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Polarization Of Cystic Fibrosis Macrophages Is Dysregulated

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POLARIZATION OF CYSTIC FIBROSIS MACROPHAGES IS DYSREGULATED

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Abstract: ACTIVATION OF CYSTIC FIBROSIS MACROPHAGES IS DYSREGULATED
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We hypothesize that intrinsic defects in cystic fibrosis (CF) macrophages affect their ability to adequately respond to extracellular stimuli, affecting their phenotypic response and contributing to a hyper-inflammatory state.

Peripheral blood-derived monocytes from healthy donors (HD) (n=10) as well as CF subjects (n=10) were differentiated into macrophages using RPMI media with 50 ng/mL of MCSF for 10 days. Cells were treated with 100 ng/mL of LPS plus 20 ng/mL of INF-gamma to activate the M1 phenotype or 20 ng/mL of IL-4 to activate the M2 phenotype. Cells were treated for 24 hours in preparation for RNA isolation and 3 hours in preparation for protein isolation. RNA was isolated and reverse transcribed to cDNA in preparation for qualitative, reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed using IL-6, IL-1B, and TNF-alpha primers as M1 markers and TGF-B1, MRC, PPAR-gamma as M2 markers. Expression levels were performed in seven experiments, each using a different set of HD and CF subjects, utilizing 8.0 x 10^5 to 1.0 x 10^6 cells per well, while three protein experiment were done using different sets of HD and CF subjects.

Stimulation of CF macrophages induces greater phosphorylation of proteins that regulate macrophage activation, as compared to HD macrophages. When CF macrophages are challenged with LPS/INF-gamma, they show greater phosphorylation of STAT1 protein as compared to HD macrophages. Similarly, when CF macrophages are challenged with IL-4, they exhibit greater phosphorylation of STAT6 protein, as compared to HD macrophages. Finally, CF macrophages show greater phosphorylation of AKT protein as compared to HD macrophages when stimulated with both M1 and M2 cytokines. As expected, HD macrophages (n=7, experiments) demonstrated appropriate plasticity and polarization with increased expression of M1 markers and decreased expression of M2 markers in response to LPS/INF-gamma. Furthermore, they demonstrated modestly increased expression of M2 markers and decreased expression of M1 markers in response to IL-4. In marked contrast, when stimulated with LPS/INF-gamma CF macrophages (n=7, experiments) demonstrated hyper-inflammation with dramatically increased expression levels of M1 markers as well as aberrant polarization as evidenced by increased expression levels of some M2 makers. Aberrant polarization was further characterized by increased expression of M1 markers in the presence of IL-4 in addition to increased expression of M2 markers.

These data suggest that lack of functioning CFTR in macrophages leads to the inability of macrophages to adequately respond to environmental cues and activate into appropriate phenotypes suggesting there is an intrinsic cellular defect. Moreover, the mechanism(s) that underlie these aberrant responses likely involve altered intracellular signal transduction which is currently under investigation.

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Table of Contents

1. Introduction 6
1.1 Cystic Fibrosis 6
1-1.1 Clinical Diagnosis 6
1-1.2 Clinical Presentation 7
1-1.3 Genetic Basis of Disease 8
1-1.4 CFTR Protein 8
1-1.5 CFTR Role in Ion Transport 9
1-1.6 Pathophysiology of Dysfunctional Ion Transport 10
1-1.6.1 Abnormal ASL 11
1-1.6.2 Dehydration Hypothesis 11
1-1.6.3 High Salt Hypothesis 12
1.2 Hyperinflammation in CF 14
1-2.1 CFTR Directly Causes Dysregulated Hyperinflammation 14
1-2.2 Inflammation in the Absence of Infection 14
1-2.3 Exaggerated, Persistent Inflammatory Response 15
1-2.4 Altered Receptor Signaling and Intracellular Pathways Cause Inflammation 15
1-2.4.1 Dysregulation of Pattern Recognition Receptors 15
1-2.4.2 NFκB Pathway Drives Inflammation 16
1-2.4.3 Other Pathways that Drive Inflammation 17
1-2.5 Innate and Adaptive Immunity may Directly Contribute to CF 17
1-2.5.1 Neutrophils 18
1-2.5.1.1 Exaggerated Neutrophil Response 18
1-2.5.1.2 Impaired Neutrophil Bacterial Killing 18
1-2.5.1.3 Neutrophil Elastase is Increased 19
1-2.5.1.4 Neutrophils Drive Inflammation with ROS 19
1-2.5.2 Lymphocytes 20
1-2.5.2.1 IL-17 Response is Dysregulated in Lymphocytes Lacking CFTR. 20
1-2.5.2.2 CF Patients Lack Functioning T-Regulatory Lymphocytes 21
1.3 Macrophages Directly Contribute To CF Pathophysiology: Importance of Macrophage dysfunction to CF lung disease 21
1-3.1 Discovery of 'Phagocytes' 21

1-3.2 Diverse Roles of Macrophages 22
1-3.3 Origins of Macrophages 23
1-3.4 Process of Differentiation 23
1-3.5 Polarization of Macrophages 24
1-3.5.1 M1 Phenotype 25
1-3.5.2 M2 Phenotype 26
1-3.6 Macrophages and CF Lung Disease 27
1-3.6.1 Macrophages Express Functioning CFTR 28
1-3.6.2 CFTR Defects Directly Affect Macrophage Function 28
1-3.6.3 Macrophages Exhibit Mixed M1/M2 Phenotype in CF 30
1-3.6.4 Mechanisms Contributing to Abnormal CF Mϕ Activity 30
2. Statement of Purpose 32
3. Methods 34
3.1 Chemicals and Reagents 34
3.2 Isolation and Culture of Human Peripheral Bone Marrow Derived Mϕs 34
3.2-1 Recruitment and Subject Profile 34
Figure 5. Subject Demographics: All CF subjects were recruited from pediatric CF clinic. 35
3-2.2 Isolation 35
3-2.3 Culture 36
3-2.4 Confirming Macrophage Population 37
3-2.4.1 Confirming CFTR Function in Macrophages 38
3.3 Cytokine treatments 39
3.4 QT-RT-PCR and Expression Analysis 40
3.5 Protein isolation and Western blot 40
3-5.1 Western Blot Antibodies 40
3.6 Statistical Analysis 41
4. Results 42
4.1 Macrophages Effectively Transport Cl- Via CFTR 42
4.2 LPS/INF-γ Stimulated CF Mϕs Exhibit Different Levels of STAT1/pSTAT1 42
4.3 LPS/INF-γ Stimulated CF Mϕs hyper-express M1 Gene Markers 44
4.4 IL-4 Stimulated CF Mϕs Exhibit Different Levels of pSTAT6/STAT6 45
4.5 IL-4 Stimulated CF Mϕs Up-regulate Expression of M2 Gene Markers 46
5. Discussion 48

6. References 52

Figure Legend

Figure 1: Polarization of Macrophages: 25

Figure 2: M1 Pathway 26

Figure 3: M2 Pathway 27

Figure 4. Isolation of Monocytes Using Ficol 35

Figure 5. Subject Demographics 36

Figure 6. Confirmation of Macrophage Population 38

Figure 7. Macrophage Culture and Treatment Protocol 39

Figure 8. MQAE Efflux in Macrophages 42

Figure 9. Differential Expression of pSTAT1/STAT1 in CF Mϕs 43

Figure 10. Stimulation with LPS/INF-γ Leads to Hyperexpression of M1 Markers in CF Mϕs 44

Figure 11. Up-regulation of M2 Markers with Stimulation of M1 Cytokine by CF Mϕs 45

Figure 12. Differential Expression of pSTAT6/STAT6 in CF Mϕs 46

Figure 13. Stimulation with IL-4 Leads to Hyper-expression of M2 Markers in CF Mϕs 47

Figure 14. AKT Serves as Master Regulator 50

1. Introduction

1.1 Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal recessive disease caused by a mutation to the gene that codes for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The disease manifests as a chronic, degenerative process that affects the upper and lower respiratory tracts, the pancreas, the hepatobiliary tree, intestinal tract, sweat glands and the reproductive tracts [1].

1-1.1 Clinical Diagnosis

As of 2015, 59.6% of patients are diagnosed through newborn screening (NBS) by measuring for uncharacteristically high levels of immunoreactive trypsinogen (IRT) in blood spot tests [2]. High levels of IRT indicate pancreatic damage. High levels are not specific to CF, and infants that screen positive for IRT require either further genetic testing or confirmation with a sweat chloride test. Classically, a diagnosis of cystic fibrosis is made when clinical features of the disease accompany a sweat chloride concentration greater than 60 mmol/L. If sweat chloride concentrations fall in the intermediate range of 30-59 mmol/L, further DNA testing can be done [3]. NBS and early diagnosis are important, as early intervention allows for improved nutrition and growth, which may play an important role in reducing disease burden. In fact, several studies have shown a correlation between improved growth rates in infancy/childhood and improved lung function later into adolescence [4, 5].

1-1.2 Clinical Presentation

CF often presents as a classical clinical sequelae of homozygous mutations within Class I-III categorizations. The neonatal period is often significant for meconium ileus, protracted jaundice, and intestinal atresia. In infancy, CF patients begin to experience failure to thrive and chronic diarrhea from pancreatic insufficiency; cholestasis, chronic infection with *Staphylococcus aureus* (*Sa*), and anemia also present. In the absence of NBS or sweat chloride testing, infants with CF can go undiagnosed, further exacerbating these manifestations. Some pathognomonic features of CF in childhood include rectal prolapse, intussusception, distal intestinal obstruction syndrome (DIOS), chronic pansinusitis or nasal polyposis, ABPA, and the beginning of liver disease. In adulthood, CF manifests as advanced lung disease often requiring transplant after 30 years of age, chronic infection with *Pseudomonas aeruginosa (Pa*), CF related diabetes (CFRD), anxiety, osteopenia, congenital bilateral absence of vas deferens in mails, digestive tract cancer, and advanced liver disease ([1, 3]). Due to the great strides made in drug development and in clinical management, more than 50% of CF patients are adults with the median predicted survival age up to 41.6 years, up from around 30 years in 1987 ([2]). As a result, our understanding of the adult disease sequelae is evolving.

1-1.3 Genetic Basis of Disease

The gene coding the CFTR protein was identified in 1989 and was localized to chromosome 7 ([6]). To date, over 1500 mutations have been identified, but the functional importance of only several mutations is understood. The deletion of the amino acid phenylalanine at position 508 on the CFTR protein (F508del) is the most common allelic mutation, with an estimated frequency of 66% that varies within populations depending on demographics. While mutations in the CFTR gene are the principal cause of disease in patients with CF, even among patients who share the same genotype on each allele, there is great variability of both clinical phenotype as well as survival outcomes. This variability suggests that other environmental and genetic factors modify disease severity. In fact, this variability can be partially explained by the presence of additional genetic polymorphisms, such as -509 and codon 10 genotype variants of the 5' end of TGFβ1, which further adversely modify disease phenotype [7].

1-1.4 CFTR Protein

Mutations in the CFTR gene lead to alterations in the synthesis, trafficking, and function of the CFTR protein, a selective anion channel for chloride, bicarbonate, and thiocyanate, that belongs to the adenine nucleotide-binding cassette (ABC) protein family. The protein contains five domains: two nucleotide-binding domains (NBDs), two transmembrane domains, and a unique R domain. Activation of the CFTR protein involves cAMP-dependent phosphorylation of the R domain by PKA first, followed by binding and hydrolysis of ATP by the two

NBDs to induce open and closed conformational changes. The two transmembrane domains line the pore and regulate the conductance and selectivity of the pore, allowing trafficking of anions down their electrochemical gradient [6, 8]. This characteristic distinguishes the CFTR protein from other ABC transporters, as the other members of this protein family utilize ATP hydrolysis to transport ions up their electrochemical gradient.

Distinct CFTR mutations affect protein function differently and can be further categorized into five classes. Class I mutations lead to defective synthesis of the protein, Class II mutations lead to defective processing/protein maturation, Class III mutations lead to defective protein regulation, Class IV mutations lead to defective ion conductance, and Class V mutations lead reduced synthesis and function [1].. As stated before, association between genotype and phenotype is incomplete as twins may display varying phenotypes, but generally speaking, severity of disease increases with ascending order of class mutation.

1-1.5 CFTR Role in Ion Transport

The CFTR protein is most commonly found on the apical membrane of epithelial cells in the airways, exocrine pancreas, gastrointestinal tract, and sweat ducts, where it is responsible for anion conductance, as well as regulation of other ion channels. This widespread distribution of protein expression explains the broad spectrum of clinical pathology, across numerous organ systems. CFTR regulates salt and water transport across epithelial membranes, and loss of function has been observed to cause decreased secretion of chloride and

bicarbonate, as well as the increased transmembrane absorption of sodium. Phosphorylation of the R domain activates the CFTR protein, opening the channel for conductance to chloride and bicarbonate secretion, which in turn creates an electrochemical driving force for parallel, paracellular diffusion of sodium and water. This mechanism for secreting fluid is physiologically vital for maintaining adequate airway surface liquid (ASL) and delivering exocrine pancreatic and antimicrobial macromolecules to luminal surfaces [9, 10].

1-1.6 Pathophysiology of Dysfunctional Ion Transport

While the clinical manifestations of CF are diverse, around 80% of cystic-fibrosis-related deaths are attributable to lung disease and pulmonary insufficiency [1]. The three hallmarks of airway disease pathophysiology in CF are chronic infection, dysregulated hyperinflammation, and airway obstruction. This pathophysiology causes lung remodeling in the form of bronchiectasis that leads to air trapping, hypercarbia, hypoxemia, and ultimately pulmonary insufficiency. Although we know that loss of CFTR function leads to this pathophysiology, and restoration of CFTR function corrects the underlying pathobiology that drives disease, we still have a limited understanding of the direct links between CFTR gene mutation and the pathophysiology of the disease.

Several hypotheses have been proposed that link dysfunctional ion transport with abnormalities in ASL that lead to thickened mucus and defective mucociliary transport (MCT). These hypotheses propose that defective MCT and

thickened mucus create environments that lead to chronic infections, hyperinflammation, and ultimately the clinical manifestations of CF.

1-1.6.1 Abnormal ASL

ASL consists of an interface between a mucus layer and a periciliary liquid layer (PCL). The mucus layer is responsible for trapping inhaled particles in carbohydrate-rich mucins [11]. The PCL is responsible for keeping cilia fully extended so that they can effectively beat and drive MCT. ASL volume is tightly regulated in order to maintain PCL height and mucous viscosity in optimal ranges. Boucher and colleagues propose that dysfunctional CFTR leads to a dehydrated ASL that osmotically drives water out of both the PCL and mucous layer. As a result, mucous viscosity increases with an increase in mucin concentration, and PCL levels fall below the height necessary to keep cilia fully extended and properly beating ([12, 13])

1-1.6.2 Dehydration Hypothesis

The “dehydration hypothesis” proposes that under physiologic conditions, airway epithelial cells regulate ASL volume through the coordination of luminal epithelial Na+ channel (ENaC) and CFTR. When ASL volume is high, increase absorption of luminal sodium via ENaC transport leads to increased transcellular H2O transport and paracellular Cl^- transport from the lumen, resulting in increased absorption of ASL fluid. Conversely, when ASL volume is low, CFTR mediated Cl^- secretion increases, which results in increased transcellular H_2O

secretion and paracellular Na^+ secretion into the lumen, resulting in increased secretion of ASL fluid. Overall, these counterbalancing processes lead to isotonic fluid transport [10, 11].

Dysfunctional CFTR disrupts the equilibrium, as CFTR-mediated ASL secretion decreases, and ENaC-mediated ASL absorption increases. This imbalance leads to a dehydration of ASL, decreasing PCL height and increasing mucus viscosity [14]. Mucus plaques develop and create hypoxic microenvironments that serve as breeding grounds for bacteria as defective MCT greatly hinders clearance. In this setting, the dehydrated airway leads to chronic infection as bacteria like *Pa* infiltrate the plaques, multiplying in nutrient rich hypoxic environments, eventually adhering to the epithelial cells as biofilms. Chronic infection provokes a hyperinflammatory response that can lead to lung remodeling, airway obstruction and pulmonary insufficiency ([11-13, 15]).

1-1.6.3 High Salt Hypothesis

The “high salt hypothesis” argues that defects in CFTR impair transcellular absorption of Cl^- and ultimately lead to inability of epithelial cells to absorb salt. Consequently, ion concentration of ASL fluid increases, while volume remains unchanged [16]. Additionally, because CFTR regulates the secretion of bicarbonate into the lumen, loss of CFTR function greatly decreases ASL fluid pH [17].

Welsh and colleagues found that the ASL fluid recovered from CF epithelia cells in culture displayed decreased bacterial killing in comparison to

WT cells. The ASL fluid of CF epithelia cells had higher concentrations of salt than WT cells, and when the fluid was diluted, the bactericidal properties were restored [18]. Furthermore, they found that when CFTR function was restored to epithelia cells, they were able to correct the high salt concentration of the ASL fluid, and restore bacterial killing properties [19].

These findings supported the concept that ASL fluid must contain anti-microbial molecules that are essential for host defense. In the absence of functional CFTR, the ASL becomes more acidic and highly concentrated with salt, which in turn neutralizes the antimicrobial molecules [17]. This work led to the discovery of specific molecules, such as human β-defensins and LL-37 that are found in the ASL, and that exhibit gram-negative, antimicrobial properties. These molecules are sensitive to both pH and salt concentration, and as a result, become less effective in ASL fluid produced by CF epithelia cells in comparison to WT cells [20, 21]. Finally, some studies have shown that decreased bicarbonate secretion that results from loss of CFTR function, can also directly affect the viscosity of the mucus layer in ASL fluid by preventing physiology expansion and rheology of mucinous proteins [22]. In conclusion, as a result of decreased pH and increased salt concentration, the ASL fluid of the CFTR deficient airway exhibits reduced bactericidal properties and thickened mucus viscosity that allows bacteria to chronically colonize the airways. This defect may be the inciting event that leads to the pathogenesis of CF lung disease.

1.2 Hyperinflammation in CF

1-2.1 CFTR Directly Causes Dysregulated Hyperinflammation

The CFTR protein acts principally as an anion channel. Therefore, it comes as no surprise that significant resources have been focused on elucidating the connections between abnormal ion transport and the pathogenesis of CF. Most of these connections assert that chronic inflammation is a secondary consequence that results when loss of CFTR function leaves the CF airway vulnerable to chronic infections. Nevertheless, a growing body of evidence supports the hypothesis that dysfunctional CFTR directly causes hyperinflammation, even in the absence of infection [23].

1-2.2 Inflammation in the Absence of Infection

This paradigm shift is supported by experiments that show abnormally increased levels of the pro-inflammatory mediator IL-8 as well as increased numbers of monocytes and immune cells in CF *ex*-vivo, uninfected human fetal tracheas cells [24]. Further studies examining the bronchoalveolar lavage fluid (BALF) of infants without evidence of infection showed increased levels of pro-inflammatory mediators TNF-α, IL-1β, and IL-8 [25, 26]. Therefore, even in the absence of pathogenic bacteria and clinically observable disease, CF airways display hyperinflammatory phenotypes. These findings indicate hyperinflammation directly results from loss of CFTR function.

1-2.3 Exaggerated, Persistent Inflammatory Response

While CF cells naïve to infection show a modestly increased basal level of pro-inflammatory cytokines, exposure to infection causes an exaggerated inflammatory response followed by a persistent increased basal secretion level of these same cytokines. CF primary cell cultures taken from chronically infected individuals display increase levels of pro-inflammatory cytokines IL-6 and IL-8. Also, these cells tend to secrete a decreased level of the anti-inflammatory mediator IL-10, in comparison to healthy controls [27, 28]. BALF from CF airways recapitulated these findings showing increased levels of TNF-α, IL-1β, IL-6, IL-8, with very low levels of IL-10 [29]. Even when immortalized epithelial cell lines with functional CFTR are treated with CFTRinh-172, a potent and specific CFTR inhibitor, they express increased levels of IL-8 in response to *Pa [30].* This basal-state imbalance provides evidence of a prolonged, persistent, and exaggerated inflammatory state in CF, that directly results from loss of CFTR function [31].

1-2.4 Altered Receptor Signaling and Intracellular Pathways Cause Inflammation

1-2.4.1 Dysregulation of Pattern Recognition Receptors

Pattern recognition receptors (PRRs) expressed on epithelial cells are the sentinels of the innate immune system, but these receptors are also expressed on monocytes, macrophages (Mϕs), lymphocytes and neutrophils [23]. PRRs include multiple receptor types, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain family, Fc receptors, and RIG-like receptors.

Airway epithelial cells primarily employ TLRs, expressing a variety of surface and intracellular iterations. Evidence shows that in CF, dysregulation of the trafficking, desensitization, internalization and degradation of PRRs leads to the activation of pro-inflammatory pathways without resolution [32]. Therefore, these receptors provide a link between epithelial cells, innate and adaptive immune cells, and systemic hyperinflammation.

1-2.4.2 NFκB Pathway Drives Inflammation

Acting downstream of PRRs, nuclear factor-kappa B (NFκB), is a pathway that is also found in all the aforementioned cell lines. TLRs are implicated in the activation of NFκB, which involves IκB kinases phosphorylating the inhibitory IκB proteins [33]. Upon activation, NFκB localizes to the nucleus and up-regulates pro-inflammatory cytokines. In primary cell cultures of CF cells, NFκB shows an increased nuclear localization in comparison to healthy donor (HD) cells [34]. Further studies show an increase of NFκB proteins in congruence with a decrease in inhibitory IκB protein and lipopolysaccharide-inducible protein A20. Human tracheal epithelial cells treated with CFTR inhibitors show an increased translocation of NFκB, followed by increased secretion of pro-inflammatory cytokines [35]. As mentioned before, the CF lung environment has increased basal levels of pro-inflammatory cytokines, such as IL-8 and decreased levels of IL-10. With respect to NFκB, IL-8 serves to activate the pathway, while IL-10 serves as an inhibitor of the pathway [36]. Thus, the imbalance of these two cytokines in the CF airway leads to excessive, persistent activation NFκB,

resulting in the further overexpression of pro-inflammatory cytokines IL-6, IL-8, TNF-α, and IL-1β [37, 38].

1-2.4.3 Other Pathways that Drive Inflammation

While the NFκB pathway has been well described as dysfunctional in CF, abnormal lipid metabolism, decreased production of nitric oxide, the misfolded protein stress response, and increased levels of ceramide are other immune pathways that are characterized as abnormal in CF [39].

1-2.5 Innate and Adaptive Immunity may Directly Contribute to CF

As evidenced by the "High Salt Hypothesis" and the "Dehydration Hypothesis", as well as *ex-vivo, in-vitro,* and primary cell experiments on naïve cells, dysfunctional CFTR in epithelial cells directly leads to hyperinflammation. Yet, cells of the innate and adaptive immune systems, not epithelial cells, have principally been described as the regulators of inflammation. The discovery that the promoter of the CFTR gene shared characteristics with housekeeping genes, inspired research exploring the expression levels of CFTR in non-epithelial cells. This research found that cells of the innate and adaptive immune systems express CFTR [40]. The confirmation of significant expression levels of CFTR in lymphocytes, neutrophils, monocytes, and dendritic cells (DCs) links our understanding of the cells that regulate inflammation and the implication that these cells directly cause the hyperinflammation found in CF as a result of CFTR loss of function.

1-2.5.1 Neutrophils

1-2.5.1.1 Exaggerated Neutrophil Response

As stated above, CF airway cells produce an abnormal amount of IL-8, a primary chemokine for attracting neutrophils. Thus, a hallmark of CF lung disease is increased neutrophil recruitment. Additionally, in CF, neutrophils do not undergo the usual cycle of recruitment, activation, phagocytosis/bacteria killing, and apoptosis with clearance, which leads to the accumulation of neutrophils [31]. As a result, CF lungs are more prone to hyperinflammation, ineffective bacterial clearance, and tissue damage and lung remodeling.

1-2.5.1.2 Impaired Neutrophil Bacterial Killing

Abnormal accumulation of neutrophils in CF has been shown in the BALF of naïve airways, airways infected with *Pa*, and the airways of adults with no signs of current exacerbations [25-28]. These elevated levels may result from the decreased effectiveness of bacterial killing. Killing deficiencies result from the inability of phagolysosomes in neutrophils that lack CFTR to adequately lower pH [32]. CF neutrophils also display decreased degranulation of antimicrobial proteins containing secondary and tertiary granules [37]. Impaired bacterial killing resulting from loss of CFTR function in neutrophils leads to the inability of the CF lung to clear infection and ultimately resolve inflammation.

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1-2.5.1.3 Neutrophil Elastase is Increased

Neutrophil elastase further compounds the deleterious effects of deficiencies in neutrophil killing. Accumulation of neutrophils leads to high concentrations of neutrophil elastase in CF airways. Left unchecked, neutrophil elastase dismantles gross lung anatomy through the degradation of collagen and elastin [37]. Also, elevated elastase disrupts the regulation of immune signaling pathways via the cleavage of plasma membrane receptors and proteins [32]. Clinicians have utilized this knowledge to use neutrophil elastase concentrations in sputum as a biomarker for decline in lung function in CF patients [41].

1-2.5.1.4 Neutrophils Drive Inflammation with ROS

Neutrophils use the anti-microbial properties of reactive oxygen species (ROS) when activated in the lung. Due to the accumulation and protracted presence of neutrophils, ASL fluid collected from CF lungs contains elevated concentrations of ROS. These levels are further exacerbated by the metabolic abnormalities in CF cells that reduce their antioxidant capabilities, as well as the repeated release of DNA by dead neutrophils that ramp up oxidative stress. Similar to neutrophil elastase, low levels of ROS are protective, but ultimately become drivers of sustained hyperinflammation in CF lungs due to their persistent, high concentrations [42]. In conclusion, neutrophils help set in motion a cycle of inflammation, and their persistent accumulation contributes to the prolonged, exaggerated state of hyperinflammation in CF lung disease.

1-2.5.2 Lymphocytes

1-2.5.2.1 IL-17 Response is Dysregulated in Lymphocytes Lacking CFTR

Certain deadly pathogens like *Pseudomonas Aeruginosa and A. fumigatus* trigger the pro-inflammatory IL-17 response – an adaptive response that recruits neutrophils and encourages phagocytes to uptake and kill these pathogens. Th17 cells, a specific helper-T cell that secretes IL-17, orchestrate this response. While initially this response is adaptive, CF patients are unable to clear these infections, for reasons described earlier, resulting in a sustained IL-17 response in CF patients. This sustained response is associated with bronchiectasis and classic airway obstruction disease [43]. The sputum collected from CF patients shows up-regulation of IL-17 secretion [44], and these results corroborated in CFTR knockout mouse that showed increased levels of IL-17 in BALF when challenged with *A. fumigatus [39, 45]*. An exaggerated Th17 response in lymphocytes lacking CFTR leads to the pathophysiology of sustained hyperinflammation and tissue remodeling that leads to obstructive lung disease in CF.

A deficiency in the enzyme indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan, may explain the increase of Th17 cells found in CF patients. IDO deficiency has been shown to be associated with patients homozygous for F508del [46]. CFTR knockout mice show correlations between IDO deficiency and an increase in Th17 cell frequency. As a result, mice are more susceptible to infection with *A. fumigatus.* When IDO is replaced in these mice, they show reduced susceptibility to *A. fumigatus* infection [46].

1-2.5.2.2 CF Patients Lack Functioning T-Regulatory Lymphocytes

Research has also recently begun on identifying defects in lymphocytes that can be attributed to CFTR loss of function. Studies show that CF patients may have both defective and overall reduced number of circulating Treg cells [47]. Tregs express CD4+, CD25+,Foxp3+ surface markers, and as a subset of CD4+ cells they are responsible for preventing autoimmunity and dampening down excess inflammation [48]. They also secrete anti-inflammatory cytokines IL-10 and TFG-β1. In CF patients colonized with *Aspergillus fumigatus (A. fumigatus)*, the subset of patients that develop allergic bronchopulmonary aspergillosis (ABPA) tend to have decreased frequency of Tregs in comparison to CF patients that are colonized with *A. fumigatus* but do not develop ABPA [48]. Also in association with lower Treg frequency was the proclivity for these patients' cells to exhibit exaggerated Th2 and Th17 responses. Therefore, the lack of Treg cells helps create the imbalanced environment found in CF lungs that tends show exaggerated pro-inflammatory IL-8 and IL-17 responses, with insufficient counterbalancing anti-inflammatory IL-10 responses.

1.3 Macrophages Directly Contribute To CF Pathophysiology: Importance of Macrophage dysfunction to CF lung disease

1-3.1 Discovery of 'Phagocytes'

Eli Metchnikoff first characterized the macrophage in the late 19th century, describing the cells as diverse agents of inflammation and host defense that, free

of anatomical restrictions, are permitted to circulate throughout the body “eating to defend”. Thus, the “phagocytosis theory” was born. Drawing on brilliant scientific intuition, Metchnikoff understood that embryologic development was rooted in Darwinism. He used “ontogenetic recapitulation to understand phylogenetic development” [49], recognizing not only that macrophages were derived from an ancient phylogenetic origin, but also understanding that they represented an end point of a process of differentiation that was put in motion from the original embryological cell division [49].

1-3.2 Diverse Roles of Macrophages

These seminal discoveries serve as the foundation for our understanding of the innate immune system and the diverse roles of macrophages. Today, we understand that macrophages are more than a specialized agent of innate immune system responsible only for elimination of pathogens and phagocytosis. Macrophages serve many essential functions in tissue homeostasis and development, releasing growth factors and directing tissue-repair through the sensing of tissue damage [50]. Macrophages also support tissue homeostasis through removal of apoptotic cells, remodeling of extracellular matrix, and serve as support cells for principal cells that execute primary tissue function roles, i.e. alveolar macrophages’ support of alveolar epithelial cells in surfactant homeostasis through recycling of surfactant [50]. Finally, pathological processes such as cancer, fibrosis, obesity, and osteoporosis often reappropriate macrophages plasticity, transforming them into drivers of disease [50].

1-3.3 Origins of Macrophages

Macrophages have distinct roles depending on functional demand, tissue location, and homeostatic state of tissues. This diversity in phenotype depends on the different embryological origin of tissue resident macrophages, deriving from the yolk sac, fetal liver, and hematopoietic stem cells in the bone marrow [51, 52]. These macrophage populations are able to remain as distinct populations due to their capability of continual self-renewal throughout the lifespan of the organism [53]. Epigenetics and enhancer-mediated gene-expression further distinguish distinct macrophage populations from each other in a tissue-specific and ontogenetic origin-specific manner.

1-3.4 Process of Differentiation

This background gives macrophages the flexibility to assume a diverse breadth of phenotypes. Deriving from different embryological backgrounds, arriving to tissues partially pre-programmed, macrophages undergo a process of activation, differentiation, and polarization [50]. Tissue-identity signals, functional-demand signals or a combination of both directs this process through signaling pathways involving hierarchically organized transcriptional regulators. In the process of maturation, progenitor cells first undergo activation, assuming a specific phenotypic endpoint, followed by reversible polarization, and concluding with stable, irreversible differentiation [50]. Maturation transitions from reversible

polarization to irreversible differentiation when the signals directing transcriptional control become more stable and consistent.

1-3.5 Polarization of Macrophages

Throughout this manuscript, the term polarization refers to the process by which activated macrophages receive regulatory signals that alter their cellular biology through differential expression of genes, translation of proteins, regulation of metabolism, and control of signaling pathways. Initially, polarization was described as the process by which macrophages assume one of two phenotypes that occupy opposite ends of a bipolar vector: M1 macrophages and M2 macrophages [52]. This concept was modeled after the dichotomous lymphocyte system of Th1 and Th2 cells. We now understand that this model was oversimplified, and as a result we did not appreciate the broad spectrum of macrophage phenotypes [53]. New descriptions of macrophage polarization account for the myriad of signals that drive macrophages to become certain phenotypes through the activation of specific pathways (Fig 1). Therefore, the original dichotomous polarization model has not been discarded, but instead expanded. As a result, the original paradigm that described macrophages as M1 or M2 is still used, but now adds further subdivisions for classification that account for the individual pathways that are activated by specific signals, as well as the idea that hybrid phenotypes exist [54, 55].

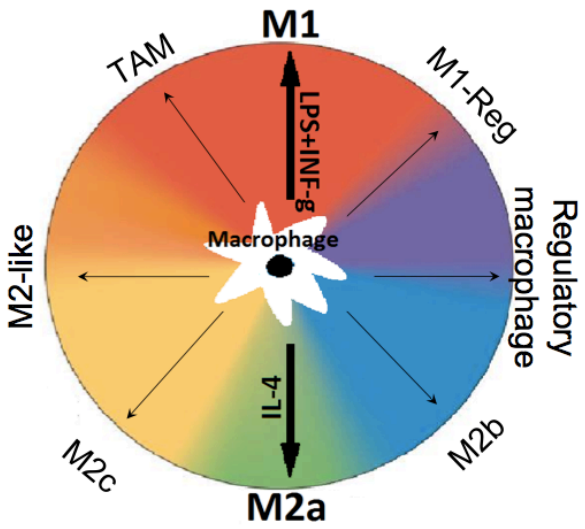

Figure 1: Polarization of Macrophages: Specific signals determine macrophage polarization and create a spectrum of phenotypes that expand beyond the original dichotomous M1 and M2 model.

1-3.5.1 M1 Phenotype

The M1 phenotype describes macrophages that express prototypic inflammatory responses and markers. The two most common stimuli that lead to the M1 phenotype are IFN-γ and LPS. IFN-γ activates the IFN-γ receptor that is formed by the IFNγR-1 and IFNγR-2 chains. Activation of this receptor recruits JAK1 and JAK2, which in turn recruits STAT1 and then activates the protein through phosphorylation. Phosphorylated STAT1 translocates to the nucleus where it is responsible for controlling the gene expression of cytokine receptors, cell activation markers, and adhesion molecules (Fig. 2). LPS signals TLRs that activate the MyD88 and TRIF proteins, which in turn activate NF-κB, IRF and AP-1. Both IFN-γ and LPS are responsible for the increased expression of pro-

inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-12, as well as the decrease in anti-inflammatory cytokine IL-10 [55].

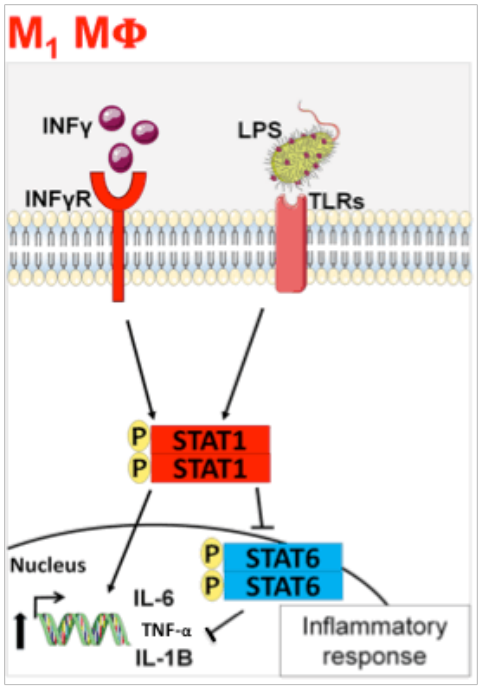

Figure 2: M1 Pathway**:** LPS and IFN-γ initiate the cascade of signaling that results in M1 polarization. Activation of INFγR and TLRs eventually lead to the phosphorylation of STAT1 which translocates to the nucleus, thereby increasing expression of pro-inflammatory cytokines IL-6, TNF-α, IL-1β, among other genes.

1-3.5.2 M2 Phenotype

The M2 phenotype refers to macrophage phenotypes that are involved in resolution of inflammation and tissue repair. The most commonly described cytokine in this subdivision is IL-4, although IL-13, IL-10, and other ligands have also been characterized [55]. IL-4 activates IL-4 receptor, which activates JAK1 and JAK3. JAK1 and JAK3 then phosphorylate STAT6, which can translocate to the nucleus upon phosphorylation. STAT 6 mediates the gene expression of anti-

inflammatory cytokines as well as receptors that mitigate the effects of pro-inflammatory cytokines [55] (Fig. 3).

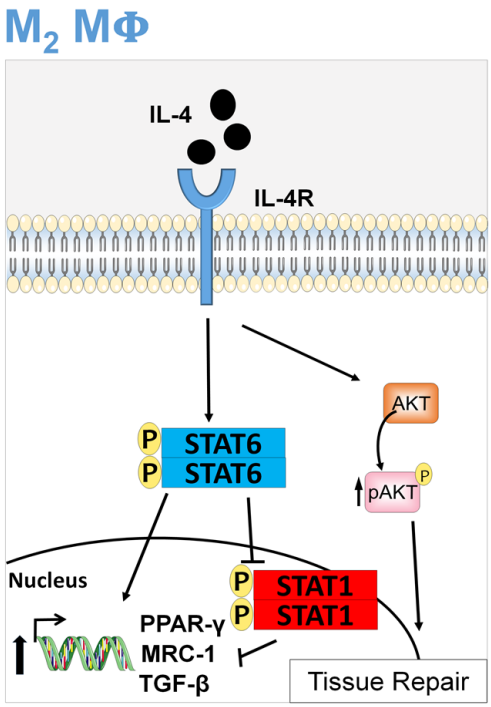

Figure 3: M2 Pathway**:** IL-4 initiates the cascade of signaling that results in M2 polarization. Activation of IL-4R eventually leads to the phosphorylation of STAT6, which translocates to the nucleus, thereby increasing expression of anti-inflammatory cytokines PPARγ, MRC-1, and TGF-β, among other genes.

1-3.6 Macrophages and CF Lung Disease

In 1982, before the CFTR gene had even been characterized, macrophages were first hypothesized to be involved in the pathogenesis of CF lung disease [56]. Although the mediators and mechanisms characterized in the original paper have not been substantiated, evidence is mounting in support of the conclusion that macrophages directly contribute to the underlying pathophysiology of CF.

1-3.6.1 Macrophages Express Functioning CFTR

Monocytes and macrophages must express CFTR in order to establish that a causal link between monocytes and CF pathogenesis exists. In 1989 the CFTR gene was identified, and in 1991 Yoshimura et al. first showed that CFTR mRNA levels in monocytes/macrophages were comparable to levels found in HeLa epithelial cells [40]. Sorio et al. followed up this finding by discovering physiologic levels of CFTR protein in monocytes/macrophages, localizing the protein to the apical surface of monocytes, and showing CFTR functionality when stimulated with forskolin/IBMX [57].

1-3.6.2 CFTR Defects Directly Affect Macrophage Function

Once the presence of functional CFTR in macrophages was confirmed, research focused on elucidating the effects of dysfunctional CFTR on macrophages. *In vivo* studies of CF alveolar macrophages taken from the BALF of mice show an exaggerated secretion of pro-inflammatory cytokines IL-1a, KC, TNF-α, IL-6 when stimulated with LPS in comparison to WT mice. These studies show that CF macrophages are hyperinflammatory in response to M1 cytokines [58].

In order to prove that the exaggerated response is intrinsic to macrophages, and independent of the potentially pro-inflammatory environment of CF lungs, *in vitro* studies were performed on macrophages derived from peripheral blood derived monocytes of CF, heterozygote (HT), and WT mice. Even after naïve bone marrow-derived cells are cultured in a neutral petri dish for

10 days, LPS evokes an exaggerated pro-inflammatory response in CF macrophages in comparison to WT macrophages. These experiments show that hyperinflammatory secretion of cytokines by CF macrophages appears to be CFTR dependent, especially when considering that heterozygotes showed an intermediate phenotype [59].

This work was further supported by experiments performed on mouse bone marrow chimeras. By first irradiating the bone marrow of WT or CF mice, and then transplanting either WT or CF bone marrow, four chimeras were created: WT marrow-WT background animals, WT marrow-CF background animals, CF marrow-WT background animals, and CF marrow-CF background animals. The transplantation of $CFTR^{+/+}$ macrophages into $CFTR^{-/-}$ background mice significantly dampened the hyperinflammatory secretion of cytokines, and converse is true as well. Therefore, independent of environment and background, hyperinflammation in macrophages appears to be intrinsic to CFTR mutations [59].

This hypothesis is also supported by work done on a conditional knockout model of CFTR using myeloid-targeted Cre-recombinase LysMCre transgene, which is exclusively expressed in neutrophils, macrophages and DCs. This model shows that mice with a conditional CFTR knockout in monocytes have worse survival outcomes, increased number of neutrophils and macrophages in BALF that persists 10 days after infection, decreased bacteria killing, and worse gross lung pathology in comparison to WT cells, even with normal epithelial cells [60]. In conclusion, while abnormal ion transport in epithelial cells secondary to

CFTR defects leads to lung disease, lack of CFTR in immune cells also directly contributes to CF lung disease.

1-3.6.3 Macrophages Exhibit Mixed M1/M2 Phenotype in CF

Once defects in CFTR were believed to directly affect macrophage function, research examined polarization profiles of human CF macrophages using cells recovered from the BALF. Initial studies showed that CF macrophages had elevated levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-8, and IL-6 [29] while also expressing decreased levels of the anti-inflammatory cytokine IL-10 [61] in comparison to HD macrophages. These studies were supported and enhanced by later work that showed human CF macrophages recovered from BALF showed increased levels of the anti-inflammatory cytokines IL-4 and IL-13, in addition to the pro-inflammatory profile described earlier [62]. These studies describe CF macrophages as possessing a mixed M1/M2 profile, which may be explained interactions between defective CF macrophages and a complex CF lung environment that simultaneously exhibits pro-inflammatory and anti-inflammatory characteristics, as evidenced by chronic inflammation and lung fibrosis.

1-3.6.4 Mechanisms Contributing to Abnormal CF Mϕ Activity

Extensive research of CF macrophages has uncovered numerous altered mechanisms that may, individually and symbiotically, contribute to abnormal CF macrophage activity. CF macrophages exhibit dysregulated signal pathway

regulation due to aberrant lipid raft composition that leads to altered levels and distribution of cholesterol, sphingolipids, and scaffolding proteins [63-66]. Defective vesicle trafficking, docking, and maturation also affect the spatial and temporal distribution of immune receptors, which increases intensity and duration of pro-inflammatory signaling pathways [64]. Defective autophagy leads to prolonged retention of TLR4, ultimately causing increased activation of the pro-inflammatory pathways like NF-kB when stimulated with LPS [58]. The NF-kB pathway is further activated in CF macrophages secondary to dysregulated nuclear receptors such as PPARγ [67]. Over-activation of the XBP-1 arm of the unfolded protein response due to mutations in CFTR also leads macrophage activity [68]. All these disturbances in CF macrophage signaling lead to persistent hyperinflammation and increased secretion of pro-inflammatory cytokines in response to external stimuli.

CF macrophages also display defects in phagocytosis and bacteria killing. Defective chemotaxis affects CF macrophages ability to localize to sites of infection. Abnormal lipid rafts affect CF macrophage surface membrane's ability to properly reorganize into formations necessary for phagocytosis [57]. Furthermore, phagolysosomes exhibit defective maturation capacity, and show inability to properly kill bacteria resulting from improper regulation of pH and ROS formation [69, 70]. Finally, macrophages do not produce normal levels of anti-bacterial molecules [71]. Ultimately, these defects lead to macrophages underperform the vital functions of bacterial killing and phagocytosis of dead cells necessary to clear infections and dampen inflammation.

2. Statement of Purpose

Even though the CFTR gene was identified in 1989 , research to establish direct links between the gene mutation and the pathophysiology of the disease still spark controversy. Up until recently, most research has focused on how defective CFTR disrupts ion transport in airway epithelia cells. Since CFTR gene expression was identified in bone marrow derived cell lines [56], mounting evidence supports the assertion that macrophages directly cause CF disease pathology due to innate defects that result from mutations in CFTR.

Macrophages serve in a variety of roles from supporting tissue development and homeostasis, to regulating inflammation, to initiating the host immune response [50]. The process of polarization describes the mechanism through which macrophages can assume a broad spectrum of phenotypes depending on tissue localization, functional demand, and embryological origin. Characterization of macrophage polarization began in the early 1990's [54, 55]. Modeled after the dichotomous lymphocyte classification system, macrophages were classified as either M1 or M2 phenotypes. Under this system, M1 macrophages refer to cells stimulated by pro-inflammatory cytokines, such as LPS and INF-γ, and initiate pathways important in killing intracellular pathogens and inflammation. M2 macrophages refer to cells stimulated by anti-inflammatory cytokines, such as IL-4 and IL-10, and initiate pathways important for tissue repair and resolution of inflammation.

As our view of macrophages has broadened from a specialized immune cell to a tissue-support cell, so has our understanding of polarization. We now characterize polarization as a dynamic, fluid process that activates macrophages into phenotypes represented better by a 360° color wheel than by a bidirectional vector.

As mentioned earlier, macrophages with defective CFTR show alterations in numerous vital pathways involved in phagocytosis, bacterial killing, and signal transduction [45]. Furthermore, polarization profiles of CF macrophages have exhibited mixed M1-M2 profiles [61, 62]. While descriptive studies have informed us of the physiological pathways invoked in M1-M2 polarization, these studies have not been reproduced in human CF macrophages.

The purpose of this study is:

1) To determine the polarization profiles of human CF macrophages in comparison to HD macrophages upon stimulation with M1 and M2 cytokines.

2) To characterize in CF macrophages the pathways previously implicated in polarization, and to compare their signaling profiles to those of HD cells.

3) To determine if the mixed phenotype attributed to CF macrophages is secondary to dysregulated polarization pathways.

3. Methods

3.1 Chemicals and Reagents

Pseudomonas aeruginosa (*Pa*) LPS (Sigma-Aldrich) was prepared in PBS at 100X stock solution and used at a concentration of 100 ng/mL. Recombinant human INF-γ (ConnStem) was prepared in 0.1% BSA at a 2000x stock solution and used at a concentration of 20 ng/mL. Recombinant human IL-4 (ConnStem) was prepared in 0.1% BSA at 500x stock solution and used at a concentration of 20 ng/mL. Recombinant human MCSF (ConnStem) was prepared in RPMI with 10% H_2O at 50x stock solution and used at a concentration of 50 ng/mL.

3.2 Isolation and Culture of Human Peripheral Bone Marrow Derived Mϕs

3.2-1 Recruitment and Subject Profile

Subjects were recruited and consented according to approved IRB protocol. All CF subjects were recruited from pediatric clinics and were under the age of 18. No patients were on Kalydeco or Orikambi at time of recruitment. All patients had moderate to severe mutations (Figure 4).

Subject #	Sex	Genotype
12	M	ΔF/G542X
23	F	ΔF/G85E
26	F	2789+57A/21841NS
34	M	ΔF/D110H
45	F	ΔF/ΔF
54	M	ΔF/R117H
58	F	ΔF/ΔF
69	M	ΔF/ΔF
78	F	ΔF/Deletion
81	F	ΔF/1444fs

Figure 4. Subject Demographics: All CF subjects were recruited from pediatric CF clinic.

3-2.2 Isolation

Using 20G needles, ~10 mL of whole blood was drawn from both CF and HD patients, and collected in tubes containing EDTA. Whole blood was then mixed in a 1:1 ratio with PBS solution, before being gently pipetted into 15 mL conical tubes containing Ficoll solution (Histopaque 1077 Sigma H8889) at 25° C, always maintaining a 2:1 ratio of whole blood-PBS mixture:Ficoll. These tubes were then centrifuged at 1100 RPMs for 30 minutes, with centrifuge settings set to “no break” and “half-speed acceleration”. After 30 minutes of density centrifugation with Ficoll, whole blood separates into buffy coat layers (Fig. 5). The layer containing the monocytes was located between the serum and Ficoll

layers, and this layer was exclusively extracted from the 15 mL conical tubes (69).

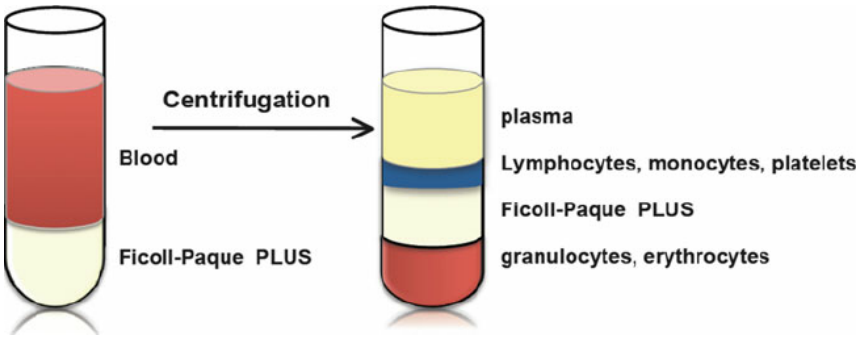

Figure 5. Isolation of Monocytes Using Ficoll**:** Ficoll solution separated whole blood into buffy coats upon centrifugation. A cloudy layer that rests above the clear Ficoll layer contains monocytes, and was isolated for culture.

Once extracted, the monocyte layer was washed in 40 mL PBS with 5 mL RPMI solution containing 50 ng/mL of MCSF, and then centrifuged at 1500 RPMs for 10 minutes with “full acceleration” and “full break” settings. Upon centrifugation, the supernatant was discarded, the pellet containing immature monocytes was dissolved in 3 mL of RPMI supplemented with 10% FBS and 50 ng/mL recombinant human MCSF per 1x10^6 cells, and then the cells were placed into 6 well plates at 3 x 10^6 cells/well.

3-2.3 Culture

Cells were cultured in 6 well plates and stored in incubators at 37°C with CO_2 5%. Cells were carefully monitored each day for changes in the pH of the media, as indicated by the media changing color from pink to yellow. This change in pH was a good surrogate for cell activity, and generally indicates that fresh media should be added to the wells. Cells usually began to attach to the bottom

of wells at around day 5, at which time cells were passed into a new well to expand the culture. Cells were cultured until all 6 wells became confluent, at about 1x10^6 cells/well, which generally took 10-14 days, at which time they were ready to be treated with cytokines for experiments.

3-2.4 Confirming Macrophage Population

` In previously studies, after 1-2 weeks, cells were characterized by flow cytometry (CD14+/CD45+) and the morphology analyzed on cytospin (figure with FACS data, and mac picture). For our studies we also relied on the knowledge that gravity dependent centrifugation using Ficoll has been an accepted method for isolating lymphocyte, platelet, and monocyte populations. Furthermore, the RPMI media supplemented with MCSF used to culture these cells, only stimulates the growth of monocyte activation into macrophages, and would not support the growth of other cell types. Additionally, when looking under the microscope, platelets and red blood cells (RBCs) could easily be differentiated from macrophages due to their red color and smaller size. After ~10 days of culture, cells appeared to settle to the bottom of the wells, with extended pseudopodia – characteristic of macrophages (Fig. 6), without visual presence of RBCs or platelets.

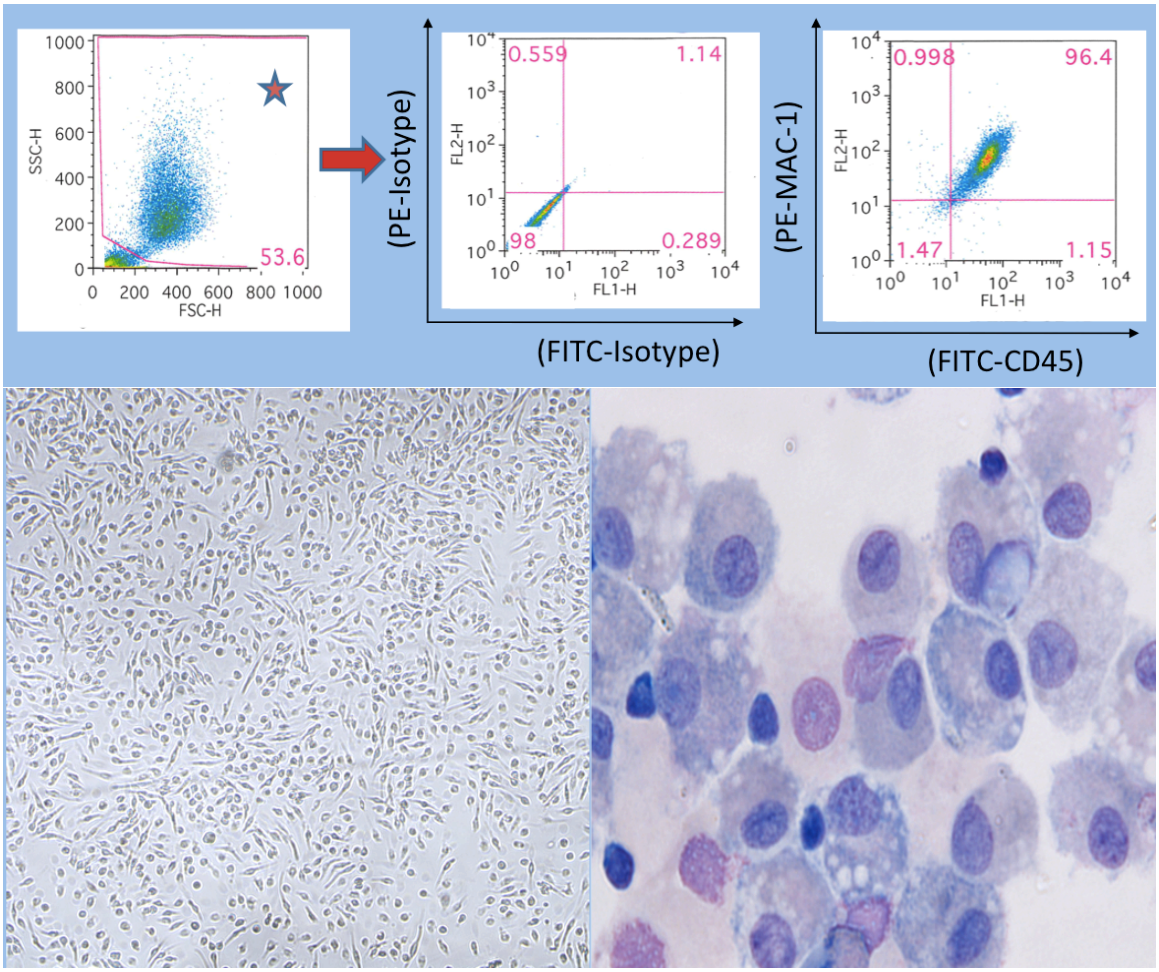

Figure 6. Confirmation of Macrophage Population**:** Top panel shows confirmation of monocyte/macrophage population using flow cytometry. Bottom left panel shows adherent macrophages as visualized under microscope. Bottom right panel shows macrophages under high-powered magnification with staining.

3-2.4.1 Confirming CFTR Function in Macrophages

Chloride efflux in macrophages was confirmed by measuring the efflux of N-[ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide (MQAE), a Cl^- -specific fluorescent dye. Under steady state, MQAE dye is up taken by the cell and does not fluoresce when it is bound to Cl^-. As cells are stimulated and secrete Cl^-, MQAE becomes unquenched and begins to emit light. Cl^-- efflux was quantified as the initial change in MQAE fluorescence over time (slope) as the cells

transitioned from Cl^--containing to Cl^--free solutions following dye excitation at 354 ± 10 nm & measurement of emitted light at 460 ± 10 nm [72].

3.3 Cytokine treatments

When cells were ready to be treated, media was aspirated and cells were washed three times with PBS solution. Cells were organized into three treatment groups: untreated, M1, or M2. Untreated groups had growth media replaced with RPMI supplemented with 50 ng/mL MCSF. M1 treatment groups had growth media replaced with RPMI supplemented with 50 ng/mL MCSF, 20 ng/mL INF-γ, 100 ng/mL of LPS. M2 treatment groups had growth media replaced with RPMI supplemented with 50 ng/mL of MCSF and 20 ng/mL of IL-4. Experiments prepared for qRT-PCR were treated for 24 hours, experiments for western blot were treated for 3 hours (Fig 7).

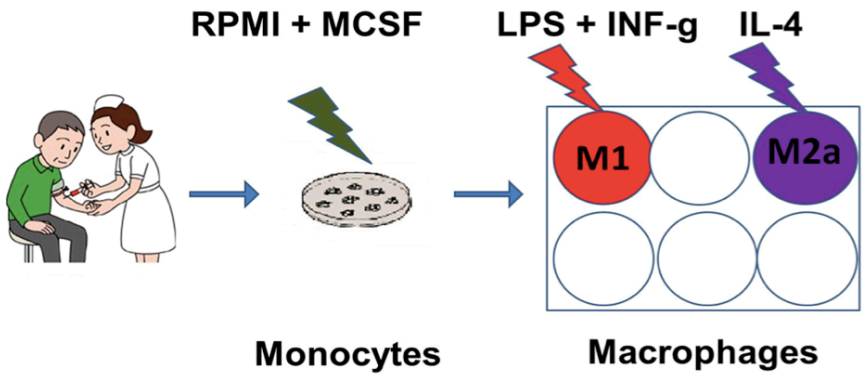

Figure 7. Macrophage Culture and Treatment Protocol**:** Macrophages were treated with either M1 cytokines, LPS and INF-γ, or with an M2 cytokine, IL-4.

3.4 QT-RT-PCR and Expression Analysis

Cells were lysed in triazol (Qiagen), and total RNA was isolated from $1x10^6$ cells using QiagenRNAMini Kits^{TM} (Qiagen), following the manufacturer's instructions. Real-time PCR analysis was performed with a Bio-Rad iCycler using TaqMan technology. Copy number was normalized by 18S and the relative expression to untreated cells was calculated by $\Delta\Delta$Ct method. M1 primers used were IL-6, TNF-α, and IL-1β. M2 primers used were MRC1, TGFβ1, and PPARγ. All TaqMan primers and probes were purchased from Applied Biosystems (Life Technology).

3.5 Protein isolation and Western blot

Cells were washed three times with PBS, and total cell lysate was extracted using RIPA lysis buffer (Cell Signaling) containing 1mM phenylmethanesulfonyl fluoride (PMSF) and protease and phosphatase inhibitor cocktails (Roche Diagnostics). Protein concentration was determined by a Bradford assay and an equal amount of protein was separated by electrophoresis on 4-15% Mini PROTEAN Gels (Bio-Rad Laboratories, CA), transferred to nitrocellulose membrane (Bio-Rad Laboratories, CA), and probed with primary antibodies using standard procedures.

3-5.1 Western Blot Antibodies

The following antibodies were used: rabbit monoclonal anti-STAT1 – D1K9Y (1:1000, Cell Signaling), rabbit monoclonal anti-phospho-STAT1 –Tyr701

-58D6 (1:1000, Cell Signaling), rabbit monoclonal anti-STAT6-D3H4 (1:1000, Cell Signaling), rabbit polyclonal anti-STAT6-phosphoY (1:1000, Abcam), rabbit polyclonal anti-AKT (1:1000, Cell Signaling), rabbit monoclonal anti-phospho-AKTSer473-D9EXP (1:2000, Cell Signaling), and rabbit-HRP anti-actin (1:5000, Santa Cruz). For detection, horseradish peroxidase was conjugated to IgG secondary antibodies (1:2000, Santa Cruz), followed by visualization using enhanced chemiluminescence (ECL). The chemiluminescence imaging system ChemiDoc (Biorad) and the Image lab software (Biorad) were used for image acquisition and for signal quantification. Protein relative expression is normalized to β-Actin. Images have been cropped for presentation.

3.6 Statistical Analysis

Statistical analyses were conducted using two-tailed two-sample t-tests or two-sample unequal variance t-tests. All experiments were performed in triplicate, unless otherwise indicated. Data are expressed as mean ± standard deviation. A P value <0.05 was considered statistically significant. Prism 7.0 (GraphPad) was used for all statistical analyses.

4. Results

4.1 Macrophages Effectively Transport Cl- Via CFTR

The MQAE results show that macrophages have expected efflux of Cl- upon stimulation with zero-Cl- solution (Fig. 8, left panel). Furthermore, as expected, CF macrophages do not show efflux of Cl- in a zero-Cl- solution due to lack of functioning CFTR (Fig. 8, right panel).

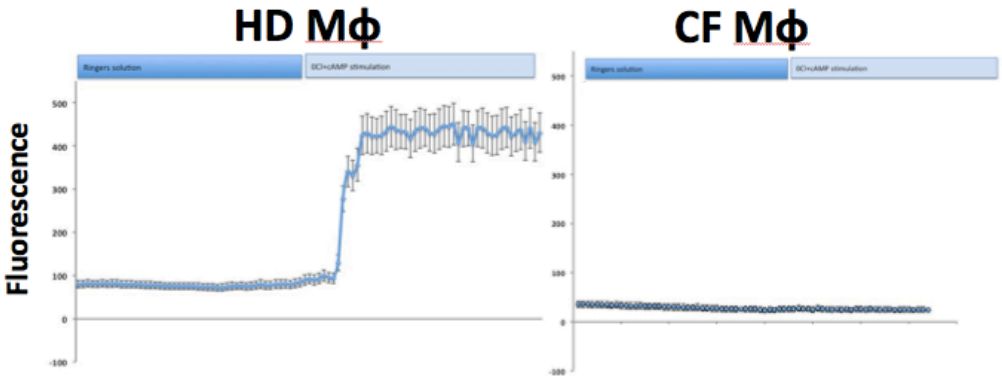

Figure 8. MQAE Efflux in Macrophages**:** The left panel shows adequate efflux of MQAE upon stimulation with zero-Cl- solution, which represents Cl- efflux via CFTR. The panel on the right shows defective Cl- efflux in CF macrophages due to lack of functioning CFTR.

4.2 LPS/INF-γ Stimulated CF Mϕs Exhibit Different Levels of STAT1/pSTAT1

In order to interrogate the pathway involved in M1 polarization, we studied phosphorylation levels of STAT1 – a protein well described in macrophage literature as a regulator of signal transduction and an activator of transcription (49-50). Upon stimulation by LPS and INF-γ, certain cell surface receptors transduce signals through the phosphorylation of STAT1. Upon phosphorylation, STAT1 localizes to the nucleus where it up-regulates the transcription of gene's

involved in activating inflammation and pathogen killing. (49,50,70). Thus cell surface signals affect metabolic activity and phenotypic changes in macrophages through STAT1 signaling. We found that when stimulated with M1 cytokines, LPS/INF-γ, CF macrophages displayed differential expression of total STAT1 proteins as well as phosphorylated STAT1 (pSTAT1) proteins. CF macrophages had decreased protein levels of total STAT1 (Fig. 9; Student TTest, n=3, $p < .05$), yet increased ratio of pSTAT1 to STAT1 protein levels at 3-hour time course, (Student TTest, n=3, $p < .05$), in comparison to HD macrophages (Fig. 9). In CF macrophages, total STAT1 protein levels were the highest in untreated cells, and decreased with stimulation by either M1 or M2 cytokines. Finally, as expected, phosphorylation rates of STAT1 were only significantly elevated when stimulated with M1 cytokines, and showed an almost lack of protein levels after M2 cytokines stimulation.

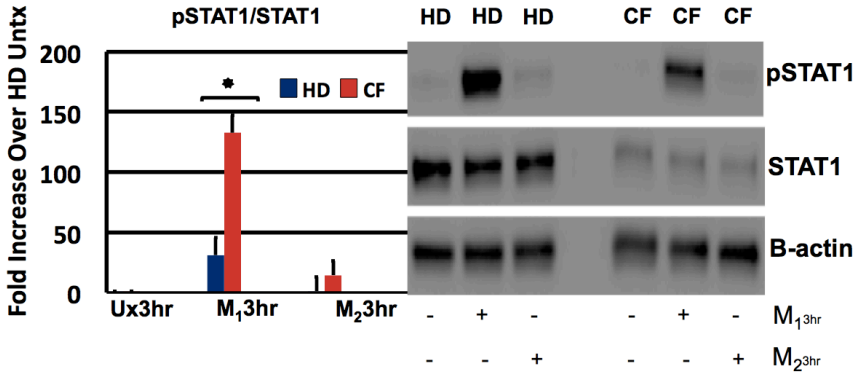

Figure 9. Differential Expression of pSTAT1/STAT1 in CF Mϕs**:** CF macrophages show elevated ratio of pSTAT1/STAT1 when stimulated with LPS/INFγ after three hours and lower expression of total STAT1.

4.3 LPS/INF-γ Stimulated CF Mϕs hyper-express M1 Gene Markers

We studied the downstream effects of STAT1 signaling by using QT-RT-PCR to examine the expression level of three M1 markers, IL-6, TNF-α, IL-1β , that are regulated by STAT1 [54, 55]. CF and HD macrophages were stimulated with M1 cytokines for 24 hours before RNA was isolated. CF macrophages hyper-expressed all three markers upon stimulation with M1 cytokines, in comparison to HD cells (Fig. 10; Student TTEST, n=8, $p < .05$). Unexpectedly, when stimulated with M1 cytokines, the M2 marker, TGF-β1, exhibited an on average, modest increase in expression levels with a broad range (0.1-16.0). In comparison, HD cells always showed down-regulation of these M2 markers upon stimulation of M1 cytokines (Fig 11).

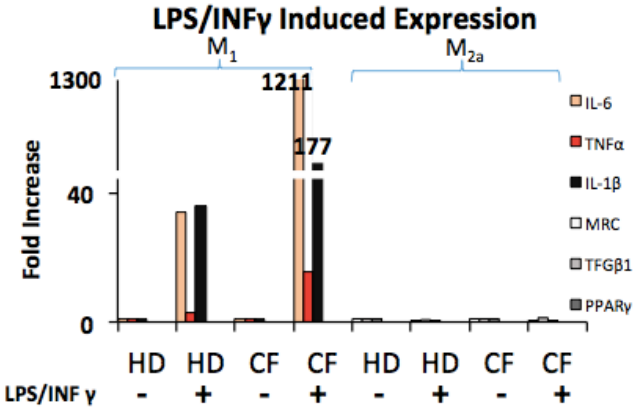

Figure 10. Stimulation with LPS/INF-γ Leads to Hyperexpression of M1 Markers in CF Mϕs**:** This figure is a representative experiment for expression profiles of HD vs CF Mϕs upon stimulation with M1 cytokines. All M1 markers showed hyper-expression in CF cells. (Student T-test, n=8, $p <.05$)

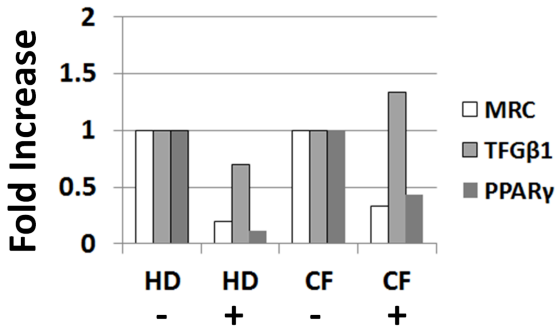

Figure 11. Up-regulation of M2 Markers with Stimulation of M1 Cytokine by CF Mϕs**:** Representative figure showing increased expression of TGF-β1 and PPARγ by CF cells.

4.4 IL-4 Stimulated CF Mϕs Exhibit Different Levels of pSTAT6/STAT6

In order to interrogate the pathway involved in M2 polarization, we studied phosphorylation levels of another signal transducer and activator of transcription, STAT6. Upon phosphorylation, STAT6 localizes to the nucleus where it up-regulates the transcription of gene's involved in activating resolving inflammation and coordinating tissue repair [54, 55, 73]. Conversely, STAT6 down-regulates M1 polarization signaling (Fig. 2). We found that when stimulated with the M2 cytokine, IL-4, CF macrophages displayed differential expression of total STAT6 proteins as well as phosphorylated STAT6 (pSTAT6) proteins. CF macrophages had decreased protein levels of total STAT6, yet increased ratio of pSTAT6 to STAT6 protein levels at 3-hour time course (Fig. 12, Student TTest, n-=3, $P < .05$), in comparison to HD macrophages. Furthermore, in comparison to HD cells, CF macrophages showed decreased levels of total STAT6 on average in untreated groups and cells treated with M1 cytokines. Finally, phosphorylation

rates of STAT1 were only significantly elevated when stimulated with M1 cytokines versus M2 cytokines, as expected (Figure 12).

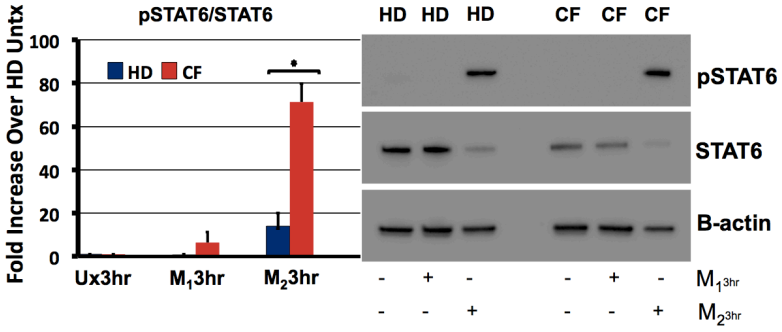

Figure 12. Differential Expression of pSTAT6/STAT6 in CF Mϕs: CF macrophages show elevated ratio of pSTAT6/STAT6 when stimulated with IL-4 after three hours and lower expression of total STAT6 (Student TTest, n=3, $p < .05$).

4.5 IL-4 Stimulated CF Mϕs Up-regulate Expression of M2 Gene Markers

We studied the downstream effects of STAT6 signaling by using QT-RT-PCR to examine the expression level of three M2 markers, MRC, TGFβ1, and PPARγ, that are regulated by STAT6 (49,50). CF and HD macrophages were stimulated with M2 cytokines for 24 hours before RNA was isolated. CF macrophages expressed higher levels of all TGFβ1 and MRC upon stimulation with M1 cytokines, in comparison to HD cells (Fig. 13; Student TTEST, n=8, $p < .05$). Even more interesting was the unexpected, aberrant increase in expression of IL-6, a M1 cytokine, that should be down-regulated upon stimulation by IL-4 (Student TTEST, n=8, $p < .05$). In comparison, HD cells show down-regulation of these M1 markers upon stimulation of M2 cytokines (Figure 13).

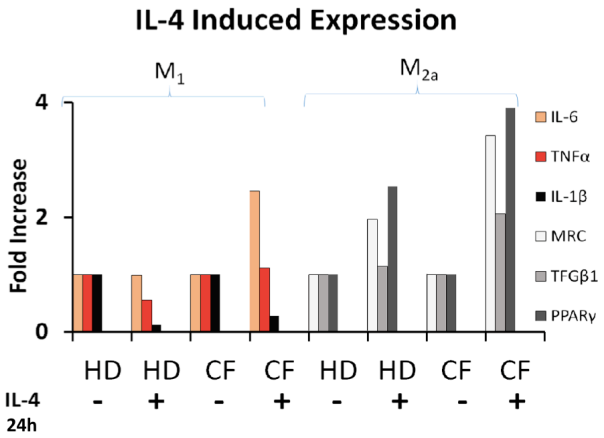

Figure 13. Stimulation with IL-4 Leads to Hyper-expression of M2 Markers in CF Mϕs**:** This figure is a representative experiment for expression profiles of HD vs CF Mϕs upon stimulation with M2 cytokines. TGFβ1 and MRC showed hyper-expression in CF cells (Student T-test, n=8, $p < .05$). PPARγ showed increase on average.

5. Discussion

Cystic Fibrosis pathophysiology involves chronic infection, hyperinflammation, and obstructive ductal/airway disease. Until recently, defective CFTR in epithelial cells was thought to be the primary underlying driver of disease. We now understand that CFTR is expressed in macrophages, and that macrophages with defective CFTR display abnormal phagocytosis, bacterial killing, and cell signaling. Furthermore, abnormal macrophage activity has been shown to persist even in lungs lacking CF disease or in an environment that lacks signaling from CF epithelial cells [59, 60]. We believe that dysfunctional CFTR in macrophages leads to intrinsic defects in cell signaling important for regulating inflammation. Therefore, CF macrophages directly contribute to hyperinflammation, chronic infection, and ultimately obstructive disease.

Our experiments showing altered regulation of phosphorylation of STAT6 and STAT 1 in CF macrophages support this conclusion that signaling pathways are affected in CF. CF macrophages exhibit increased phosphorylation rates of STAT1 and STAT6 in comparison to HD cells, which indicates that they are unable to appropriately control activation pathways. Furthermore, CF macrophages have lower expression levels of total STAT proteins. Untreated CF cells have the highest expression of total STAT1, and these levels decrease with stimulation by either M1 or M2 cytokines. This finding may represent an attempt by cell machinery to down-regulate these pathways, or maybe transcription of new STAT proteins cannot keep pace with the rate of phosphorylation.

Ultimately, the higher ratio of phosphorylated proteins and the decrease in total protein suggest that the pathways are dysregulated in CF macrophages.

As STAT proteins traffic to the nucleus and affect gene expression when phosphorylated, the increased ratio of phosphorylated STAT1 and STAT6 proteins may also explain the elevated levels of expression of M1 and M2 genetic markers in response to M1 and M2 cytokines. We expected macrophages to show up-regulation of M1 cytokines in response to LPS/INFγ, but the CF macrophages showed expression levels that were dramatically higher than the HD cells. This finding is consistent with the hyperinflammatory state found in patients with CF, and suggests that CF macrophages may be driving persistent hyperinflammation in CF patients through up-regulation of expression of M1 cytokines.

When stimulated with IL-4, CF macrophages showed a higher expression level of M2 markers. Another unexpected finding, though, was that M2 markers were up-regulated in response to M1 cytokines. Based on previous descriptions of STAT1 and STAT6 regulation of gene expression markers, we would expect M1 cytokines to only up-regulate expression of M1 markers while down-regulating M2 markers. The converse should be true as well. This inability for CF macrophages to assume one definitive phenotype when receiving stimulation by either M1 or M2 cytokines implies that the previously observed mixed phenotypes of alveolar macrophages from BALF result from intrinsic defects that result from defective CFTR, in addition to the influence from the complex CF lung environment.

The intrinsic mixed phenotype of CF macrophages in the context of dysregulated STAT1 and STAT6 phosphorylation, as well as the different levels of total protein, suggests that a master regulator may be implicated in the aberrant signal pathways. Previous research has explored the role AKT and phosphorylated AKT play on macrophage polarization. AKT has been shown to act as a master regulator, affecting cell signaling in ways that up-regulate one pathway while down-regulating opposing pathways [74]. In this way, AKT can be seen as applying the gas to one pathway, M1 for example, while applying the breaks to another pathway, M2 for example (Fig. 14). The mixed phenotypes observed in CF macrophages may result from abnormal regulation of opposing pathways by a master regulator such as AKT.

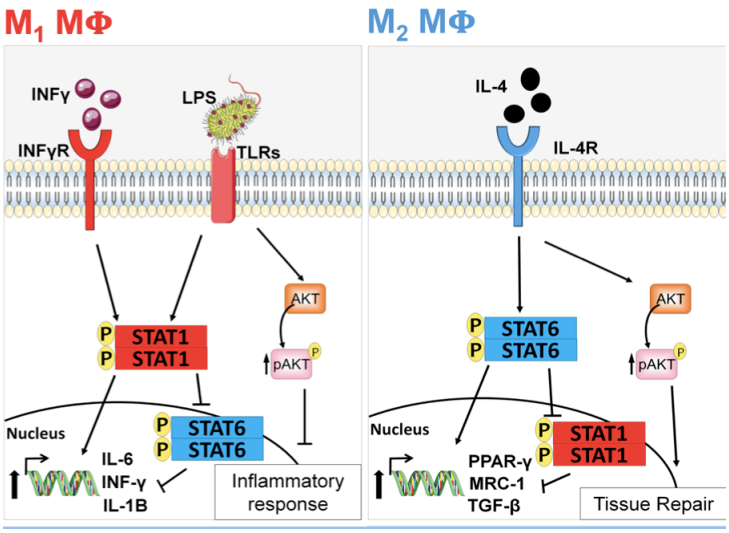

Figure 14. AKT Serves as Master Regulator**:** AKT directs polarization by inhibiting M1 pathway signaling and enhancing M2 pathway signaling, thus acting as a master regulator.

Finally, dysregulated AKT signaling has been exhibited in macrophage polarization pathways in connection with decreased levels of insulin growth factor-1 (IGF-1). Studies show that appropriate levels of IGF-1 are required in order to allow AKT signaling to completely up-regulate the M2 pathway while down-regulating the M1 pathway [75]. Furthermore, studies have shown decreased levels of IGF-1 in the serum of CF patients experiencing pulmonary exacerbations. These clinical findings might explain why human CF macrophages show mixed phenotypes with dysregulated activation pathways, and could explain a mechanism underlying hyperinflammaiton.

6. References

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