

Aspergillus fumigatus Generates an Enhanced Th2-Biased Immune Response in Mice with Defective Cystic Fibrosis Transmembrane Conductance Regulator¹

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Cystic fibrosis (CF) lung disease is characterized by persistent airway inflammation and airway infection that ultimately leads to respiratory failure. *Aspergillus sp.* are present in the airways of 20–40% of CF patients and are of unclear clinical significance. In this study, we demonstrate that CF transmembrane conductance regulator (CFTR)-deficient (CFTR knockout, *Cftr*^{tm1Unc}-TgN(fatty acid-binding protein)CFTR) and mutant (Δ F508) mice develop profound lung inflammation in response to *Aspergillus fumigatus* hyphal Ag exposure. CFTR-deficient mice also develop an enhanced Th2 inflammatory response to *A. fumigatus*, characterized by elevated IL-4 in the lung and IgE and IgG1 in serum. In contrast, CFTR deficiency does not promote a Th1 immune response. Furthermore, we demonstrate that CD4⁺ T cells from naive CFTR-deficient mice produce higher levels of IL-4 in response to TCR ligation than wild-type CD4⁺ T cells. The Th2 bias of CD4⁺ T cells in the absence of functional CFTR correlates with elevated nuclear levels of NFAT. Thus, CFTR is important to maintain the Th1/Th2 balance in CD4⁺ T cells. *The Journal of Immunology*, 2006, 177: 5186–5194.

Cystic fibrosis (CF)³ is the most common, lethal genetic disease in Caucasians with much of the morbidity and nearly all of the mortality resulting from progressive lung disease and respiratory failure. CF transmembrane conductance regulator (CFTR) is the abnormal gene product in CF, with hundreds of mutations described (1). CFTR is a cAMP-regulated chloride channel and is expressed by many cell types. Among them, airway epithelial cells have received the most extensive study; these cells demonstrate increased Na⁺ absorption with depletion of the airway surface liquid layer in the absence of CFTR expression (2–4). Despite efforts to date, a clear understanding of the pathogenesis of CF lung disease remains incomplete, as much of the phenotype cannot be explained simply by a defect in ion transport by epithelial cells.

CF lung disease is characterized by persistent airway inflammation and airway infection. Neutrophils are the major cell type

within the CF airway but both B and T lymphocytes are found in large aggregates beneath the surface epithelium (5, 6). In infants with CF, exaggerated production of proinflammatory cytokines is observed in lower airways (7, 8), yet the majority of CF patients will eventually become colonized with *Pseudomonas aeruginosa* and complete eradication of this organism is uncommon (9). Additionally, as many as 20–40% of CF patients have chronic airway colonization with *Aspergillus sp.* (10–14). Although *P. aeruginosa* is treated aggressively in CF, the clinical significance of persistent *Aspergillus fumigatus* colonization is unclear. Although CF patients have an intact immune system and are able to generate an exuberant inflammatory response, the nature of this response may be inappropriate as it does not eradicate the organisms to which it is directed and may itself be responsible for significant airway damage.

A Th2 immune response has been associated with both asthma and allergic bronchopulmonary aspergillosis (ABPA). In these settings, lung disease is associated with the release of several cytokines which stimulate maturation and differentiation of eosinophils (IL-4 and IL-5) (15), mucus hypersecretion (IL-13) (16, 17), and are potent inducers of Ab production by B cells, particularly IgE and IgG1 (18). Patients with CF have a high prevalence of asthma (>30%) and ABPA (>10%), suggesting that Th2 inflammation may be important (9, 19–25). Thus, it is possible that both lung pathology and the inability of CF patients to control and eradicate airway *Aspergillus* colonization may in part be due to a Th2-biased inflammatory response.

Although CFTR has been found to be expressed on T cells (26), the role of CFTR in T cell immune responses has not been investigated. In this study, we examined the role of CFTR in the differentiation of CD4⁺ T cells into Th1/Th2 effector cells. We demonstrate that naive CD4⁺ T cells from mice deficient in CFTR have a spontaneous Th2 bias in vitro. Furthermore, we show that the absence of CFTR leads to an excessive Th2-biased inflammatory response to *A. fumigatus* Ags in vivo. Thus, CFTR is an important factor that influences CD4⁺ T effector cell differentiation and potentially the effectiveness of the immune response to *A.*

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Received for publication November 29, 2005. Accepted for publication July 25, 2006.

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¹ This work was supported by the Center for Biomedical Research Excellence Program of the National Center for Research Resources Grant P20RR15557, the Cystic Fibrosis Foundation and a Biomedical Research Infrastructure Network award from the National Center for Research Resources, and National Institutes of Health Grant RO1 AI 51468 (to K.A.M.).

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³ Abbreviations used in this paper: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; ABPA, allergic bronchopulmonary aspergillosis; AHA, *A. fumigatus* hyphal Ag; KO, knockout; WT, wild type; alum, aluminum hydroxide; BAL, bronchoalveolar lavage; HPRT, hypoxanthine phosphoribosyltransferase; C_T, threshold cycle.

fumigatus. A better understanding of how CFTR regulates CD4⁺ T cell development is critical to improving our understanding of the pathogenesis of CF lung disease. We speculate that CF patients develop an exuberant Th2-biased inflammatory response to *A. fumigatus* that directly contributes to airway inflammation and damage.

Materials and Methods

Reagents

Anti-CD3 mAb and anti-CD28 mAb (BD Pharmingen) were used for CD4⁺ T cell activation. *A. fumigatus* hyphal Ags (AHA) were generated by culture of Af293 isolate (5 days, 37°C) in RPMI 1640 plus 10% FCS. The hyphal mat was harvested, sequentially washed with PBS, and disrupted by vortexing with glass beads. The slurry was subjected to paraformaldehyde (1%) for microbial inactivation. Protein concentration was measured using the Bradford assay (Bio-Rad) according to manufacturer's instructions. The product was concentrated using an endotoxin-free dialysis membrane with 10-kDa pore size (Pierce Biotechnology) to achieve a final protein concentration of 800–1000 µg/ml.

Mice

ΔF508 mice, on a C57BL/6 background (27), provided by Dr. W. Colledge (University of Cambridge, Cambridge, U.K.), and CFTR knockout (KO) (28), from Case Western Reserve University (Cleveland, OH), on a mixed genetic background were used in all studies. Age-matched wild-type (WT) littermates were used as controls for the ΔF508 mice in all experiments and age-matched C57BL/6J mice were used as controls in all experiments for the CFTR KO animals. All mice were 8–16 wk of age. All procedures that involved mice were approved by the University of Vermont Institutional Animal Care and Use Committee. Mice were anesthetized with i.p. pentobarbital sodium (70–90 mg/kg), followed by nonsurvival surgery.

A. fumigatus airway exposure protocol

Mice were briefly anesthetized (isoflurane by inhalation) and intranasally exposed to 20 µg of AHA on day 0, 7, 14, 15, and 16.

OVA sensitization protocol

Mice were immunized i.p. with a 100-µl injection containing 20 µg of OVA (grade V; Sigma-Aldrich) adsorbed to 50 µg of aluminum hydroxide (alum; Pierce) or 50 µl of CFA (Pierce). The OVA/alum immunization was performed on days 0 and 7 with spleen and lymph node harvest on day 14. The OVA/CFA immunization was performed on day 0 with spleen and lymph node harvest on day 7.

Determination of serum Ig levels

Serum was obtained by right heart puncture of anesthetized mice at the time of lung and lymph node harvest. Total serum IgE and IgG1 was determined by coating 96-well plates with anti-IgE or anti-IgG1 (2 µg/ml) and respective Ig levels were measured by ELISA using biotinylated anti-IgE or anti-IgG1 (2 µg/ml; BD Pharmingen), according to the manufacturer's instructions.

Bronchoalveolar lavage (BAL)

Tracheas were cannulated with 2 cm of 22-gauge polyethylene tubing attached to a 23-gauge needle, 1 ml of cold PBS was instilled into the lungs, and the BAL fluid was collected. Cells were pelleted and counted using an Advia cell counter (Bayer). Cells (50 × 10³) were cytospun onto glass slides and stained with Hema-3 (Biochemical Sciences). Two hundred cells per slide were counted and scored as macrophages, eosinophils, neutrophils, or lymphocytes based on characteristic morphology and staining.

Quantitative PCR analysis of gene expression

The right lung from each experimental animal was removed and snap-frozen in liquid nitrogen. It was then ground to a fine powder using a chilled mortar and pestle and total RNA was extracted using TRIzol and further purified using an RNeasy kit (Qiagen). First-strand cDNA was synthesized using random primers (Invitrogen Life Technologies) and Superscript II reverse transcriptase (Invitrogen Life Technologies). Quantitative PCR was performed on cDNA with Assays on Demand predesigned IL-4, IFN-γ, and hypoxanthine phosphoribosyltransferase (HPRT) primer/probe sets (Applied Biosystems), using TaqMan Universal PCR Master Mix and the Applied Biosystems PRISM 7700 Sequence Detection System. The level of *IL-4* or *IFN-γ* expression was normalized to *HPRT* housekeeping

gene levels and relative IL-4 or IFN-γ mRNA levels were determined according to the comparative cycle threshold method (ABI Prism 7700 Sequence Detection System, User Bulletin No. 2; Applied Biosystems). Briefly, the threshold cycle (C_T) was determined for IL-4, IFN-γ, and HPRT in each sample. The ΔC_T was calculated for each sample by subtracting the C_T of HPRT from the C_T of IL-4 or IFN-γ. The baseline ΔC_T was assigned to non-AHA-exposed WT mice. The $\Delta\Delta C_T$ values were calculated by subtracting the ΔC_T of the baseline from the ΔC_T of the experimental samples. The $\Delta\Delta C_T$ values were transformed into absolute values using the equation $2^{-\Delta\Delta C_T}$.

Lung histology

Lungs were processed for histological analysis as described previously (29) and stained with H&E according to routine procedures.

Cell preparation and activation

CD4⁺ T cells were isolated from the spleen and lymph nodes (inguinal and mesenteric) by negative selection as previously described (30), using mAbs to CD8 (clone 53-6.72 and clone 2.43) (31), class II MHC I-A^d (clone 212.A1) (31), anti-NK1.1 (PK136; American Type Culture Collection) and anti-Mac-1 (BD Pharmingen) mAbs and anti-Ig-coated magnetic beads (Qiagen). Isolated CD4⁺ T cells (1 × 10⁶ cells/ml) were activated either by: 1) plate-bound anti-CD3 mAb (10 µg/ml; BD Pharmingen) and soluble anti-CD28 mAb (1 µg/ml; BD Pharmingen) for 24, 48, and 72 h; 2) AHA (5 µg/ml) and syngeneic APCs (4 × 10⁶ cells/ml) for 72 h; or 3) OVA (100 µg/ml; Sigma-Aldrich) and syngeneic APCs for 72 h.

APCs were obtained by splenic T cell depletion with negative selection using Abs to CD4 (GK1.5) (32), CD8, and Thy-1 (33), and treatment with rabbit complement. The remaining cells were then treated with mitomycin C, as previously described (34).

After in vivo AHA exposure, mediastinal lymph nodes (MLN) were isolated at the time of lung harvest and activated (1 × 10⁶ cells/ml) with plate-bound anti-CD3 (10 µg/ml) and soluble anti-CD28 (1 µg/ml) mAbs (BD Pharmingen) for 24 h.

Determination of CD4⁺ T cell proliferation

Proliferation of CD4⁺ T cells after activation (coated anti-CD3 and soluble anti-CD28 mAbs as above) was determined by 18 h of [³H]thymidine incorporation after 3 days in culture. Plates were harvested and counted using a Tomtec Harvester 96, according to manufacturer's recommendations.

Determination of cytokine production by CD4⁺ T cells

ELISAs were performed on the cell culture supernatant for IL-4 and IFN-γ using Duosets (R&D Systems) according to manufacturer's recommendations.

Determination of NFAT binding

Nuclear extracts were prepared from stimulated (for 72 h) CD4⁺ T cells as described previously (35). Binding reactions were performed using 2 µg of nuclear proteins and a [³²P]dCTP end-labeled double-stranded oligonucleotide probe containing an NFAT binding site from the proximal *IL-4* gene promoter (36, 37) or a CREB protein site from the somatostatin gene promoter (38, 39). Anti-NFATc2 Ab (Upstate Biotechnology) was present during the binding reactions for supershift analysis. Samples were electrophoresed under nondenaturing conditions and exposed to film for autoradiography.

Statistical analysis

Determination of statistical significance for serum Ig levels between WT and CFTR KO and WT and ΔF508 mice was conducted using two-way ANOVA for multiple points of comparison. For all other experiments, statistical significance was determined by the Student *t* test. For all analyses, *p* < 0.05 was considered statistically significant.

Results

Exposure of CFTR KO mice to AHA results in enhanced airway and lung tissue inflammation

A. fumigatus airway colonization is common in patients with CF and may play a role in the chronic airway inflammation characteristic of CF lung disease. We investigated whether CFTR deficiency influences the immune response to *A. fumigatus* exposure in a mouse model. To perform these studies, we used a gut-corrected

CFTR KO mouse (28), which has intestinal epithelial expression of human CFTR to decrease the high mortality from bowel obstruction seen in non-gut-corrected CFTR KO mice (28). CFTR KO mice and WT controls were exposed to AHA on days 0, 7, 14, 15, and 16. Seventy-two hours after the last exposure, airway inflammation was determined by BAL. The nonexposed WT and CFTR KO mice did not have spontaneous airway inflammation (data not shown), as has been previously reported (28). In both the nonexposed CFTR KO and WT animals, >98% of the BAL cells were macrophages (data not shown). WT mice exposed to AHA had evidence of airway inflammation, as determined by BAL cell number, but the exposed CFTR KO mice had significantly increased airway inflammation (Fig. 1A). Airway cell differential analysis showed an eosinophil predominance in both the WT and CFTR KO mice with greater numbers of macrophages, neutrophils, and eosinophils present within the CFTR KO airway compared with WT exposed to AHA (Fig. 1B).

We also evaluated lung tissue inflammation by formalin fixation and H&E staining after AHA exposure. As anticipated, the nonexposed CFTR KO and WT mice had no significant inflammation

on H&E staining (Fig. 1C). The AHA-exposed WT mice had eosinophilic peribronchial and perivascular inflammation. However, lung inflammation was significantly greater in the AHA-exposed CFTR KO mice (Fig. 1C). Therefore, we conclude that CFTR deficiency causes an enhanced inflammatory response to AHA in the lung.

Elevated Th2-mediated Ab production in CF mice following exposure to AHA

Accumulation of eosinophils is normally associated with the development of a Th2-biased immune response as Th2 cytokines mediate the proliferation and recruitment of eosinophils (15). Release of IL-4 by Th2 lymphocytes also results in class switching of B cells to produce IgE and IgG1. To confirm the Th2 bias in CFTR-deficient mice following *A. fumigatus* exposure, we assessed serum Ig (IgE and IgG1) production in WT and CFTR KO mice. WT or CFTR KO mice were intranasally exposed to AHA, as described above. Seventy-two hours after the last AHA exposure, total IgE and total IgG1 levels in serum were determined by ELISA. As expected, low levels of serum IgE were detected from the nonexposed WT and CFTR KO mice (Fig. 2A). Although WT mice exposed to AHA had substantial levels of serum IgE, a significantly higher level of serum IgE was detected in AHA-exposed CFTR KO mice (Fig. 2A). We also determined total serum IgG1, another hallmark of a Th2 response. AHA-exposed CFTR KO mice had a greater serum titer of IgG1 than the AHA-exposed WT mice (Fig. 2B). Thus, AHA exposure in CFTR-deficient mice results in accentuated Th2-mediated Ig production.

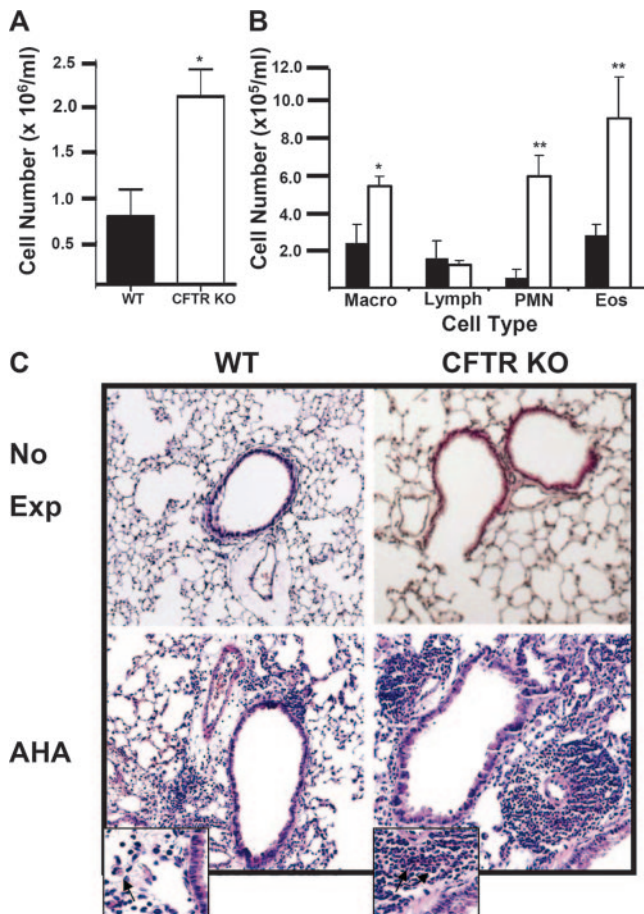


FIGURE 1. A, BAL cell counts after intranasal exposure to AHA. B, BAL cell number by cell type as determined by manual differential cell count of macrophages (macro), lymphocytes (lymph), neutrophils (PMN), and eosinophils (eos). Values represent the mean $\pm$ SEM of three mice per group and are representative of experiments performed twice. Statistical significance was determined by a Student's *t* test. *, $p < 0.05$; **, $p < 0.01$ compared with values in WT AHA-exposed mice. C, WT and CFTR KO mice were intranasally exposed to AHA on days 0, 7, 14, 15, and 16. Seventy-two hours after the last AHA exposure, lungs were sectioned, and stained with H&E. Photomicrographs are original magnification, $\times 100$ with $\times 400$ inlay. Arrows highlight lung tissue eosinophils.

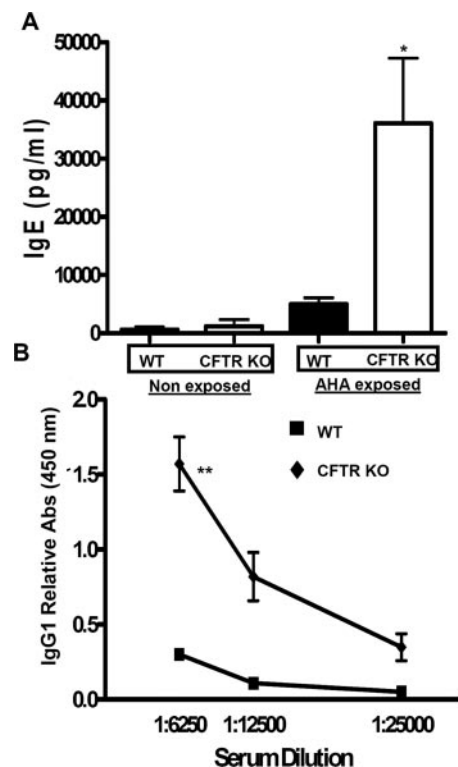


FIGURE 2. Mice were exposed to AHA as described for Fig. 1. Total serum IgE (A) in naive and AHA-exposed mice and total serum IgG1 (B) in AHA-exposed mice was determined by ELISA. Values are the means ($\pm$ SEM) of one representative experiment of two with three mice per group. Statistical significance was determined by two-way ANOVA. *, $p < 0.05$; **, $p < 0.001$ compared with values in WT AHA-exposed mice.

Exposure of CFTR-deficient mice to AHA results in increased levels of IL-4 in the lung

Eosinophilic airway and tissue inflammation and the elevated production of IgE and IgG1 following AHA exposure of CFTR KO mice could be due to increased Th2 cytokine production. Therefore, we exposed WT or CFTR KO mice intranasally to AHA (as above) and 72 h after the last AHA exposure, lungs were excised and RNA was isolated. mRNA expression of IL-4 and IFN- γ was quantified using real-time RT-PCR. IL-4 expression was clearly detected in AHA-exposed WT mice, but expression of IL-4 was significantly greater in AHA-exposed CFTR KO mice (Fig. 3A). In contrast, lung IFN- γ expression in the AHA-exposed WT and CFTR KO mice was similar (Fig. 3A). Thus, consistent with a Th2-biased immune response, AHA exposure of CFTR-deficient mice results in an increased expression of IL-4.

Because T cells are an important source of IL-4 and IFN- γ , we next determined the cytokine response of activated lymphocytes from the AHA-exposed WT and CFTR KO mice. After exposure to AHA, the MLN cells were stimulated *in vitro* for 24 h with anti-CD3 and anti-CD28 mAbs. IL-4 and IFN- γ levels in supernatant were determined by ELISA. Activated MLN cells from AHA-exposed CFTR KO mice produced higher levels of IL-4 than the MLN cells of the AHA-exposed WT mice (Fig. 3B). However, MLN cells from CFTR KO AHA-exposed mice produced similar levels of IFN- γ as WT AHA-exposed

mice (Fig. 3B). To examine the AHA-specific response *in vitro*, we isolated CD4⁺ T cells from WT and CFTR KO AHA-exposed mice and restimulated them for 72 h with AHA and APCs derived from syngeneic, mitomycin-treated, and T cell-depleted WT splenocytes. IL-4 and IFN- γ levels were determined in cell culture supernatant at the end of the culture period. As expected, we found no detectable IL-4 or IFN- γ production by the CD4⁺ T cells from the nonexposed WT and CFTR KO mice. CD4⁺ T cells derived from AHA-exposed WT animals produced detectable levels of IL-4, but CD4⁺ T cells derived from AHA-exposed CFTR KO mice produced significantly more IL-4 (Fig. 3C) while IFN- γ production was similar (Fig. 3C). These results indicate that CFTR-deficient mice develop an enhanced CD4⁺ T cell-mediated Th2 immune response to AHA.

CD4⁺ T cells from naive CFTR KO mice have a Th2 bias

Although the above results suggested that CFTR regulates cytokine production in CD4⁺ T cells, it was also possible that the Th2 bias during *ex vivo* restimulation could be due to defective

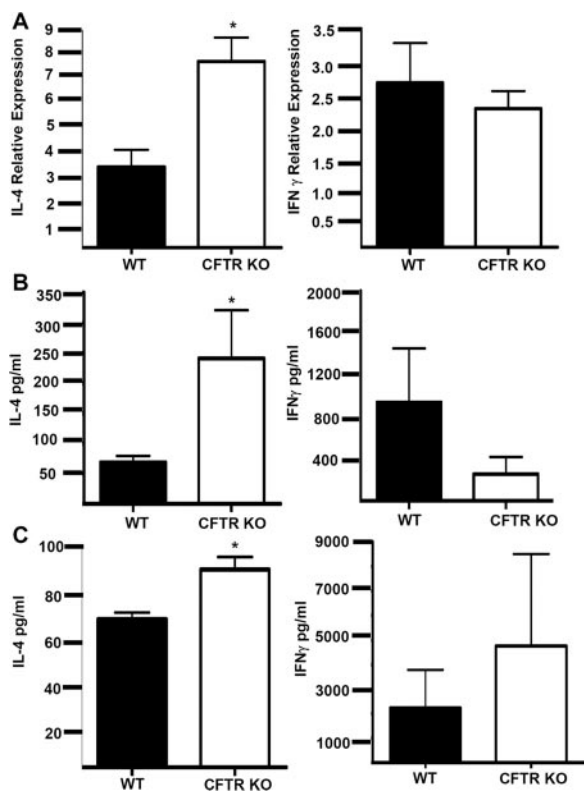


FIGURE 3. A, Total lung real-time RT-PCR for IL-4 and IFN- γ after intranasal AHA exposure. IL-4 and IFN- γ expression is referenced to non-exposed WT mice. B, Cytokine production by MLN cells isolated from mice after intranasal AHA exposure were *in vitro* activated with anti-CD3 and anti-CD28 mAbs. C, Cytokine production by *ex vivo* AHA-activated CD4 T cells after *in vivo* intranasal AHA exposure. Data presented are the means $\pm$ SEM of one experiment of two with three mice per group. Statistical significance was determined by the Student *t* test. *, *p* < 0.05 compared with values in WT AHA-exposed mice.

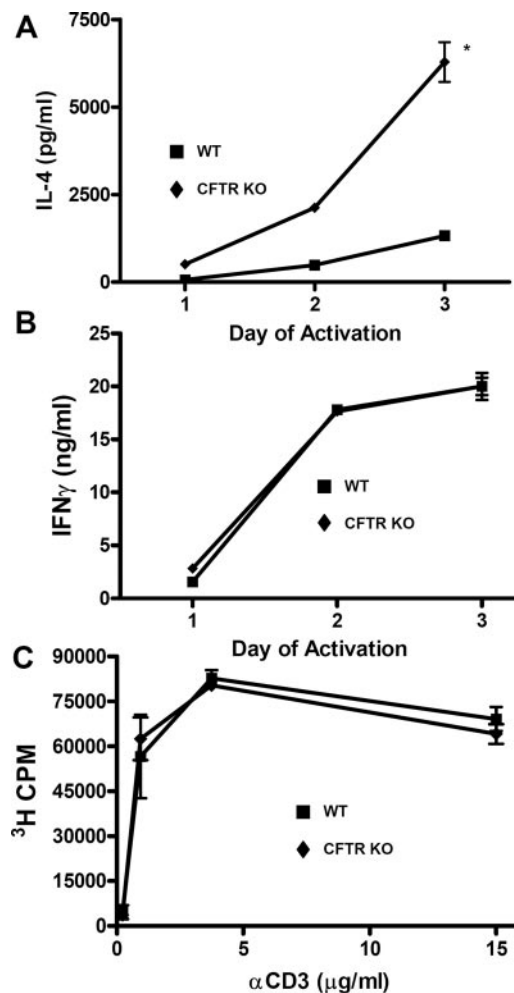


FIGURE 4. A, IL-4 (A) and IFN- γ (B) production by naive CD4 T cells after activation with anti-CD3 and anti-CD28 mAbs as determined by ELISA. C, Proliferation of naive CD4 T cells after activation with anti-CD3 and anti-CD28 mAbs as determined by [³H]thymidine incorporation. *In vitro* data presented are the means $\pm$ SEM of one representative experiment of two with cultures performed in triplicate. Statistical significance was determined by two-way ANOVA. *, *p* < 0.0001 compared with values in WT AHA-exposed mice.

CFTR during *in vivo* priming. To determine whether CFTR plays a role in CD4⁺ T effector cell differentiation, we examined IL-4 and IFN- γ production after anti-CD3 and anti-CD28 mAbs stimulation of CD4⁺ T cells isolated from the spleens of WT and CFTR KO mice. CD4⁺ T cells from WT mice had detectable levels of IL-4. However, CD4⁺ T cells from CFTR KO mice produced higher levels of IL-4 after activation (Fig. 4A). In contrast, IFN- γ production by the WT and CFTR KO CD4⁺ T cells was similar after activation (Fig. 4B). IL-2 production and proliferation of WT and CFTR KO CD4⁺ T cells were also no different (Fig. 4C). Thus, CFTR influences cytokine production by CD4⁺ T cells in AHA-naïve mice and CFTR deficiency may augment IL-4 production.

*Δ F508 mice generate an enhanced Th2-biased immune response to *in vivo* AHA exposure*

To further confirm the Th2-biased immune response to AHA in another mouse model of functional CFTR deficiency, we examined the immune response of AHA-exposed Δ F508 mice. The Δ F508 mice have a defect in CFTR that results in inappropriate shuttling of the protein to the cell surface (27) and like the CFTR KO mice, do not develop significant spontaneous lung disease (27) (Fig. 5A). The airway inflammatory response was significantly greater in AHA-exposed Δ F508 mice than in AHA-exposed WT mice (Fig. 5A). As with the CFTR KO mice, the Δ F508 mice had a predominance of airway eosinophils (data not shown). In addition, increased perivascular and peribronchial inflammation was present in the AHA-exposed Δ F508 mice compared with AHA-exposed WT mice (Fig. 5B). We also examined the serum IgE and IgG1 levels in AHA-exposed WT and Δ F508 mice. As expected, there were low levels of serum IgE in the nonexposed WT and Δ F508 mice (Fig. 5C). As observed in the CFTR KO mice, higher levels of serum IgE and IgG1 were detected in AHA-exposed Δ F508 mice than in AHA-

exposed WT mice (Fig. 5C). Thus, these data further demonstrate that CFTR deficiency results in an enhanced, Th2-biased immune response to *A. fumigatus*.

CD4 T cells isolated from naïve Δ F508 mice have a Th2 bias

To further delineate the role that CFTR plays in CD4⁺ effector cell differentiation, we examined IL-4 and IFN- γ production after anti-CD3 and anti-CD28 mAbs stimulation of CD4⁺ T cells isolated from naïve WT and Δ F508 mice. As was seen for the CFTR KO mice (Fig. 4, A and B), CD4⁺ T cells from Δ F508 mice produced higher levels of IL-4 compared with WT CD4⁺ T cells (Fig. 6A). No difference in IFN- γ production (Fig. 6B), proliferation (Fig. 6C) nor CD25 expression (Table I) were observed between WT and Δ F508 CD4⁺ T cells. Thus, these results provide further evidence that CFTR regulates cytokine production by CD4⁺ T cells.

CFTR deficiency enhances Th2, but not Th1, immune responses

The above results indicate that CFTR deficiencies affect IL-4 but not Th1 cytokines, such as IFN- γ . To further demonstrate the selective involvement of CFTR in Th2 differentiation *in vivo*, we performed immunization studies with OVA in the presence of CFA, as an adjuvant to promote Th1 development, or alum, as an adjuvant to promote Th2 development. After immunization, CD4⁺ T cells were isolated and restimulated *in vitro* with OVA for 72 h and IL-4 and IFN- γ production was determined by ELISA. Similar to the AHA exposure, we found that CD4⁺ T cells from OVA/alum-immunized Δ F508 mice produced more IL-4 than OVA/alum-immunized WT CD4⁺ T cells (Fig. 7A). In contrast, CD4⁺ T cells from OVA/CFA-immunized Δ F508 mice produced less IFN- γ than OVA/CFA-immunized WT CD4⁺ T cells (Fig. 7B). These results indicate

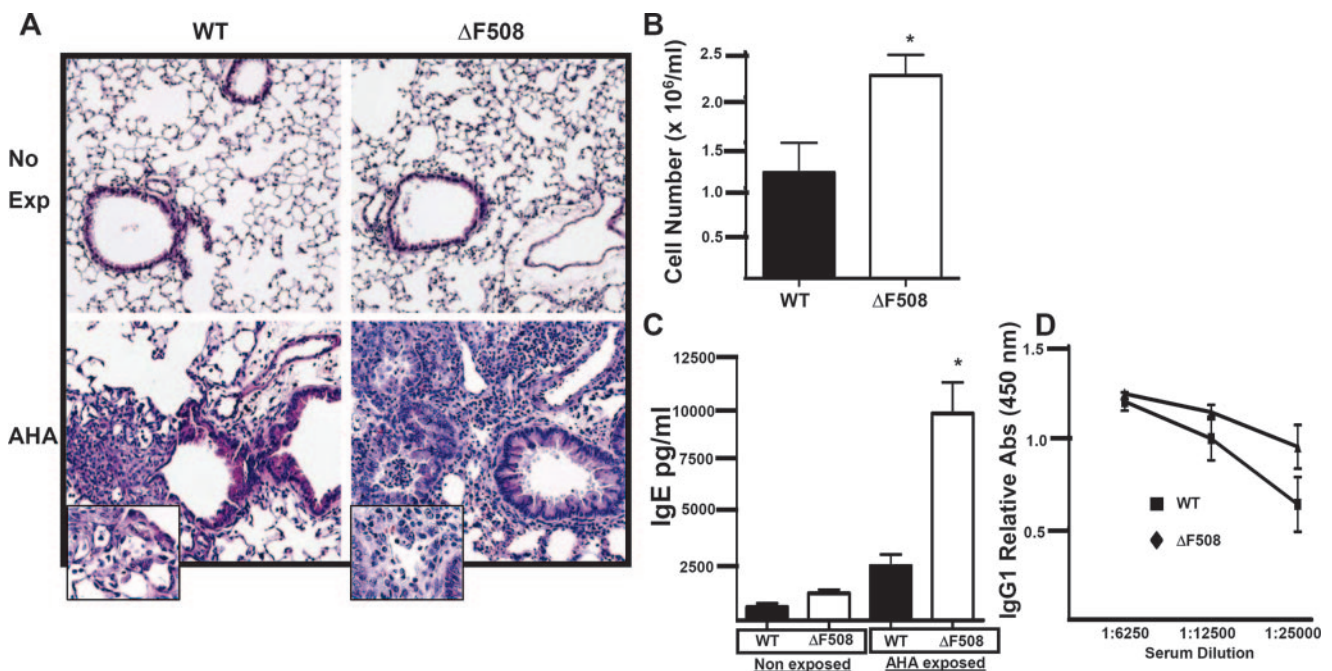


FIGURE 5. A, WT and Δ F508 mice were intranasally exposed to AHA on days 0, 7, 14, 15, and 16. Forty-eight hours after the last AHA exposure, lungs were sectioned and stained with H&E. B, BAL cell count after intranasal exposure to AHA. Total serum IgE (C) and IgG1 (D) after intranasal AHA exposure. The values represent the mean $\pm$ SEM of four mice per group and are representative of experiments performed twice. Statistical significance was determined by the Student *t* test for BAL cell number and by two-way ANOVA for serum IgE and IgG1. *, *p* < 0.05; **, *p* < 0.001 compared with values in WT AHA-exposed mice.

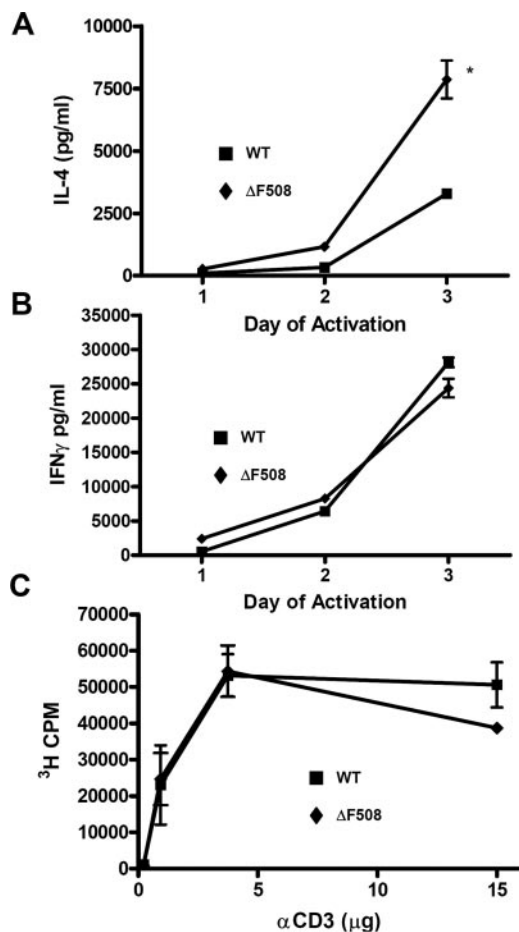


FIGURE 6. IL-4 (A) and IFN- γ (B) production by naive CD4 T cells after activation with anti-CD3 and anti-CD28 mAbs as determined by ELISA. C, Proliferation of naive CD4 T cells after activation with anti-CD3 and anti-CD28 mAbs as determined by [³H]thymidine incorporation. In vitro data presented are the means $\pm$ SEM of one representative experiment of three with cultures performed in triplicate. Statistical significance was determined by two-way ANOVA. *, $p < 0.001$ compared with values in WT AHA-exposed mice.

that CFTR deficiency does not cause a lack of general immunosuppression and an amplification of a general immune response. Instead, they further confirm the selective effect of CFTR on differentiation of CD4⁺ T cells into Th2 effector cells.

CD4⁺ T cells isolated from naive ΔF508 and CFTR mice have increased NFAT binding

CFTR is a cAMP-regulated chloride channel that can function as a conductance regulator of other cell surface channels (40).

Table I. Activated CD4⁺ T cells from WT and ΔF508 mice have equivalent CD25 expression^a

Mouse Strain	CD4 ⁺ /CD25 ⁺ by Day of Activation		
	Day 1 (%)	Day 2 (%)	Day 3 (%)
WT	49	66	61
ΔF508	48	65	63

^a Expression of CD25 by activated (anti-CD3/anti-CD28 mAbs) CD4⁺ T cells isolated from the spleen of WT or ΔF508 mice. Data presented are the results of one representative experiment of three.

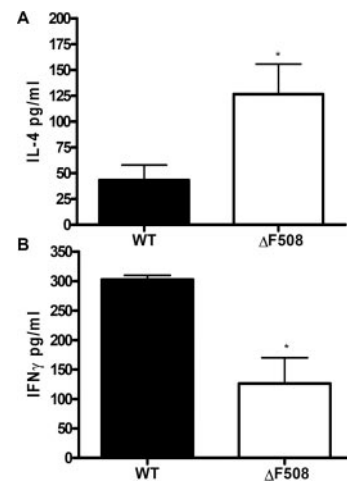


FIGURE 7. IL-4 (A) production by OVA-stimulated CD4⁺ T cells from OVA/alum-immunized ΔF508 and WT mice and IFN- γ (B) production by OVA-stimulated CD4⁺ T cells from OVA/CFA-immunized ΔF508 and WT mice. IL-4 was not detected in the OVA/CFA-immunized mice (both WT and ΔF508). Statistical significance was determined by the Student t test for IL-4 and IFN- γ production. *, $p < 0.05$ compared with values in WT OVA-immunized mice.

Increased calcium signals have been reported in airway epithelial cells from CF patients and are associated with increased inflammatory cytokine production (41, 42). NFAT is a transcription factor whose activation is heavily dependent on increased intracellular calcium due to its dephosphorylation and nuclear trafficking by the calcium-dependent phosphatase, calcineurin (39). NFAT is involved in the regulation of IL-4 and both NFATc1 and NFATc2 have been shown to bind to the *IL-4* locus and promote gene expression (43). We have shown that activation of NFAT in T cells is required for Th2 immune responses and allergic airway inflammation (44). Therefore, we tested whether the lack of functional CFTR could result in increased NFAT activity that could lead to increased IL-4. ΔF508 and WT CD4⁺ T cells were activated with anti-CD3 and anti-CD28 mAbs for 24 h. Nuclear extracts were generated and used to examine NFAT DNA binding by EMSA. The level of NFAT DNA binding found in the activated ΔF508 CD4⁺ T cells was substantially higher compared with WT CD4⁺ T cells (Fig. 8A). To further confirm the affect of CFTR deficiency on NFAT activation, an additional experiment including CFTR KO CD4⁺ T cells was performed. Increased levels of NFAT DNA binding were observed in both the ΔF508 and CFTR KO CD4⁺ T cells compared with WT CD4⁺ T cells (Fig. 8, B and C). As control for protein loading and nuclear extract quality, we also examined the DNA binding of CREB, a transcription factor that is not substantially affected by TCR activation. Unlike NFAT, the levels of nuclear CREB complexes in CD4⁺ T cells from CFTR KO and ΔF508 were not increased relative to those in WT CD4⁺ T cells (Fig. 8, B and C). Because NFATc2 is the predominant member of the NFAT nuclear complexes in primary activated CD4⁺ T cells (39), we performed competition analysis using the anti-NFATc2 Ab during the binding reaction. Most NFAT complexes were supershifted with the anti-NFATc2 Ab as expected, but increased noncompeted complexes (likely containing NFATc1) could be observed in both CFTR KO and ΔF508 CD4⁺ T cells (Fig. 8B). Thus, the absence of a functional CFTR in CD4⁺ T cells increases NFAT nuclear complexes following stimulation, suggesting that CFTR can regulate IL-4 through NFAT.

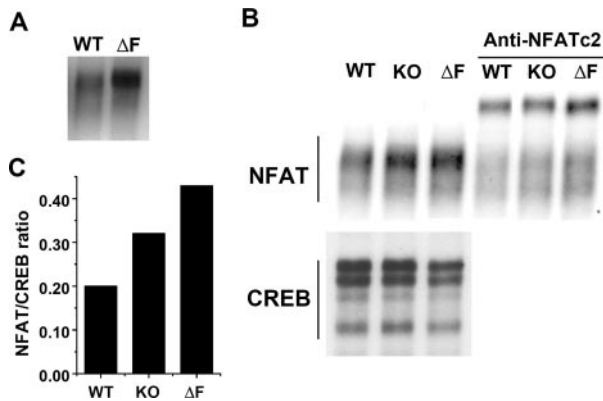


FIGURE 8. A, CD4⁺ T cells from wild-type (WT) and Δ F508 (Δ F) mice were activated for 24 h in the presence of anti-CD3 and anti-CD28 mAbs. Nuclear extracts were prepared and examined for NFAT DNA-binding activity by EMSA. B, CD4⁺ T cells from WT, CFTR KO (KO), and Δ F508 (Δ F) mice were activated as in A and nuclear extracts were prepared and examined for NFAT DNA-binding activity in the presence or absence of an anti-NFATc2 Ab by EMSA. CREB DNA binding was also examined as a control for protein loading. C, The densitometric ratio of NFAT complexes to CREB complexes.

Discussion

Most studies addressing the mechanisms of CF airway disease pathogenesis have focused on epithelial cells and neutrophils (4, 45–50). These cell types are important contributors to the altered airway environment. The lung disease in CF is characterized by persistent inflammation, infection, and mucus hypersecretion (51) and studies thus far have not yielded a clear picture of disease pathogenesis. Lymphocytes express CFTR and therefore may be affected by defects in this channel (26, 52–56). Lymphocytes are also present in sizable aggregates within the subepithelial space of the CF airway (6), positioning them to function as important regulators of airway inflammation. It is clear that the immune response contributes to the overall CF disease pathogenesis, but few studies have approached this aspect directly. The results of this study suggest that CFTR affects the differentiation and effector cell function of CD4⁺ T cells and therefore influences lung inflammation.

CF patients have an increased prevalence of asthma (9) and ABPA (19), both of which are diseases mediated by CD4⁺ Th2 cell activation (19–25, 57). It is unclear whether the increased prevalence of Th2-mediated diseases is a consequence of altered CD4⁺ T cell function or a consequence of the environment in which the CD4⁺ T cell is activated. Recent studies of *P. aeruginosa* infection in CFTR-deficient mice suggest that bone marrow-derived cells are more relevant than the airway environment in generating excessive inflammation (58). In this study, results suggest that CFTR-deficient mice that lack spontaneous lung disease develop increased airway and tissue Th2-biased inflammation in response to *A. fumigatus* hyphal Ag exposure.

Although the in vivo immune response to *A. fumigatus* Ags and OVA in CFTR-deficient mice is Th2 biased, this could arise from altered cytokine production during Ag presentation at the time of CD4⁺ T cell activation. Dendritic cells and macrophages can influence CD4⁺ T cell differentiation in this way and may themselves be altered by defects in CFTR. However, here we show that CD4⁺ T cells from naive CFTR-deficient mice exhibit a Th2 bias, suggesting that CFTR-deficient CD4⁺ T cells more readily become Th2 cells. It is unclear whether this

is due to an intrinsic T cell defect or their response to all or certain Ags. This suggests that the Th2-biased response to *A. fumigatus* seen in vivo is partially due to the Th2-biased phenotype of the CD4⁺ T cell and that CFTR may be regulating IL-4 production by these cells. The results from the immunization with OVA in the presence of either a Th1- or Th2-biasing adjuvant further support this model. We show that CFTR deficiency increases IL-4 production by in vivo OVA-specific Th2 cells, but does not affect or even inhibit IFN- γ production by OVA-specific Th1 cells. Thus, CFTR selectively regulates Th2 differentiation and does not affect Th1 differentiation.

No mechanisms for how CFTR affects cytokine gene expression have been proposed and this is the first study showing a direct effect of CFTR deficiency leading to altered cytokine production by CD4⁺ T cells. It is well-accepted that IL-4 promotes Th2 differentiation through activation of Gata-3 and Stat-6 (59). However, we show here that CD4⁺ T cells from CFTR-deficient mice have a Th2 bias upon TCR activation even in the absence of exogenous IL-4, suggesting that TCR signaling in these cells favors IL-4 production. Another transcription factor that is involved in *IL-4* gene expression and is regulated by TCR signaling is NFAT. Both NFATc2 and NFATc1 bind to the *IL-4* locus and enhance gene expression (43). We previously have shown that inhibition of NFAT in CD4⁺ T cells interferes with Th2 immune response and the development of allergic airway inflammation (44). In addition, inhibition of NFAT in CD4⁺ T cells does not interfere with IFN- γ production and Th1 cell development (M. Rincon, S. Diehl, and J. Argueta, unpublished observation). Because we show here an increased accumulation of NFAT nuclear complexes in CD4⁺ T cells from both CFTR KO and Δ F508 mice, we propose that CFTR deficiency up-regulates IL-4 production through NFAT. The mechanism by which CFTR regulates NFAT nuclear accumulation remains to be determined. NFAT nuclear translocation is dependent on increased intracellular calcium levels and activation of calcineurin. CFTR is a chloride channel that can also affect other ion channels, such as calcium channels. It has been shown that CFTR on airway epithelium regulates intracellular calcium, such that a deficiency of CFTR leads to higher intracellular calcium (60). Thus, we propose that CFTR may be an indirect modulator of calcium channels on CD4⁺ T cells and in the absence of a functional CFTR, calcium levels may be persistently elevated, leading to enhanced NFAT activation. Future studies would be needed to confirm this mechanism.

P. aeruginosa is the primary bacterial pathogen within the CF airway and its presence is associated with poor clinical outcomes (61). The clinical significance of *A. fumigatus*, a common colonizer of the CF airway, is much less clear. *Aspergillus* sp. are found in the sputum cultures of 20–40% (10, 13, 14, 24) of CF patients and is not currently treated outside of the coexisting diagnosis of ABPA. Although conidia are the morphotype of *Aspergillus* normally inhaled, the antigenic components of the fungus exist in the hyphal form (19). In our study, we show that CFTR-deficient mice develop excessive airway and tissue inflammation in response to AHA exposure suggesting that the presence of *A. fumigatus* within the CF airway may not be a benign secondary effect of mucus retention, but may in fact contribute to the chronic, persistent airway inflammation that is characteristic of CF.

In conclusion, we demonstrate for the first time that CFTR-deficient mice develop excessive, Th2-biased airway and lung tissue inflammation in response to *A. fumigatus* Ag exposure. We also show for the first time that activated CD4⁺ T cells from

naive CFTR-deficient mice produce a more potent Th2 response. Taken together, these results suggest that *A. fumigatus* airway colonization and the abnormal immune response that it generates may be important in contributing to CF lung disease pathogenesis. Further studies in humans with CF should be undertaken to determine whether treatment of this organism, or the immune response it generates, is beneficial.

Disclosures

The authors have no financial conflict of interest.

Acknowledgments

We thank Benjamin Suratt for his assistance in manuscript preparation.

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