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The effect of cystic fibrosis environment on the bactericidal and phagocytic abilities of macrophages

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An abstract of
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Abstract

By Melissa Shenep

Alveolar cystic fibrosis (CF) macrophages have decreased innate bactericidal abilities. Compared to non-CF macrophages in the airway, CF macrophages are surrounded with an overabundance of cytokines and a thick layer of dehydrated mucous. To test the effect of the CF environment on the bactericidal and phagocytic ability of the macrophage, we conducted *in vitro* bactericidal assays. We infected mouse leukaemic monocyte macrophage cell line (RAW 264.7) macrophages with *Pseudomonas aeruginosa* (PA01) after conditioning the cells with CF plasma, non-CF plasma, tumor necrosis factor α (TNFα), interleukin-8 (IL-8), or CF sputum. Additionally, we performed an *in vivo* bactericidal test in which we infected CF and wild-type mice with PA01 and measured bacterial proliferation in the bronchoalveolar fluid (BAF) and lungs. Furthermore, we tested the respiratory burst of primary CF and non-CF monocytes, measured the level of pro-inflammatory cytokines TNFα and IL-6 released by primary CF monocytes after infection with PA01, and observed the internalization of PA01 by CF macrophages. Although the CF monocytes appeared to be able to internalize bacteria, produce reactive oxygen species, and signal other cells via cytokines or chemokines, they exhibited decreased bactericidal abilities when tested *in vivo* or with TNFα or IL-8. Thus, we conclude that the inflammatory nature of the CF environment inhibits the bactericidal abilities of macrophages.

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Introduction

Lung infection remains the primary cause of morbidity and mortality for cystic fibrosis (CF) patients. CF is an autosomal recessive disorder that affects 1 in 4000 people in the United States (1, 2). In the lungs, a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) channel causes a build-up of mucous and excessive inflammation from the overproduction of cytokines, promoting bacterial colonization, which leads to impaired respiratory function and death (3). The ABC-transporter class CFTR ion channel normally facilitates transport of chloride out of epithelial cells, accompanied by sodium and water, thereby increasing the fluidity of lung mucous. Mutations in the CFTR protein diminish chloride transport and dehydrate the airway surface liquid (ASL) (4, 5). In addition to the dehydration of ASL, the associated inflammation causes cells in the airway to lose their cilia; therefore, the bacteria that are normally cleared by the cilia become trapped in the thickened mucosal layer (3). While the inflammatory condition of the lungs increases the likelihood of infection, the defective CFTR channel might also affect the ability of immune cells to effectively clear the infection.

Although it is known that the innate immunity of a CF patient is defective in general, the combined effect of the CF inflammatory environment and the defective CFTR channel on pulmonary macrophage function is yet to be clarified. Circulating blood monocytes mature into pulmonary macrophages as they enter the lung tissue to fight infection. Overproduction of cytokines by macrophages in CF mice has been shown to directly contribute to inflammation (6). Normally, when an infection is present, the epithelium activates macrophages, which in turn signal neutrophils with cytokines and chemokines, resulting in an inflammatory response (7). In addition, to decrease the inflammatory response and help in further controlling the infection, macrophages will ingest the dead or dying neutrophils that have engulfed bacteria themselves

(7). In CF patients there is a remarkable increase in the number of airway neutrophils (8, 9). The increased presence of neutrophils in CF patients suggests a problem in the immunological responses of CF lungs. Some possible defects that could contribute to this phenomenon are defects in the CF macrophage's ability to phagocytosize neutrophils, in the programmed cell death of neutrophils, or in the cell signaling responses of macrophages to recruit neutrophils to the site of infection. In patients with defective CFTR, the innate immune response differs from that of subjects with normal CFTR through the over-recruitment of neutrophils to the airway due to excessive production of cytokines by macrophages and possibly due to diminished macrophage phagocytosis.

The main function of macrophages is to signal other cells to the site of infection, engulf and destroy the surrounding bacteria or dead and dying cells, and eventually convert into an antigen-presenting cell to activate T cells, which in turn activate B cells to make antibodies to the invading bacteria. To destroy the engulfed bacteria, macrophages envelop the bacteria into a phagolysosome and bombard the bacteria with oxygen radicals, hypochlorous acid, lysozyme, reactive oxygen species, and other toxic molecules (10, 11). Some studies suggest that CFTR-deficient ($CFTR^{-/-}$) macrophages are unable to effectively destroy bacteria due to the loss of their acidic nature, possibly due to the mutated chlorine channel, which would normally induce a strong proton gradient (12, 13). However, other studies propose functional acidification in $CFTR^{-/-}$ macrophages, and thus, suggest that additional factors are involved in inhibiting the functionality of CF macrophages (11, 14, 15). For example, Vandivier *et al*. found that the ability of macrophages to perform efferocytosis, engulfing and destroying apoptotic cells, has been compromised in CF macrophages (16).

While the reason for the defective nature of the CF macrophage remains unclear, *in vitro* studies on CF macrophages have shown an increased survival rate of ingested bacteria compared to normal macrophages infected with the same load of bacteria (4). Moreover, the loss of function of the macrophages due to CF may disrupt the coordination between neutrophils and macrophages. This dysfunction may allow infectious agents, such as *Pseudomonas aeruginosa*, to persist in the respiratory system, eventually destroying the lungs. In addition, the CF lungs are more prone to infection, because the receptors on epithelial cells that bind to *P. aeruginosa* are two times more prevalent in CF lungs than non-CF lungs (3, 17).

There is also strong evidence that CF macrophages have an overreactive inflammatory response and are unable to properly dispose of an infectious agent due to the inflammation caused by the presence of bacteria. Heeckerson *et al.* demonstrate that CF mice have increased inflammation compared to wild type mice after infection with *P. aeruginosa* (18). The hyperinflammatory response to infection in part is elicited from the innate properties of *P. aeruginosa* (19). *P. aeruginosa* flagellins increase the inflammatory response through initiating production of pro-inflammatory cytokines (19). In addition, alginate production from *P. aeruginosa* prohibits macrophage phagocytosis and bacterial opsonization (20).

Pro-inflammatory cytokines, mucous build-up, and an increase of various glycoproteins contribute to the increase of *P. aeruginosa* infections in CF lungs compared to non-CF lungs. In particular, elevated levels of cytokine tumor necrosis factor alpha (TNFα) and interleukin- 6 (IL-6), and chemokine interleukin-8 (IL-8) are found in the lungs of CF patients (3, 21, 22, 23). Increased levels of IL-6, which regulates chemokines such as IL-8, were found in CF patients compared to healthy patients (23). While the main function of IL-8 is to recruit neutrophils to the site of infection, an increase in production of IL-8 is directly correlated to lung deterioration

and colonization by *P. aeruginosa* (3, 22). Increased levels of TNFα, which induce apoptosis and recruit macrophages and other cells to the infection site, are also correlated with symptoms of lung disease in CF patients (22, 24). While the defective CFTR channel causes a decrease in bactericidal abilities of macrophages, the potential additional effects of the external, inflammation-induced environment upon the macrophage's ability to clear bacteria remain relatively unexplored (4). We hypothesized that the environment in CF lungs will cause a decrease in bactericidal abilities, an increase in production of reactive oxygen species, and an impairment of overall phagocytic abilities in both normal and CF macrophages. While several studies have examined CF and non-CF macrophages under strictly *in vitro* conditions, understanding the role of the environment surrounding defective CFTR macrophages is a crucial, yet widely unrecognized, point of interest for future treatments of CF.

Additionally, while the reasons for increased bacterial susceptibility are unclear, applying gene therapy to CF monocytes has been proposed to restore expression of functional CFTR protein and potentially increase their ability to clear infection. Today, the focus of gene therapy for CF patients usually involves repairing the damaged lung epithelium (25). However, while studies show that restoring CFTR function to the lung epithelium can combat the inflammatory response initiated due to defective CFTR, this process proves difficult due to the lung's innate resistance to viral infection, thus resistance to the vectors that transport the gene therapy (26). While gene therapy of the lung epithelium is explored in great depth despite its complications, restoring CFTR function in macrophages still remains a relatively unexplored field (27). Reinstating the normal function of macrophages could prove crucial in the treatment of CF due to the major role macrophages play in clearing infection and their ability to function as pro- and anti- inflammatory cell (8, 27). Thus, before exploring the option of gene therapy, we must

explore the causes for the defective nature of CF macrophages. Determining the effect of the environment in a CF lung on the macrophage's bactericidal ability will inform the potential usefulness of myeloid targeted genetic therapy for CF patients and allow us to better understand the pathology of CF on macrophages.

Materials and Methods

Primary CF and THP-1 monocytes

Normal cell line human monocytic cells (human acute monocytic leukemia cell line (THP-1)) and primary CF monocytic cells were grown in Roswell Park Memorial Institute medium (RPMI) enhanced with nonessential amino acids, 10% fetal calf serum, glutamine, sodium pyruvate, and penicillin/streptomycin. The cells were suspended and set at a density of around 1 x 10^6 cells/ml in a small tissue culture flask and incubated at 37°C with 5% CO2.

Growing RAW 264 and RAW GFP LC3 macrophages

Mouse leukaemic monocyte macrophage cell line (RAW 264.17) and RAW macrophages that have been transfected with green fluorescent protein gene attached to human 1 light chain 3 beta gene (RAW GFP LC3) were grown in Dulbecco's Modified Eagle Medium (DMEM) 1x enhanced with nonessential amino acids, 10% fetal calf serum, glutamine, sodium pyruvate, and penicillin/streptomycin. The cells were established at a density of around 1 x 10^6 cells/ml in a small tissue culture flask and incubated at 37°C with 5% CO2.

Growing *Pseudomonas aeruginosa* (GFP labeled)

With a 10 µl inoculate loop, we isolated *P. aeruginosa* (PA01) which was transfected with green fluorescent protein (GFP), provided by Dr. G. O'Toole, Dartmouth, from blood and simple agar plates onto a Lysogeny Broth (LB) agar plate with ampicillin. Ampicillin was used

to ensure pure isolates were cultured, for the GFP tagged *P. aeruginosa* was ampicillin resistant. The plates were left overnight at 37°C and 5% CO2. After obtaining a pure streak of *P. aeruginosa,* we gathered isolated colonies with a 10 µl loop into 15 ml of LB broth in a 50 ml test tube, leaving the bacteria to grow overnight with the lid loosened in 37°C. For the *in vivo* bactericidal assay, before culturing dilutions of PA01, we washed the inoculum by spinning the inoculum at 15,000 rpm and resuspending the pellet in 10 ml of PBS or medium without antibiotics. For the *in vitro* bactericidal assays, directly after incubation, we cultured 100 µl of various dilutions of the PA01 inoculum onto the LB ampicillin plates to obtain an estimate of the colony forming units (CFU) and put the plates in 37°C and 5% CO2 overnight to count the colonies the next day. To keep the bacteria from further multiplying, we placed the culture in a 4°C refrigerator, and after analyzing the colony counts, we obtained the number of bacteria per ml of LB broth in the inoculum. We then assessed the bacterial counts from the resulting stock solution.

Opsonized zymosan

According to the Allen (1986) method, a stock solution of opsonized zymosan at 2.5 mg/ml was made and used at a final concentration 500 µg/ml. Zymosan was opsonized with freshly collected human plasma obtained from healthy non-CF donors.

Luminol probe

To make the luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) probe for the respiratory burst assay, 3.5 mg of luminol (Sigma Chemical Company, St. Louis) was suspended in 1 ml of dimethyl sulfoxide (DMSO) and stored in the dark at 4°C. After diluting the luminol to 3.5 mM in distilled water, 200 µl of luminol was added to 2 ml of cell suspension to detect the oxygen radicals produced in the cells after introduction to opsonized zymosan or *P. aeruginosa.*

The resultant chemiluminescence produced by the luminol was measured in a BioTek (Winooski, VA) plate reader.

Preparing trypsin

To prepare the trypsin that was used to break down the sputum, 15 ml of 0.25% trypsin, which was stored at 4°C, was thawed at 37°C in a water bath. After incubation, 11.2 µl of trypsin was added to 1 ml of sputum for a final concentration of 0.0028% trypsin/ml of sputum per the Fuller *et al.* (2004) method.

Obtaining and preparing CF sputum

CF sputum was obtained from the CF Biospecimen Registry, a core facility of the Emory+Children's Center for CF Research. Three ml of sputum were collected and processed without sputolysin to mechanically dissociate mucous then divided into 1 ml aliquots and designated as plain CF sputum, boiled CF sputum, or trypsinized CF sputum. As a negative control, 1 ml of CF sputum was degraded in 0.0028% trypsin. Following the Fuller *et al*. (2004) method, 1ml of sputum was incubated at 37°C and 5% CO2 with 0.0028% trypsin and another 2 ml of plain CF sputum was incubated for 5 hours. Then 1 ml of plain sputum was boiled for 30 min alongside the 1 ml of sputum that contained 0.0028% trypsin. All 3 samples of sputum were stored at 20°C for 0-48 hours until used in the experiments.

Imaging of CF, non-CF, THP-1, and RAW macrophages

Primary CF and non-CF monocytes were infected with PA01 for 45 minutes and then examined under the total internal reflection fluorescence (TIRF) microscope that scanned the surface of the monocytes. Additionally, the monocytes were examined under a confocal microscope that allowed observation of the inside of the monocytes after infection. Due to the green fluorescent protein expressed by the PA01, the bacteria were easily identified.

Photographs of THP-1 human monocytes were taken using bright field imaging and snapshots were taken of RAW macrophage fluorescence after the macrophages were infected with 10^8 CFU of PA01, 10^4 CFU of PA01, or no PA01.

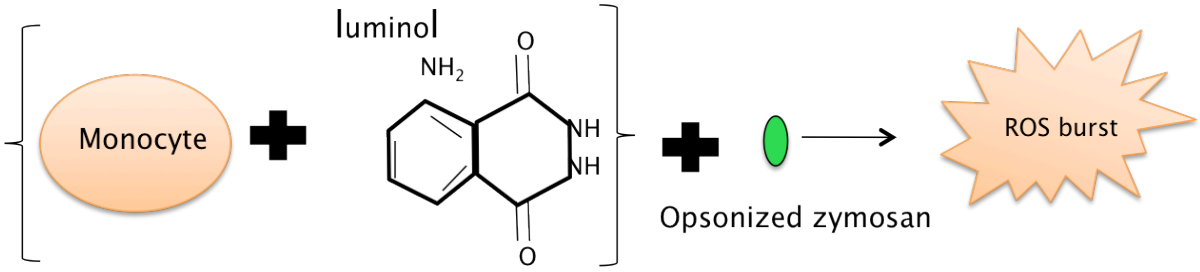


Figure 1: Diagram of Respiratory Burst

Testing the respiratory burst ability of CF monocytes

To test the macrophages' ability to produce reactive oxygen species in the presence of foreign particulates, opsonized zymosan was used to induce the respiratory burst in primary CF and non-CF monocytes. Before testing the respiratory burst, the cells were maintained at 37°C and 5% CO2 in an incubator. Adjusting the cell concentration to around 1 x 10^6 cells per ml, a luminol probe was added to the cell suspension at 100 µl/ml of cells. Then 150 µl of the cell suspension was added to a white 96 well plate in triplicate. As a negative control, two rows of the 96 well plate contained 50 µl of PBS in place of zymosan. Immediately following the addition of 1 mg/ml or 0.5 mg/ml of opsonized zymosan to trigger the respiratory burst response, the 96 well plate was put into a Biotek (Winooski, VA) plate reader to measure the luminol's chemiluminescence release as the probe detected oxygen radicals (see Figure 1). The ROS release was observed every 6 minutes for over one hour. The chemiluminescence, which was measured in relative light units (RLU), was used as a measurement of the number of photons generated at each reaction time. Calculations were made via Microsoft Excel (Seattle, WA) through plotting the average ROS release on a time course.

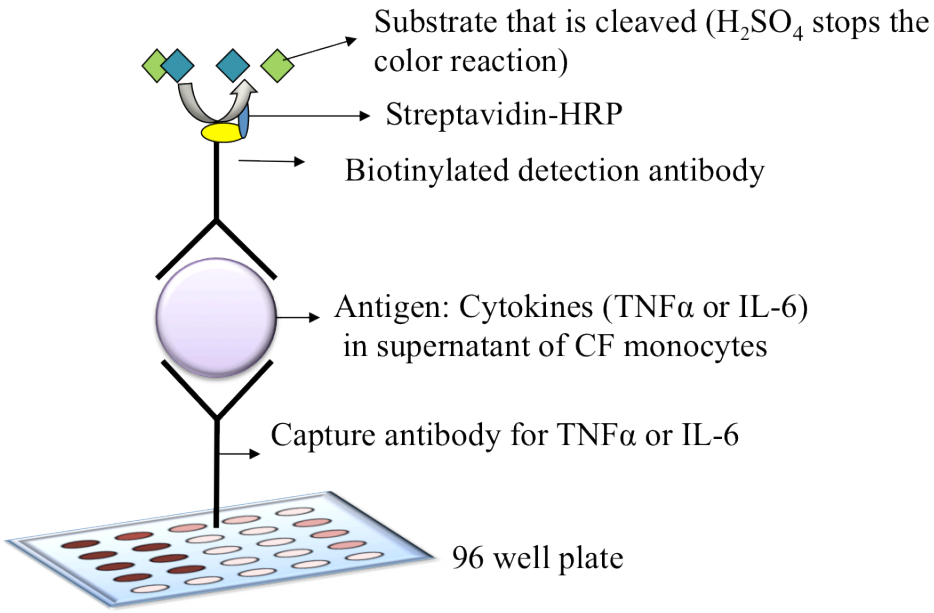


Figure 2: Diagram of ELISA assay

Determining the cell-signaling abilities of CF monocytes

To test for the efficacy of cell-signaling in CF monocytes, an enzyme-linked immunsorbent assay (ELISA) was used to test for the production of cytokines TNFα and IL-6 in response to GFP-PA01 following the Zughaier *et al*. (1999) methods (see Figure 2). Cytokines DouSet ELISA kits were purchased from R&D Systems, Minneapolis, MN. In two Maxisorp (Fischer Scientific Company, Suwanee, GA) 96 well plates, 100 µl per well of a mixture (56 µl of capture antibody/100 ml of PBS) was added. The plates were covered overnight at room temperature. The 96 well plates were washed with 300 µl washing buffer (0.05% Tween 20 in PBS), then blotted on tissue paper to decant the liquid, and 300 µl of blocking buffer (1% BSA, 5% sucrose, 0.05% NaN_3 in PBS) was added to each well. After adding the blocking buffer, the plate was left for one hour at room temperature. To create the TNFα standard for the assay, 1000 pg/ml (14.5 µl of the standard and 985 µl of reagent solution (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline)) of the standard was added to the first two wells on the top row of the 96

well plate and a 2-fold serial dilution was made across the top row in duplicate. On another plate the standard IL-6 was created in the same method as TNFα standard. After the standards were created, 100 µl of supernatant from CF macrophages that were exposed to formalin fixed PA01 for 24 hours was diluted 1:1 in reagent diluents and added in duplicate at minimum. Afterwards, 100 µl of detection antibody (56 µl of biotinylated antibody (R&D Systems, Minneapolis, MN) in 10 ml of reagent diluent) was added to each well and the plate was left at room temperature for 2 hours then washed two times with washing buffer. After incubation at room temperature, 100 µl of streptavidin-HRP (R&D Systems, Minneapolis, MN) was added to each well. Then the plates were left at room temperature for 20 minutes, and washed twice. Following washing, 100 µl of substrate was added to each well. The reaction was then stopped through the addition of 50 µl of H_2SO_4 to each well and the plates read in a 96 well BioTek reader (Winooski, VA) at 450 nm. Calculations were made via Microsoft Excel (Seattle, WA) through plotting the log of optical density (OD) verses log of TNFα or IL-6 concentration.

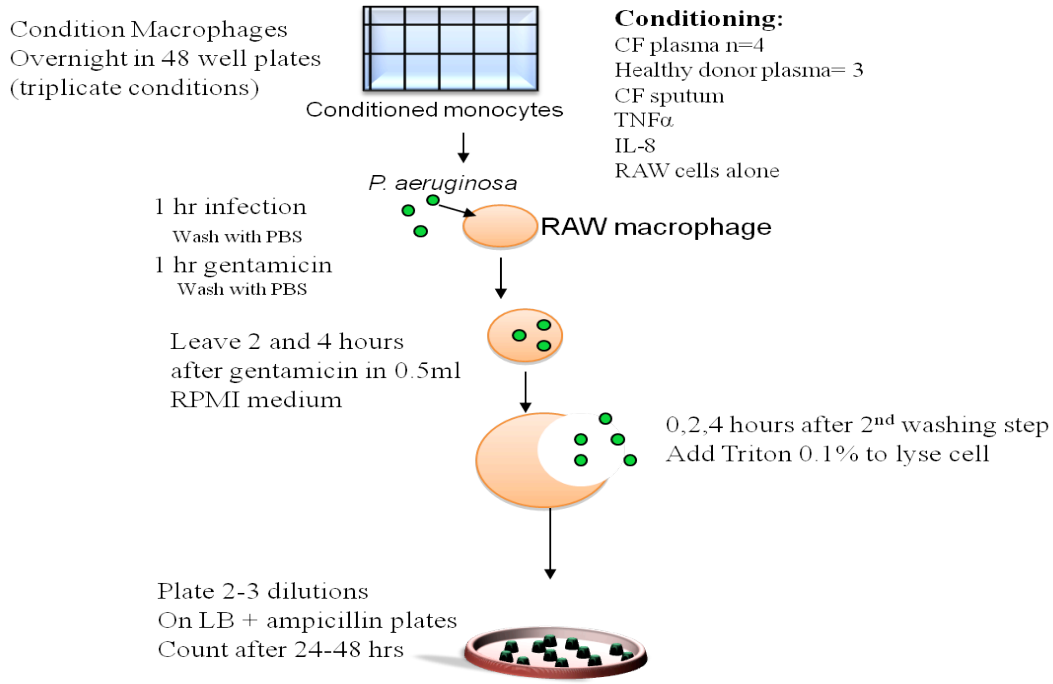


Figure 3: Diagram of *in vitro* bactericidal assay

***In vitro* bactericidal assay: RAW macrophages with CF plasma, non-CF plasma, TNFα, or IL-8**

To test the effect of the individual components of the CF environment on the bactericidal abilities of macrophages, RAW macrophages were challenged under various conditions that mimicked the CF environment (see Figure 3). RAW macrophages were first counted with a hemacytometer and adjusted to around 1 x 10^6 cells per well. To mimic the various conditions that monocytes experience *in vivo*, the monocytes were conditioned in TNFα, IL-8, CF plasma, or healthy donor plasma overnight at 37°C and 5% CO2 on a 48 well plate and left to incubate overnight at 37°C and 5% CO2. After incubation, the monocytes were infected with either 5, 10, or 25 bacteria for every macrophage at 37°C and 5% CO2 for one hour and then incubated for another hour in the presence of 0.5 ml of 0.5 mg/ml gentamicin to kill extracellular bacteria. Following incubation with gentamicin, at time 0, some monocytes were lysed with 0.1% Triton, incubated for 5 minutes in 37°C and 5% CO2, and plated on LB plates containing ampicillin to select for the GFP-PA01, which is resistant to ampicillin. The other monocytes were lysed and plated after an additional incubation period of 2 and 4 hours, respectively. The plates were then incubated at 37°C overnight and the colonies were counted after 24 to 48 hours.

***In vitro* bactericidal assay: RAW macrophages with CF sputum**

The overall effect of the CF environment on the bactericidal abilities of macrophages was tested through challenging RAW macrophages with CF sputum (see Figure 3). The method used in the previous bactericidal assay was utilized except the macrophages were conditioned in 10% plain CF sputum, boiled CF sputum, CF sputum treated with trypsin, or RPMI medium alone.

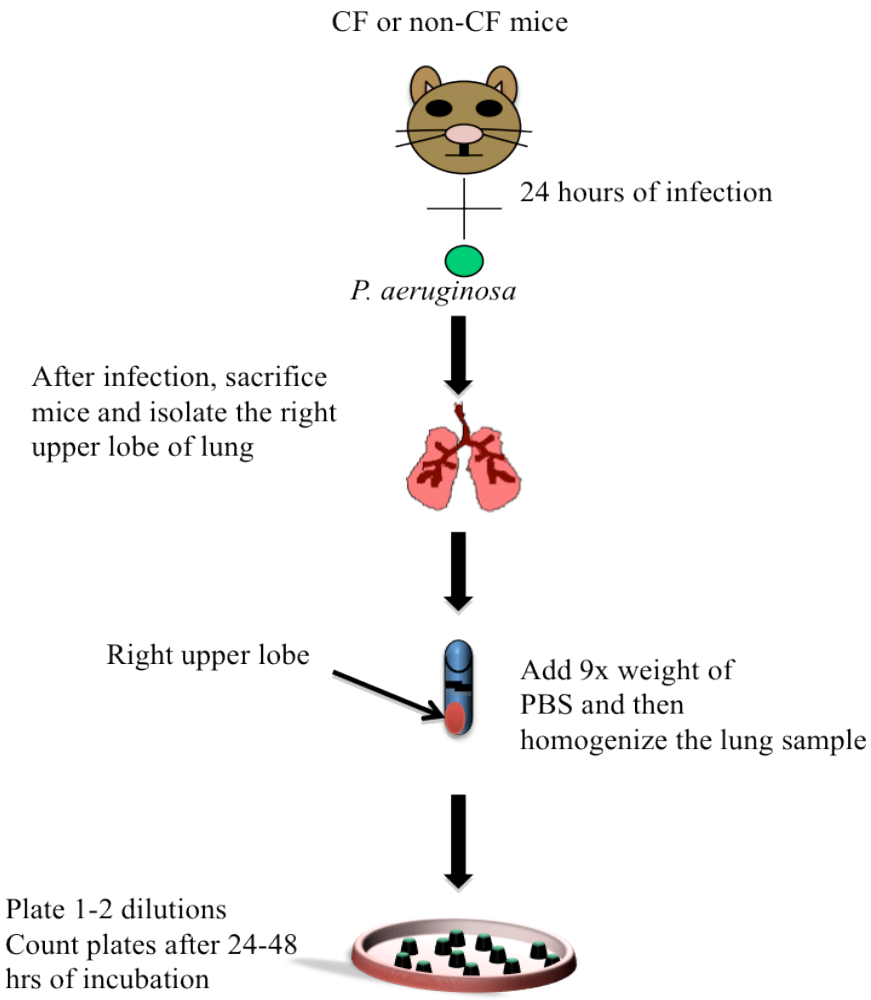


Figure 4: Diagram of *in vivo* bactericidal assay

***In vivo* bactericidal assay**

To test the bactericidal abilities of CF versus non-CF monocytes *in vivo*, 10 wild type mice and 5 CFTR knockout ($CFTR^{-/-}$) mice from the Jackson Laboratories were bred and maintained at the CF Mouse Models Core at Emory+Children's Center for Cystic Fibrosis Research (Atlanta, GA) and infected with PA01 (see Figure 4). Using the method described in Helms *et al*. (2010), the mice were infected with 75 µl of 1.52×10^7 CFU/ml, 4.9×10^6 CFU/ml, 2.968×10^6 CFU/ml, or 1×10^6 CFU/ml of PA01 inoculum.

18-24 hours after infecting the mice with PA01, the mice were sacrificed, and a bronchoalveolar lavage (BAL) was performed to collect the bronchoalveolar fluid (BAF); in addition, the right upper lobe of the lung was extracted to test for the presence of bacteria. The BAF was obtained through instilling 1 ml of PBS directly into the lungs with a syringe and then pulling back to collect the liquid from the lungs. Then 25µl of BAF was plated onto an LB + ampicillin plate, incubated at 37°C for 24-48 hours, and the CFUs on the plate were counted after incubation. To measure the bacteria in the lungs, the right upper lobe of the lung was weighed and 9x the amount of weight was added in ml of PBS. Then the lung biopsy was homogenized and dilutions were plated onto an LB + ampicillin plate, incubated at 37°C for 24-48 hours, and the CFUs on the plate were counted after incubation. The averages of all the colony counts/ml for the CF and wild-type mice were then graphed in Microsoft Excel (Seattle, WA).

Results

Examining the phagocytosis of primary CF monocytes infected with *P. aeruginosa*

We infected primary CF and non-CF monocytes with *P. aeruginosa* (PA01) that was expressing green fluorescent protein as seen in Figure 5. The top panel of Figure 6 shows PA01 on the surface of both the CF and NS cells. The bottom panel of Figure 6 exhibits the internalized PA01 on the CF and NS cells as the confocal microscope view a cross section of the macrophages. Figure 6 effectively shows that CF monocytes attach to and engulf PA01.

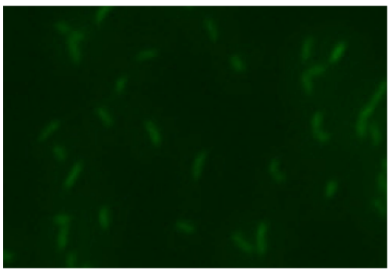


Figure. 5 *Pseudomonas aeruginosa* expressing GFP (PA01).

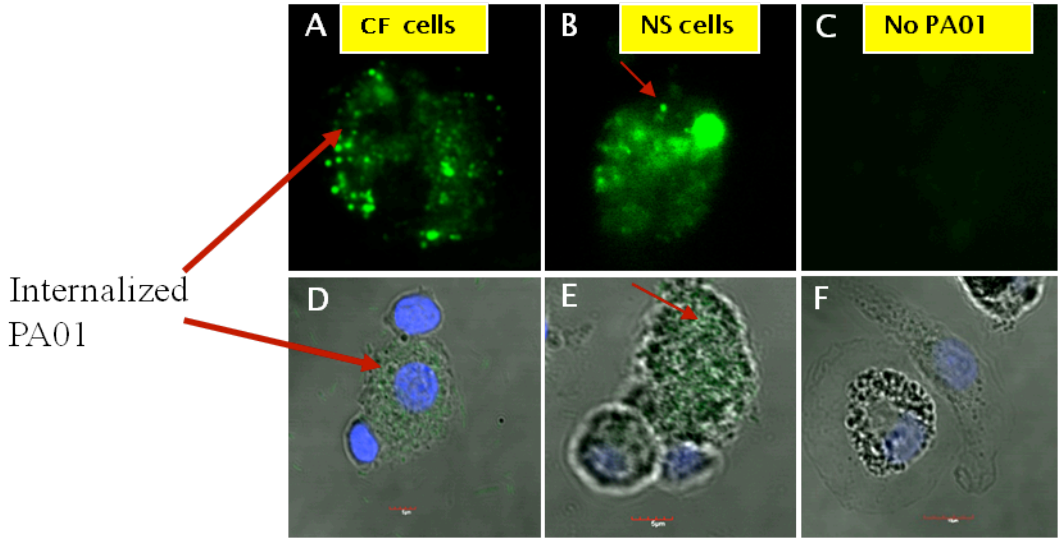


Figure 6: Phagocytosis of fluorescent *P. aeruginosa* by CF monocytes. Top images are taken with TIRF and show internalized PA01 in CF monocytes. **A.** CF, **B.** normal subject (NS), **C.** non-infected NS monocytes with red arrow signaling GFP-PA01. Bottom images show confocal images with the nucleus staining blue and the GFP-PA01 showing green pointed out by red arrows. **D.** CF, **E.** NS, and **F.** non-infected CF monocytes.

Imaging of infected THP-1 and RAW264 monocytes

To examine the morphological changes that monocytes experience after infection, PA01 was used to infect human and murine macrophages, THP-1 and RAW264 cells, respectively. The top panel of Figure 7 shows the phenotypical changes that THP-1 monocytes exhibit after infection. The bottom panel of Figure 7 indicates the internalized PA01 in RAW264 monocytes. The ability of the monocytes to change in morphology in response to infection indicates that they are able to become antigen-presenting cells.

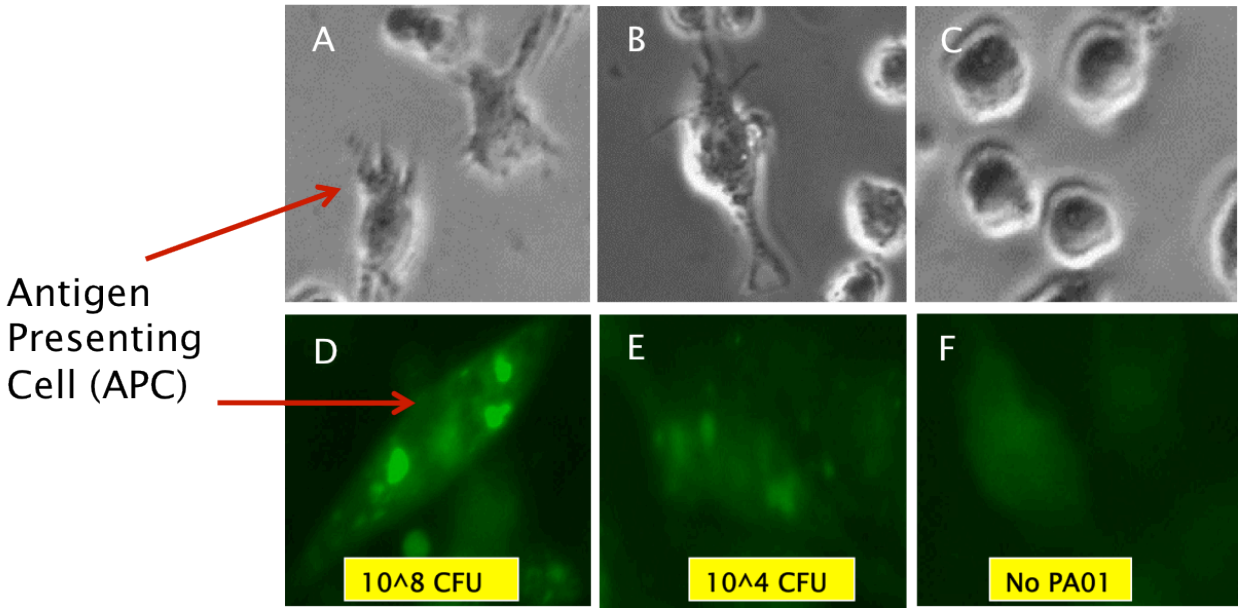


Figure 7. Morphological change in THP-1 and murine RAW264 monocytes upon infection with live *P. aeruginosa* (PA01) indicating ability to become antigen presenting cells. Top images are THP-1 cells under regular lighting and bottom images are murine RAW264 cells under fluorescent lighting taken with an inverted fluorescent microscope. **A.** 10^8 CFU of PA01 , **B.** 10^4 CFU of PA01, **C.** non-infected THP-1 monocytes. **D.** 10^8 CFU of PA01, **E.** 10^4 CFU of PA01 , and **F.** non-infected RAW murine monocytes.

Respiratory burst of primary CF and non-CF monocytes

Primary CF and non-CF monocytes were examined in a BioTek plate reader after being introduced to different dilutions of opsonized zymosan. In response to foreign particulates, monocytes normally produce reactive oxygen species (ROS) in order to break up the foreign matter. As seen in Figure 8, both the CF and non-CF monocytes exhibited ROS release in response to zymosan. However, primary CF monocytes appear to produce more reactive oxygen species and respond faster than primary non-CF monocytes (Figure 8). Nevertheless, after 20 minutes, primary CF monocytes exhibit a decrease in ROS release, but primary non-CF monocytes appear to continue to slowly increase the release of ROS over time in response to opsonized zymosan (see Figure 8).

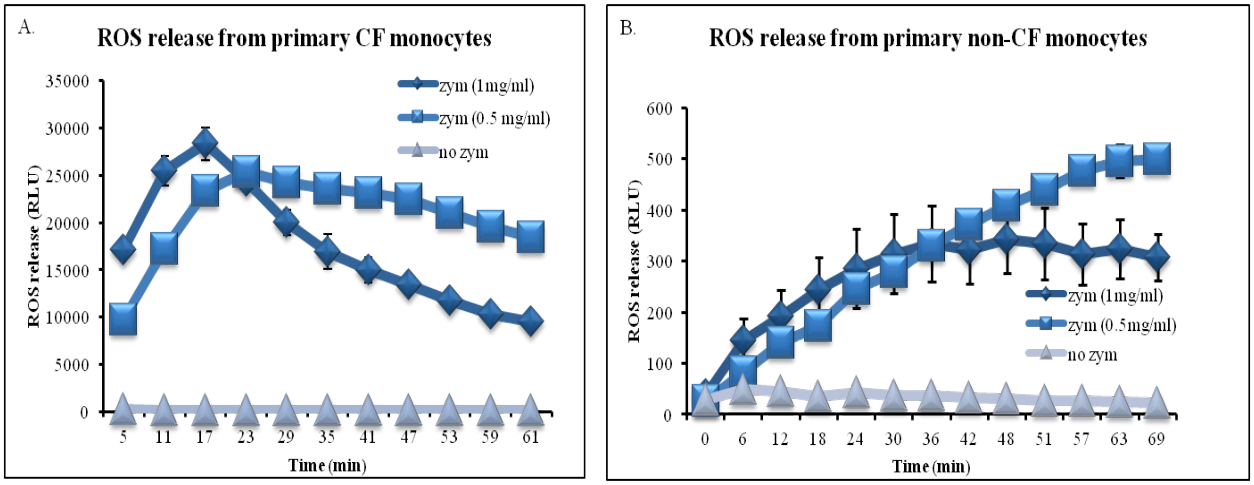
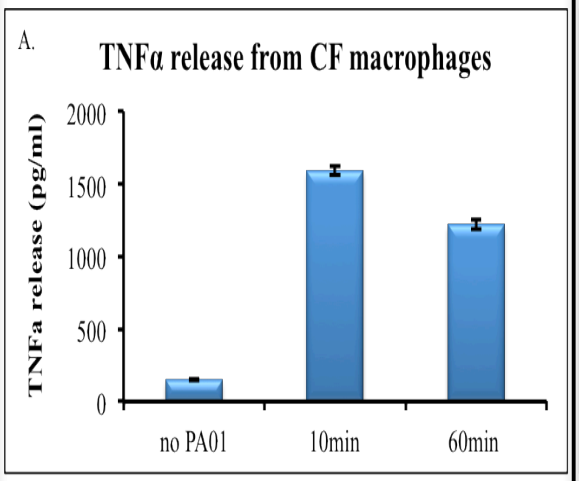


Figure 8. Normal respiratory burst of primary CF monocytes triggered with opsonized zymosan. **A.** Primary CF monocytes were analyzed for 61 minutes with a luminol probe after triggering with opsonized zymosan. Error bars show standard deviation for n= 3. **B.** ROS release from primary non-CF monocytes was measured for chemiluminescence over 69 minutes after triggering with opsonized zymosan. Error bars show standard deviation for n= 6.

Measuring the cell-signaling abilities of primary CF monocytes

An enzyme-linked immunosorbent assay (ELISA) was used to determine the ability of CF monocytes to produce cytokines after infection with PA01. CF monocytes appeared to produce cytokines in response to PA01 in a time-dependent (Figure 9) and somewhat dose-dependent (Figure 10) manner. The CF monocytes appeared to release less TNFα and IL-6 after 60 minutes of infection compared to 10 minutes of infection (Figure 9). Additionally, when the monocytes were infected with more PA01, they responded by releasing more cytokines (Figure 10). The supernatant from CF macrophages that were infected with 100ul of stock PA01 per ml of cells contained more cytokines than supernatant from CF macrophages that were infected with a 1x dilution (50ul of PA01 and 50ul of PBS) or 2x dilution (25ul of PA01 and 75ul of PBS).



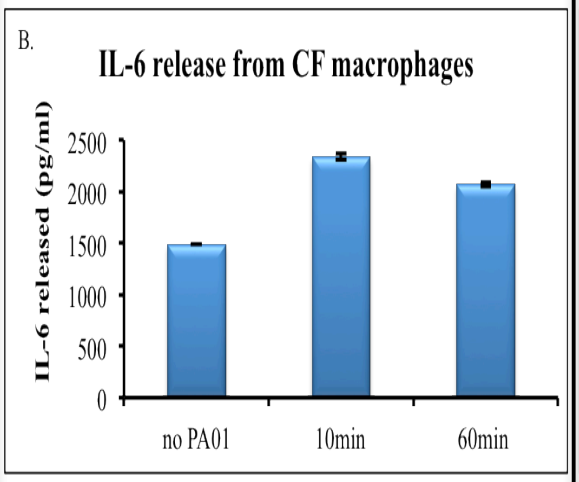
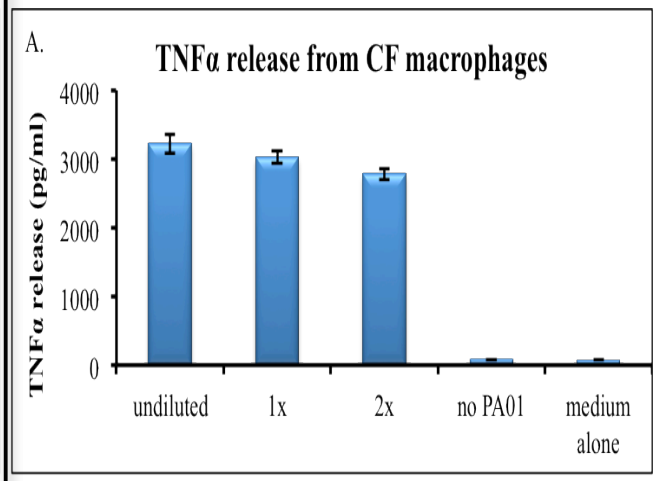


Figure 9. Cytokine production in CF macrophages upon infection with formalin fixed *P. aeruginosa*. A. TNFα and **B.** IL-6 cytokines are all detected in CF macrophages after infection with PA01. Error bars show standard deviation for n=4.



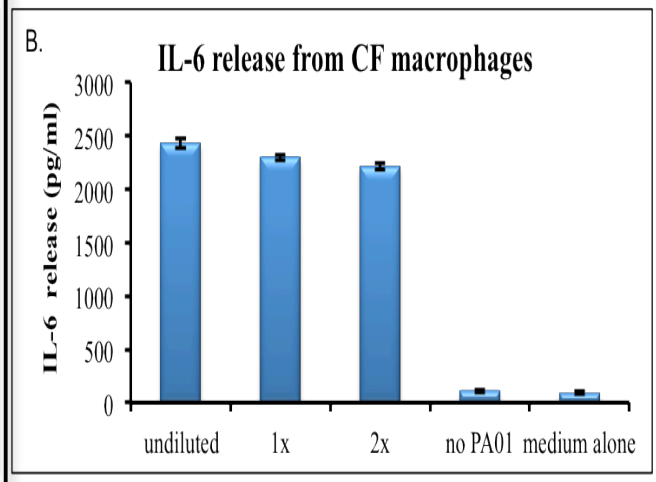


Figure 10. Cytokine production in CF macrophages upon infection with live *P. aeruginosa*. A. TNFα and **B.** IL-6 cytokines are all detected in the supernatant of CF macrophages after infection with PA01. Error bars show standard deviation for n=4.

In vitro bactericidal assay in RAW264 macrophages

The bactericidal ability of murine RAW264 macrophages was tested when cells were conditioned with CF and non-CF plasma, TNFα, IL-8, or medium alone overnight. When conditioned with CF or non-CF plasma, the cells exhibited increased bacterial survival compared

to RAW macrophages exposed to medium alone (Figure 11B). When the RAW macrophages were conditioned with cytokine TNFα or chemokine IL-8, there was an increase in bacterial proliferation compared to cells in medium alone as seen in Figure 11A. After 4 hours of infection, the RAW macrophages exhibited an increase in bacterial load compared with macrophages after 2 hours of infection, indicating an inability to kill bacteria (Figure 11). Conditioning the cells with a cytokine or chemokine appeared to cause an increase in bacterial growth; after 4 hours of infection the RAW macrophages conditioned with IL-8 appear to have a significant increase in the amount of colony forming units compared with 2 hours of infection (Figure 11).

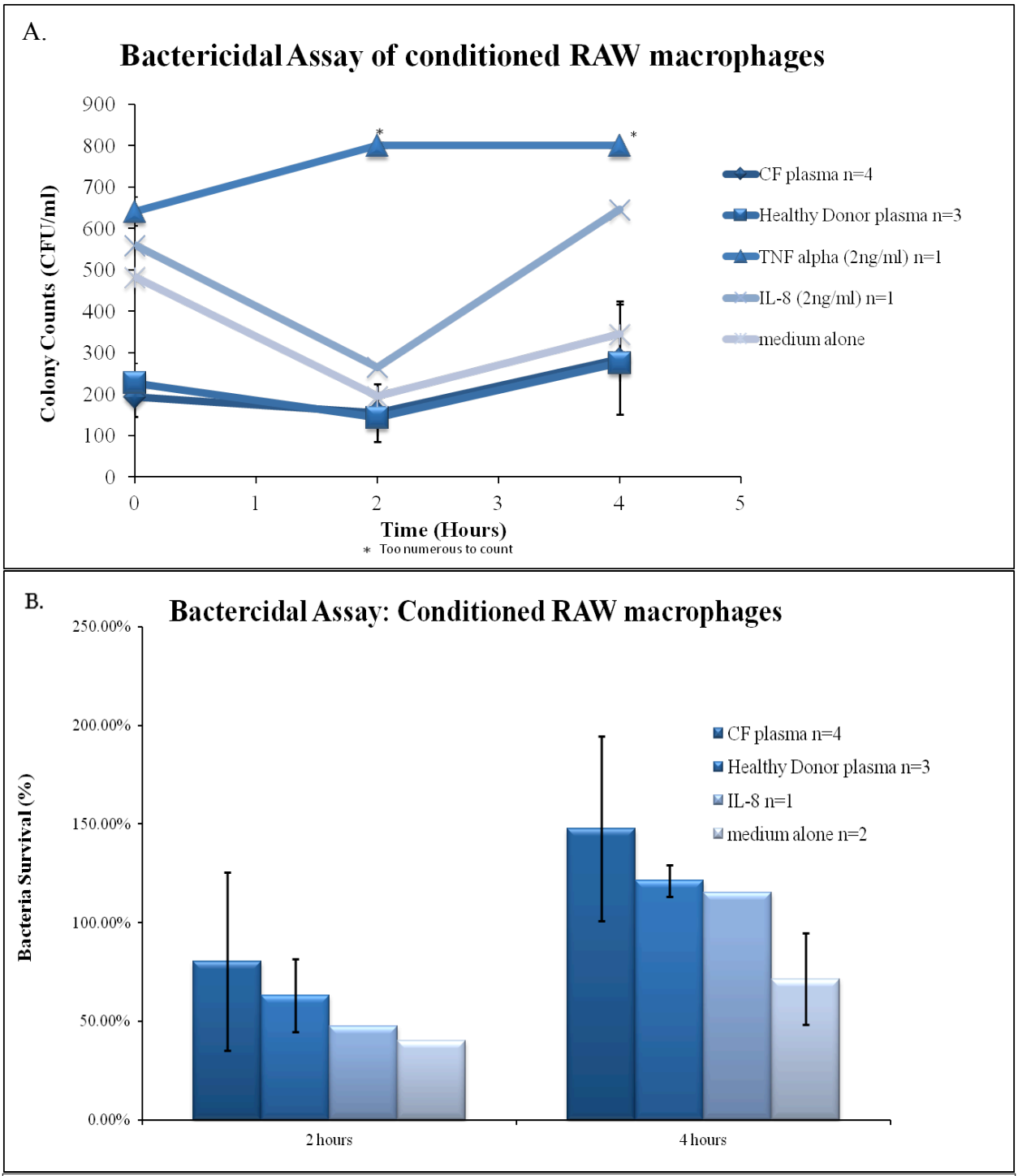


Figure 11. Bactericidal assay of conditioned murine RAW264 macrophages. Prior to exposure to PA01, RAW264 macrophages were conditioned with CF and non-CF plasma, TNFα, IL-8, or medium alone. **A.** The colony forming units of bacteria at time 0, 2, and 4 hours were counted after lysing the cells and plating the lysates on LB agar. **B.** The bacteria survival was calculated through using the 0 hour as the initial count of bacteria and dividing time 2 and 4 hour counts by time 0 counts.

***In vivo* bactericidal assay with CF and wild-type mice**

In order to test the ability of the CF mice to clear a bacterial infection, wild type and CF mice were infected with PA01 for 18 hours and cultures (Ctx) of the lungs and BAF were taken to assess the bacterial load. Although the amount of bacteria in the BAF was similar in both the CF and wild type mice, the lungs of the CF mice appeared to have a higher bacterial load after infection for 18 to 24 hours compared to the wild type mice (Figure 12). Figure 12 demonstrates a compilation of 4 independent experiments and the average of the colony forming units per ml of lung or BAF culture in the CF and wild type mice.

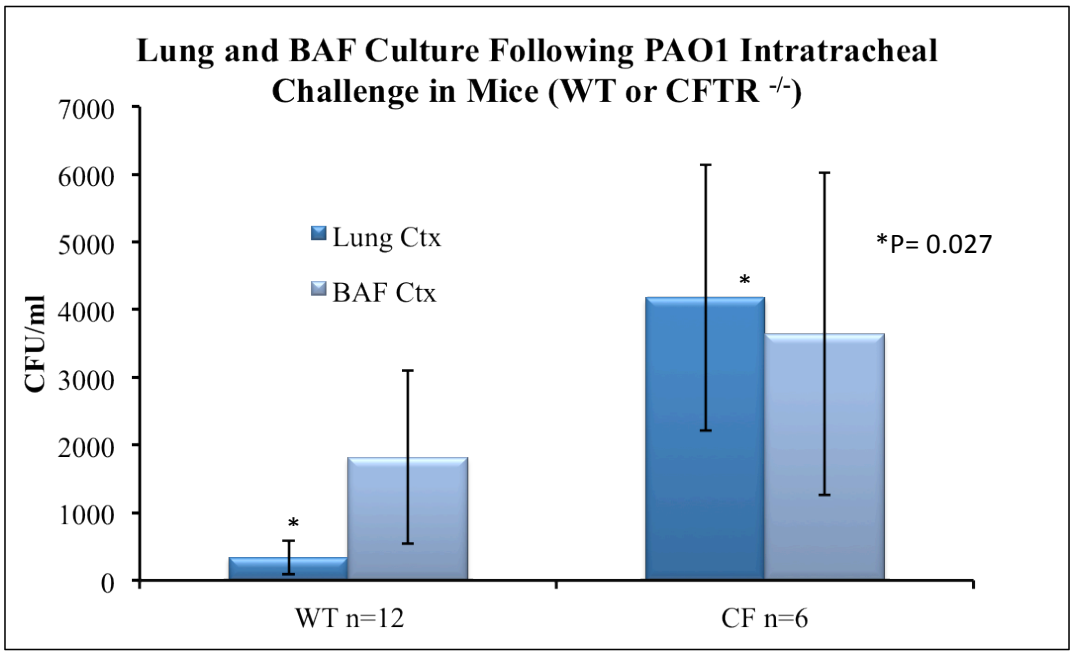


Figure 12. Lung and BAF bacterial load following PA01 instillation in CFTR$^{-/-}$ and wild-type mice. Cultures (Ctx) of the right upper lobe lung piece and the bronchoalveolar fluid (BAF) were taken in four different experiments and compiled in the graph above. In all, twelve wild type (WT) and six CF mice were used in this experiment and the averages of the colony forming units in the airway microbe clearance challenge were assessed. The error bars represent the means of the Standard Error and the P value for the lung cultures is based upon the Mann-Whitney test.

Discussion

It is thought that the inflammatory environment that CF perpetuates contributes to the inability of the CF lungs to effectively clear *P. aeruginosa* colonization and thus, to the deterioration of the lungs, which eventually leads to respiratory failure and death (33). CF monocytes have been found to have an innate defect in bacterial clearance (4). Although Heeckerson *et al*. found the bacterial burden of the lungs of CF verses non-CF mice were not different after 3 days of infection with *P. aeruginosa* laden beads (17), more recent studies demonstrate an inability of CF lungs to clear bacteria (4, 8, 26, 27). The differences between the two studies could have resulted from the use of the bacterial beads, which elicit an inflammatory response in wild-type mice as well as CF mice (8). Our *in vivo* bactericidal experiments demonstrated that CF mice exhibited a defect in airway microbial clearance upon exposure of *P. aeruginosa* compared to wild-type mice. Additionally, the *in vitro* bactericidal experiment revealed that if macrophages were exposed to cytokine TNFα or chemokine IL-8 before they were challenged with *P. aeruginosa,* more bacterial proliferation occurred. The results from the *in vitro* experiment also suggest that CF and healthy donor plasma have some antimicrobial activity as seen through the reduced bacterial proliferation in the cells at the beginning of infection compared with the cells subjected to other conditions. However, after 4 hours, the plasma appeared to inhibit the ability of the macrophages to effectively clear bacteria as more bacterial growth was observed over time. Thus, the inflammatory CF environment inhibits the bactericidal abilities of macrophages.

To determine if the inability of the CF macrophage to clear infection was due to the CF environment or genetics, the ability of the CF monocyte to respond to bacteria was evaluated in various milieus. The innate immune responses of CF monocytes were examined to determine if

the CF monocytes were defective in phagocytosis or cytokine/chemokine production under the varying conditions of our assays. The innate responses of CF macrophages were demonstrably different in magnitude and kinetics from their normal counterparts, although not necessarily diminished (Figure 8). The reason for this occurrence could be due to the defect in the CFTR channel, or due to the different environment each macrophage experienced. To determine if the environment or genetics was responsible for the defective phagocytic abilities of CF macrophage, a cell line would have to be constructed with one cell line containing the defect in the CFTR channel and the other without. Each cell line would then be subjected to either the same conditions or to CFTR conditioned and the phagocytic abilities would be measured.

To examine the ability of the primary CF macrophage to release oxygen radicals, ROS release from CF macrophages was compared to the ROS release from primary non-CF macrophages. The primary CF monocytes released more ROS and responded faster than primary non-CF monocytes to the presence of bacteria. However, after 20 minutes, the ROS release of the CF monocyte began to decrease, while the non-CF monocyte continued to respond to the *P. aeruginosa*. The increased response to infection indicates that CF monocytes are primed and in a constant pro-inflammatory state. On the other hand, the quick decrease in ROS after a short time of infection suggests that the CF monocytes deplete their resources faster and thus are unable to clear infection effectively.

Additionally, the CF monocytes produced cytokines in response to *P. aeruginosa* in a time-dependent and possibly dose-dependent manner. Although the amount of cytokine levels decreased after a two-fold reduction in bacterial infection, the levels did not decrease two-fold as well. One explanation for this phenomenon is that the cells were already saturated to their full capacity to react and produce cytokines. Moreover, after 60 minutes of infection, the CF

monocytes experienced a decrease in the amount of cytokine release of TNFα and IL-6, further indicating that after exposure to infection, CF monocytes become frustrated quickly and therefore ineffectively clear bacteria.

Although CF monocytes are defective in killing bacteria, their ability to ingest bacteria remains intact as seen in the robust ability of the macrophages to ingest *P. aeruginosa* assayed through the confocal and TIRF imaging of the monocytes after infection. Thus, a combination of genetics and environmental factors are the most likely contributor to the inability of macrophages to clear bacteria in the CF environment.

Future Direction

Our next step in examining the bactericidal and phagocytic abilities of CF and wild-type macrophages in a CF environment will be to examine the bactericidal activity of primary CF and non-CF macrophages after conditioning with CF sputum and combined cytokines.

Due to the complex nature of the bactericidal assays, the experiments that have been conducted with CF sputum to date have failed to produce consistent results. The viscous nature of human CF sputum and possible cross-reactivity of human cytokines with murine macrophages might have been the source of complication. Thus, the experiments will be repeated after modification. One possible modification to make would be to the preparation of the sputum through using deoxyribonuclease (DNase) to break the DNA fibrin complexes, which could be a contributing factor to the clumping of the cells. In addition, replacing the use of the 48 well plates with tubes in order to centrifuge the samples will combat the clumping effect of the macrophages and may provide a way forward. Additionally, the use of human CF monocytes in place of the RAW macrophages will be considered for future experiments in order to make the experiments more applicable to the conditions that CF patients experience.

Furthermore, the 96 well plate format in the bactericidal challenge might be used in future experiments in order to better perform the bactericidal experiments. Using 96 well plates will allow the monocytes and bacteria to be spun in a centrifuge in order to better infect the monocytes. Through the centrifugal force, the bacteria would be more likely to come in contact with the macrophages and represent a more accurate model of infection. In addition, the optical density will be able to be measured in place of plating the bacteria to assess the bacterial count. Due to the fluorescent properties of the *P. aeruginosa*, the fluorescence will also be gauged as an additional measurement of the amount of surviving bacteria.

In an effort to review if the exaggerated ROS response of the CF macrophages to infection does frustrate the cells more over time compared to non-CF macrophages, the respiratory burst assay will need to be repeated with primary CF and non-CF macrophages in the same experiment. In addition, the respiratory burst of the monocytes after conditioning with different doses of cytokines or sputum can be assessed to determine if the CF environment does elicit more ROS release and stress on the macrophages in response to infection.

Moreover, the cell signaling abilities of CF monocytes and non-CF monocytes should be tested and compared. In order to see if the cell signaling abilities of monocytes are dose-dependent, the amount of bacteria or detection antibody should be significantly reduced to assess the macrophages under non-saturated conditions. In addition, cytokine levels in monocytes should also be recorded after conditioning the monocytes with CF sputum in order to determine if the CF environment elicits cytokine production.

Due to the hardships we faced in obtaining CF and non-CF macrophages, the use of harvesting peritoneal or bone marrow derived mouse macrophages per the Fortier and Falk

method (1994) as described in the Zhang X *et al*. (2010) paper should be considered for future experiments.

We will further test the macrophages' bactericidal, phagocytic, and cell-signaling properties after gene therapy is applied to the CF monocytes. Through transfecting the bone marrow derived cells of CF mice with a construct expressing corrected CFTR protein, the macrophages will regain CFTR function. Comparing CF mice, genetically modified CF mice, and wild-type mice in a bactericidal challenge, ROS response, and cell-signaling assay, we will be able to determine the potential usefulness of bone marrow derived genetic therapy for cystic fibrosis patients.

References

1. **Wilfond BS and Taussig LM**. Cystic Fibrosis: General Overview. *Pediatric Respiratory Medicine*. 66: 982-1075, 1999.

2. **Ratjen F, Barker A, O'Donnell AE**. Case Study Investigations: Integrating Clinical Advances to Optimize Pharmacologic Management of CF and Non-CF Bronchiectasis. *Gilead Sciences Medical Affairs*. 3-28, 2001.

3. **Leigh MW, Knowles MR, and Boucher RC**. Cystic Fibrosis: Genetics and Disease Mechanism. *Pediatric Respiratory Medicine*. 67: 991- 1099, 1999.

4. **Porto PD, Cifani N, Guarnieri S, Di Domenico EG, *et al.*** Dysfunctional CFTR Alters the Bactericidal Activity of Human Macrophages against *Pseudomonas aeruginosa*. *PLoS One*. 6(5): e19970. doi:10.1371/journal.pone.0019970, 2001.

5. **Matsui H, Grubb BR, Tarran R, Randelll SH, Gatzy JT, *et al***. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Science.* 95: 1005–1010, 1998.

6. **Bruscia EM, *et al***. Macrophages Directly Contribute to the Exaggerated Inflammatory Response in Cystic Fibrosis Transmembrane Conductance Regulator -/- Mice. *Am J Resp Cell and Mole Biol*. 40: 295-304, 2004.

7. **Silvia, MT**. Macrophage phagocytosis of neutrophiles at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. *J Leukoc Biol*. [ahead of print] 2010.

8. **Ratner D and Mueller C**. Immune responses in Cystic Fibrosis; are they intrinsically defective?. *Am J Respir Cell Mol Biol*. [ahead of print] 2012.

9. **Sajjan U, Thanassoulis G, Cherapanov V, Lu A, Sjolin C, Steer B, Wu, YJ, Rotstein OD, Kent G, McKerlie C, Forstner J, and Downey GP**. Enhanced Susceptibility to Pulmoary Infection with *Burkholderia cepacia* in Cftr -/- Mice. *Infect Immun*. 69(8): 5138-5150, 2001.

10. **Delves PJ and Roitt IM**. The immune system. First of two parts. *N Engl J Med*. 343(1): 37-49, 2000.

11. **Haggie PM and Verkman AS**. Cystic Fibrosis Transmembrane Conductance Regulator-independent Phagosomal Acidification in Macrophages. *The Journal of Biological Chemistry*. 282(43): 31422-31428, 2007.

12. **Di A, *et al*.** CFTR regulates phagosome acidification in macrophages and alters bactericidal acitivity. *Nat Cell Biol*. 8(9): 933-944, 2006.

13. **Zhang Y, Li X, Grassmé H, Döring G, and Gulbins E.** Alterations in ceramide concentration and pH determine the release of reactive oxygen species by Cftr-deficient macrophages on infection. *J Immunol*. 184 (1): 5104-5111, 2010.

14. **DiCiccio JE and Steinberg BE**. Lysosomal pH and analysis of the counter ion pathways that support acidification. *JGP*. 137 (4): 385-390, 2011.

15. **Haggie PM and Verkman AS.** Defective organellar acidification as a cause of cystic fibrosis lung disease: reexamination of a recurring hypothesis. *Am J Physiol Lung Cell Mol Physiol*. 296: L859-L867, 2009.

16. **Vandivier WR, *et al*.** Dysfunctional cystic fibrosis transmembrane conductance regulator inhibits phagocytosis or apoptotic cells with proinflammatory consequences. *Am J Physiol Lung Cell Mol Physiol*. 297: L677-L686, 2009.

17. **Saiman L, Cacalano G, Gruenert D, Prince A**. Comparison of adherence of *Pseudomonas aeruginosa* to respiratory epithetial cells from cystic fibrosis patients and healthy subjects. *Infect Immun.* 60(7): 2808-2814, 1992.

18. **Heeckeren A, Walenga R, Konstan MW, Bonfield T, Davis PB, Ferkol T.** Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with Pseudomonas aeruginosa. *J Clin Invest*. 100 (11): 2810-2815, 1997.

19. **Machen TE**. Innate immune response in CF airway epithelia: hyperinflammatory?. *Am j Physiol Cell Physiol*. 291(2): C218-C230, 2006.

20. **Oliver EM and Weir DM**. The effect of *Pseudomonas* alginate on rat alveolar macrophage phagocytosis and bacterial opsonization. *Clin. exp. Immunol.* 59: 190-196, 1985.

21. **Greally P, Hussein MJ, Cook AJ, Sampson AP, Piper PJ, and Price JF.** Sputum tumour necrosis factor-α and leukotriene concentrations in cystic fibrosis. *Archives of Disease in Childhood.* 68: 389-392, 1993.

22. **Karpati F, Hjelte FL, and Wrelind B**. TNF-alpha and IL-8 in consecutive sputum samples from cystic fibrosis patients during antibiotic treatment. *Scand J Infect Dis*. 32(1):75-79, 2003.

23. **Brennan S, Sly PD, Gangell CL, Sturges N, Winfield K, Wilstron M, Gard S, Upham JW**. Alveolar macrophages and CC cehmokines are increased in children with cystic fibrosis. *ERJ*. 34(3): 655-661, 2009.

24. **Yarden J, Radojkovic D, De Boeck K, Macek M Jr, Zemkova D, Vavrora V, Vlietinck R, Cassiman JJ, and Cuppens H**. Association of tumour necrosis factor alpha variants with the CF pulmonary phenotype. *Thorax*. 4: 320-325, 2005.

25. **Loi R, *et al***. Limited Restoration of Cystic Fibrosis Lung Epithelium In Vivo with Adult Bone Marrow-derived Cells. *Am J Repir Crit Care Med*. 173: 171-179, 2006.

26. **Heeckeren AM, *et al***. Role of Cftr in the response to chronic *Pseudomonas aeruginosa* lung infection in mice. *Am J Physiol Lung Cell Mon Physiol* 287: L944-L952, 2004.

27. **Sorio C and Melotti P**. Editorial: The role of macrophages and their scavenger receptors in cystic fibrosis. *Journal of Leukocyte Biology*. 86 (3): 465-468, 2009.

28. **Allen RC**. Phagocytic leukocyte oxygenation activities and chemiluminescence: a kinetic approach to analysis. *Methods Enzymol*. 133: 449-493, 1986.

29. **Fuller MD, Zhang Z, Cui G, Kubanek J, McCarty NA**. Inhibition of CFTR channels by a peptide toxin of scorpion venom. *Am J Physiol Cell Physiol*. 287: C1328-C1341, 2004.

30. **Zughaier SM, Ryley HC, Jackson SK**. Lipopolysaccharide (LPS) from Burkholderia cepacia is more active than LPS from Pseudomonas aeruginosa and Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes. *Infect Immun*. 67(3): 1505-1507, 1999.

31. **Zughaier SM, Ryley HC, Jackson SK**. A melanin pigment purified from an epidemic strain of Burkholderia cepacia attenuates monocyte respiratory burst activity by scavenging superoxide anion. *Infect Immun*. 67(2): 908- 913, 1999.

32. **Helms MN, Torres-Gonzalez E, Goodson P, Rojas M**. Direct tracheal instillation of solutes into mouse lung. *J Vis Exp*. 42: 1941, 2001.

33. **Aitken ML, Fiel SB, Stern RC**. Cystic Fibrosis: Respiratory Manifestations. *Pediatric Respiratory Medicine*. 68: 1009-1032, 1999.

34. **Fortier AH and Falk LA**. Isolation of Murine Macrophages. *Curr Protoc Immunol*. 14(1): 1-9, 1994.

35. **Zhang X, Goncalves R, and Mosser DM**. The Isolation and Characterization of Murine Macrophages. *Curr Protoc Immunol*. 14(1): 1-18, 2010