Lack of Cystic Fibrosis Transmembrane Conductance Regulator in CD3⁺ Lymphocytes Leads to Aberrant Cytokine Secretion and Hyperinflammatory Adaptive Immune Responses

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Cystic fibrosis (CF), the most common fatal monogenic disease in the United States, results from mutations in CF transmembrane conductance regulator (CFTR), a chloride channel. The mechanisms by which CFTR mutations cause lung disease in CF are not fully defined but may include altered ion and water transport across the airway epithelium and aberrant inflammatory and immune responses to pathogens within the airways. We have shown that *Cftr*^{-/-} mice mount an exaggerated IgE response toward *Aspergillus fumigatus*, with higher levels of IL-13 and IL-4, mimicking both the T helper cell type 2-biased immune responses seen in patients with CF. Herein, we demonstrate that these aberrations are primarily due to *Cftr* deficiency in lymphocytes rather than in the epithelium. Adoptive transfer experiments with CF splenocytes confer a higher IgE response to *Aspergillus fumigatus* compared with hosts receiving wild-type splenocytes. The predilection of *Cftr*-deficient lymphocytes to mount T helper cell type 2 responses with high IL-13 and IL-4 was confirmed by *in vitro* antigen recall experiments. Conclusive data on this phenomenon were obtained with conditional *Cftr* knockout mice, where mice lacking *Cftr* in T cell lineages developed higher IgE than their wild-type control littermates. Further analysis of *Cftr*-deficient lymphocytes revealed an enhanced intracellular Ca²⁺ flux in response to T cell receptor activation. This was accompanied by an increase in nuclear localization of the calcium-sensitive transcription factor, nuclear factor of activated T cell, which could drive the IL-13 response. In summary, our data identified that CFTR dysfunction in T cells can lead directly to aberrant immune responses. These findings implicate the lymphocyte population as a potentially important target for CF therapeutics.

Keywords: cystic fibrosis transmembrane conductance regulator; lymphocytes; allergy; intracellular calcium; nuclear factor of activated T cell

Cystic fibrosis (CF) is the most common autosomal recessive genetic disease in the United States, affecting 1 in 3,300 live births. CF is the result of mutations in CF transmembrane conductance regulator (CFTR), a chloride channel and regulator of other ion channels. Patients with CF suffer a wide range of clinical consequences from the disease, including pancreatic insufficiency, with subsequent malabsorption and impaired nutrition, and chronic airway infection with *Pseudomonas aeruginosa* and

other pathogens (1–4). The mechanisms by which CFTR mutations cause chronic lung disease in CF are not fully defined, but may include the combined effects of altered ion and water transport across the airway epithelium (5–7), increased binding or decreased clearance of *P. aeruginosa* (8, 9), as well as increased proinflammatory cytokine production in the CF airway (10–14). CF cell lines demonstrate increased NF- κ B activation and increased IL-8 secretion in response to *P. aeruginosa* exposure as compared with control cells (14, 15). Furthermore, CFTR mutant mice demonstrate a greater cytokine response (keratinocyte chemoattractant [KC], macrophage inflammatory protein-2 [MIP2], IL-1 β), greater mortality, and greater weight loss after airway challenge with a *P. aeruginosa*-agarose bead slurry as compared with control mice (16–19).

Allergic bronchopulmonary aspergillosis (ABPA) is a clinical syndrome characterized by recurrent wheezing and pulmonary infiltrates, an excessively high total serum IgE, and high IgE and IgG antibodies directed against *Aspergillus fumigatus* (*Af*), which is usually found to be colonizing the airways of these patients. ABPA is very common in CF, affecting approximately 15% of all patients (20, 21). It is occasionally seen in patients with asthma, although some studies have shown that up to 50% of patients with asthma with ABPA have at least one mutation in the CFTR gene (22–25). The immune response in ABPA appears to exemplify the IL-4-driven, T helper cell (Th) type 2-predominant response that is seen in patients with CF and in the *Cftr* mutant mouse expression profiling studies (26–29). Based on this observation, our laboratory has recently described an ABPA-like model in CF mice (30). In subsequent studies, we observed divergent cytokine production in splenocytes from the *Cftr* mutant mice challenged with *Af* antigen (31). This led us to question whether the differences in inflammatory signaling that are apparent in CF mice are due to the direct or indirect effects of *Cftr* mutations within nonepithelial cell types, such as lymphocytes.

This question has been raised a number of times in previous work (32, 33). Studies conducted immediately after the discovery of CFTR, indicated that lymphocyte chloride transport was defective in CF, and that this could affect function under certain circumstances (34). A number of other studies have shown that CFTR gene replacement could restore lymphocyte channel activity to normal (35, 36). Finally, the Th2 bias of CF lymphocytes has been confirmed by a number of investigators (27, 28). In this study, we investigated whether there are *primary* defects in lymphocytes lacking CFTR function. A number of experimental systems, including conditional *Cftr* knockout mice, demonstrated a hyperinflammatory adaptive immune response that was dependent upon the *Cftr* genotype of CD3⁺CD4⁺ lymphocytes. Furthermore, we propose a possible mechanism for this increased response in lymphocytes, in which aberrant calcium fluxes lead to an increase in the nuclear localization of nuclear factor of activated T cell (NFAT), a transcriptional regulator of cytokines driving the Th2-biased response.

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MATERIALS AND METHODS

Mouse Strains

The *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 (51), and then modified with the transgenic overexpression of gut-specific expression of human *CFTR* from the fatty acid binding protein (FABP) promoter to prevent intestinal obstruction and improve viability (52). The other mouse strain used is *Cftr*-ΔF508. Both mouse strains have been backcrossed 10 generations onto a C57BL/6 mouse. For experiments on conditional knockout mice, the recently developed floxed *Cftr* mouse (37) was crossed with the C57BL/6 mice expressing CRE recombinase under the control of the leukocyte-specific protein tyrosine kinase (Lck) promoter.

Aspergillus Sensitization and Challenge

Animals were sensitized to *Af*-crude protein extract (*Af*-cpe) (Greer Laboratories, Lenoir, NC). Briefly, animals were administered intraperitoneal injections of 200 μg *Af*-cpe extract dissolved in 100 μl PBS 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 (Midlothian, VA) with an airflow of 6 L/min on Days 28, 29, and 30.

Adoptive Transfers

Adoptive transfers were performed by harvesting either *Af*-cpe-sensitized or -naïve splenocytes from *Cftr*-ΔF508 (*Cftr*^{-/-}) or wild-type control littermates. Briefly, spleens were disaggregated in Hanks' buffered saline solution and passed through a 20-μm mesh. Cells were then resuspended in PBS at a concentration of 4.5×10^8 cell/ml. Rag^{-/-} mice on a C57BL/6 background were then injected intraperitoneally with 100 μl of the suspension. A total of 8 weeks was allowed for engraftment before either challenging or sensitizing and challenging Rag^{-/-} mice.

Figure 1. Total serum IgE levels in congenic C57BL/6-cystic fibrosis (CF) transmembrane conductance regulator (CFTR)^{-/-} mice and their littermates after *Aspergillus fumigatus* (*Af*) sensitization and challenge. Mice were sensitized with 200 μg of *Af* crude extract dissolved in 100 μl of PBS on Days 1 and 14. Blood samples were collected on Day 32, 48 hours after the third aerosol *Af* challenge. Total serum IgE in the *Af*-sensitized mice was measured by ELISA. Data are shown as group averages (±SEM).

Antigen Recall

Spleens were harvested and CD4 T cells and CD11b cells were separated using the AutoMACs pro (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were counted and plated in 96-well, round-

Figure 2. Adoptive transfer of CF splenocytes confers elevated IgE phenotype. *CFTR*-ΔF508 (*CFTR*^{-/-}) mice on the B6 background or matched control C57BL/6 mice (*CFTR*^{+/+}) were used as donors for splenocyte adoptive transfer into B6-Rag^{-/-} mice. Total serum IgE (A and C) and IgM (B and D) were measured after *Af* challenge. (A and B) *CFTR*-ΔF508 (*n* = 6) and C57BL/6 littermates (*n* = 6) were sensitized with *Af*-crude protein extract (*Af*-cpe), and 2 weeks after sensitization their splenocytes were transferred into Rag^{-/-} mice, where they were allowed to engraft for 8 weeks before airway challenging the mice. (C and D) Naïve *CFTR*-ΔF508 (*n* = 10) and naïve C57BL/6 littermates (*n* = 10) were used to harvest naïve splenocytes for transfer into Rag^{-/-} mice. Splenocytes were allowed to engraft for 8 weeks, and then Rag^{-/-} mice were sensitized and airway challenged with *Af*-cpe.

bottom plates so that there were 1×10^5 CD4 T cells and 1×10^5 CD11b-positive cells for a total of 2×10^5 cells per well. Cells were cultured in media that contained 10 mg/ml albumin (Sigma, St. Louis, MO). After 3 days, supernatants were removed and frozen for cytokine analysis using Luminex Technology (Bio-Rad, Hercules CA).

Intracellular Calcium Flux

Spleens were harvested and CD4 T cells were separated using AutoMACS pro (Miltenyi Biotec). Intracellular calcium (iCa^{2+}) staining was done as previously described (53).

NFAT Studies

Splenocytes from *Cftr*- $\Delta F508$ (*Cftr*^{-/-}) or wild-type control littermates were plated on paired, 96-well, round-bottom plates with RPMI 1,640 10% FBS and 1% penicillin/streptomycin at a concentration of 1×10^5 cells/well. The cells were then stimulated with CD3/CD28 antibody cocktail, and wells from one plate were harvested at 10, 30, and 90 minutes for nuclear protein extraction, while the other was used to obtain cell culture supernatants at 1.5, 3, 6, and 24 hours for cytokine analysis. Nuclear NFAT translocation was assayed from the nuclear extracts using the modified kit, TransAM NFATc1 (Active Motif, Carlsbad, CA). Cell culture supernatants were analyzed for cytokine secretion profiles using Luminex Technology (Bio-Rad).

RESULTS

Cftr Deficiency and Th2-Skewed Adaptive Immune Responses

In previous studies, we have shown that *Cftr*^{-/-} mice on a mixed background had IgE levels that were two- to fivefold higher than their controls when sensitized and airway challenged with *Af*-cpe

(30). Furthermore, this phenotype was partially corrected after *Cftr* gene replacement with recombinant adeno-associated vectors (31). Here, we further establish this phenotype by comparing total serum IgE levels in *Cftr*^{-/-} mice that are on a congenic C57BL6 background. *Cftr*^{-/-} and *Cftr*^{+/+} littermates were sensitized and challenged with *Af*-cpe, as previously described (30). Total serum IgE from congenic mouse littermates challenged with *Af*-cpe were, on average, twofold higher in *Cftr* deficient mice compared to wildtype mice (Figure 1).

Adoptive Transfer Confers CF-Like Phenotype

To determine if the altered immune milieu imparted by *Cftr*^{-/-} lung epithelial cells seen during the aerosol challenge was involved in generating the twofold higher IgE response toward *Af*-cpe, we designed adoptive transfer experiments into Rag^{-/-} mouse hosts that were otherwise normal for *Cftr*. In the first set of experiments, *Cftr*^{-/-} mice with a deletion of phenylalanine at amino acid position 508 of the *Cftr* gene ($\Delta F508$, the most common mutation in the CF population) were sensitized, but not airway challenged, with *Af*-cpe. We then harvested splenocytes from these mice and their wild-type control littermates, and adoptively transferred them via an intraperitoneal injection into Rag^{-/-} mice of the same C57BL6 genetic background. At 8 weeks after transfer, the Rag^{-/-} mice were airway challenged with aerosolized *Af*-cpe. Because Rag^{-/-} mice are deficient in T and B cells, and cannot produce IgM, total circulating IgM levels were measured on these mice to verify the engraftment of the adoptive transfer (Figure 2B). To determine if sensitized spleno-

Figure 3. *In vitro* antigen recall with mixed cell populations. Splenocytes from congenic CFTR^{-/-} or CFTR^{+/+} mice sensitized to ovalbumin (OVA) were separated into both CD11b⁺ and CD4⁺ cell populations with magnetic beads. Cells were mixed and “crossed” to make four groups of cell preparations: (1) CF CD11b⁺ with CF CD4⁺ cells; (2) CF CD11b⁺ with wild-type (WT) CD4⁺ cells; (3) WT CD11b⁺ with CF CD4⁺ cells; and (4) WT CD11b⁺ with WT CD4⁺ cells. Cells were then stimulated with OVA for 5 days. The cell culture supernatants were analyzed for cytokine profiles at Day 5. Data are shown as group averages ($\pm$ SEM).

cytes from CF mice alone were able to recapitulate the high IgE phenotype in the presence of a wild-type (*Cftr*^{+/+}) airway, sera from the adoptively transferred Rag^{-/-} mice were checked for IgE levels. The results indicate that the twofold-enhanced IgE phenotype is transferred into the host purely by the adoptive transfer of sensitized splenocytes (Figure 2B). To rule out further the possibility of other *Cftr*-deficient tissues having an effect on the sensitization of the immune cells that were transferred, we repeated these experiments by transferring naive splenocytes into naive Rag^{-/-} mice. At 8 weeks after the naive splenocyte transfer, Rag^{-/-} mice were sensitized and airway challenged with *Af*-cpe. Once again, the experiments recapitulated the hyper-IgE phenotype, as serum levels of Rag^{-/-} mice receiving CF splenocytes were also twofold higher than Rag^{-/-} mice receiving wild-type splenocytes (Figure 2C). In contrast, IgM levels in these two groups were not different, confirming that the difference in IgE antibody levels was not due to differences in B-cell engraftment (Figure 2D).

Purified CF Cell Populations Demonstrate CF Phenotype *Ex Vivo*

We then designed a series of antigen recall experiments to begin determining what cell population of the splenocytes was responsible for polarizing the adaptive immune responses along a Th2 pathway, ultimately leading to the enhanced IgE response seen with the adoptive transfers. In these experiments, splenocytes from mice that had been sensitized to ovalbumin (OVA) were magnetically sorted into either CD4⁺ lymphocytes or CD11b⁺ monocytes. The rationale was that, upon OVA stimulation *in vitro*, the monocytes would process the antigen and present it to the OVA-specific T cells, allowing one to monitor the cytokine profiles secreted in response to a specific antigen. To determine if the Th2 polarization seen with CF splenocytes was due to either antigen presentation or to the antigen-specific response by T cells, the magnetically sorted cell populations were paired in the following configuration: CF monocytes with CF T cells; CF monocytes with wild-type T cells; wild-type monocytes with CF T cells; or wild-type monocytes with wild-type T cells. These experiments uncovered three main cytokine secretion patterns. The first one is evidenced with the Th2 cyto-

kines IL-4 and IL-13, where the elevated secretion seen with CF splenocytes was dependent on whether the stimulated cells had CF CD4⁺ T cells, and was independent of the monocyte source (Figures 3A and 3B). A different cytokine secretion pattern was observed for IL-17 and IL-5; this one was synergistic, and dependent on whether both the antigen-presenting cell population (CD11b⁺) and the effector cell population (CD4⁺) were *Cftr*^{-/-} (Figures 3C and 3D). Finally, IL-2 secretion was similar among all combinations, demonstrating that the altered cytokine secretion patterns are not an experimental artifact resulting from the cell mixtures (Figure 3E).

Aberrant Immune Responses Result from *Cftr*-Deficient Lymphocytes in a Conditional Knockout Model

To confirm the hypothesis that deficiency of a functional CFTR channel in T cells results in Th2-biased adaptive immune response, and leads to higher IgE levels, we used conditional *Cftr* knockout mice. These studies were performed by crossing the recently described *Cftr* floxed mice (37) with mice expressing Cre recombinase under the control of the Lck promoter, which drives expression of Cre recombinase in CD3⁺ lymphocytes, resulting in the knockout of the *Cftr* gene in both CD4⁺ and CD8⁺ T cells.

The floxed *Cftr* gene was maintained as a homozygous allele, and mice were crossed to yield both mice that were Lck-Cre⁺, and thus *Cftr* deficient in the T cell population, and mice that were Cre⁻, and thus maintaining the functional floxed *Cftr* gene in all tissues and cell types. Interestingly, measurement of IgE levels in naive mice that were Lck-*Cftr*^{-/-} showed a significant up-regulation of serum IgE, even at basal levels, when compared with their control littermates (Figure 4). This suggests an inherently Th2-biased commitment of *Cftr*-deficient T cells, even in the absence of stimulation. Next, we sensitized and airway challenged these mice along with their control littermates with *Af*-cpe. The results further confirmed a role for *Cftr* expression in T cells, as evidence by the divergence in serum IgE levels developing at Day 21 (1 wk after the second intraperitoneal injection) and through Day 32 (48 h after the final aerosol challenge) (Figure 5).

Figure 4. Total serum IgE levels in naive conditional CFTR knockout mice. T cell conditional knockout mice created by crossing the floxed *Cftr* mouse with mice expressing the Cre recombinase expressed from the leukocyte-specific protein tyrosine kinase (Lck) promoter were compared with control animals that were *Cftr* floxed, but Cre recombinase negative. Basal serum IgE levels were analyzed by ELISA. Data are shown as group averages ($\pm$ SEM).

Figure 5. Total serum IgE levels in *Af*-cpe-sensitized conditional CFTR knockout mice. T cell *Cftr* knockout mice (*Cftr* floxed and Lck Cre⁺) along with WT controls (*Cftr* floxed and Cre⁻) were sensitized with 200 μ g of *Af* crude extract dissolved in 100 μ l of PBS on Days 1 and 14. Blood samples were collected on Days 21, 28, and 32. Total serum IgE in the *Af*-sensitized mice was measured by ELISA. Data are shown as group averages ($\pm$ SEM).

Altered Calcium Signaling and NFAT Activation

To begin to elucidate possible mechanisms for the CFTR channel altering lymphocyte activation, we turned to a recently described model suggesting a link between CFTR and iCa^{2+} . Although iCa^{2+} concentration can be controlled by a myriad of mechanisms that may involve calcium channels, plasma membrane Ca^{2+} ATPase pumps, and potassium channels, among others. The model linking the *Cftr* mutation to altered iCa^{2+} and its eventual signaling and inflammation suggests that CFTR, through its effect on cell membrane potentials, alters the electrical driving force for Ca^{2+} to enter the cells. In *Cftr*^{+/+} T84 intestinal epithelial cells, it has been demonstrated that changes in membrane potentials caused expected changes in iCa^{2+} during agonist activation of calcium entry pathways (38, 39). Thus, with a reduction in Cl^- permeability in *Cftr*^{-/-} cells, their membranes can hyperpolarize, and it is therefore predicted that Ca^{2+} entry across the cell membrane would be increased. Alterations in this pathway are important, because, in lymphocytes, iCa^{2+} initiates a signal transduction pathway through calmodulin and calcineurin, eventually leading to the activation of NFAT, which has been shown to enhance gene expression of IL-4, IL-13, IL-5, and TNF- α cytokines, among others (40).

To investigate this hypothesis and whether the absence of the CFTR channel in T cells would have an effect on iCa^{2+} signaling through the T cell receptor (TCR), we measured iCa^{2+} fluxes in CD4⁺ T cells in response to CD3/CD28 stimulation of the TCR. Measurements of iCa^{2+} in CD4⁺ T cells using the calcium-sensitive Indo-1 dye revealed a significantly greater and enhanced calcium flux response in *Cftr*-deficient T cells (Figure 6).

To show that the increased iCa^{2+} fluxes seen in TCR activation in response to CD3/CD28 results in nuclear accu-

mulation of NFAT, we performed a time course where we stimulated T cells from splenocyte preparations with the same CD3/CD28 antibody cocktail. Nuclear extracts from the stimulated cells were prepared and used to measure NFAT levels. The results confirm that NFAT is translocated to the nucleus in a time-sensitive manner after TCR activation by CD3/CD28, and that this nuclear localization is increased in *Cftr*-deficient T cells (Figure 7).

To show directly if this nuclear translocation of NFAT would also translate to an up-regulation and increased secretion of IL-13 in *Cftr*^{-/-} T cells, we measured the cytokine levels in the supernatants of CD3/CD28-stimulated cells. The data show that the secretion pattern of TNF- α , an early-activated cytokine known to respond to iCa^{2+} signaling through the TCR (41), mimicked the pattern seen with the nuclear translocation of NFAT, and was secreted at significantly higher levels from *Cftr*^{-/-} T cells (Figure 7B). Although IL-13 was below detection in the earlier time points, it was found to be expressed at significantly higher levels from *Cftr*^{-/-} T cells in supernatants collected 24 hours after stimulation (Figure 7C).

DISCUSSION

In the present study, we demonstrate, for the first time, that the absence of CFTR in lymphocytes leads to an inherent divergence in the adaptive immune response *in vivo*. Specifically, in this case, it is characterized by an aberrant Th2-biased immune response to *Af* that is dependent on CFTR function in lymphocytes alone. We demonstrate that sensitization and aerosol challenge of *Cftr*^{-/-} mice and their wild-type controls leads to an enhanced-

Figure 6. Intracellular calcium (iCa^{2+}) flux. Changes in the concentration of intracellular free Ca^{2+} ions are measured by monitoring the change in the emission spectrum of Indo-1 dye from blue to violet upon binding to Ca^{2+} . The blue emission is measured through a 530/30 BP filter, and the violet through a 405/20 BP and 405 LP filter; thus, a shift in the violet:blue ratio over time is a reflection of the increase in iCa^{2+} concentration. CD4⁺ cells were enriched (see inset, top left) from *Cftr*- Δ F508 and *Cftr*^{+/+} mice and stimulated with anti-CD3/CD28 antibodies (Abs), followed by 500 ng/ml of ionomycin (as a positive control; see inset on bottom right). (A) Representative iCa^{2+} flux for *Cftr*- Δ F508 and *Cftr*^{+/+} CD4⁺ T cells. (B) The area under the curve for the traces from *Cftr*- Δ F508 ($n = 6$) and *Cftr*^{+/+} ($n = 6$) in response to CD3/CD28 Abs. (C) Slopes for the traces from *Cftr*- Δ F508 ($n = 6$) and *Cftr*^{+/+} ($n = 6$) in response to CD3/CD28 Abs.

Figure 7. Nuclear factor of activated T cell (NFAT) c1 nuclear translocation and cytokine secretion in *Cfr*^{-/-} and *Cfr*^{+/+} mice after T cell receptor stimulation. (A) Nuclear translocation of NFATc1 was measured using a modified ELISA from nuclear splenocyte extracts after CD3/CD28 stimulation at 10, 30, and 90 minutes after stimulation. Parallel plated cells were assessed for cytokine expression after CD3/CD28 stimulation at 1.5, 3, 6, and 24 hours. (B) Time course expression for TNF- α . (C) Expression of IL-13 at 24 hours after stimulation. Data are shown as group averages ($\pm$ SEM) for a total of $n = 5$.

IgE response in *Cfr*^{-/-} mice that is reminiscent of a CF-related allergic asthmatic condition known as allergic ABPA. Although ABPA remains rare outside of the CF population, it is very common among patients with CF, affecting approximately 15% of them (20, 21). Interestingly, we demonstrate that the transfer of naive *Cfr*-deficient splenocytes into congenic Rag^{-/-} mice was enough to confer the high-IgE response to the Rag^{-/-} mice. These experiments revealed that a CFTR-dependent phenotype can be transferred from CF mice into CFTR-sufficient hosts purely through splenocytes.

The expression of CFTR in lymphocytes has been well characterized throughout the years (33, 36, 42); however, the physiological relevance of CFTR expression in lymphocytes is less clear, with some studies implicating it with volume regulation and cytolysis regulation of CD8⁺ T cells (43–46). Other models suggest that activation of Cl⁻ currents by CFTR in response to nitric oxide via a cyclic GMP-dependent mechanism is defective in T cells from patients with CF (47). Importantly, electrophysiological studies document a functional role for CFTR in lymphocytes by recording a defect in cyclic AMP-dependent Cl⁻ currents in CF-derived lymphocytes using whole-cell patch clamp techniques (33, 48). These studies demonstrate the presence and function of CFTR within lymphocytes, and lend some credence to the long-standing yet controversial view that a primary immune abnormality is associated with CF. To date, it has been difficult to separate aberrant immune response observed in CF from the disease phenotype imparted by epithelial cell dysfunction. Our adoptive cell transfer experiments indicate that a primary immune abnormality was indeed a possibility. Namely, the transfer of naive splenocytes was able to recapitulate the twofold-higher IgE response seen in *Cfr*^{-/-} mice. We further investigated what specific cell type from the splenocyte pool was responsible for this phenotype with *in vitro* assays. Because the IgE response is known to be driven by Th2 cytokines, and we have previously

demonstrated that IL-13 is up-regulated in *Cfr*^{-/-} mice challenged with *Af*-cpe, we designed a series of experiments to analyze the secretion of Th2 cytokines by activated T cells in response to antigen. According to our data, a marked difference in the secretion of IL-4, -13, -5, and -17 was observed from CD4⁺ and CD11b⁺ cells that were *Cfr* deficient. Interestingly, antigen-specific responses characterized by increased secretion of IL-4 and IL-13 were only observed in the presence of *Cfr*^{-/-} CD4⁺ T cells, whereas the increased secretion of IL-5 and IL-17 seemed to be dependent on a synergistic interaction between both *Cfr*^{-/-} CD11b⁺ and CD4⁺ cells. Taken together with the adoptive transfer experiments, these data suggest that *Cfr*-deficient T cells imparted a Th2-skewed response, characterized by increased secretion of IL-4 and IL-13, ultimately leading to the high IgE response directed against *Af*-cpe. However, based on the secretion of IL-5 and IL-17 in the antigen recall experiments, it is conceivable that skewed responses may be due to the interaction of various *Cfr*-deficient immune cells, such as macrophages, dendritic cells, and T cells.

To determine directly whether this phenotype can be attributed to *Cfr*-deficient T cells, we created T cell-specific *Cfr* knockout mice by using a floxed *Cfr* mouse expressing Cre recombinase under the control of the LCK promoter. These mice presented with an increase in basal IgE levels in the absence of any exogenous antigenic stimulus. These elevated basal IgE circulating antibody levels are consistent with a previous observation in which we recorded higher IgE levels in *Cfr*^{-/-} mice as compared with controls that had been mock PBS-sensitized and challenged with *Af*-cpe (30). Furthermore, these T cell *Cfr*-conditional knockout mice went on to develop dramatically different IgE responses to *Af*-cpe, as was characteristic of *Cfr*^{-/-} mice. With these experiments, we were able to demonstrate finally, without any confounding variables, a primary

immune abnormality associated with CF *in vivo*, which, to our knowledge has never been shown before.

A consequence of *Cftr* deficiency in lymphocytes is the reduction in Cl^- permeability, which, in turn, may hyperpolarize their membranes. It is hypothesized that the altered membrane potential could then alter the electrical driving force for Ca^{2+} to enter the cells. Here, we also show that iCa^{2+} fluxes in *Cftr*^{-/-} CD4⁺ T cells are increased in response to CD3/CD28 stimulation, as determined by the area under the curve and the slopes of the Ca^{2+} flux response when compared with wild-type CD4⁺ T cells (Figure 6). The relevance of this altered calcium flux in *Cftr*^{-/-} T cells is that it may lead to increased transcription activity of Ca^{2+} -regulated transcription factors. NFAT activity is modulated by cytoplasmic Ca^{2+} concentration through various Ca^{2+} -associated signaling pathways. Ultimately, increases in cytoplasmic Ca^{2+} concentration induce NFAT dephosphorylation and NFAT translocation to the nucleus, where it binds to *cis* regulatory elements of target genes as a monomer (49). This signaling cascade proceeds through calmodulin and calcineurin, a cytoplasmic serine/threonine phosphatase. Calcineurin up-regulates NFAT by dephosphorylating serines in the serine, proline repeats and in the serine-rich N terminus region of NFAT, exposing the nuclear localization signal, allowing NFAT to translocate to the nucleus (49). In T cells, TCR stimulation causes an increase in iCa^{2+} concentration, depleting Ca^{2+} -sequestered ions in the endoplasmic reticulum, followed by an influx of extracellular calcium ions from Ca^{2+} release-activated Ca^{2+} channels (49). Although NFAT activation regulates a variety of immune processes, NFAT has been found to regulate a number of other promoters for cytokine genes, including those involved in the regulation of Th2 immune responses as IL-4, -13, -5, and TNF- α (50). In agreement with the results for the iCa^{2+} flux experiments, we observed a significantly greater increase in the nuclear translocation of NFAT after TCR activation with CD3/CD28 in *Cftr*^{-/-} cells (Figure 7). Parallel cell experiments demonstrated that, after NFAT nuclear translocation, *Cftr*^{-/-} cells had significantly higher expression of TNF- α and the key Th2 effector cytokine, IL-13. These data suggest that a possible mechanism responsible for the *in vivo* immune aberration observed in the conditional *Cftr* T cell knockout mice may be directly related to the more vigorous TCR-mediated calcium flux responses, leading to an increased translocation of NFAT and higher induction of IL-13 in *Cftr*^{-/-} T cells. Exactly how CFTR dysfunction affects Ca^{2+} signaling in lymphocytes warrants further investigation, and the convergence or overlap of other dysregulated pathways related to *Cftr* deficiency cannot be ruled out. There is some evidence of CFTR dysfunction imparting elevated antibody secretion in B-cell hybridomas. Although these data do not explicitly assess CFTR function in B cells, they do suggest that, regardless of aberrant or normal B-cell function in CF, the upstream events of T cell activation and helper function alone are enough to impart a polarized antibody response, even in the presence of B cells with intact CFTR function.

The main pathological features associated with CF are pancreatic cysts that lead to a reduction in the secretion of digestive enzymes, as well as chronic airway infections, most notably by *Pseudomonas*. Although it has been well established and recognized that impaired CFTR function adversely affects the secretory epithelium, the role of CFTR in nonepithelial cells has received less attention, or has largely eluded investigators. Certainly, if the CFTR alters the function of immune cells, it should be expected to result in an aberrant immune response, which could further compromise patients. In summary, our data identify that CFTR dysfunction in T cells can, in and of itself, lead to aberrant immune responses. Specifically, we show how it skews responses to Af, leading to a higher-than-normal IgE

response. This observation, itself, is reminiscent ABPA, an otherwise rare but prevalent condition in the CF population. These findings represent a new and important cell population to investigate to try to prevent or ameliorate aberrant immune responses in people with CF. Thus, current and future drugs targeting CF should determine if their benefits extend to this cell population. Alternatively, these results also open a new avenue to test small molecule modulators of immune response as a potential therapy for CF.

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