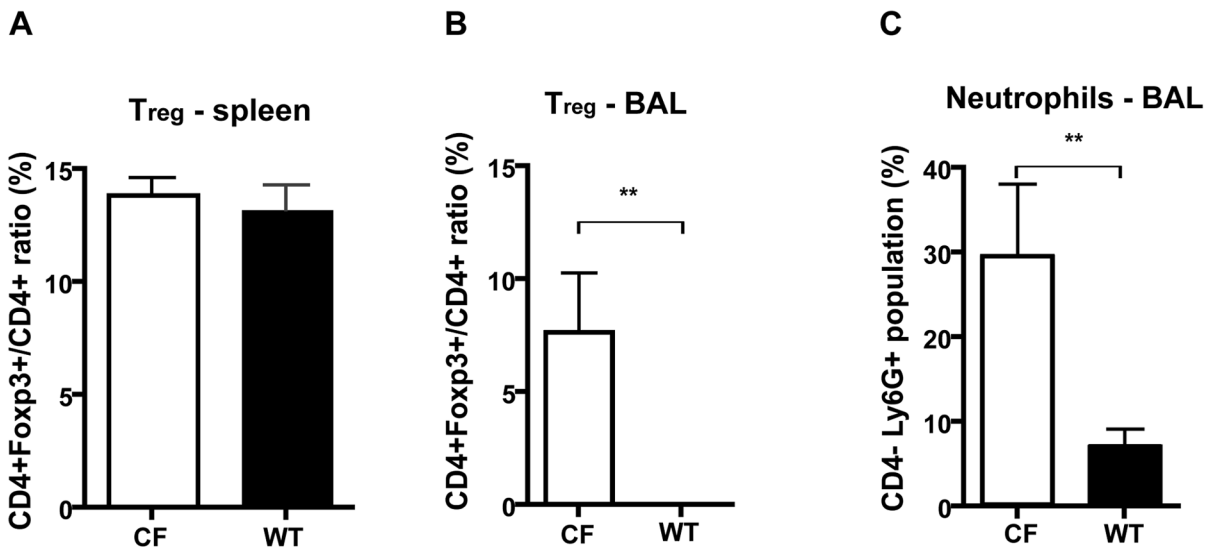

Figure 1.

Comparison of T_{reg} and neutrophil frequencies in CF and WT mice. (A) Splenocytes and (B, C) BAL fluid cells harvested from CF mice and WT mice were analyzed by FACS. The ratio of CD4+Foxp3+/ CD4+ reflects the frequency of T_{reg} as the percentage of the CD4 positive population. Data are presented as the average of 3 mice. Error bar denotes SEM. **P < 0.01, T test. CF: cystic fibrosis, WT: wild type.

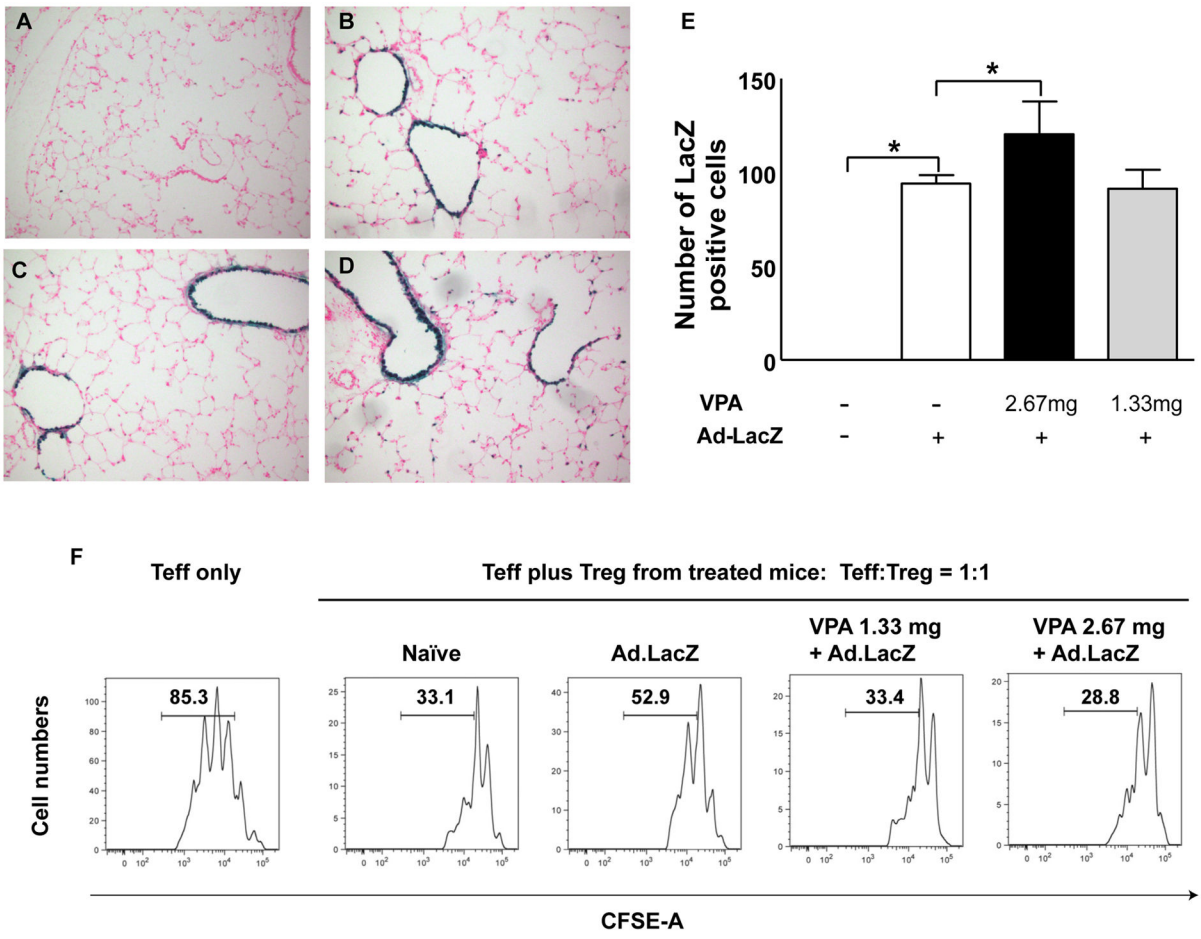

Figure 2.

Adenovirus-mediated LacZ gene expression in CF mouse lung. CF mice were subjected to VPA treatment and dosed with $5x10^{10}$ of the Ad.LacZ vector IN. 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 3 mice. Error bar denotes SD. *P < 0.05, Dunnett's multiple comparison test. (F) T_{reg} function in CF mice treated with the Ad.LacZ vector and VPA. CFSE-labeled T_{eff} cells were isolated from the spleen and incubated with T_{reg} at the indicated ratios. The proliferative fraction of T_{eff} cells is shown. CF: cystic fibrosis, WT: wild type

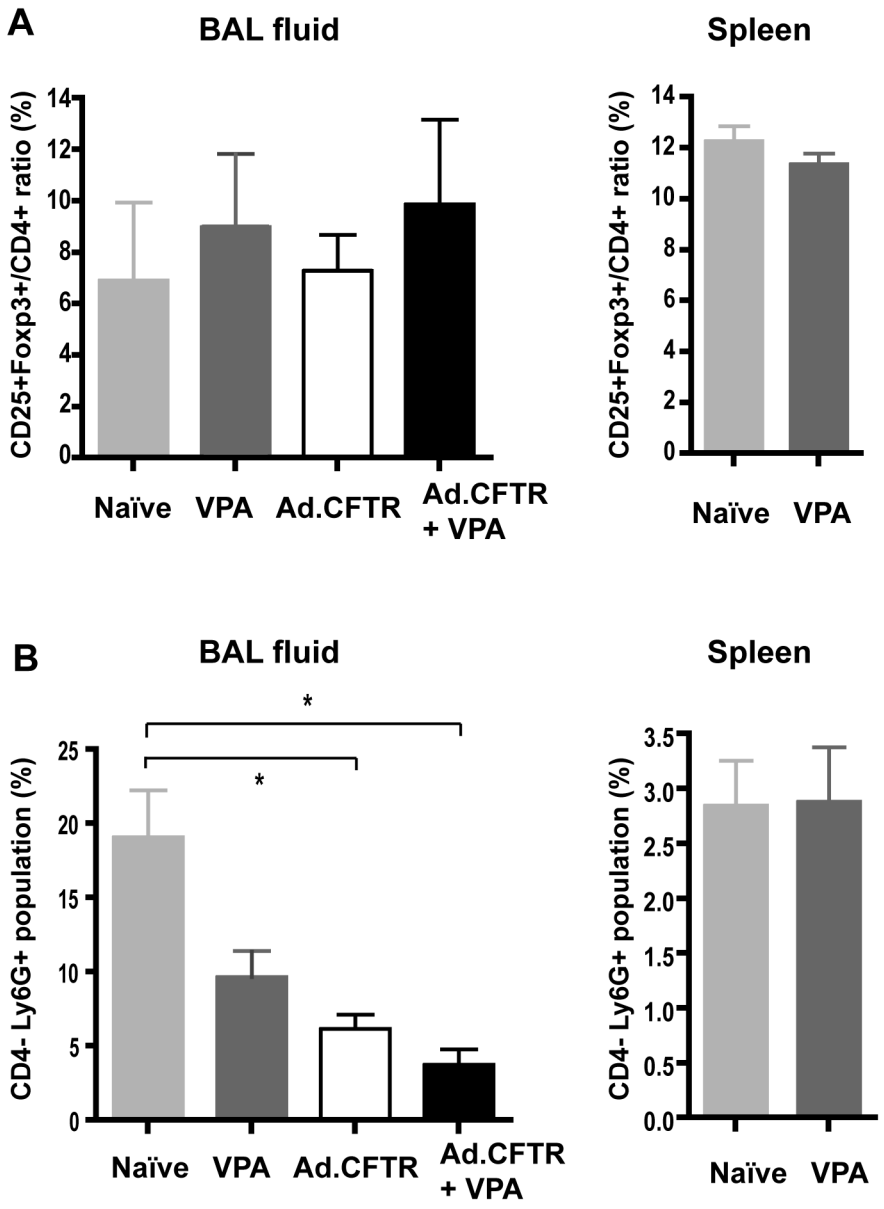

Figure 3.

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} and (B) neutrophils was analyzed using FACS. * $P < 0.05$, t-test.

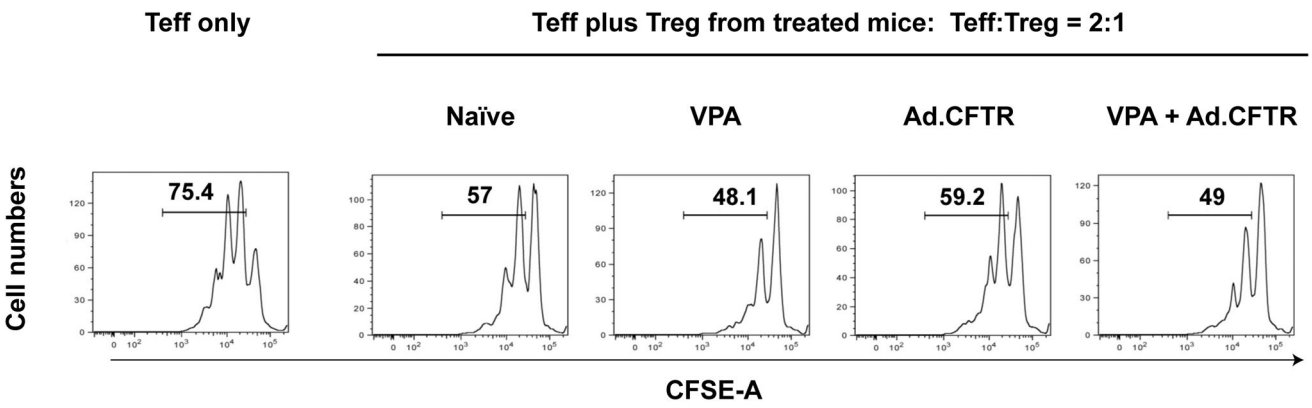

Figure 4.

VPA enhances T_{reg} function. CFSE-labeled T_{eff} cells were co-cultured with T_{reg} 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 was calculated is presented.