

## Mesenchymal Stem Cell Soluble Mediators and Cystic Fibrosis

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### Abstract

Human Mesenchymal stem cells (hMSCs) secrete products (supernatants) that are anti-inflammatory and antimicrobial. We have previously shown that hMSCs decrease inflammation and *Pseudomonas aeruginosa* infection in the *in vivo* murine model of Cystic Fibrosis (CF). CF is a genetic disease in which pulmonary infection and inflammation becomes the major cause of morbidity and mortality. Our studies focus on determining how hMSCs contribute to improved outcomes in the CF mouse model centering on how the hMSCs impact the inflammatory response to pathogenic organisms. We hypothesize that hMSCs secrete products that are anti-inflammatory in scenarios of chronic pulmonary infections using the murine model of infection and inflammation with a specific interest in *Pseudomonas aeruginosa* (gram negative). Further, our studies will identify whether the hMSCs are impacting this inflammatory response through the regulation of peroxisome proliferator activator receptor gamma (PPAR $\gamma$ ) which aides in decreasing inflammation.

**Keywords:** Mesenchymal stem cells; Chemokines; Cytokines; Anti-inflammatory cytokines; Anti-inflammation; Chemotaxis; PPAR $\gamma$ ; Cystic fibrosis

### Introduction

Mesenchymal Stem Cells (hMSCs) have been shown to be anti-inflammatory and anti-microbial *in vitro* as well as *in vivo* using the murine model of CF lung infection and inflammation [1-3]. Cystic Fibrosis (CF) is a genetically inherited fatal disease in which the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene is mutated resulting in defective protein causing patients to have failure to thrive, lung congestion and infection with a variety of bacteria [4]. Defective CFTR results in impeded mucociliary clearance, which is associated with increased susceptibility to bacterial infection requiring treatment with antibiotics as well as mucus thinning reagents [5]. *Pseudomonas aeruginosa* is the most prevalent bacteria in CF infections, but is not the only bacteria involved in creating serious infection and damage in the CF lung. Organisms such as *Streptococcus pneumoniae* and *Staphylococcus aureus* create a complex pulmonary niche and microbiome [6,7]. In the end, despite new developments in small molecular correctors and CFTR expression enhancers, the ensuing inflammatory response, pulmonary failure continue to be a serious issue leading to significant morbidity and eventual mortality in CF [8]. The immune response in CF individuals has been shown to be excessive, and ultimately contributing to lung damage. In healthy lungs, bacterial infiltration results in acute neutrophil influx and eradication of bacteria followed by inflammation resolution. However, in CF patients, the neutrophil influx is excessive with increased release of neutrophils containing Neutrophil Elastase (NE), which is extremely damaging to the lung milieu. NE induces an increased gland secretion, and an increase in bacterial binding to the airway surface [9]. Increased levels of neutrophils also lead to an influx in oxygen radical production which induces chemokine productin including interleukin 8 (IL-8) which perpetuates further recruitment of neutrophils. hMSCs secrete products that are anti-inflammatory, anti-microbial, angiogenic, chemotactic, anti-apoptotic, and anti-scarring [10,11]. hMSCs have

been shown to have therapeutic potential in redirecting the excessive CF and inflammatory based pathophysiology [12,13]. In these studies we studied hMSC efficacy in the infection/inflammation using the murine model of cystic fibrosis (CF) [14]. Wild Type (WT) and cystic fibrosis transmembrane Conductance Regulator (Cftr) Deficient Mice (CF) mice were chronically infected with *Pseudomonas aeruginosa* (PA) and followed for several days for clinical score, survival and weight loss kinetics. At euthanasia, Bronchoalveolar Lavage (BAL) showed that hMSCs shifted the pulmonary differential away from neutrophils towards macrophages which was statistically significant for CF animals since they traditionally have an augmented neutrophilic response. In the studies reported here, we have begun to pursue the mechanisms associated with the anti-inflammatory function of hMSCs in the Cftr deficient murine models. We report that the anti-inflammatory potency of the hMSCs can affect cell recruitment and aide in attenuating the robust response inherent in CF pathophysiology. Our data also suggest that the mechanism of anti-inflammatory action is through regulating the inflammatory transcription regulator PPAR $\gamma$  as well as the production of chemokines ultimately impacting the phenotype of the inflammatory response. We further provide data to suggest that deficient CFTR function alters hMSC's activity implicating the need for allogeneic sources of hMSCs for clinical trials. The novelty of the results

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**Received** August 27, 2017; **Accepted** September 18, 2017; **Published** September 22, 2017

**Citation:** Sutton MT, Fletcher D, Episalla N, Auster L, Folz M, et al. (2017) Mesenchymal Stem Cell Soluble Mediators and Cystic Fibrosis. J Stem Cell Res Ther 7: 400. doi: [10.4172/2157-7633.1000400](https://doi.org/10.4172/2157-7633.1000400)

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in this manuscript demonstrate the unique niche which defines the function of hMSCs and their powerful therapeutic potential for diseases associated with chronic inflammation, in the context of unrelenting infection. Further we have begun to define the mechanistic impact of the hMSCs in the context of inflammation and how it might be impacted in scenarios of deficient CFTR activity.

## Methods

### Human cell preparations

**hMSCs:** hMSC preparations were obtained from the National Center for Regenerative Medicine, Case Comprehensive Cancer Center, and SCC Cellular Therapy Laboratory. The hMSCs were isolated and grown following the standard operating procedures associated with hMSCs in clinical trials [15,16]. hMSCs were expanded and grown in hMSC condition media treated with 10% fetal calf serum. hMSCs were taken from first or second passage.

**PBMCs:** Peripheral Blood Mononuclear Cells (PBMCs) were isolated from healthy control individuals as previously described using Institutional IRB approval [17]. We experimented with the protocol to optimize the yield of monocytes in anticipation of obtaining limited blood volumes from CF patients. The isolated PBMCs were either cultured with control media (RPMI 1641) or hMSC preparations (1:1). Control conditions of these cultures were left unstimulated and experimental conditions were stimulated with Lipopolysaccharide (LPS) at 10 µg/ml. There was a 24-hour incubation period at 37°C.

**Animal studies:** All Animal Studies were done with approval by Case Western Reserve IACUC committee and approval of application 2011-0146. Cystic Fibrosis transmembrane receptor deficient animals (Cftrtm1Kth, CF) and congenic background controls (C57BL/6J, WT) were anesthetized and inoculated with agarose beads embedded with 106 *Pseudomonas Aeruginosa* (PA-M5715) or *Staphylococcus Aureus* (ATCC# SA 29523).

Animals were followed with daily weights and clinical scores out to 10 days. Animals were euthanized and evaluated for lung inflammation by bronchoalveolar lavage (BAL) for cytokines, chemokines, cellular recruitment and differentials as described previously [3,18].

**Gene expression:** hMSCs that were used both in the *in vivo* murine CF model and the *in vitro* bactericidal and anti-inflammatory assays were processed for messenger ribonucleic acids (mRNA) followed by synthesis of complementary deoxyribonucleic acid (cDNA) for chemokine gene expression by real-time-PCR [19]. hMSCs were grown in the presence and absence of CFTR inhibitor (I-172, 1 µg/ml) to mimic CF hMSCs 3. Both baseline and CFTR inhibited hMSCs were stimulated with and without lipopolysaccharide (LPS, 10 µg/ml) to mimic exposure to gram negative bacteria. The quality of mRNA and synthesized cDNA was assessed using Nanodrop spectrophotometry technology (optimal threshold 260-280 nm) as well as validated using peptidyl prolyl isomerase A (PPIA) as the human ubiquitous housekeeping gene. We evaluated the expression of interleukin-8 (IL-8), interleukin 6 (IL-6), chemokine ligand 2(CCL-2), chemokine ligand 20 (CCL20) and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) as a comparison of PPIA ubiquitous gene expression. In the animal studies, gene expression was compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All data is expressed as deltaCT response using PPIA (hMSCs) or GAPDH (RAW 642.7 cells) as the ubiquitous control genes respectively ( $\Delta$ CT value; n=10 Mean  $\pm$  SEM). The fold change for each target genes threshold cycle ( $\Delta$ Ct) value followed by the normalization to the baseline control to measure the overall response as previously described [20].

**Luminex studies:** hMSC supernatants or PBMC supernatants were evaluated for cytokines and chemokines using Luminex multi-bead array technology (R&D Systems, Minneapolis, MN) as previously described [21,22], using a multi-array purchased which focused on IL-8, IL-6, macrophage inflammatory protein 1a(MIP-1a), CCL-2, and stem cell factor 1 (SCF-1). Briefly, supernatants from samples were interfaced with luminescence beads with ratios of proprietary fluorophores. Each bead is specific for a cytokine or chemokine. As the sample interacts with the beads, the capture antibodies conjugated to the beads binds to the cytokine/chemokine which is followed by a second antibodies labeled with Phycoerythrin (PE). The detection of the cytokine/chemokine is based upon detection of the unique bead idea and the detection of the PA using a dual laser system with a lower end sensitivity of 3.2 pg/ml. Each cytokine/chemokine is compared to its specific standard curve, also done in multiplex. Data is expressed as mean (pg/ml  $\pm$  SEM, n=10).

**Aushon bioscience ciraplex studies:** The Peripheral Blood Mononuclear Cells (PBMC) studies were evaluated using Aushon Bioscience Ciraplex<sup>®</sup> Technology in which capture antibodies were spotted as a solid array chip, with the read-out being exquisitely sensitive chemi-luminescence. The advantage of this system was the small volume of sample required, the lower end sensitivity and the ease of the assay system. The sensitivity of the study was 1.0 pg/ml, n=10; MSC preparations and n=4 PBMC preparations (see below). For the PBMC gene expression data, cells were cultured *in vitro* with and without LPS and hMSC supernatants. After 24 hours, cells were removed and supernatants were evaluated for cytokine and chemokines using the Aushon Ciraplex<sup>®</sup> multi-array technology using Trizol<sup>®</sup> RNA extraction and TaqMan<sup>®</sup> real-time PCR assays.

**Chemotaxis:** PBMCs at  $1 \times 10^6$  cells/ml were put into the top chamber of a 0.4 µl transwell tissue culture plate. In the bottom well, 0.5mls of fresh hMSC supernatant utilized as previously described 20. After 24 hours, the PBMCs in the top and the cells recruited into the bottom were counted for total cell count and made into cytopins to determine the cell differential. Cytopins were dried followed by staining with Wright Giemsa, which stains lymphocytes, monocytes, neutrophils, eosinophils and basophiles with a specific pattern.

**PPAR $\gamma$  studies:** The *in vitro* assay for measuring the overall impact of hMSCs on PPAR $\gamma$  was done using RAW-267 cells, a macrophage cell line with known parameters for expressing PPAR $\gamma$ . Gene expression was done using real-time PCR as previously described [23].

**Data evaluation and statistics:**  $\Delta$ CTs were calculated by subtracting the relative cycling number of the probed sample (i.e. samples probed with IL-8, IL-6, CCL20 and CCL2) from the cycling number of the same sample probed with the background or housekeeping gene PPIA. In each group there were 10 different donor MSC preparations.  $\Delta\Delta$  CTs were calculated by subtracting the  $\Delta$ CT of stimulated samples (i.e. LPS, I-172, I-172 LPS) from the unstimulated samples  $\Delta$ CT for each preparation. Statistics were performed using GraphPad Prism 3.0 technology using non-parametric ANOVA analysis and standard T-cells when appropriate. Data is expressed as a Mean  $\pm$  Standard Error of the Mean (SEM) through non-parametric Mann-Whitney tests. P<0.05 denotes significance.

## Results

### *In vivo* potency and efficacy

We have studied hMSC therapeutic potential in the infection/inflammation murine model of inflammation and infection focusing on chronic *Pseudomonas aeruginosa* and *Staphylococcus aureus*

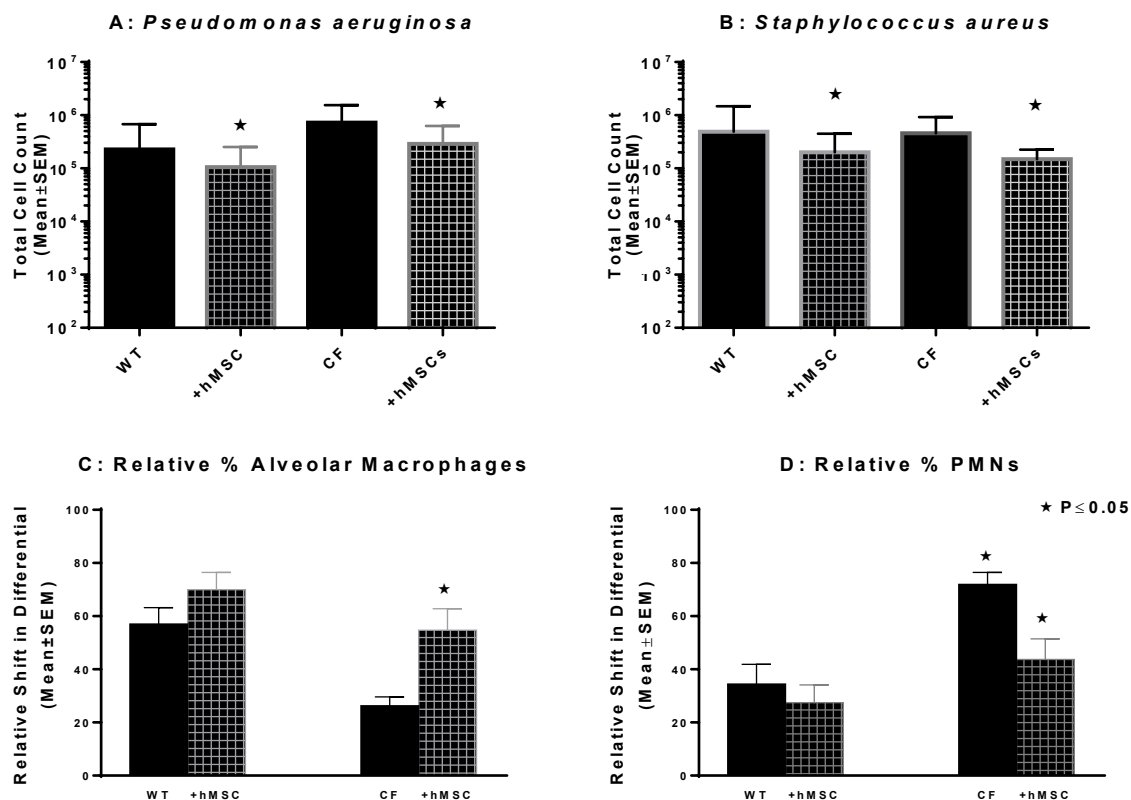
pneumonia in WT and CF mice. Animals were infected followed 24 hours later by  $10^6$  hMSCs infused through the retro-orbital sinus and then monitored for 10 days prior to euthanasia for Bronchoalveolar Lavage (BAL). The CF BAL fluid had significantly more Colony Forming Units (CFUs) than the WT BAL fluid regardless of whether it was a gram negative (*Pseudomonas aeruginosa*) or gram positive (*Staphylococcus aureus*) infection ( $p \leq 0.05$ ,  $n=4$  experiments, 10 mice per condition) [3]. When evaluating the inflammatory infiltrate of each of the models, both *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections increased the total cell counts (Figure 1A and 1B respectively), which was decreased with hMSC treatment ( $p \leq 0.05$ ,  $n=4$  experiments, 10 mice per condition). When looking at the phenotype of the cells in the infection models, as previously described neutrophil recruitment was significantly higher in the CF model as compared to the WT controls (Figure 1A and 1B,  $p < 0.05$  for each,  $n=4$  experiments, 10 mice per condition). Treatment of each of the infection modeling systems with hMSCs resulted in a shift in the BAL differentials changing the lung milieu to preferentially attract macrophages (Figure 1C  $p \leq 0.05$ ,  $n=4$  experiments, 10 mice per condition) while decreasing the number of neutrophils (Figure 1D,  $p \leq 0.05$ ,  $n=4$  experiments, 10 mice per condition). Neutrophils are known to participate in the pathophysiologic process associated with CF lung disease. Macrophages are known to be important in infection resolution and attenuation of the inflammatory response.

### hMSCs are active at promoting cellular recruitment of a specific phenotype

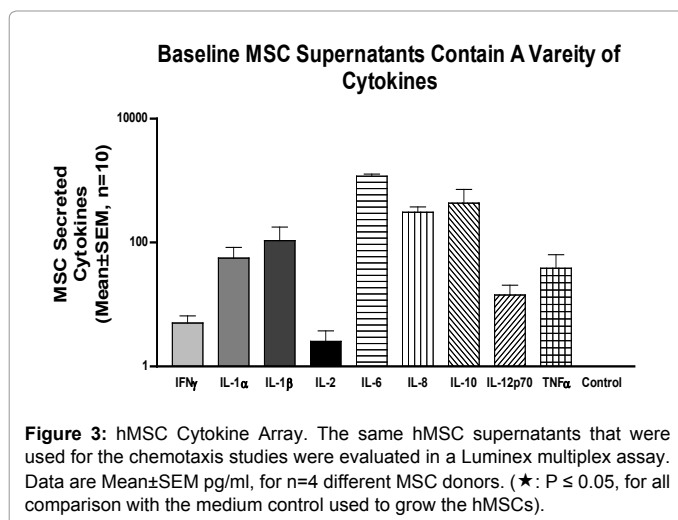
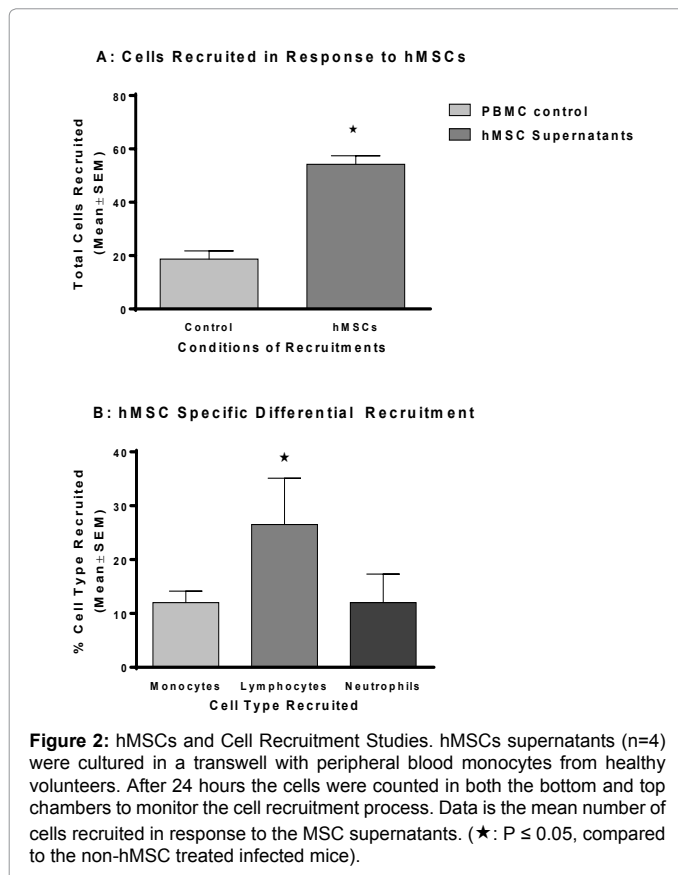
hMSCs enhance cellular recruitment overall, which could ultimately be beneficial or detrimental, depending on the cell type. In these studies, we monitored the ability of the hMSCs to recruit cells from peripheral blood mononuclear cells obtained from healthy volunteers using transwell plates of  $0.45 \mu\text{M}$ . The hMSC supernatants significantly recruited cells from the upper chamber (Figure 2A,  $p \leq 0.05$ ,  $n=4$ ). Lymphocytes were most heavily recruited (Figure 2B, F variance of  $p=0.05$ ), followed by monocytes and lastly neutrophils. The preferential recruitment of lymphocytes may aid in lowering neutrophil elastase, translating to less lung damage.

### hMSC soluble mediators

To determine how the hMSCs are contributing to the specific recruitment of cells, hMSCs were evaluated for cytokine secretion. The same supernatants that were utilized in the chemotaxis studies were evaluated in Luminex multiplex assays to determine their chemokine/cytokine profile (Figure 3). The MSCs secreted a variety of cytokines/chemokines that define the potential of the cells to contribute to their environment including  $\text{IFN}\gamma$ ,  $\text{IL-1}\alpha$ ,  $\text{IL-1}\beta$ ,  $\text{IL-2}$ ,  $\text{IL-6}$ ,  $\text{IL-8}$ ,  $\text{IL-10}$ ,  $\text{IL-12p70}$ ,  $\text{TNF}\alpha$  where we all significantly elevated relative to the control medium ( $p \leq 0.05$  for all  $n=4$ ). The cytokines that were generated in the



**Figure 1:** *In Vivo* Potency and Efficacy: *Cftr*<sup>tm1Kth</sup> (CF) and C57BL/6 (WT) mice were infected with either  $10^6$  CFUs of *Pseudomonas aeruginosa* (A) or *Staphylococcus aureus* (B) and monitored for 10 days. At day 10 the total cell count (1A and 1B) were determined by bronchoalveolar lavage (BAL) using cytoSpin and H&E staining from each of the different models. These studies were done 4 times with  $n=10$  animals in each group using 4 different hMSC donors. Figure 1C and 1D focus on the differentials specific for the *Pseudomonas aeruginosa* model since it is prototypic of CF lung infection and inflammation. These data demonstrate that part of the impact of hMSCs is on re-directing the inflammatory infiltrate away from neutrophils toward having more macrophages. (\*:  $P \leq 0.05$ , compared to the non-hMSC treated infected mice).



largest amount even without stimulation were IL-6 and IL-8 followed by IL-1 $\beta$  and IL-10 which were significantly higher than the medium control that is used to grow the hMSCs. The next series of studies focus on the expression of the chemokines that might predict how the hMSCs contribute to their environment and how the chemokine expression profile of the hMSCs may be impacted by the functionality of CFTR.

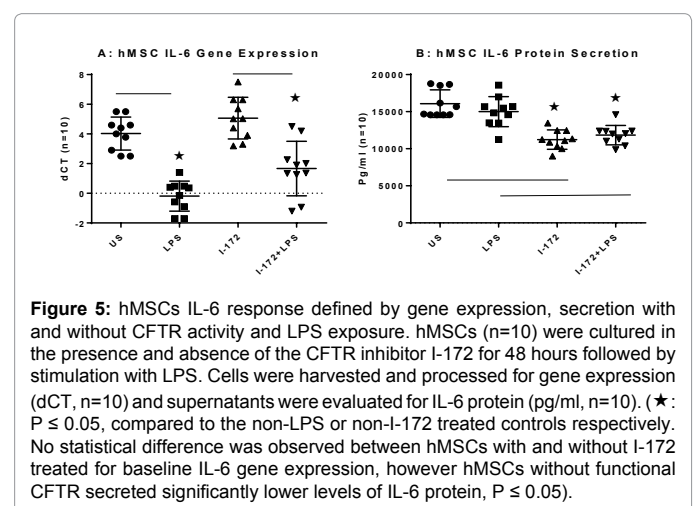
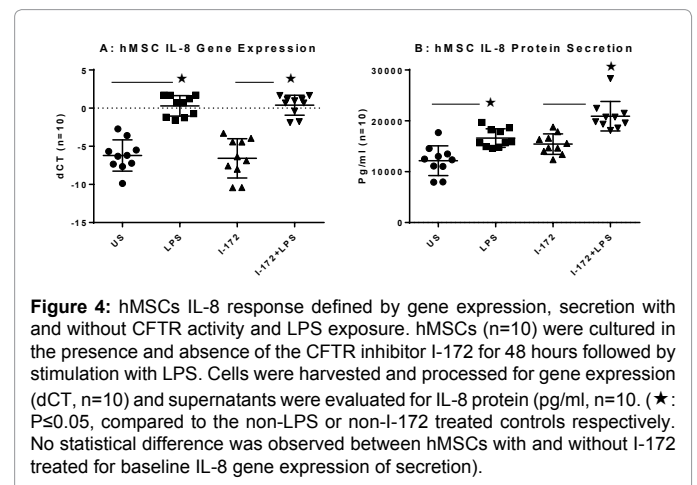
### hMSC IL-8 production and impact of CFTR

IL-8 induces chemotaxis in target cells, shifting toward acute neutrophil influx at the site of infection [24]. IL-8 has been shown to be significantly expressed in the absence of CFTR activity in a variety of other cells

including epithelial cells [25]. hMSCs also secrete excessive levels of IL-8 when CFTR is inhibited as compared to control CFTR sufficient hMSCs (Figure 4A,  $-6.58 \pm 2.57$  versus  $6.21 \pm 0.29$  respectively;  $p \leq 0.05$ ,  $n=10$ ). Stimulation with LPS causes an increase in IL-8 expression in comparison to control mRNA, with CFTR inhibition causing a further increase (LPS :  $0.29 \pm 1.35$  and LPS I-172:  $0.40 \pm -1.31$ ;  $p \leq 0.05$  for each;  $n = 10$ ). Similar findings were determined through Luminex technology on secreted products, with differences between I-172 and LPS with I-172 also showing statistical significance (Figure 4B  $p \leq 0.05$ ,  $n=10$ ).

### hMSC IL-6 production and impact of CFTR

IL-6 is an acute phase protein, meaning that it induces other chemokines, such as IL-10. IL-6 has extensive anti-inflammatory functions, many of which could be beneficial in scenarios of chronic infection and inflammation such as CF [26]. IL-6 is downregulated in CFTR deficient cells in comparison to unstimulated mRNA, a difference of 1.04 cycles in ABI Taqman (Figure 5A,  $p \leq 0.05$ ,  $n=10$ ). Similarly, in comparing LPS stimulated hMSC mRNA, CFTR deficient cells expressed significantly less IL-6 as opposed to LPS stimulated mRNA (1.84 cycles difference;  $p \leq 0.05$ ,  $n=10$ ). Similar results were obtained in secreted product data, with IL-6 downregulated in I-172 and LPS I-172 conditions in comparison to unstimulated controls (Figure 5B,  $11225 \pm 13$ ,  $11834 \pm 25$  and  $12705 \pm 12.893$  respectively;  $p \leq 0.05$ ,  $n=10$ ).



### hMSCs CCL2 gene expression and secretion and the impact of CFTR activity

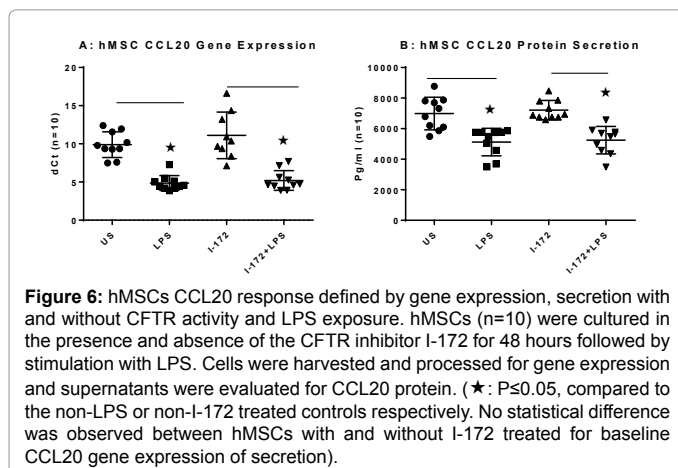
CCL2 is a chemokine involved in the recruitment of monocytes and helper-T-cells, both of which are beneficial in scenarios of chronic inflammation [27]. CCL2 expression was significantly lowered with the addition of LPS, both in the case of otherwise unstimulated cells and in cells with I-172 inhibitor (Figure 6A,  $-0.03 \pm 1.0$  and  $0.73 \pm 1.5$  respectively as compared to US:  $4.49 \pm 0.98$  ( $p \leq 0.05$ ,  $n=10$ ). CCL2 expression was significantly lowered with the addition of LPS, both in the case of otherwise unstimulated supernatant and in supernatant with I-172 inhibitor ( $5402 \pm 22.$ ,  $5480 \pm 15.$  respectively as compared to US:  $6683 \pm 27$ ,  $p \leq 0.05$ ,  $n=10$ ). Though not statistically significant, I-172 stimulation of hMSCs alone decreased CCL2 expression in Luminex (Figure 6B,  $6337 \pm 14$  versus US:  $6683 \pm 27$ ;  $p \leq 0.05$ ,  $n=10$ ).

### hMSCs CCL20 (MIP-3 $\alpha$ ) gene expression and secretion and the impact of CFTR activity

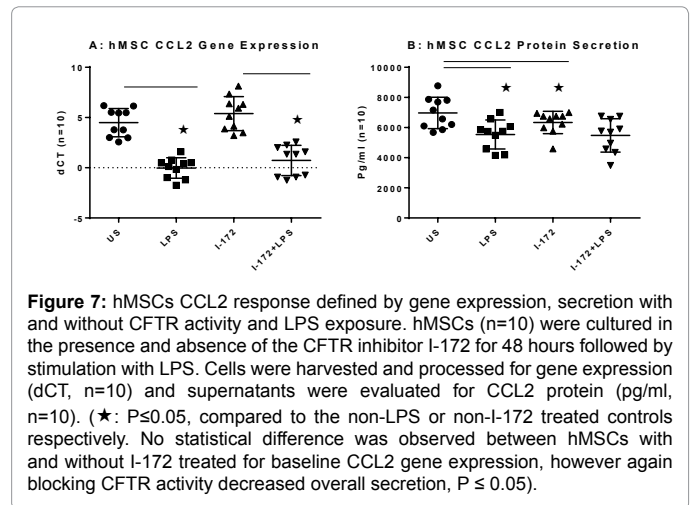
CCL20 is chemotactic for lymphocytes and dendritic cells, while weakly recruiting neutrophils. CCL20 also has antimicrobial properties that would be beneficial in scenarios of acute infection. CCL20 is dependent on activation of amplified IL-6. There was a large decrease in expression between LPS stimulated cells and control cells, both with and without I-172 stimulations (Figure 7A,  $9.9 \pm 1.7$ ,  $4.9 \pm 0.98$ ,  $5.2 \pm 1.3$  respectively;  $p \leq 0.05$ ,  $n=10$ ). There was no significant difference in CCL20 expression in hMSCs between unstimulated and I-172 cells ( $p \leq 0.05$ ,  $n=10$ ). Similar data was determined in secreted products, with LPS stimulation providing significant decreases in expression of CCL20 chemokine as compared to control supernatant (Figure 7B,  $p \leq 0.05$ ;  $n=10$ ).

### hMSCs impact on LPS stimulated PBMC cytokine profile

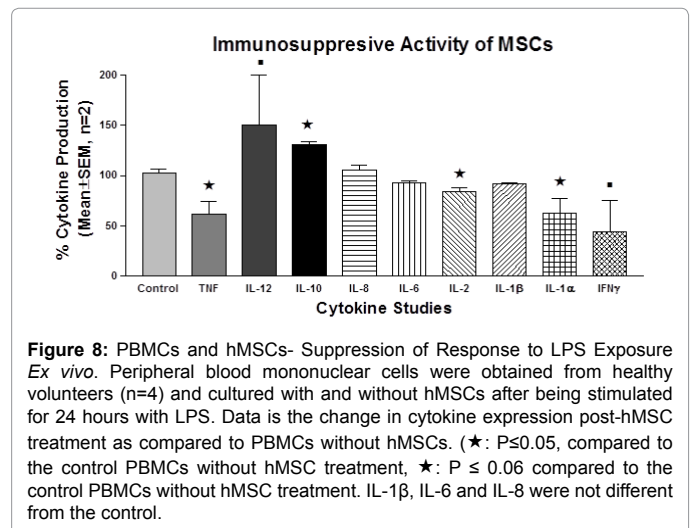
PBMCs stimulated with LPS produced significant levels of IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10 IL-12p70 and TNF $\alpha$  ( $p \leq 0.05$  for all) relative to control unstimulated PBMCs (data not shown). Pro-inflammatory and lymphocyte activating cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 IL-6, TNF-  $\alpha$ , IFN-  $\gamma$  were decreased when the PBMCs were cultured in the presence of hMSCs (Figure 8). Cytokine TNF $\alpha$  was decreased from  $216. \pm 4.3$  to  $140 \pm 3.4$  pg/ML with hMSC stimulation post LPS stimulation. Cytokine IFN $\gamma$  decreased with hMSC stimulation post LPS stimulation from  $98 \pm 2$  to  $83 \pm 3.3$ . Cytokines were minimal in the non -bacterial stimulated samples (no LPS), with levels similarly minimal in both unstimulated hMSC and control conditions. hMSCs down-regulate the inflammatory response of monocytes exposed to bacterial toxin such as LPS which is summarized in Table 1.



**Figure 6:** hMSCs CCL20 response defined by gene expression, secretion with and without CFTR activity and LPS exposure. hMSCs ( $n=10$ ) were cultured in the presence and absence of the CFTR inhibitor I-172 for 48 hours followed by stimulation with LPS. Cells were harvested and processed for gene expression and supernatants were evaluated for CCL20 protein. (★:  $P \leq 0.05$ , compared to the non-LPS or non-I-172 treated controls respectively. No statistical difference was observed between hMSCs with and without I-172 treated for baseline CCL20 gene expression or secretion).



**Figure 7:** hMSCs CCL2 response defined by gene expression, secretion with and without CFTR activity and LPS exposure. hMSCs ( $n=10$ ) were cultured in the presence and absence of the CFTR inhibitor I-172 for 48 hours followed by stimulation with LPS. Cells were harvested and processed for gene expression (dCT,  $n=10$ ) and supernatants were evaluated for CCL2 protein (pg/ml,  $n=10$ ). (★:  $P \leq 0.05$ , compared to the non-LPS or non-I-172 treated controls respectively. No statistical difference was observed between hMSCs with and without I-172 treated for baseline CCL2 gene expression, however again blocking CFTR activity decreased overall secretion,  $P \leq 0.05$ ).



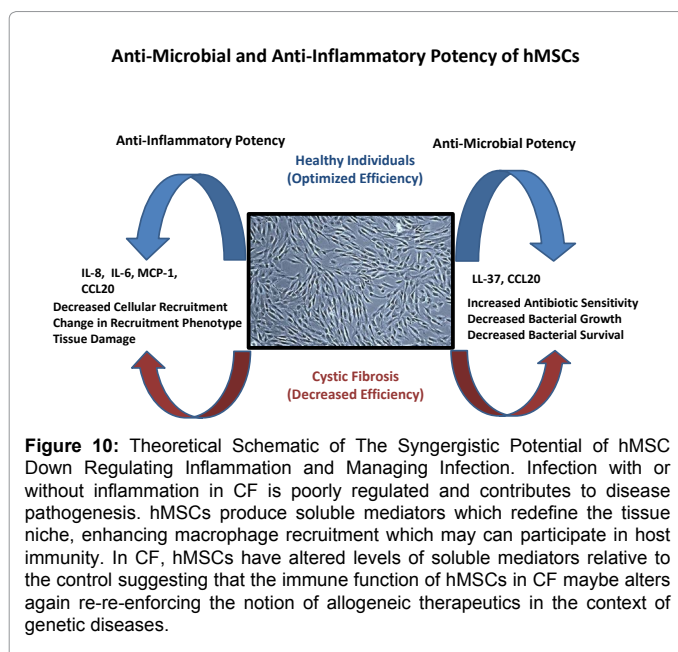
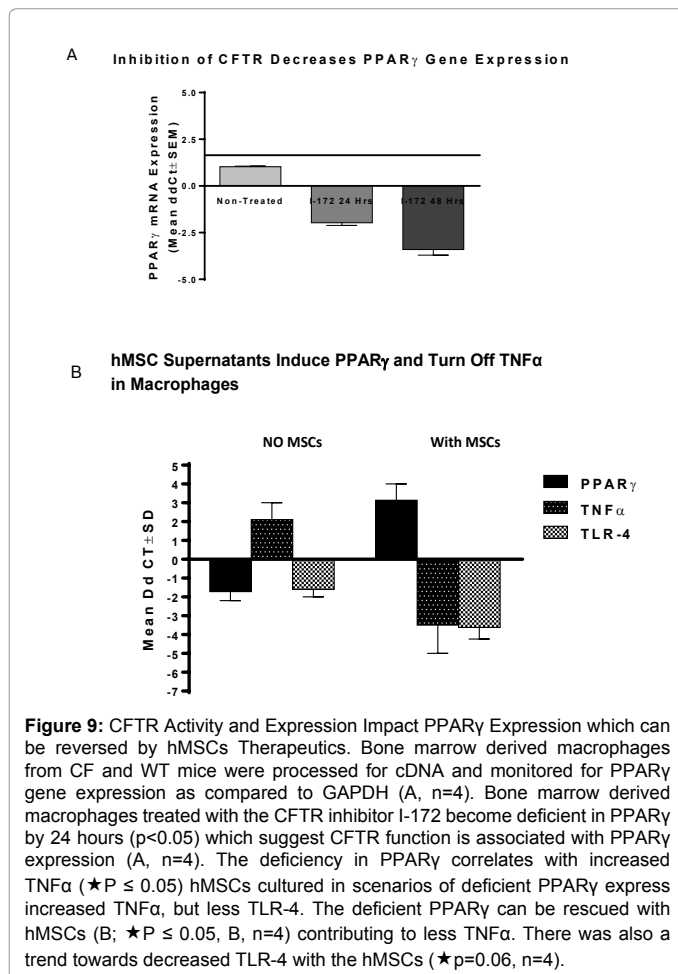
**Figure 8:** PBMCs and hMSCs- Suppression of Response to LPS Exposure *Ex vivo*. Peripheral blood mononuclear cells were obtained from healthy volunteers ( $n=4$ ) and cultured with and without hMSCs after being stimulated for 24 hours with LPS. Data is the change in cytokine expression post-hMSC treatment as compared to PBMCs without hMSCs. (★:  $P \leq 0.05$ , compared to the control PBMCs without hMSC treatment, ★:  $P \leq 0.06$  compared to the control PBMCs without hMSC treatment. IL-1 $\beta$ , IL-6 and IL-8 were not different from the control).

Cytokine	Concentration Change
<b>Pro- Inflammatory</b>	
IL-1alpha	-11.50%
IL-1Beta	-8.25%
IL-6	-6.85%
TNF-alpha	-39.20%
<b>TH1 Activators</b>	
IL-2	-16.40%
IFN-gamma	-45.85%
<b>Anti-Inflammatory</b>	
IL-10	31.00%

**Table 1:** Immune Redirection by hMSCs.

### The absence of CFTR decreases PPAR $\gamma$ gene expression which can be repaired by hMSCs

PPAR $\gamma$  is constitutively expressed in alveolar macrophages to attenuate the immune response to constant bombardment by environmental antigens [28,29]. In scenarios of chronic inflammation, PPAR $\gamma$  becomes deficient [30]. PPAR $\gamma$  has been shown to be deficient in macrophages and epithelial cells [31-35]. We generated a model of this observation using the CFTR blocker I-172, which decreased PPAR $\gamma$  in WT macrophages (Figure 9A). When CFTR activity is blocked PPAR $\gamma$



levels decrease as shown in the comparison of treated (I-172 inhibited) versus non-treated mRNA PPAR<sub>γ</sub> expression at 24 and 48 hours post treatment with inhibitor. In Figure 9, the comparison of the non-

treated ddCT value of  $0.925 \pm 0.3$ , 24 hours and 48-hour treatment to the treated groups, there was significantly less PPAR<sub>γ</sub> ( $-2.12 \pm 0.3$  and  $-3.52 \pm 0.2$  respectively for 24 and 48 hours,  $p \leq 0.05$ ). We have further established that hMSCs have the capacity to rescue deficient PPAR<sub>γ</sub> expression whether the deficient is due to endotoxin induced down-regulation (Figure 9B) or deficient CFTR activity. Bone marrow derive macrophages were treated with LPS which resulted in decreased PPAR<sub>γ</sub>, TLR-4 expression while TNF<sub>α</sub> expression is increased as predicted. When hMSCs are added, PPAR<sub>γ</sub> expression was increased while turning down the expression of both TNF<sub>α</sub> and TLR-4, decreasing the pro-inflammatory phenotype of the macrophages. The increase in TNF<sub>α</sub> specifically correlated with the decrease in PPAR<sub>γ</sub> ddCT value (normalized to initial start time CT value) from 6 to 24 hours ( $-1.87 \pm 0.74$  and  $3.35 \pm 0.46$  respectively; Mean  $\pm$  SEM,  $p \leq 0.05$ ).

## Discussion

We and others have previously demonstrated that hMSCs secrete products that are anti-inflammatory and antimicrobial, making them a suitable candidate to treat diseases associated with severe chronic infection and inflammation such as CF. Defects in the CFTR gene in CF result in increased susceptibility to pulmonary infections from *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumonia* [24]. The inability to resolve these infections and the ensuing over-zealous inflammatory response is the major cause of morbidity and mortality in CF. Our previous data has shown that hMSCs decrease inflammation in the *in vivo* murine model of CF chronic *Pseudomonas aeruginosa* infection and inflammation. The studies outlined in this manuscript focus on understanding the diversity in the hMSCs anti-inflammatory effectiveness. We have previously shown that growth conditions impact the overall antimicrobial effectiveness of hMSCs and that deficient CFTR function can also decrease antimicrobial potency [24]. Furthering these studies on hMSC phenotype, we have demonstrated that hMSCs are potent inflammatory mediators, and have contributed to defining the complexity of these cells. Our studies demonstrated that: 1) hMSCs have the capacity to alter the inflammatory response in both a gram negative (*Pseudomonas aeruginosa*) and gram positive (*Staphylococcus aureus*) murine modeling system of CF; 2) hMSCs have the capacity to alter their environment by directly recruiting cells of a specific type recruiting more lymphocytes than monocytes or neutrophils; 3) hMSCs produce a wide variety of factors that have the capacity to define the host response; 4) hMSCs are anti-inflammatory, producing anti-inflammatory chemokines, increasing IL-6 and CCL2 expression, while decreasing IL-8 expression; 5) hMSCs also produce the chemokine CCL20 (MIP- $\alpha$ ) which has not only chemotactic properties but also has been shown to be antimicrobial; 6) Deficient CFTR activity alters the expression of all of these cytokines as well as the capacity of the hMSCs to respond to LPS and gram negative bacteria; 7) Deficient CFTR results in the deficiency of the anti-inflammatory transcriptional regulator PPAR<sub>γ</sub> contributing to inflammation by producing factors such as TNF<sub>α</sub>; 8) hMSCs have the capacity to rescue PPAR<sub>γ</sub> expression as a potential mechanism associated with the MSC anti-inflammatory effectiveness in CF and other chronic inflammatory diseases.

Previous studies have implicated the anti-inflammatory and antimicrobial activity of the hMSCs in both animal models and in preliminary clinical trials [36-38]. In our own studies, we have been able to demonstrate that not only do hMSCs impact inflammation and infection they also alter the direction of inflammatory cell recruitment. In WT and CF mice infected with *Pseudomonas aeruginosa*, hMSCs decreased both bacterial burden and inflammation [14]. Mice given

the hMSCs had significantly less bacterial burden than mice treated with saline along with attenuated inflammation. In the studies presented in this manuscript, we have demonstrated that hMSCs are active contributors to the host environment by producing cytokines and chemokines that participate, in the host response to pathogen exposure. We believe that the contribution of the hMSCs will be disease specific since in our hands using the same hMSC preparations we have been able to identify very different end-points of hMSC effectiveness whether it is in the context of infection (as in the CF studies) or in acute inflammation or chronic asthma [38]. Certainly our studies demonstrate the unique capacity of the hMSCs to alter inflammation regardless of the inciting source of the pathophysiology.

There are several aspects that need to be considered when thinking about hMSC-based therapeutics including how the cells are cultured and administered [36,39] but also important to consider the source of the hMSC preparation. Several reviews have outlined the unique attributes of MSCs and their specific source and processing and the outcome on clinical potency and efficacy [37,40]. In our studies, we have demonstrated previously that hMSCs deficient in CFTR activity have decreased anti-microbial potency and efficacy [3] which may have important implications into whether the autologous therapeutics can be utilized for CF hMSC clinical trials. This may also relate to other diseases in which there is a genetic association with the pathogenicity and may be the reasoning behind the overall variability in hMSC clinical efficacy in clinical trials. The results outlined in this manuscript explore the issue of CFTR expression on hMSC anti-inflammatory potency. Our data indicate that CF hMSCs do not behave similarly to healthy hMSCs in response to bacterial stimulation with healthy and CF hMSCs express different amounts of IL-6, CCL2, CCL20 and IL-8 [41,42]. With bacterial stimulation, CF cells express a greater amount of IL-8 than healthy hMSCs when both are exposed to bacteria. The CF hMSCs express less IL-6, CCL2 and CCL20 than healthy hMSCs with bacterial stimulation. Although CF hMSCs and healthy hMSCs express different amounts of IL-6, CCL2, and IL-8, both types of MSCs express roughly equal amounts of CCL20 which may be important in the chemotactic and antimicrobial functions of these chemokine/cytokines [27,43]. The production of chemokines and cytokine mediators will have a contributory role in defining the tissue niche as a response to the inflammatory stimulus. The contributory fingerprints of the hMSCs secreted products will ultimately change how the host will manage the inflammatory process.

The difference in expression of cytokines between healthy and CF hMSCs in response to bacterial stimulation indicates that autologous hMSCs are unlikely to be a successful form of therapy for CF patients. The data also suggests the impact of CFTR deficient activity throughout CF patient's body, not only at the level of the immune system but also the functionality of the autologous hMSCs. This implies an additional contribution of deficient CFTR on CF pathophysiology, encompassing not only the primary major consequence of the defect on the epithelium, but also the additive impact on the CF immune system and the multipotent cell source of hMSCs

In the pursuit of the mechanistic action of the hMSCs as an anti-inflammatory adjuvant therapeutic, we have begun to identify the potential of hMSCs to alter PPAR $\gamma$  expression in macrophages which is an important regulator of inflammation in chronic inflammation [44]. In the lung, PPAR $\gamma$  is constitutively expressed in alveolar macrophages in order to attenuate the host response to the continuous exposure to environmental insult [23,33]. PPAR $\gamma$  quiets inflammation through transcriptional repression of NF $\kappa$ B preventing the ability of NF $\kappa$ B to initiating transcription from its promoter regions which are

involved with pro-inflammatory gene expression [45]. In scenarios of chronic inflammation, PPAR $\gamma$  becomes deficient in macrophages which contributes to the uncontrolled expression of pro-inflammatory genes by NF $\kappa$ B [35], a pathophysiological mechanism described in a variety of chronic inflammatory diseases [46,47]. Like our studies it has been shown that CF macrophages are deficient in PPAR $\gamma$  [35]. We have been able to demonstrate that hMSCs can up-regulate the expression of PPAR $\gamma$ , in macrophages which in turn has the capacity to down-regulate inflammation regardless of the routes associated with the down-regulation of this inflammation rheostat. Theoretically, the hMSCs likely produce soluble mediators which alter the macrophage phenotype, enhancing the PPAR $\gamma$  expression, allowing for efficient modification of pro-inflammatory cytokine production through the prevention of NF $\kappa$ B promoter activation. In either case of LPS stimulated mouse bone marrow derived macrophages or human peripheral blood mononuclear cells, hMSCs decreased pro-inflammatory cytokine production. The direct mechanisms of these effects are the focus of on-going work in our laboratory.

hMSCs have anti-inflammatory potential which has been documented in several different *in vivo* and *in vitro* modeling systems [48]. We propose that one of the mechanisms associated with this hMSC function is the capacity to up-regulate the expression of PPAR $\gamma$ . How CFTR might impact this effect of hMSCs will be pursued in the context of our investigator initiated clinical trial of hMSCs in CF. Further, these studies are consistent with previous benefits of hMSCs in scenarios of Acute Lung Injury (ALI) which is a disease of significant morbidity and mortality in intensive care patients [37]. ALI is defined by the acute onset of pulmonary infiltrates with severe hypoxemia. In a mouse model of ALI, Lipopolysaccharide (LPS) derived from the bacterium *Escherichia coli* endotoxin was put into the lung followed by administration of murine hMSCs several hours later. Mice treated with murine MSCs had decreased inflammation [49,50]. The pro-inflammatory response to the LPS endotoxin was decreased, while the resolution response and anti-inflammatory cytokines were enhanced [18]. These studies suggest the innovative use of adult human MSCs in the treatment of lung diseases associated with infection and inflammation.

## Conclusions

hMSCs are active in their environment secreting products that define the host milieu and capacity to regulate immunity and cellular response. The capacity to down-regulate inflammation is reproducible regardless of the modeling system using mouse bone marrow derived macrophages or human peripheral blood mononuclear cells going across species with hMSCs. The capacity of the hMSCs to secrete cytokines and respond to pathogen endotoxin like LPS is impacted by CFTR activity, suggesting that CF hMSCs may not be appropriate for autologous therapy. The inefficiency of the CF hMSCs in these systems along with the established antimicrobial activity, implies that innate hMSC function may be altered in CF, potentially contributing to disease. hMSCs are environmentally sensitive and environmentally pro-active emphasizing the complexity of their function and potential therapeutic impact. Future studies will pursue the impact of CFTR on hMSC activity and how hMSC regulates the combined anti-inflammatory and antimicrobial milieu *in vivo*.

## Acknowledgements

We appreciate the expertise and the services provided by the CTSC Bioanalyte Core and the CF Animal Core. The funding for this research was graciously provided by the David and Virginia Baldwin Fund, The National Center for Stem Cell and Regenerative Medicine, The Case

Western Reserve Vision Fund, Rainbow Babies and Children's Hospital Summer Programs in Undergraduate Research, Rainbow Babies and Children's Hospitals Strategic Innovator Award and National Institutes of Health HL: R21:104362.

## Author Contributions

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Lauren Auster: Provision of Study Materials, Manuscript Review.

Michael Folz: Manuscript Review Data Analysis and Interpretation.

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## Disclosure of Potential Conflicts of Interest

The authors listed have no conflicts of interest to report.

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