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## Lack of CFTR in CD3+ Lymphocytes Leads to Aberrant Cytokine Secretion and Hyper-Inflammatory Adaptive Immune Responses: A Master's Thesis

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**LACK OF CFTR IN CD3+ LYMPHOCYTES LEADS TO ABERRANT  
CYTOKINE SECRETION AND HYPER-INFLAMMATORY ADAPTIVE IMMUNE  
RESPONSES**

A Master's Thesis Presented

By

**Christian Mueller**

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
In partial fulfillment of the requirements for the degree of

**MASTER OF SCIENCE**

April 24, 2012

Masters in Clinical Investigation

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signifies that the student has met all master's degree graduation requirements of  
the school.

Anthony Carruthers, Ph.D,  
Dean of the Graduate School of Biomedical Sciences

Masters in Clinical Investigation

April 24 2012

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## Abstract

*Background:* Cystic fibrosis (CF) remains the most common fatal monogenic disease in the US, affecting 1 in 3,300 live births. CF is the result of mutations in CFTR, a chloride channel and regulator of other ion channels. 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 and aberrant inflammatory and immune responses to pathogens within the airways. We have shown that Cfr<sup>-/-</sup> mice mount an exaggerated IgE response towards *Aspergillus fumigatus* (Af) when compared to Cfr<sup>+/+</sup> mice. Along with the increased IgE levels, the Cfr<sup>-/-</sup> mice had higher levels of IL-13 and IL-4, mimicking both the Th-2 biased immune responses and predilection to mounting Af-specific IgE seen in CF patients. Herein we hypothesize that these immune aberrations are primarily due to the lack of Cfr expression in lymphocytes rather than with Cfr deficiency in the epithelium.

*Results:* Our results indicate that adoptive transfer experiments with Cf splenocytes confer higher IgE response to Af in host mice as compared to hosts receiving wild-type splenocytes. The predilection of Cfr-deficient lymphocytes to mount Th2 responses was confirmed by *in vitro* antigen recall experiments, where higher levels of IL-13 and IL-4 were seen only in the presence of Cfr-deficient lymphocytes. Conclusive data on this phenomenon were obtained with conditional Cfr knockout mice, where mice lacking Cfr in T-cell lineages developed the higher IgE titers as compared to their wild-type littermate controls.

Further analysis of Cftr-deficient lymphocytes revealed an enhanced intracellular  $\text{Ca}^{2+}$  flux in response to T cell receptor activation as compared to normal lymphocytes. This was accompanied by a significant increase in nuclear localization of the calcium-sensitive transcription factor NFAT, which could contribute to the enhanced secretion of IL-13 and other cytokines. *Conclusions:* In summary, our data identified that CFTR dysfunction in T cells can lead directly to aberrant immune responses. This is the first instance that a CF related phenotype has been entirely modeled in vivo by selectively knocking out CFTR in the immune system. Specifically, Cftr deficient lymphocytes directed skewed responses to *Aspergillus fumigatus*, leading to a higher than normal IgE response. These findings implicate the lymphocyte population as a potentially important target for therapeutics directed to the treatment of CF lung disease.

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## Preface

The work and ideas presented in this thesis has resulted in the publication of the two manuscripts cited below.

1. **Mueller C**, Braag SA, Keeler A, Hodges C, Drumm M, Flotte TR. *Lack of Cfr in CD3+ Lymphocytes Leads to Aberrant Cytokine Secretion and Hyper-inflammatory Adaptive Immune Responses*. Am J Respir Cell Mol Biol. 2010 Aug 19.: PMID:20724552
2. Ratner D, **Mueller C**. Immune responses in Cystic Fibrosis; are they intrinsically defective? Am J Respir Cell Mol Biol. 2012 Mar 8. [Epub ahead of print] PMID: 22403802

## CHAPTER I: Introduction

Cystic fibrosis (CF) is the most common autosomal recessive genetic disease in the US, affecting 1 in 3,300 live births. CFTR is a chloride channel and a regulator of other ion channels, and many aspects of the CF phenotype are directly related to ion channel abnormalities attributable to Cfr mutation. Initially, CF was described as a constellation of pancreatic pathology and recurrent lung infections, but it was soon realized that the clinical and pathological findings could be better explained as a generalized defect in all exocrine glands. The manifestation of abnormal exocrine gland function can be divided into three types: 1, those that become blocked by viscid or solid eosinophilic material in the lumen (i.e. pancreas, intestinal glands, intrahepatic bile ducts, gallbladder, submaxillary glands); 2, those glands that produce excess of secretions (i.e. tracheobronchial and Brunner glands); and 3, those glands that are histologically normal yet secrete excessive amounts of electrolytes (i.e., sweat, parotid, and small salivary glands) <sup>1,2</sup>. Lung disease is the most common limitation to the quantity and quality of life for CF patients. In prenatal life, the lung manifestations of CF are extremely subtle, although hyperplasia of submucosal glands has been reported. Postnatally, CF patients develop evidence of small airways obstruction and inflammation, most often beginning within the first two years of life. Patients typically develop airway infection with certain species of bacteria, often beginning with *Staphylococcus aureus* and typically progressing to infection with *Pseudomonas aeruginosa*<sup>3-6</sup>. Coincident with airway infection, an exuberant

inflammatory response occurs in the airways of CF patients. This response is dominated by neutrophil infiltration, although adaptive immune responses to the infecting organisms are typical as well. It is not entirely clear why CF patients are particularly prone to airway infection with *P. aeruginosa* and the other organisms mentioned above. 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<sup>7-9</sup>, increased binding or decreased clearance of *Pseudomonas aeruginosa*<sup>10,11</sup> as well as increased pro-inflammatory cytokine production in the CF airway<sup>12-16</sup>. CF cell lines demonstrate increased NFkappaB activation and increased IL-8 secretion in response to *P. aeruginosa* exposure, as compared with control cells<sup>16,17</sup>. Furthermore, CFTR mutant mice demonstrate a greater cytokine response (KC, MIP2, IL1-beta), greater mortality, and weight loss after airway challenge with a *P. aeruginosa*-agarose bead slurry, as compared with control mice<sup>18-21</sup>.

Although it is not clear whether the CFTR defect itself initiates inflammation, a widely held point of view is explained by the "Isotonic Low Volume Hypothesis". It states that the water permeable airway epithelia regulate the volume of the ASL. In the CF airways the abnormal sodium absorption in combination with the failure to secrete chloride leads to an overall loss of water and hence a depletion in the volume of the ASL<sup>22</sup>. The cAMP-dependent Cl<sup>-</sup> channel activity of CFTR facilitates the release of ATP, which is involved in the down-regulation of the epithelial sodium channel (ENaC), which accounts for the bulk of salt and water

transport in the airways<sup>23-25</sup>. ENaC is expressed in the airways and the alveolar epithelium and it is the channel responsible for sodium reabsorption, helping maintain a constant depth of the airway surface fluid<sup>26</sup>. As the imbalance in sodium is created toward the inside of the epithelial cells, water follows the osmotic gradient depleting the ASL. This leaves a dehydrated low volume ASL that is characterized by thickened mucus and impaired ciliary clearance. It also provides bacteria with tenacious mucus layer to colonize consequently leading to chronic infection<sup>8,27,28</sup> (FIGURE 1). Although there is strong evidence for underhydration of the sol layer of the airway surface liquid, there is not an obvious reason why *P. aeruginosa* would infect an underhydrated airway surface preferentially. A number of studies have shown qualitative alterations in mucus glycoproteins as well, including changes in sulfation, fucosylation, and sialylation. Some studies have shown an increase in *P. aeruginosa* binding in CF epithelial cells, while others have shown a decrease in cellular internalization of bacterial pathogens in CF.

However what still remains enigmatic is the observed alterations in innate and adaptive immune responses to certain pathogens. Specifically altered responses to *Pseudomonas aeruginosa* and *Aspergillus fumigatus*, with a peculiar increase in neutrophil chemo-attractants in the former case, and a hyper-IgE-like state in the latter are common in CF which predisposes these patients to Allergic broncho-pulmonary aspergillosis (ABPA). This 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<sup>29,30</sup>. It is occasionally seen in patients with asthma, although some studies have shown that up to 50% of asthma patients with ABPA have at least one mutation in the CFTR gene<sup>31-34</sup>. The immune response in ABPA appears to exemplify the IL-4-driven, Th2-predominant response that is seen in CF patients and in the *Cftr* mutant mouse expression profiling studies<sup>35-38</sup>. Based on this observation, we have recently described an ABPA-like model in CF mice<sup>39</sup>. In subsequent studies we observed divergent cytokine production in splenocytes from the *Cftr* mutant mice challenged with Af antigen<sup>40</sup>. 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 non-epithelial cell types, such as lymphocytes.

This question has been raised a number of times in previous work<sup>41,42</sup>. Studies conducted immediately following the discovery of CFTR, indicated that lymphocyte chloride transport was defective in CF and that this could affect function under certain circumstances<sup>43</sup>. A number of other studies have shown that CFTR gene replacement could restore lymphocyte channel activity to normal<sup>44,45</sup>. Finally, the Th2-bias of CF lymphocytes has been confirmed by a number of investigators<sup>36,37</sup>. The unresponsiveness of CF lymphocytes against *P. aeruginosa* has been known since the late 1970s<sup>46-52</sup>, and recent insights are

beginning to shed some light on these observations. Central to the CF T-cell phenotype appears to be a predilection to mount a type 2 helper T-cell (Th2) response <sup>36</sup>, which is pro-allergic and appropriate for fighting parasites, but not pathogens such as *P. aeruginosa*. Indeed, clinical data shows that approximately 10% of CF patients develop Allergic Bronchopulmonary Aspergillosis (ABPA) upon exposure to *A. fumigatus* <sup>33,53</sup>, with increased levels of IL-4, IL-13, and IgE in what appears to be a hyper-inflammatory Th2-dominated immune response <sup>54</sup>. As mentioned above in CF mice, increased levels of IgE are observed compared to controls after exposure to *A. fumigatus* <sup>39,55</sup>, accompanied by a significant shift to a predominantly IL-4 and IL-13 cytokine profile, and a greater sensitivity of CF B-cells to IL-4 stimulation <sup>37</sup>.

There is some evidence that T-cell and macrophage unresponsiveness to *P. aeruginosa* may be the result of Th2 skewing in CF. Moss et al showed that helper T-cells from CF patients produce lower levels of IFN-g, a Th1 cytokine, and higher levels of IL-10, a Th2 cytokine <sup>56</sup>. IL-10 expression in CF has been a subject of debate, but it has been confirmed to be up-regulated by Casaulta et al <sup>57</sup>. This is interesting because the ability of IL-10 to down-regulate IFN-gamma production and decrease co-stimulatory molecules on macrophages can hinder antigen presentation and a proper immune response to *P. aeruginosa*, *A. fumigatus*, and other pathogenic species. Further studies are needed to establish if antigen-presentation by macrophages is indeed compromised.

A Th1 immune response may in fact be critical for successfully clearing *P. aeruginosa* infection. Moser et al showed that when CFTR<sup>-/-</sup> mice are repeatedly infected with *P. aeruginosa*, recovery from infection is accompanied by a shift to a Th1 response and increased levels of IL-12, but no change in IgG levels<sup>58</sup>. The surviving mice are resistant to re-infection and have improved mortality. Interestingly, CF mice colonized with *P. aeruginosa* actually have a higher antibody titer against *P. aeruginosa* antigens than do colonized wild-type mice, but still develop chronic infection<sup>59</sup>. This suggests that a TH1-type immune response is protective against *P. aeruginosa* infection independent of antibody production, and compels us to examine the mechanism of a CFTR-mediated defect in T-cells which seems to favor inappropriate Th2 differentiation.

One emerging explanation for the phenomenon of Th2 skewing is the hypothesis that mutant *Cftr* causes increased Ca<sup>2+</sup> flux across the T-cell membrane, thereby perturbing Ca<sup>2+</sup> sensitive gene expression pathways. Ca<sup>2+</sup> influx is known to be critical for T-cell activation, and is tightly regulated by a number of ion channels, including *Cftr*<sup>60</sup>.

While the downstream effects of *Cftr* dysfunction are important, it is events at the cell membrane that ultimately drive the *Cftr*-mediated gene expression aberrations in T-cells and warrant further attention. In 2001, Fanger et al<sup>61</sup> showed that Ca<sup>2+</sup> entry in Jurkat T-cells results in activation of KCa channels, creating an efflux of K<sup>+</sup> ions which prevents cell membrane depolarization and allows continued Ca<sup>2+</sup> entry; these Ca<sup>2+</sup> levels are sufficiently high to cause the

signaling necessary for transcriptional activation. If so, then defective Cftr function and subsequent intracellular retention of Cl<sup>-</sup> ions would be expected to further hyperpolarize the T-cell membrane and augment Ca<sup>2+</sup> activated signaling. It is worth noting that the Orai1/Stim1 complex, is also present in the T-cell membrane; studies are needed to determine whether it is similarly affected by mutant Cftr and leading to abnormal gene expression.

Recent insights in T-cell electrophysiology are also giving new perspective to local Cftr functions in the T-cell membrane. According to Cahalan & Chandy<sup>62</sup>, some ion channels (Orai1, STIM1, Kv1.3, and KCa3.1) cluster at the antigen presentation site following contact with an antigen-presenting cell (APC). They hypothesize that this clustering could be important for stabilizing the interaction between the T-cell and the APC, and also in generating large extracellular K<sup>+</sup> concentrations sufficient to depolarize both the APC and the T-cell. This may be important for efficient antigen presentation, as MHC class II molecule expression on dendritic cells has been shown to double within a minute of K<sup>+</sup> induced depolarization<sup>63</sup>. The downstream effects of this may be speculated, yet regardless, these mechanisms may be affected by the lack of functional Cftr at the cell membrane. The work presented in this thesis begins to explore the role of CFTR in T-cell as it relates to the development of allergic responses to *aspergillus fumigatus*, in addition it attempts to provide a possible mechanism for the difference in T-cell responses seen in Cftr deficient lymphocytes.



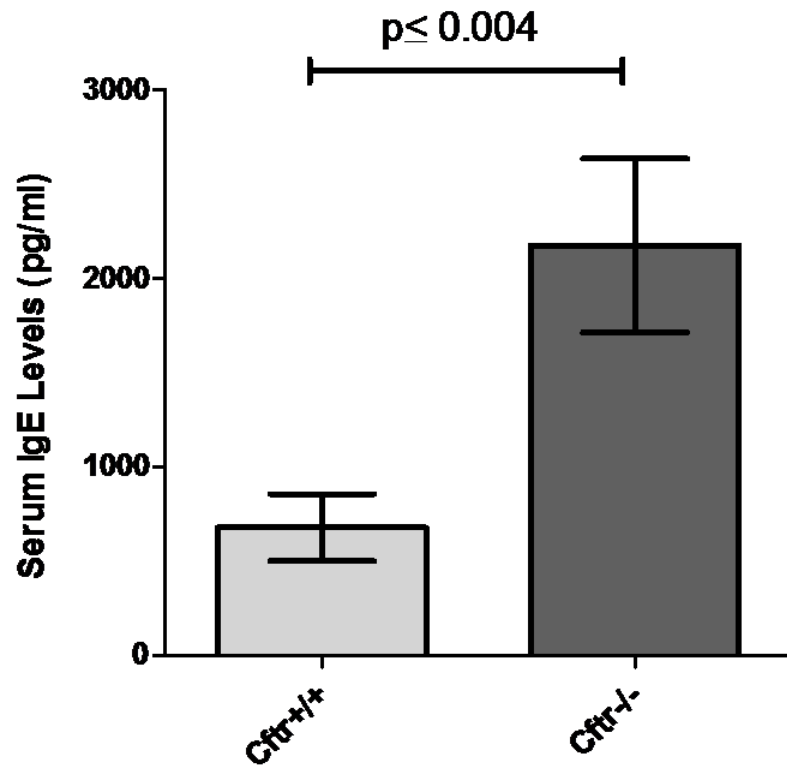
## TRANSITION TO RESULTS AND DATA

### CHAPTER II: Results

#### *Cftr deficiency and Th2 skewed adaptive immune responses*

In previous studies we have shown that *Cftr*<sup>-/-</sup> mice on a mixed background had IgE levels that were 2-5 fold higher than their controls when sensitized and airway challenged with *Aspergillus fumigatus* crude protein extract (Af-cpe)<sup>39</sup>. Furthermore, this phenotype was partially corrected after *Cftr* gene replacement with recombinant adeno-associated vectors<sup>40</sup>. Here we further establish this phenotype by comparing total serum IgE levels in *Cftr*<sup>-/-</sup> mice that are on a congenic C57BL6 background. *Cftr*<sup>-/-</sup> and *Cftr*<sup>+/+</sup> littermates were sensitized and challenged with Af-cpe as previously described. Total serum IgE from congenic mice littermates challenged with Af-cpe were on average two-fold higher for *Cftr*<sup>-/-</sup> mice (figure 1).

**Figure 1.** *Total serum IgE levels in congenic C57B6-CFTR<sup>-/-</sup> mice and their littermates after Af sensitization and challenge.* Mice were sensitized with 200ug of Af crude extract dissolved in 100ul of PBS on day 1 and day 14. Blood samples were collected on day 32; 48hrs after the 3rd aerosol Af challenge. Total Serum IgE in the Af-sensitized mice were measured by ELISA. Data is shown as group averages + S.E.M

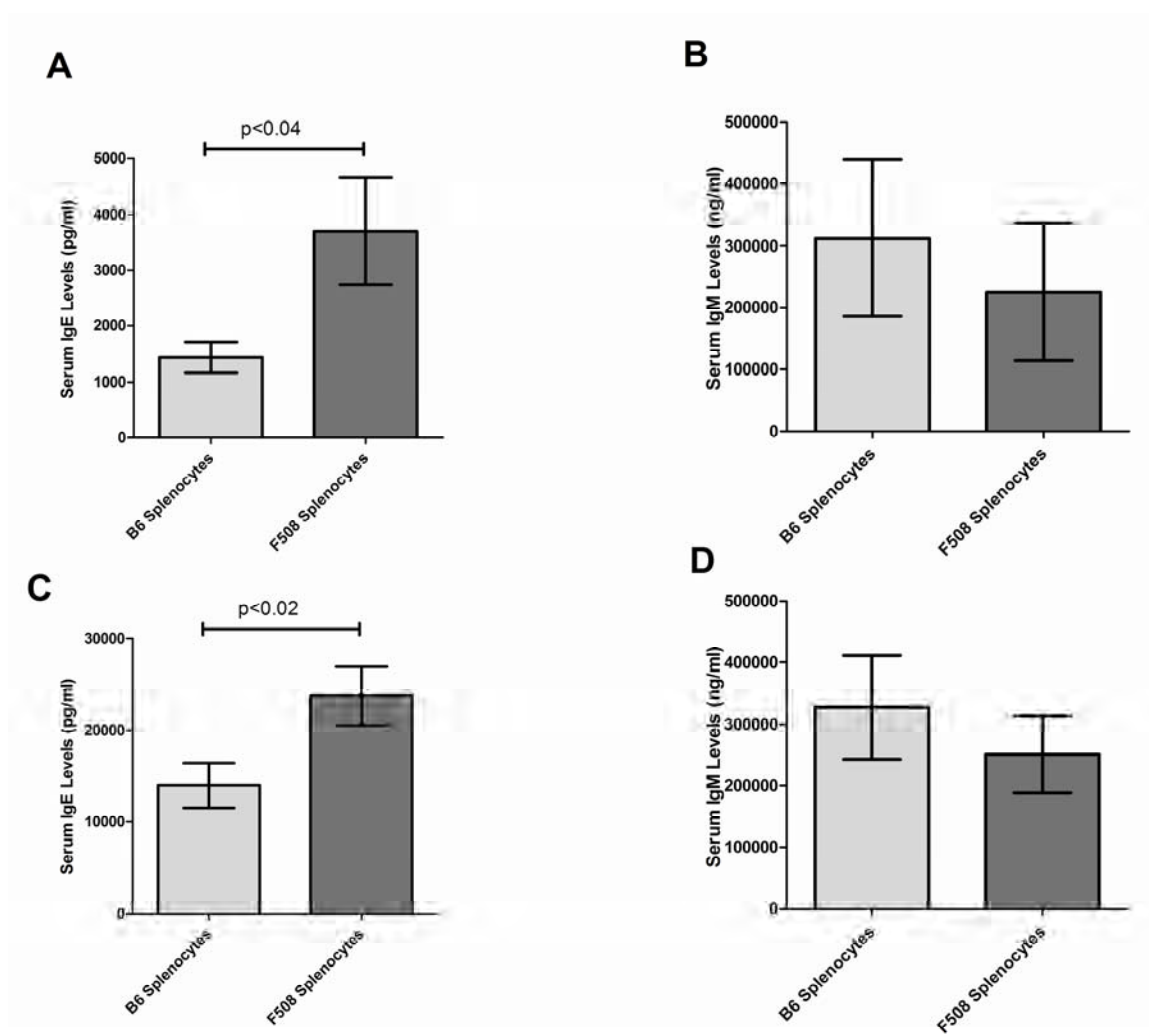


*Adoptive transfer confers CF-like phenotype*

To determine if the altered immune milieu imparted by Cfr<sup>-/-</sup> lung epithelial cells seen during the aerosol challenge was involved in generating the two-fold higher IgE response towards Af-cpe, we designed adoptive transfer experiments into Rag<sup>-/-</sup> mice hosts that were otherwise normal for Cfr. In the first set of experiments Cfr<sup>-/-</sup> mice with a deletion of phenylalanine at amino acid position 508 of the Cfr 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 wildtype littermate controls and adoptively transferred them via an intra-peritoneal injection into Rag<sup>-/-</sup> mice of the same C57BL6 genetic background. Eight weeks post transfer the Rag<sup>-/-</sup> mice were airway challenged with aerosolized Af-cpe. Since Rag<sup>-/-</sup> are deficient in T and B cells and can not produce IgM, total circulating IgM levels were measured on these mice to verify the engraftment of the adoptive transfer (Figure 2b). In order to determine if sensitized splenocytes from CF mice alone were able to recapitulate the high IgE phenotype in the presence of a wildtype (Cfr<sup>+/+</sup>) airway, sera from the adoptively transferred Rag<sup>-/-</sup> mice were checked for IgE levels. The results indicate that the two-fold enhanced IgE phenotype is transferred into the host purely by the adoptive transfer of sensitized splenocytes (Figure 2a). To further rule out the possibility of other Cfr-deficient tissues having an effect on the sensitization of the immune cells that were transferred, we repeated these experiments by transferring naïve splenocytes into naïve Rag<sup>-/-</sup>

mice. Eight weeks after the naïve splenocyte transfer, Rag<sup>-/-</sup> mice were sensitized and airway challenged with Af-cpe. Once again the experiments recapitulated the hyper-IgE phenotype as serum levels of Rag<sup>-/-</sup> mice receiving CF splenocytes were also two-fold higher than Rag<sup>-/-</sup> mice receiving wildtype splenocytes (Figure 2c). In contrast, IgM levels in these two groups were not different, confirming that the difference in IgE antibodies levels was not due to differences in B-cell engraftment (Figure 2d).

**Figure 2.** *Adoptive transfer of CF splenocytes confers elevated IgE phenotype.* CFTR- $\Delta$ F508 (CFTR<sup>-/-</sup>) mice on the B6 background or matched control C57BL/6 mice (CFTR<sup>+/+</sup>) were used as donors for splenocyte adoptive transfer into B6-Rag<sup>-/-</sup> mice. Total serum IgE (A, C) and IgM (B, D) were measured after Af challenge. A,B) CFTR- $\Delta$ F508 (n=6) and C57BL/6 littermates (n=6) were sensitized with Af-cpe, two weeks after sensitization their splenocytes were transferred into Rag<sup>-/-</sup> mice where they were allowed to engraft for 8 weeks prior to airway challenging the mice. C, D) Naïve CFTR- $\Delta$ F508 (n=10) and naïve C57BL/6 littermates (n=10) were used to harvest naïve splenocytes for transfer into Rag<sup>-/-</sup> mice. Splenocytes were allowed to engraft for eight weeks and then Rag<sup>-/-</sup> mice were sensitized and airway challenged with Af-cpe.



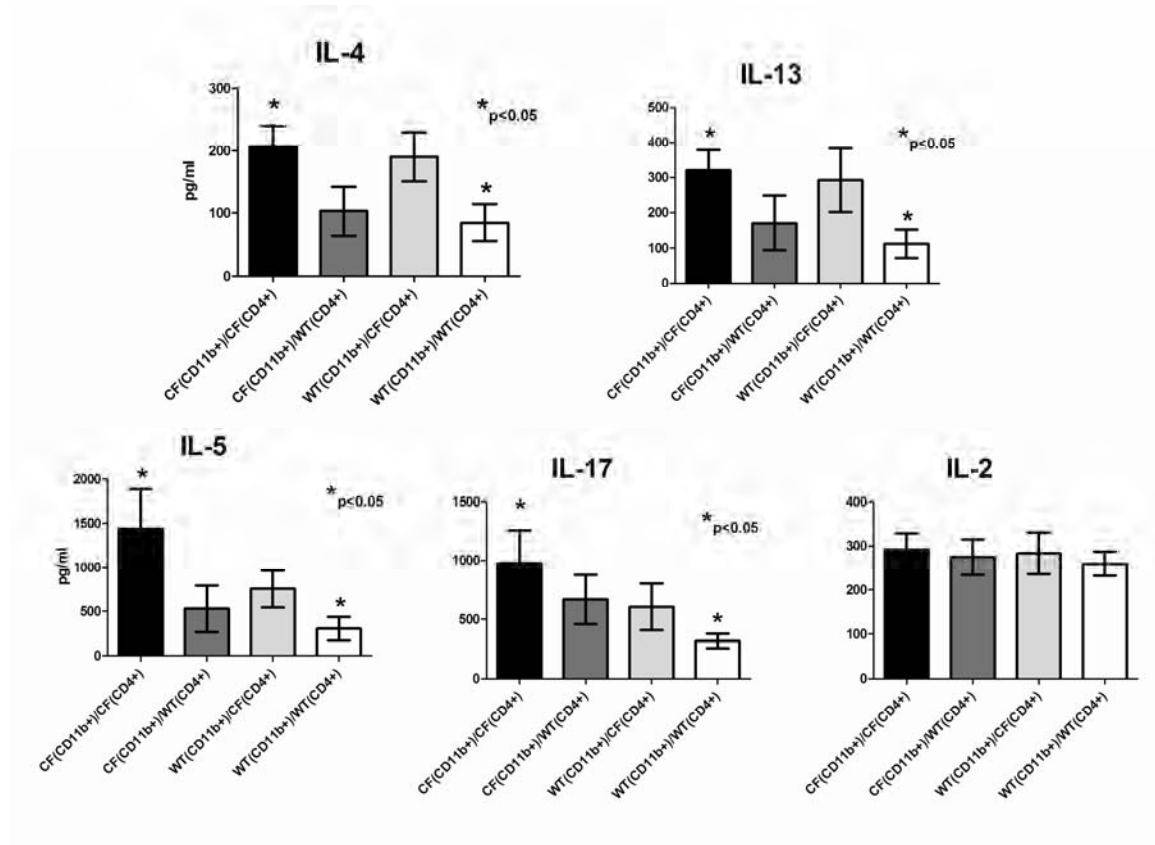
*Purified CF cell populations demonstrate CF phenotype ex vivo*

We then designed a series of antigen recall experiments in order to begin determining what cell population of the splenocytes was responsible for polarizing the adaptive immune responses along aTh2 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<sup>+</sup> lymphocytes or CD11b<sup>+</sup> 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 wildtype T-cells, wildtype monocytes with CF T-cells or wildtype monocytes with wildtype T-cells. These experiments uncovered three main cytokine secretion patterns, the first one is evidenced with the Th2 cytokines IL-4 and IL-13, where the elevated secretion seen with CF splenocytes was dependent on whether the stimulated cells had CF CD4<sup>+</sup> T cells and was independent of the monocyte source (figure 3a &b). A different cytokine secretion pattern was observed for IL-17 and IL-5; this one was synergistic and dependent on whether both the antigen present cell population (Cd11b<sup>+</sup>) and the effector cell population (CD4<sup>+</sup>) were Cfr<sup>-/-</sup> (figure 3c & d).



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).

**Figure 3.** *In vitro Antigen Recall with mixed cell populations.* Splenocytes from congenic CFTR<sup>-/-</sup> or CFTR<sup>+/+</sup> mice sensitized to ovalbumin were separated into both CD11b<sup>+</sup> and CD4<sup>+</sup> cell population with magnetic beads. Cells were mixed and 'crossed' to make four groups of cell preparations; 1) CF CD11b<sup>+</sup> with CF CD4<sup>+</sup> cells, 2) CF CD11b<sup>+</sup> with Wt CD4<sup>+</sup> cells, 3) Wt CD11b<sup>+</sup> with CF CD4<sup>+</sup> cells and 4) Wt CD11b<sup>+</sup> with Wt CD4<sup>+</sup> cells. Cells were then stimulated with OVA for 5 days. The cell culture supernatants were analyzed for cytokine profiles at day 5. Data is shown as group averages + S.E.M

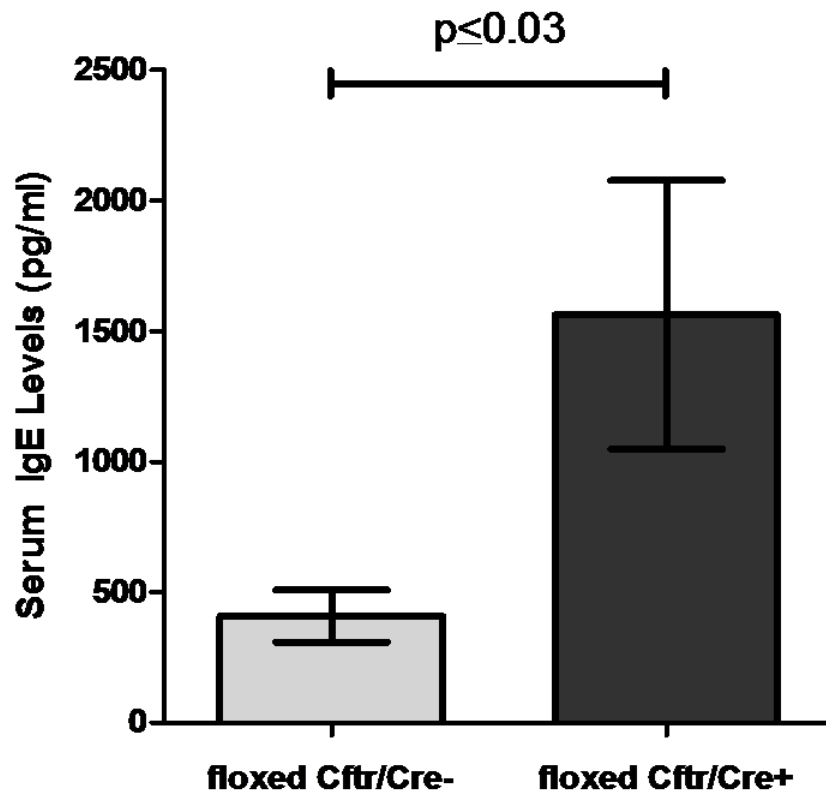


*Aberrant immune responses result from Cftr-deficient lymphocytes in a conditional knockout model*

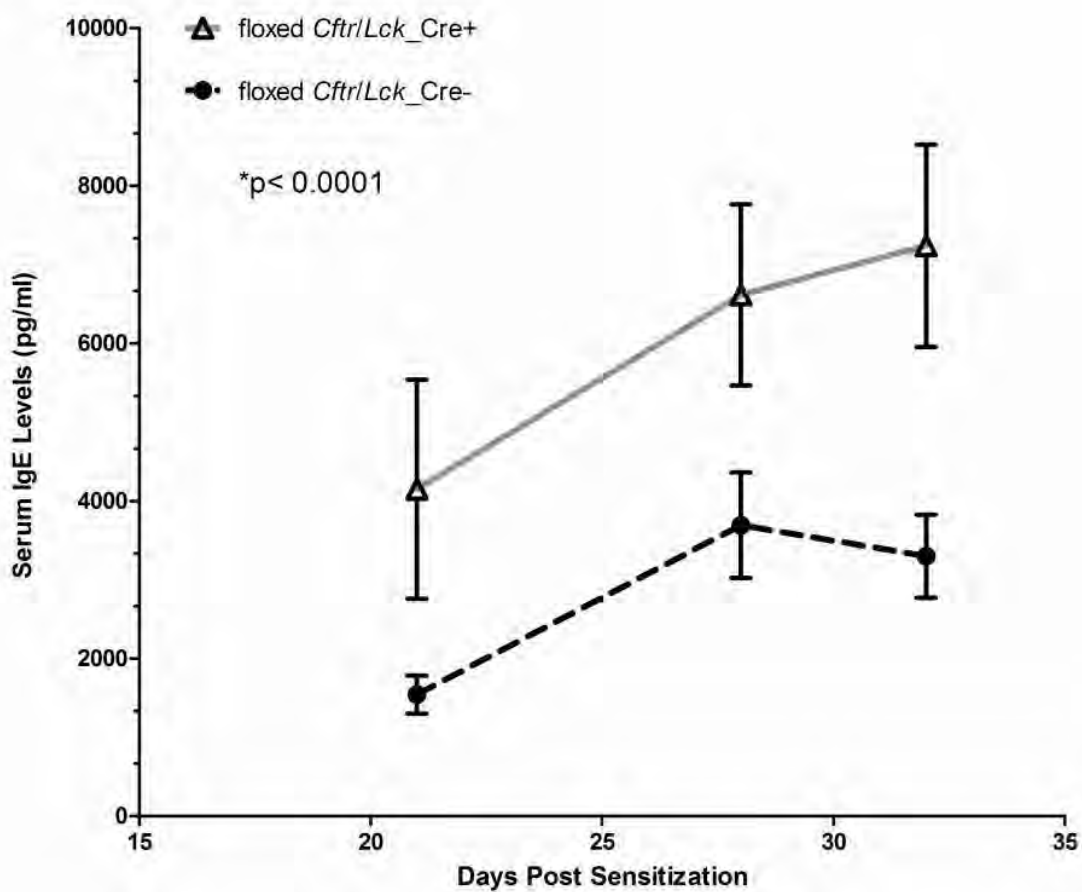
To confirm the hypothesis that deficiency of a functional CFTR channel in T cells results in Th2 biased adaptive immune response and leads to higher IgE levels, we used conditional Cftr knockout mice. These studies were carried out by crossing the recently described Cftr floxed mice<sup>64</sup> with mice expressing Cre recombinase under the control of the leukocyte-specific protein tyrosine kinase (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 naïve mice that were Lck-Cftr<sup>-/-</sup> showed a significant upregulation of serum IgE even at basal levels when compared to their littermate controls (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 littermate controls 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 week after the 2nd i.p injection) and through day 32 (48 hrs after the final aerosol challenge) (figure 5).

**Figure 4.** *Total Serum IgE Levels in Naïve Conditional CFTR Knockout Mice.* T-cell conditional knockout mice created by crossing the floxed Cftr mouse with mice expressing the Cre recombinase from an LCK promoter were compared to controls that were Cftr floxed but Cre recombinase negative. Basal serum IgE levels were analyzed by ELISA. Data is shown as group averages + S.E.M



**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 wildtype controls (Cftr floxed and Cre-) were sensitized with 200ug of Af crude extract dissolved in 100ul of PBS on day 1 and day 14. Blood samples were collected on days 21, 28 and 32. Total Serum IgE in the Af-sensitized mice were measured by ELISA. Data is shown as group averages + S.E.M





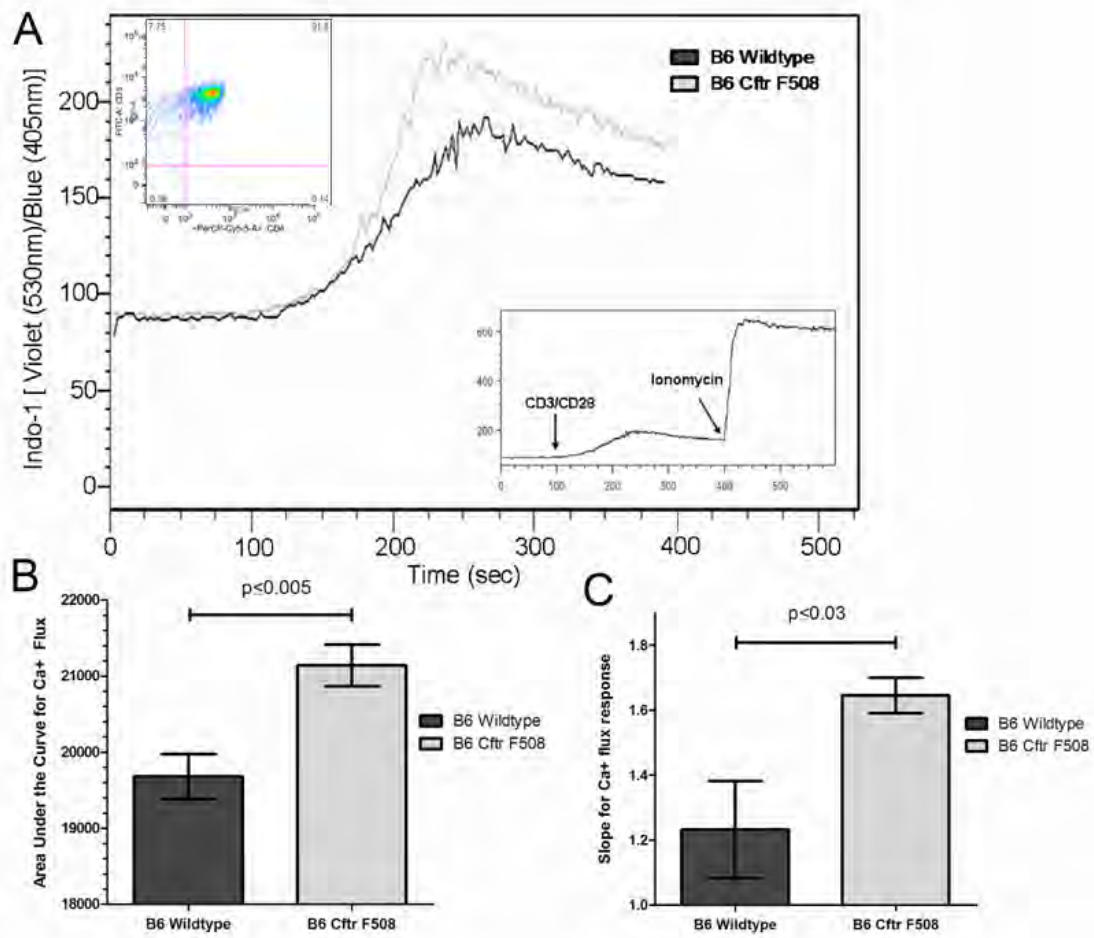
### Altered calcium signaling and NFAT activation

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 intracellular calcium ( $iCa^{2+}$ ). While,  $iCa^{2+}$  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 Cfr 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 Cfr<sup>+/+</sup> 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<sup>65,66</sup>. Thus, with a reduction in Cl<sup>-</sup> permeability in Cfr<sup>-/-</sup> 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 Nuclear factor of activated T-cells (NFAT), which has been shown to enhance gene expression of IL-4, IL-13, IL-5, TNF-alpha cytokines among others<sup>67</sup>.

To investigate this hypothesis and whether the absence of the CFTR channel in T cells would have an effect  $iCa^{2+}$  signaling through the T cell receptor (TCR), we measured intracellular calcium fluxes in CD4<sup>+</sup> T-cells in

response to CD3/CD28 stimulation of the TCR. Measurements of intracellular calcium in CD4<sup>+</sup> 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).

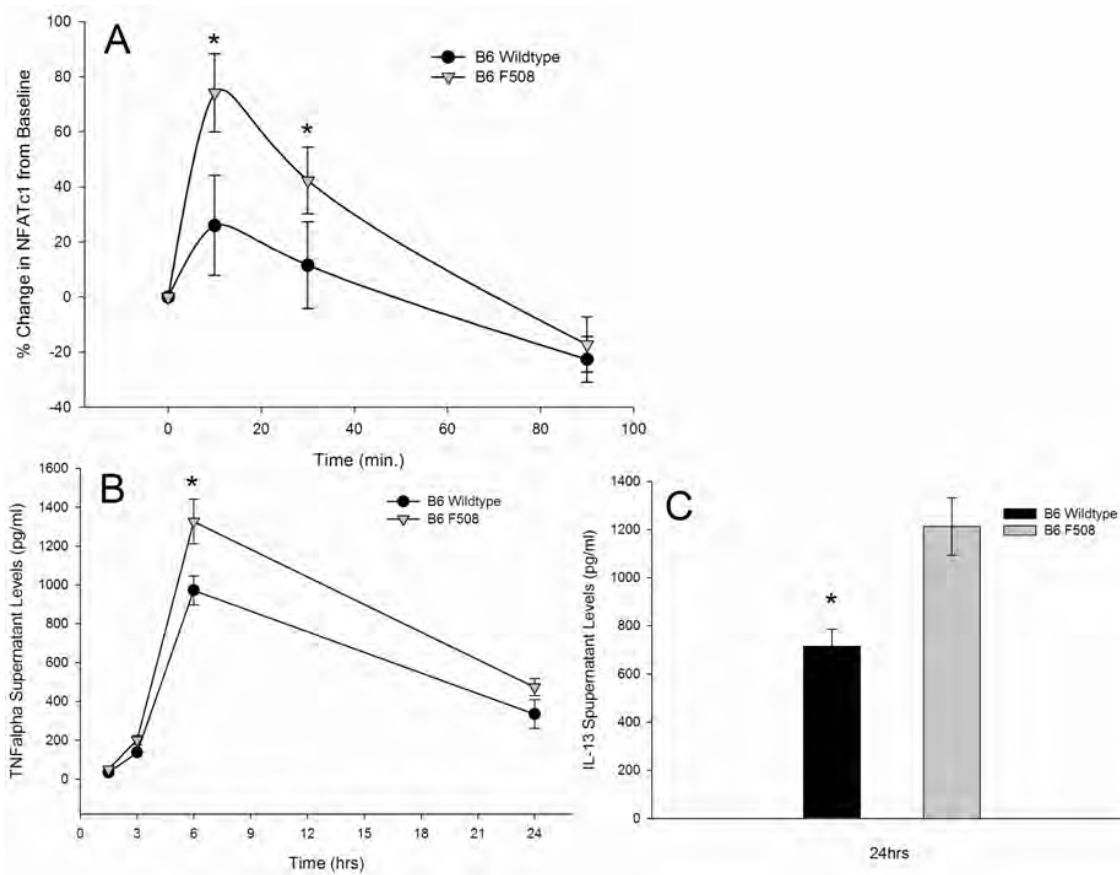
**Figure 6. Intracellular Calcium Flux.** Changes in the concentration of intracellular free  $\text{Ca}^{2+}$  ions are measured by monitoring the change in its emission spectrum of Indo-1 dye from blue to violet upon binding to  $\text{Ca}^{2+}$ . The blue emission is measured through a 530/30 BP filter 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 intracellular  $\text{Ca}^{2+}$  concentration.  $\text{CD4}^{+}$  cells were enriched (see inset panel top left) from  $\text{Cftr-}\Delta\text{F508}$  and  $\text{Cftr}^{+/+}$  mice and stimulated with anti- $\text{CD3/CD28}$  antibodies followed by 500ng/ml of ionomycin (as a positive control see inset on bottom right). A) Representative intracellular calcium flux for  $\text{Cftr-}\Delta\text{F508}$  and  $\text{Cftr}^{+/+}$   $\text{CD4}^{+}$  T cells. B) The area under the curve for the traces from  $\text{Cftr-}\Delta\text{F508}$  (n=6) and  $\text{Cftr}^{+/+}$  (n=6) in response to  $\text{CD3/CD28}$  Abs. C) The slopes for the traces from  $\text{Cftr-}\Delta\text{F508}$  (n=6) and  $\text{Cftr}^{+/+}$  (n=6) in response to  $\text{CD3/CD28}$  Abs.



To show that the increased intracellular calcium fluxes seen in TCR activation in response to CD3/CD28 results in nuclear accumulation 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 directly show if this nuclear translocation of NFAT would also translate to an upregulation and increased secretion of IL-13 in *Cftr*<sup>-/-</sup> T-cells, we measured the cytokine levels in the supernatants of CD3/CD28 stimulated cells. The data showed that the secretion pattern of TNF- $\alpha$ , an early activated cytokine known to respond to  $iCa^{2+}$  signaling through the T cell receptor<sup>68</sup> mimicked the pattern seen with the nuclear translocation of NFAT and was secreted at significantly higher levels from *Cftr*<sup>-/-</sup> T cells (figure 7b). While IL-13 was below detection in the earlier time points it was found to be expressed at significantly higher levels from *Cftr*<sup>-/-</sup> T-cells in supernatants collected 24hrs after stimulation (figure 7c).

**Figure 7.** *NFATc1 Nuclear translocation and cytokine secretion in *Cftr*<sup>-/-</sup> and *Cftr*<sup>+/+</sup> 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, 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-alpha. C) Expression of IL-13 at 24hrs post stimulation. Data is shown as group averages + S.E.M for a total of N=5.



### CHAPTER III: 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 *aspergillus fumigatus* that is dependent on CFTR function in lymphocytes alone. We demonstrated that sensitization and aerosol challenge of *Cftr*<sup>-/-</sup> mice and their wildtype controls leads to an enhanced-IgE response in *Cftr*<sup>-/-</sup> mice that is reminiscent of CF related allergic asthmatic condition known as allergic broncho-pulmonary aspergillosis (ABPA). While ABPA remains rare outside of the CF population it is very common among CF patients, affecting approximately 15% of them<sup>29,30</sup>. Interestingly, we demonstrated that the transfer of naive *Cftr*-deficient splenocytes into congenic *Rag*<sup>-/-</sup> mice was enough to confer the high IgE response to the *Rag*<sup>-/-</sup> 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,<sup>42,45,69</sup> however the physiological relevance of CFTR expression in lymphocytes is less clear, with some studies implicating it with volume regulation and cytotoxicity regulation of CD8<sup>+</sup> T-cells<sup>70-73</sup>. Other models suggest that activation of Cl<sup>-</sup> currents by CFTR in response to nitric oxide via a cyclic GMP-dependent mechanism are defective in T cells from CF patients<sup>74</sup>. Importantly, electrophysiological studies document a functional role for CFTR in lymphocytes



by recording a defect in cAMP-dependent Cl<sup>-</sup> currents in CF- derived lymphocytes using whole cell patch clamp techniques<sup>42,75</sup>. These studies demonstrate the presence and function of CFTR within lymphocytes and lend some credence to the longstanding yet controversial view that a primary immune abnormality is associated with cystic fibrosis. 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 indicated that a primary immune abnormality was indeed a possibility. Namely, the transfer of naïve splenocytes was able to recapitulate the 2-fold 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. Since the IgE response is known to be driven by Th2 cytokines and we have previously demonstrated that IL-13 is upregulated 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, IL-13, IL-5 and IL-17 was observed from CD4<sup>+</sup> and CD11b<sup>+</sup> 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<sup>+</sup> T cells, whereas the increased secretion of IL-5 and IL-17 seemed to be dependent on a synergistic interaction between both Cfr-/- Cd11b<sup>+</sup> and CD4<sup>+</sup> cells. Taken together with the adoptive transfer experiments these data suggested 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 *Aspergillus fumigatus* crude protein extract. 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 Cftr deficient immune cells, such as macrophages, dendritic cells and T-cells.

In order to directly determine whether this phenotype can be attributed to Cftr deficient T-cells we created T-cell specific Cftr knockout mice by using a floxed Cftr 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 Cftr<sup>-/-</sup> mice, as compared to controls which had been mocked-PBS sensitized and challenged with Af-cpe<sup>39</sup>. Furthermore, these T-cell Cftr conditional knockout mice went on to develop dramatically different IgE responses to Af-cpe as was characteristic of Cftr<sup>-/-</sup> mice. With these experiments we were able to finally demonstrate without any confounding variables a primary immune abnormality associated with cystic fibrosis in vivo, which, to our knowledge has never been shown before.

A consequence of Cftr deficiency in lymphocytes is the reduction in Cl<sup>-</sup> permeability which in turn may hyperpolarize their membranes. Its hypothesized that the altered membrane potential could then alter the electrical driving force for

Ca<sup>2+</sup> to enter the cells. Here we also show that intracellular calcium fluxes in C<sub>fr</sub><sup>-/-</sup> CD4<sup>+</sup> T cells are increased in response to CD3/CD28 stimulation as determined by the area under the curve and the slopes of the Ca<sup>2+</sup> flux response when compared to wildtype CD4<sup>+</sup> T cells (figure 6). The relevance of this altered calcium flux in C<sub>fr</sub><sup>-/-</sup> T-cells is that it may lead to increased transcription activity of Ca<sup>2+</sup> regulated transcription factors. Nuclear factor of activated T-cells (NFAT) activity is modulated by cytoplasmic Ca<sup>2+</sup> concentration through various Ca<sup>2+</sup> associated signaling pathways. Ultimately, increases in cytoplasmic Ca<sup>2+</sup> concentration induce NFAT dephosphorylation and NFAT translocation to the nucleus where it binds to cis regulatory elements of target genes as a monomer<sup>76</sup>. This signaling cascade proceeds through calmodulin and calcineurin, a cytoplasmic serine/threonine phosphatase. Calcineurin upregulates NFAT by dephosphorylating serines in the SP-repeats and in the serine rich N terminus region of NFAT exposing the nuclear localization signal, allowing NFAT to translocate to the nucleus<sup>76</sup>. In T cells, TCR stimulation causes an increase in intracellular Ca<sup>2+</sup> concentration depleting Ca<sup>2+</sup> sequestered ions in the ER, followed by an influx of extra-cellular calcium ions from Ca<sup>2+</sup> release activated Ca<sup>2+</sup> channels (CRAC)<sup>76</sup>. While 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, IL-13, IL-5 and TNF- $\alpha$ <sup>77</sup>. In agreement with the results for the intracellular Ca<sup>2+</sup> flux experiments we observed a significantly greater

increase in the nuclear translocation of NFAT after TCR activation with CD3/CD28 in *Cftr*<sup>-/-</sup> cells (figure 7). Parallel cell experiments demonstrated that following NFAT nuclear translocation *Cftr*<sup>-/-</sup> cells had significantly higher expression of TNF-alpha and the key Th2 effector cytokine IL-13. These data suggests 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 increase translocation of NFAT and higher induction of IL-13 in *Cftr*<sup>-/-</sup> T-cells. Exactly how CFTR dysfunction affects Ca<sup>2+</sup> signaling in lymphocytes warrants further investigation and the convergence or overlap of other dysregulated pathways related to *Cftr* deficiency can not be ruled out. There is some evidence of CFTR dysfunction imparting elevated antibody secretion in B cell hybridomas; while 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 cell with intact CFTR function.

The main pathological features associated with cystic fibrosis are pancreatic cysts that lead to a reduction in the secretion of digestive enzymes as well as chronic airway infections most notably by pseudomonas. While it has been well established and recognized that impaired CFTR function adversely affects the secretory epithelial, the role of CFTR in non-epithelial 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 identifies that CFTR dysfunction in T cells can in of itself lead to aberrant immune responses. Specifically we show how it skews responses to *Aspergillus fumigatus*, 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 in order to try to prevent or ameliorate aberrant immune responses in people with cystic fibrosis. Thus, current and future drugs targeting cystic fibrosis should determine if their benefits extend to this cell population, alternatively this also opens a new avenue to test small molecule modulators of immune response as a potential therapy for cystic fibrosis.

## CHAPTER IV: Materials and Methods

### *Mouse strains*

The Cftr knock-out strain used for these studies was the CFTR S489X  $-/-$  neo insertion in C57BL/6 mice developed initially at the University of North Carolina<sup>78</sup> and then modified with the transgenic over expression of gut-specific expression of human CFTR from the fatty acid binding protein (FABP)- promoter in order to prevent intestinal obstruction and improve viability<sup>79</sup>. The other mouse strain used is Cftr- $\Delta$ F508 mouse, 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<sup>64</sup> 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 aspergillus fumigatus crude protein extract (Af-cpe) (Greer Laboratories). Briefly, animals were administered with intraperitoneal (i.p.) injections of 200ug of Af-cpe extract dissolved in 100ul of PBS on days 0 and 14. Aerosol challenge was performed with 0.25 % Af-cpe for 20 min in a 30x30x20 cm acrylic chamber using a jet nebulizer Pari model LC-D with an air flow of 6 liters/min on days 28, 29 and 30.

### *Adoptive Transfers*

Adoptive transfer were performed by harvesting either Af-cpe sensitized or naïve splenocytes from Cftr- $\Delta$ F508 (Cftr $-/-$ ) or wildtype littermate controls. Briefly,

spleens were disaggregated in hanks buffer saline and passed through a 20um mesh. Cells were then resuspended in PBS at a concentration of  $4.5 \times 10^8$  cell/ml. Rag<sup>-/-</sup> mice on a C57BL/6 background were then injected intra-peritoneally with 100ul of the suspension. Eight weeks were allowed for engraftment before either challenging or sensitizing and challenging Rag<sup>-/-</sup> mice.

#### *Antigen Recall*

Spleens were harvested and CD4 T cells and CD11b cells were separated using the AutoMACs pro (Miltenyi Biotec). Cells were counted and plated in 96 well round bottom plates so that there were  $1 \times 10^5$  CD4 T cells and  $1 \times 10^5$  CD11b positive cells for a total of  $2 \times 10^5$  cells per well. Cells were cultured in media that contained 10mg/ml Albumin (Sigma). 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 staining was done as previously described<sup>80</sup>.

#### *NFAT Studies*

Splenocytes from Cfr- $\Delta$ F508 (Cfr<sup>-/-</sup>) or wildtype littermate controls were plated on paired round bottom 96 well plates with RPMI 1640 10% FBS and 1% Pen/Strep at a concentration of  $1 \times 10^5$  cells/well. The cells were then stimulated with CD3/CD28 antibody cocktail and wells from one plate were harvested at (10,

30 and 90 min.). For nuclear protein extraction while the other was used to obtain cell culture supernatants at (1.5, 3, 6 and 24 hrs) for cytokine analysis. Nuclear NFAT translocation was assayed from the nuclear extracts using the modified kit TransAM NFATc1 (Active Motif). Cell culture supernatants were analyzed for cytokine secretion profiles using Luminex Technology (Bio-Rad, Hercules CA).



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