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Yash Patel April 12, 2017

Impact of Activation and Airway Transmigration on Human Neutrophil Metabolism

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An abstract of
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Abstract

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Recent studies of dendritic cells and macrophages have shown that these critical regulators of innate and adaptive immunity undergo profound metabolic reprogramming during activation. Neutrophils are the most abundant leukocyte subset in human blood and the first line of defense against pathogens in the body. Prior studies in inflammatory diseases such as cystic fibrosis (CF) suggest that similar metabolic changes are seen upon neutrophil activation to those described in activated dendritic cells and macrophages. Specifically, in the context of CF, neutrophils migrate into the lung lumen in large numbers and display an array of new functions therein, suggesting metabolic licensing, i.e., the ability to use exogenous nutrients to adapt to a new environment. However, metabolic changes in activated neutrophils remain poorly characterized so far, and this study proposes to tackle this current gap in knowledge. The significance of this study is that neutrophils are recognized to play a critical role in the progressive destruction of the CF lungs via pathological reprogramming, and that a better understanding of their metabolic regulation will bring about potential new avenues for therapy. We hypothesize that upon blood neutrophil exposure to pro-inflammatory mediators (PMA, fMLF, LPS, LTB4) there will be an increase in oxygen consumption rate (OCR, reflecting NADPH oxidase activity), and in extracellular acidification rate (ECAR, reflecting extracellular lactate production by glycolysis). Furthermore, we hypothesize that these effects will be further amplified in neutrophils transmigrated *in vitro* to mimic those found in the lumen of the lung. Understanding the effect of agonists on metabolism of PMNs, the most abundant leukocyte subset in humans, is an ongoing endeavor. Here in this study, we provide proof of concept for the use of the Seahorse bio-analyzer to study the impact of various mediators on PMN metabolism (measured as changes in extracellular acidification rate, ECAR, and oxygen consumption rate, OCR). Also we are able to show pilot data suggesting PMNs have the ability to be further activated after having been submitted to recruitment and activation by CF ASN in the transmigration model which was not the prior understanding.

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Finally, I would like to thank the Emory Children's Center for providing me with the resources for my experiments.

Table of Contents

List of Abbreviations

ALI, air-liquid interface

ASL, Airway surface liquid

ASN, Airway supernatant

ATP, Adenosine triphosphate

CF, Cystic Fibrosis

CFTR, Cystic Fibrosis Transmembrane conductance Regulator

COPD, Chronic obstructive pulmonary disease

DPI, Diphenyleneiodonium

F, Phenylalanine

FACS, Fluorescence-activated cell sorting

fMLF, formyl-methionyl-leucyl phenylalanine

GSH, Glutathione

HC, Healthy control

LTB4, Leukotriene B4

LPS, Lipopolysaccharide

NE, Neutrophil Elastase

PMA, Phorbol 12-myristate acetate

PMN, Polymorphonuclear neutrophils

SCN, Thiocyanate

2-DG, 2-Deoxy-D-glucose

I. Introduction

Disease burden of cystic fibrosis (CF):

Cystic Fibrosis (CF) is the most common fatal genetic disease among Caucasians. CF is best characterized as an exocrinopathy involving a disturbance in fluid and electrolyte transport eliciting multi-organ dysfunction[1]. There are currently approximately 70,000 individuals living with CF worldwide and 1,000 new CF cases diagnosed each year in the United States[2]. Between 2000 and 2010, the number of patients represented in the Cystic Fibrosis Foundation Patient Registry (CFFPR) increased from 21,000 to 26,000. Pulling from this same registry, median age increased from 14.3 to 16.7 years and adjusted mortality decreased by 1.8% per year. Furthermore, median survival of children born and diagnosed with CF in 2010 was projected to be 37 years for females and 40 years for males if mortality remained at levels observed in 2000. However, if mortality continues to decrease at the rate observed between 2000 and 2010, median survival for males and females may exceed 50 years implicating a growing population of CF adults[3]. To help this growing population, further research is needed to achieve better care.

Genetic basis of CF and pathophysiology:

Cystic Fibrosis is an autosomal recessive disorder caused by mutations in a single gene on the long arm of chromosome 7 which encodes the cystic fibrosis transmembrane regulator protein (CFTR)[4]. CFTR is an ATP- and phosphorylation-regulated anion channel[5]. The CFTR protein is composed of five domains: two nucleotide-binding domains, two six-transmembrane domains, and a regulatory region[6]. Fundamentally, the CFTR acts to maintain the osmolar, pH, and redox balance of the fluids lining the epithelial surfaces of the sweat glands, genital tract, digestive system, sweat glands, and lung by allowing a route for chloride, bicarbonate,

thiocyanate (SCN), and glutathione (GSH) ions to follow their respective concentration gradients from the cytosol into the lumen[7]. The CF gene is large, spanning 250 kb, and is composed of 27 exons. Since the identification of the gene, over 1,800 disease-causing mutations in the CF gene have been reported to the CF Genetic Analysis Consortium database. Of these 1,800 mutations reported, only 22 mutations have been identified with a frequency of at least 0.1% of known alleles and the remaining mutations are very rare, being limited to a few individuals. The ΔF508 mutation, specifically, is a three base pair deletion that codes for phenylalanine (F) at position 508 of the CFTR protein and is the most common mutation accounting for 70% of alleles[8]. Ultimately, mutations of the CFTR can elicit abnormal folding, defective membrane insertion, and/or conductance disrupting movement of anions, such as chloride, bicarbonate, SCN, and GSH. Within CF airways, the disruption of CFTR function is thought to lead to abnormal airway surface liquid (ASL) properties. However, it remains unclear whether an anomaly in the osmolarity / hydration / viscosity, pH, redox balance, or another property of the ASL begins the pathological process observed in CF airways[10]. As of today, CF airway disease is best understood as a triad of inflammation, obstruction, and infection (**Figure 1**), with no clear understanding of the initiating factor(s)[11]. Although the primary focus of the Tirouvanziam lab at Emory is to defeat airway disease, which is the main cause of morbidity and mortality in CF, this disease also adversely affects other organs, including but not limited to the following symptoms: chronic sinusitis, impaired digestive function, impaired pancreatic activity, and osteoporosis[12].

PMN-mediated inflammation in CF:

Impaired mucociliary clearance, bacterial infection, and neutrophilic inflammation are all hallmarks of CF airway disease[13]. Zeroing in on the inflammatory aspect of CF, the recruitment

of polymorphonuclear neutrophils (PMN) is the primary driving force. PMNs represent 50-70% of leukocytes in the human blood and form the first barrier during host defense, participating in diverse processes of the inflammatory response[14]. Blood PMNs are characterized by the presence of cytoplasmic granules replete with proteolytic and oxidative enzymes that allow them to kill pathogens intracellularly after uptake by phagocytosis, or extracellularly upon degranulation. Furthermore, recent discoveries have shown that PMNs have the ability to release DNA-based neutrophil extracellular traps (NETs) in a process dubbed "NETosis"[15]. During NETosis, DNA is decondensed, released along with histones, and combined with cationic primary granule proteins ultimately forming extracellular traps with antimicrobial activities[16]. The recruitment of blood PMNs into CF lungs coincides with the failure to clear bacteria, including *Staphylococcus aureus* and *Pseudomonas aeruginosa.* In CF, PMN-dominated inflammation does not halt upon failure to clear bacteria, but rather continues with the constant recruitment of new waves of blood PMNs to the airways. This leads to proteolytic destruction of the airway tissue by PMN proteases, progressively leading to respiratory failure[17]. This relentless, non-resolving PMN recruitment is believed to be modulated by altered responsiveness to host damage signals, protein and lipid chemoattractants and/or microbial colonization[18]. Prior understanding of PMNs in CF was that they acted as mere bystanders, unable to carry out their normal antimicrobial function and dying rapidly within the airways. However, recent fluorescence-activated cell sorting (FACS) analyses of CF airway fluid by the Tirouvanziam group have disproved this conventional paradigm and showed that PMNs remain alive after recruitment to the CF airway lumen. Rather than rapid death, PMNs are functionally and metabolically reprogrammed[19], upending our understanding of both the onset and progression of CF disease, and strategies for therapeutic targeting.

Metabolic licensing of neutrophils:

The CF airway lumen presents a distinct microenvironment with regards to its oxygen and metabolite contents, eliciting several recruiting and activating mechanisms in incoming PMNs. In contrast to the normal oxygen-rich airway lumen displayed in healthy subjects, diseased areas within CF airways are filled by PMN clusters, bacterial/fungal colonies, inspissated extracellular scaffolds of mucus, DNA, and actin, all leading to significant oxygen depletion[20,21]. These localized hypoxic conditions can trigger profound inflammation in response to Damage Associated Molecular Patterns (DAMPs) released upon hypoxic necrosis of host epithelial cells. Animal studies suggest that there is an increased abundance of necrotic epithelial cells in mucus-obstructed airways of CF mice. These results suggest a potential role of hypoxic epithelial necrosis in the pathogenesis of PMN inflammation, independently of, and possibly prior to, microbial infection[22]. Beyond local hypoxic conditions, the CF airway environment also displays a unique metabolite composition which can be linked to the lack of CFTR function. As discussed above the CFTR was first discovered to be an apical chloride and bicarbonate channel, but after further research has been shown to be involved in the transport/maintenance of the redox intermediates GSH and SCN[23].

Fundamental alterations in the composition the CF airway microenvironment is believed to provide the driving force for the observed adaptive reprogramming of PMNs affecting their recruitment and function. *At baseline*, PMNs primarily use aerobic glycolysis as their main pathway of ATP production, due to their relative lack of mitochondrial metabolism, thus consuming little oxygen[24]. However, upon activation by agonists (discussed further in the next section) or phagocytosis of antibody-coated particles, PMNs increase their consumption of oxygen, along with their uptake of glucose[25]. The influx of glucose in activated PMNs supports

an increase in glycolysis. The influx of oxygen is not used for oxidative phosphorylation in the mitochondria, but rather is seen to fuel the oxidation by NOX enzymes of NADPH produced by glycolysis, leading to reactive oxygen species production. Examples of reactive oxygen species produced by activated PMNs include hydrogen peroxide and hypochlorous acid (the active component of bleach), a byproduct of the enzyme myeloperoxidase exocytosed from primary granules (**Figure 3**), at the same time as NE[20,26]. Oxidation of the CF airway microenvironment affects GSH, which is a critical antioxidant therein. Indeed, studies have shown that GSH concentration was lower in bronchoalveolar lavage from children with CF, whereas GSH sulfonamide, a specific oxidation product of hypochlorous acid, was higher[27].

Study rationale:

In the context of CF, PMNs migrate into the lung lumen in large numbers and display an array of new functions therein, suggesting metabolic licensing, i.e., the ability to use an array of exogenous nutrients to adapt to a new environment[11,14]. However, metabolic changes in activated PMNs remain poorly characterized so far, and this study proposes to tackle this current gap in knowledge. PMNs are recognized to play a critical role in the progressive destruction of CF airways, via pathological reprogramming processes discussed above.

In order to explore metabolic reprogramming triggers and respective reactions in PMNs, we studied the effects of specific agonists and chemoattractants in the Seahorse energetic bio-analyzer. Leukotriene B4 (LTB4), phorbol 12-myristate 13-acetate (PMA), formyl-methionyl-leucyl phenylalanine (fMLF), and lipopolysaccharide (LPS) were used as prototypical "environmental cues" and metabolic changes taking place upon PMN activation (post-injection of metabolic modulators in the bio-analyzer) were measured. In brief, endogenous ligands

leading to PMN activation include LTB4, known to stimulate PMN migration, aggregation, and to a lesser degree, degranulation[28]; and PMA, a protein kinase C agonist that activates Nox activity in PMNs[30]. Exogenous, microbial ligands were represented by fMLF, a bacterial tripeptide known to attract and activate circulating blood PMNs by binding to specific G-protein coupled receptors[31]; and LPS, an endotoxin present in the outer membrane of gram-negative bacteria that induce a spectrum of biological effects on host cells, including PMNs[31]. Metabolic modulators used in the energetic bio-analyzer includes diphenyleneiodonium (DPI), a Nox inhibitor; 2-deoxyglucose (2-DG), a glucose analog with the 2-hydroxyl group replaced by hydrogen, leading to inhibition of glucose-6-phosphate, the rate-limiting enzyme in glycolysis[32].

We hypothesized that blood PMN exposure to agonists (PMA, fMLF, LTB4, LPS) would increase oxygen consumption rate (OCR, reflecting Nox activity), and extracellular acidification rate (ECAR, reflecting extracellular lactate production linked to glycolysis). Furthermore, we hypothesized that these effects would be further amplified in PMNs transmigrated *in vitro* to mimic the reprogramming observed in CF airways *in vivo*.

II. Materials and Methods

Sample collection and processing:

Venous blood was drawn from healthy donors in sterile vacutainer blood collection tubes containing potassium EDTA on the day of the planned Seahorse assay. 5 mL of blood was layered onto 5 mL of Polymorphprep (Nycomed, Zürich, Switzerland) [active ingredients: sodium diatrizoate (13.8% w/v) and dextran 500 (8.0% w/v); density:1.113±0.001g;], in a sterile plastic tube. Blood was then spun for 45 minutes at 350G at 20ºC to create a density gradient (**Figure 2**). Using a sterile suction dropper, the PMN-containing layer was transferred to a sterile

50 mL tube and sterile 0.45% NaCl was added to the PMNs in 1:1 ratio to restore osmolarity. PMNs were centrifuged for 10 minutes at 350 G at 20ºC again and the supernatant was decanted. To lyse remaining erythrocytes in solution, 12 mL of sterile ice cold water was added and gently mixed with cell pellet and within exactly 30 seconds, 12 mL of ice-cold sterile 1.8% NaCl solution was added. Subsequently, a 5-minute spin at 350 G at 4ºC was performed and the lysis step was repeated until the pellet was visibly white, and devoid of contaminating erythrocytes. After decanting the supernatant, isolated PMNs were resuspended in 4 mL of assay medium (20 mL XF base medium + 400 µL 200 mM glutamine + 44 µL 2.5 M glucose) and counted on a hemacytometer[19]. To this end, 20 µL of the PMN suspension in assay medium were stained with 20 µL of Trypan blue in an Eppendorf tube to assess cell viability. The hemacytometer slide and cover slip were rinsed with 70-95% ethanol and gently wiped with Kimwipes to ensure maximal cleanliness. The clean coverslip was placed on top of the dry slide to cover the reflective surfaces (Figure 3). Using a micropipette, 10 µL of the cell suspension was quickly and smoothly added to the v-shaped groove on each side of the hemacytometer. Using a compound microscope, the cells appearing as green dots were counted as live PMNs in each quadrant. The resulting average number of alive PMNs in the 4 quadrants was multiplied by 2 due to the dilution factor and then by 10^4 to determine the concentration of live PMNs per ml of initial resuspension.

Seahorse energetic bio-analyzer assay:

One day prior to the assay, XFp sensor cartridges were hydrated in XF calibrant. First, the utility plate (clear) and sensor cartridge (green) were separated and the sensor cartridge (green) was placed upside down on the bench (**Figure 6**). Each well of the utility plate (clear) was filled with 200 µL of XF calibrant. The moats surrounding the wells were each filled with

400 µL of XF calibrant. The sensor cartridge (green) and utility plate (clear) containing the calibrant were put together again and stored in a 37ºC, non-CO_2 incubator overnight. In addition, XFp microplates were coated with Cell-Tak (**Figure 4**). Immediately before adding to the plate, 295 µL of .1M sodium bicarbonate, 1.6 µL of 1N NaOH, and 3.8 µL of Cell-Tak (stock at 2.03 µg/µL) were mixed for a final concentration of 22.4 µg/mL of Cell-Tak. Ten µL of this working concentration were added to each well of the XFp microplate.

On the day of the assay, the first step was to prepare the assay medium to be used as diluent throughout the experiment. 20 mL of of XF base medium, 400 µL of 200 mM glutamine (final concentration: 4 mM), and 44 µL of 2.5 M glucose (final concentration: 5.5 mM) were mixed and adjusted to a pH of 7.4 with .1 N NaOH (~1drop). Polymorphprep was performed as described above and the purified blood PMNs were resuspended in assay medium at 75,000 cells/well or $1.5x10^6$ /mL, and 50 µL of PMNs were added to each well of the XFp microplate pre-coated with Cell-Tak (**Figure 4**). The XFp microplate with the newly added PMNs was centrifuged at 300g for 1 minute with no brake, and 130 µL of assay medium was added to each well, for a total volume of 180 µL, and placed in a 37ºC non-CO_2 incubator for 45-60 minutes.

During this incubation period, stocks of metabolic mediators intended to be injected in the Seahorse energetic bio-analyzer were prepared for addition to the injection ports in the sensor cartridge (**Figure 5**). After these mediators were diluted in assay medium to their appropriate concentrations, they were added into the ports. In order to maintain a sufficient concentration within the cell culture microplate: 20 µL of compound 1 was added to port A, 22 µL of compound 2 was added to port B, 25 µL of compound 3 was added to port C, and finally 27.5 µL of compound 4 was added to port D. The hydrated utility plate with the now loaded sensor cartridge was loaded onto the instrument tray of the Seahorse machine. The cartridge lid was

removed and the orientation was checked in order to make sure everything matched up with the programmed map of the cartridge on the machine. The sensor cartridge was subsequently taken into the machine for calibration and once the calibration completed, the hydrated utility plate was removed off the tray while the sensor cartridge was still within the machine. The prepared XFp microplate stored in the 37ºC non-CO_2 incubator was placed on the tray with the correct orientation. Plate maps varied for each experiment relative to the metabolic mediators , concentrations, and conditions used. Wells A and E were always set as control wells, leaving wells B-G for experimental use.

Transmigration model:

To mimic PMN migration into CF small airways, the Tirouvanziam lab developed a 3D model of human epithelium. This model is based on the growth of the human Club cell line H441 as a monolayer maintained at air-liquid interface (ALI) on top of an Alvetex (Reinnervate) 200 µm-thick inert 3D scaffold, amenable to PMN loading onto the basal side (lamina propria), followed by transepithelial migration into the apical side (lumen). Alvetex scaffolds are at >90% porosity, with pore sizes ranging from 36-40 µm, with interconnects of 12-14 µm.

The first step was to remove Alvetex inserts from the package and gently snap off their prongs such that they fit into a non-tissue culture-treated 12-well plate. Using a few drops of 70% ethanol, the inserts were activated, rendering the Alvetex membrane hydrophilic. Alvetex membranes were then coated overnight at 37^{o}C with rat tail collagen (3 mg/mL, Sigma) and seeded with H441 cells at 0.25 million cells per insert. Seeded H441 cells were then allowed to grow and expand while submerged in DMEM/F12 supplemented with 10% heat-inactivated serum, penicillin, and streptomycin. After 2 days, cells were supplemented basally with serum-

free DMEM/F12 with 10% Ultroser G (Pall Life Sciences) to establish ALI. Cultures were then grown for 2 weeks at ALI and supplemented basally with fresh medium every 48 hours.

After 2 weeks at ALI, Alvetex inserts were inverted and placed with the apical compartment exposed to either RPMI, LTB4 (100 nM) or CF airway supernatant (ASN, corresponding to expectorated sputum expectoration, doubly centrifuged at 800G and 3000G to remove host cells and microbes). Then, 0.5-1x10^6 PMNs were loaded onto the basal compartment of the Alvetex scaffold (situated upside) and allowed to migrate at 37°C at 5% CO_2 through the collagen and epithelial layer (**Figure 11**), into the apical compartment (situated downside) in order to mimic the physiologically relevant basolateral-to-apical migration of PMNs. Transmigrated PMNs were collected from the apical compartment into RPMI and counted before being re-suspended in Seahorse assay medium for analysis, as discussed above.

III. Results:

LPS and fMLF do not robustly increase OCR and ECAR in healthy PMNs.

LPS is a component of the outer membrane of Gram negative bacteria and can crosslink receptors on immune cells. fMLF is a bacterial and mitochondrial component that also binds to specific receptors to activate immune cells. LPS followed by fMLF is believed to induce the sequential priming and activation of PMNs. DPI is a direct inhibitor of Nox, and 2-DG is a glucose analog with the 2-hydroxyl group replaced by hydrogen, which blocks glycolysis. As shown in **Figure 7**, no increase in ECAR was seen upon sequential LPS and fMLF stimulation, suggesting a lack of stimulated increase in PMN glycolysis **(A)**. No increase in OCR was observed either, suggesting lack of activation of Nox activity **(B)**.

PMA robustly and transiently increases OCR and ECAR in healthy PMNs.

As shown in **Figure 8,** PMA (a specific activator of protein kinase C, which can increase nutrient uptake and glycolytic enzyme levels) induced a sharp yet transient increase in ECAR was observed after PMA injection (experimental wells, in green), suggesting a significant increase in glycolytic activity **(A)**. A very sharp increase in OCR was seen after PMA injection **(B)** (experimental wells, in green), suggesting a concomitant increase in oxygen consumption.

Priming by LTB4 prior to LPS stimulation increases OCR and ECAR in healthy PMNs.

LTB4 is one of the most potent endogenous mediators of inflammation serving as both a strong chemotactic agent and promoter of degranulation in PMNs. As shown in **Figure 9**, incubation of PMNs in LTB4 (green) for 12 minutes in a FACS tube prior to the assay enable priming of these cells, leading to greater increases in ECAR **(A)**, and OCR **(B)** upon LPS activation compared to unprimed, LPS-activated PMNs, suggesting priming effects of LTB4. Qualitatively similar results were expected when LTB4 was used as a priming agent before PMN activation by fMLF**,** a bacterial and mitochondrial component that also binds to specific receptors to activate PMNs. However, ECAR and OCR readings were very low for this experimental combination (**Figure 10**).

LTB4 priming and LPS activation are both capable of eliciting substantial responses in PMNs transmigrated into CF ASN to mimic in vivo reprogramming.

Blood PMNs were transmigrated to CF ASN for 10 hours, mimicking their migration into the airway lumen in CF patients (**Figure 9**). After transmigration, PMN were analyzed in the Seahorse bio-analyzer in response to LPS, with or without prior priming by LTB4 (**Figure 13**).

To this end, transmigrated PMNs were collected, resuspended in assay medium, and either incubated with LTB4 (green) for 12 minutes or left not incubated (blue). LTB4 priming elicited faster increases and higher maximal values in ECR **(A)** and OCR **(B)** after LPS stimulation by transmigrated PMNs. This occurred despite the high level of activation already experienced by PMNs transmigrated to CF ASN, highlighting the strong priming effects of LTB4, and capacity of LPS to further increase activation in these cells.

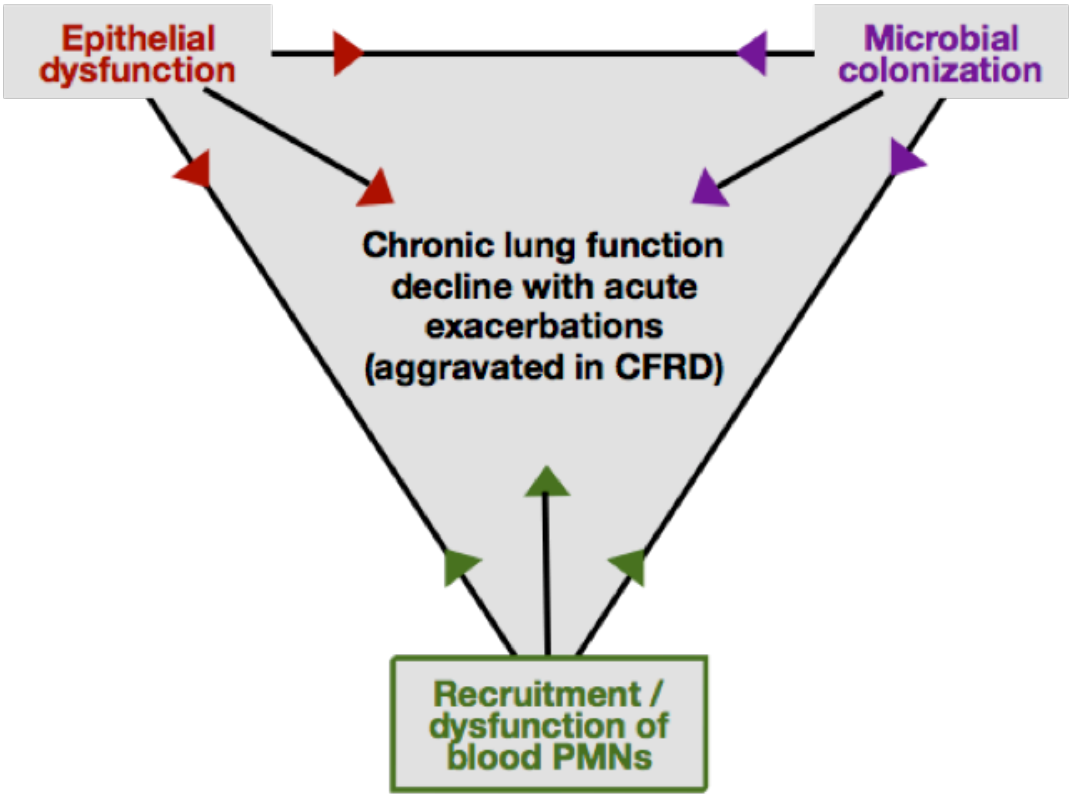
IV. Discussion:

Understanding the effect of agonists on metabolism of PMNs, the most abundant leukocyte subset in humans, is an ongoing endeavor. Here, we provide proof of concept for the use of the Seahorse bio-analyzer to study the impact of various mediators on PMN metabolism (measured as changes in extracellular acidification rate, ECAR, and oxygen consumption rate, OCR).

First, we used PMA, a powerful PMN agonist, as a positive control. Experiments were conducted to determine the concentration needed to elicit a reaction, yielding robust (albeit transient) increases in both OCR and ECAR relative to untreated PMNs. This set of experiments also allowed us to confirm the functions of DPI (Nox inhibitor) and 2-DG (glycolysis inhibitor). To reflect the environment of CF airways more closely, we used bacterial byproducts LPS and fMLF instead of PMA in subsequent experiments. Also in regards to applicability to CF, LTB4 was introduced as a priming agent, as it is a prominent mediator of CF inflammation, acting as a chemoattractant and agonist of PMNs. After determining relevant LTB4 concentrations and incubation methods, we successfully showed that LTB4 primes PMNs for higher ECAR and OCR upon LPS stimulation. However, fMLF did not result in as robust an activation of ECAR and OCR as LPS, which may be due to a technical issue (to be confirmed in follow-up assays).

To gain insight into airway PMN metabolism in CF, we used the transmigration model recently developed by the Tirouvanziam lab. This model mimics the migration that PMNs undergo when recruited from blood into the airway lumen of CF patients. In this model, the apical milieu into which PMNs migrate is CF ASN (airway supernatant corresponding to sputum, devoid of cells and bacteria). CF ASN contains many pro-inflammatory mediators such as LTB4, IL-8, and TNFalpha, and PMNs transmigrated into it undergo significant increases in ECAR and OCR compared to those transmigrated to LTB4 alone (**Figure 12,** unpublished data by Osric Forrest, graduate student in the Tirouvanziam lab). In pilot experiments conducted here, we showed that PMNs transmigrated to CF airway ASN could be further activated by subsequent stimulation by LPS, and that LTB4 priming further potentiated the effects of LPS stimulation on ECAR and OCR (faster responses, and increased magnitudes). Thus, PMNs have the ability to be further activated after having been submitted to recruitment and activation by CF ASN in the transmigration model. Our preliminary data also suggest that the magnitude of ECAR and OCR responses obtained with transmigrated PMNs (**Figure 13)** was higher than that with blood PMNs (**Figure 9**). These results confirm the activated nature of transmigrated CF airway PMNs, and suggest that they remain activatable beyond their high baseline activation, which has important implications for CF research.

Further steps include changes in the timing, concentration, and combinations of agonists used in the Seahorse bio-analyzer assays. In addition, experiments assessing the ability of LPS- and/or fMLF-stimulated transmigrated PMNs, with or without LTB4 priming, to take up and kill bacteria would help link changes in metabolic capacity with changes in functional ability of these cells. Such experiments will help advance our basic knowledge of PMN reprogramming in CF airway inflammation, and advance toward better therapies for this fatal disease.



Adapted from Dr. Rabin Tirouvanziam.

Figure 1: CF airway disease is associated with a pathological triad of epithelial dysfunction (leading to inspissated mucus), inflammation (mostly with PMNs recruited from blood), and microbial colonization.

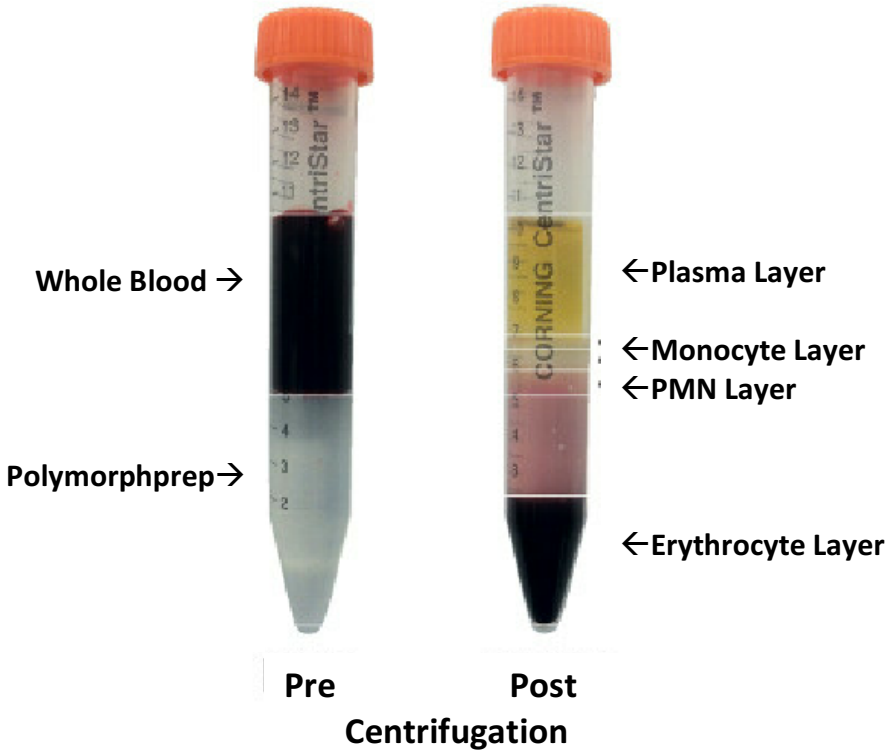


Figure 2: Diagram of blood layers following gradient density centrifugation.

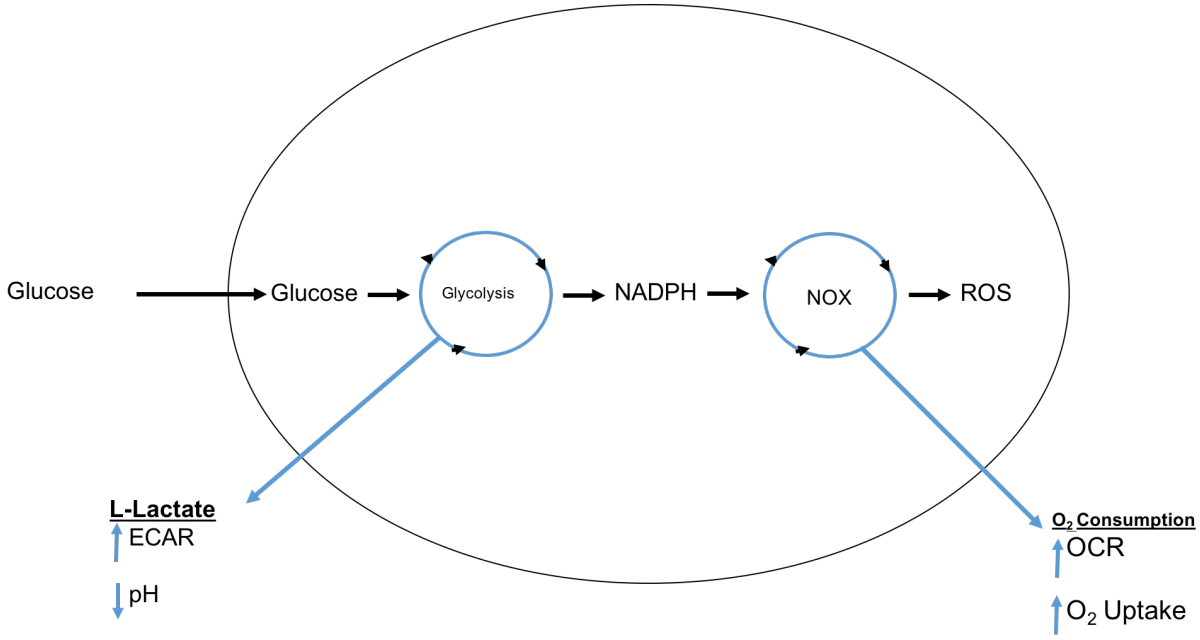
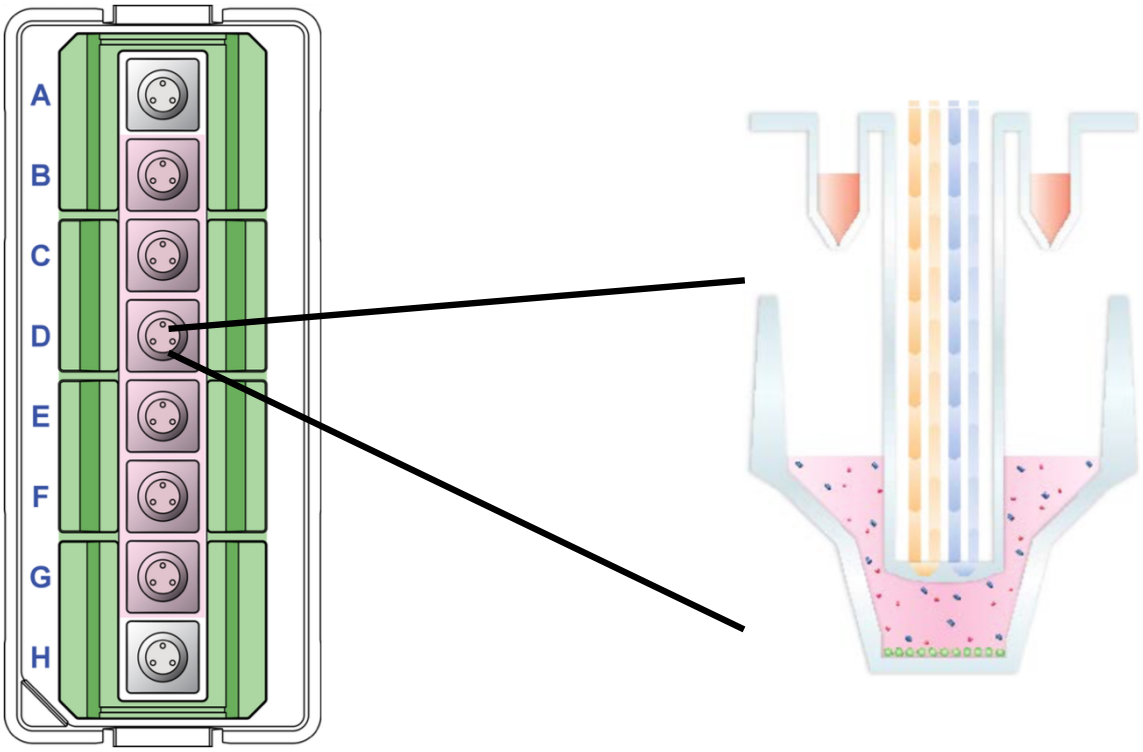
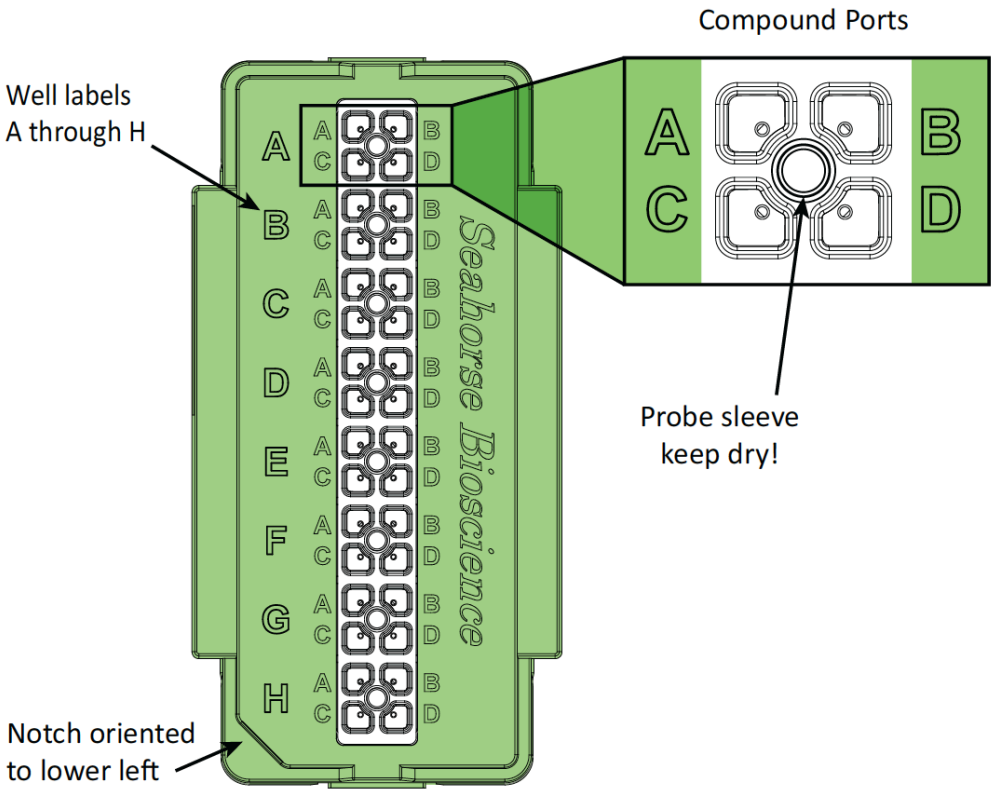


Figure 3: PMN metabolism and function in relation to extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)



Adapted from Agilent Technologies.

Figure 4: Diagram of XFp microplate and well.



Adapted from Agilent Technologies.

Figure 5: Diagram of sensor cartridge

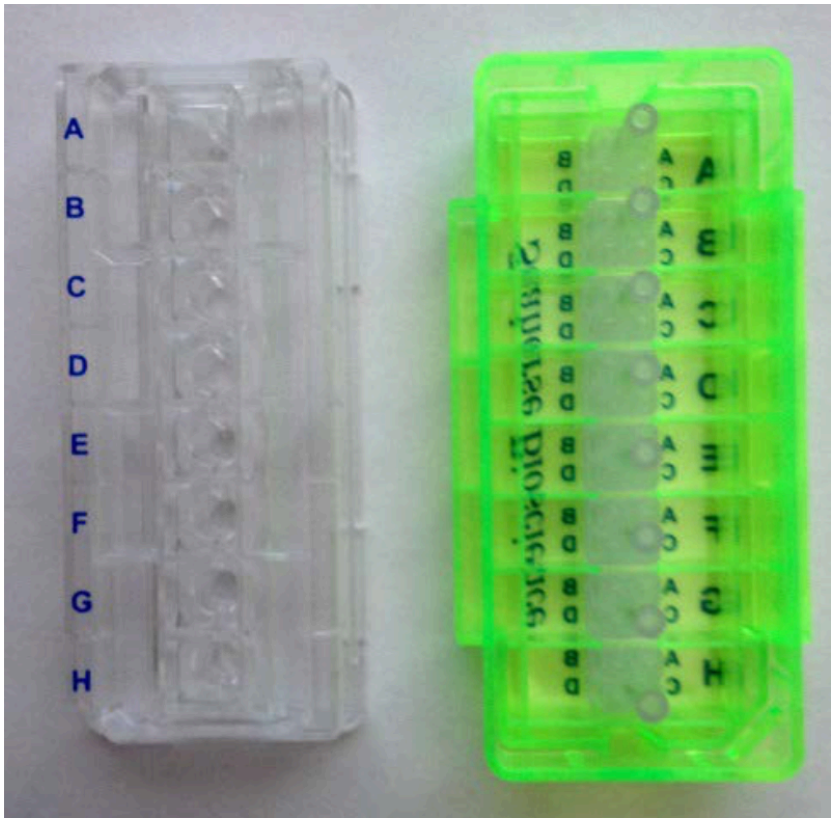
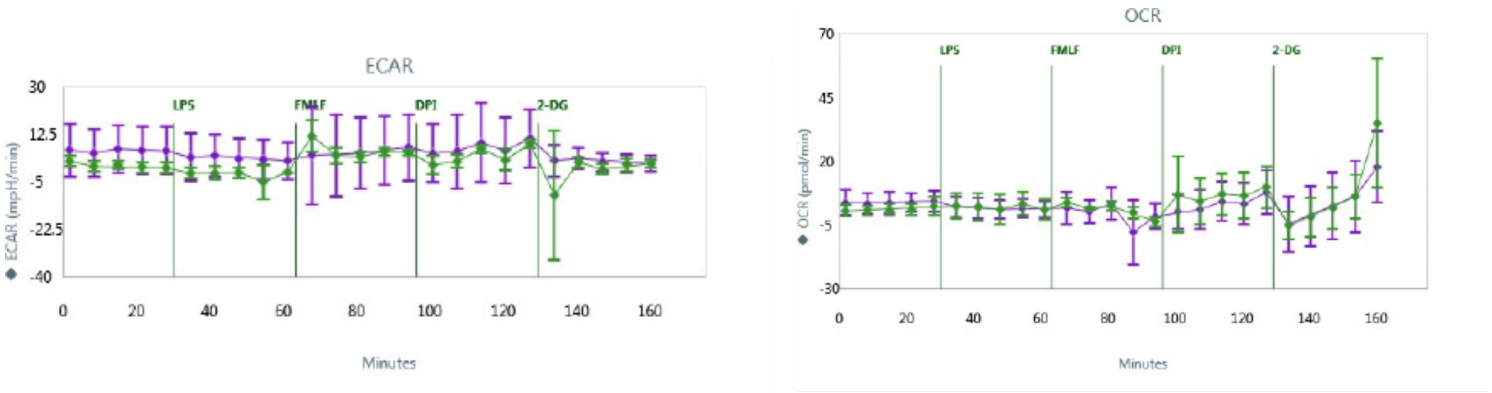
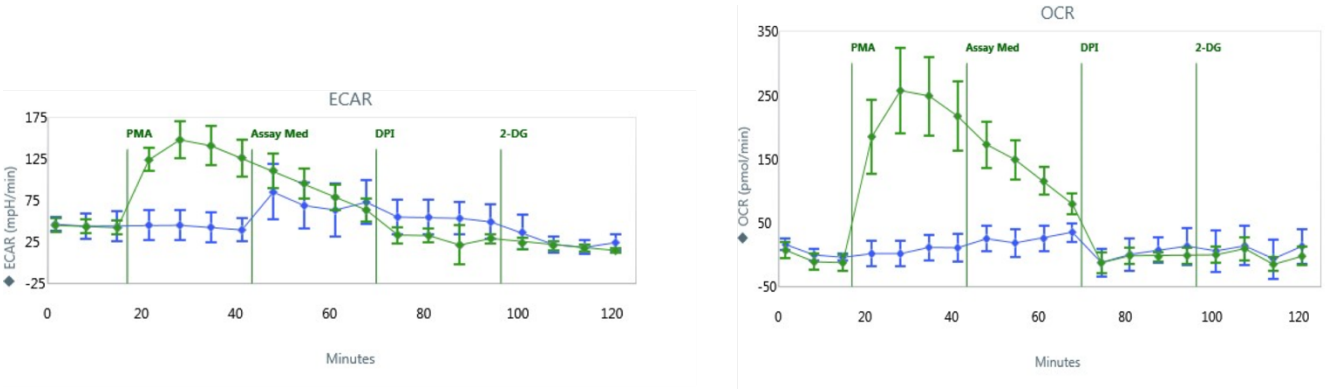


Figure 6: Picture of utility plate (white) and sensor cartridge (green)



(A) **(B)**

Figure 7: LPS and fMLF do not robustly increase ECAR and OCR in PMNs. The oxygen consumption rate (OCR, primarily a reflection of Nox activity in PMNs), was measured in a Seahorse XFp system upon sequential injections of LPS (500 ng/mL) (all wells), fMLF (500 nM) (experimental wells, green) or assay medium (control wells, purple), DPI (all wells), 2-DG (all wells), as shown in this representative tracing **(A)**. Glycolytic activity, reflected by the extracellular acidification rate (ECAR), was also measured, as shown in this representative tracing **(B)**.



(A) **(B)**

Figure 8: PMA robustly and transiently increases ECAR and OCAR in PMNs. The oxygen consumption rate (OCR, primarily a reflection of Nox activity in PMNs), was measured in a Seahorse XFp system upon sequential injections of PMA (100 ng/mL) (experimental wells, green) or assay medium (controls wells, in blue), assay medium, DPI, and 2DG, as shown in this representative tracing (A). Glycolytic activity, reflected by the extracellular acidification rate (ECAR), was also measured as shown in this representative tracing (B).

(A)

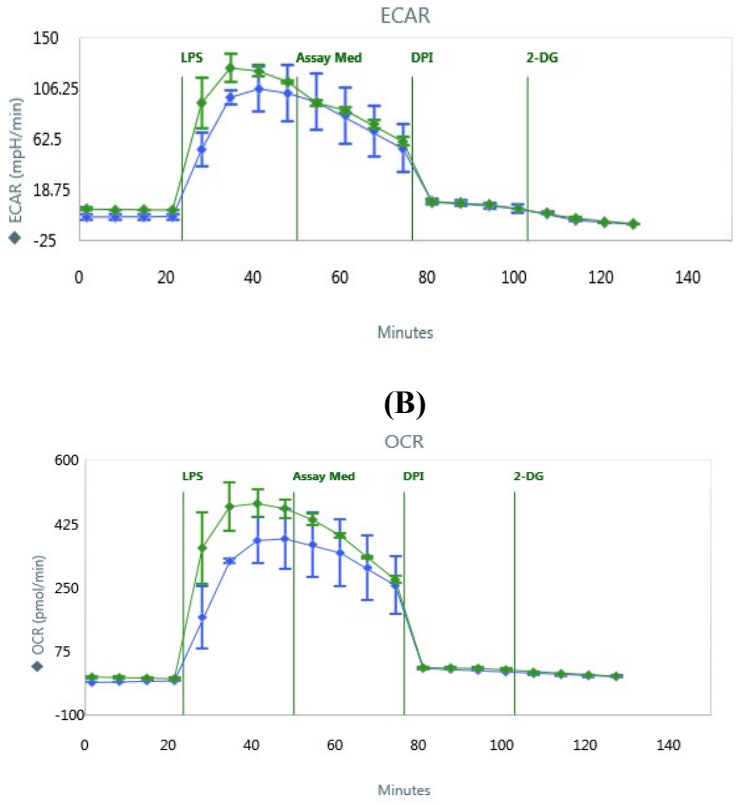
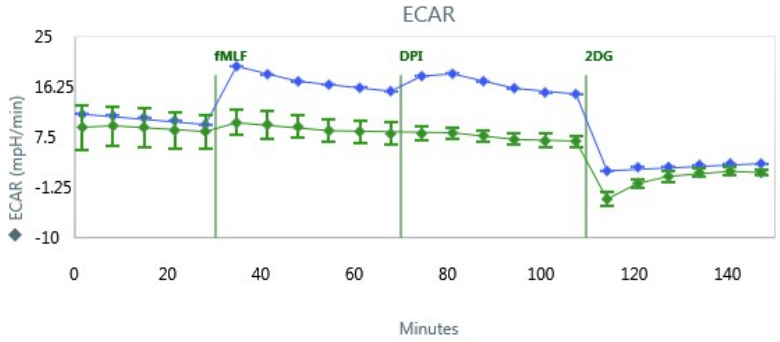


Figure 9: Priming by LTB4 prior to LPS stimulation increases ECAR and OCR in healthy PMNs. PMNs were incubated in LTB4 (100nM) diluted in assay medium for 12 minutes at room temperature and plated in experimental wells (green), or left untreated and plated in the control wells (blue). ECAR **(A)**, and OCR **(B)**, were measured upon sequential injections of LPS (500 ng/mL), assay medium, DPI, and 2DG.

(A)



(B)

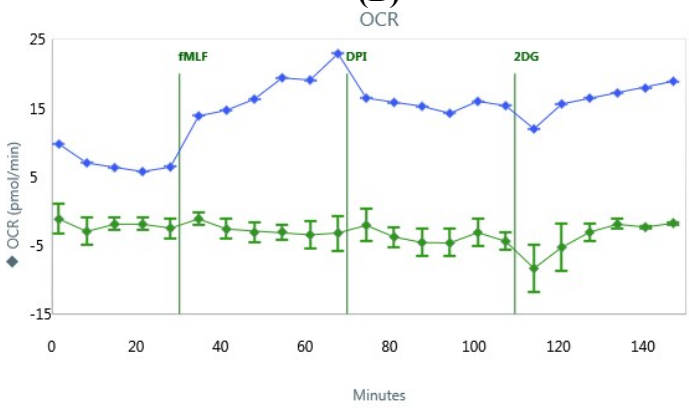


Figure 10: Priming by LTB4 prior to LPS stimulation increases ECAR and OCR in healthy PMNs. PMNs were incubated in LTB4 (100nM) diluted in assay medium for 12 minutes at room temperature and plated in experimental wells (green), or left untreated and plated in the control wells (blue). ECAR **(A)**, and OCR **(B)**, were measured upon sequential injections of fMLF (500 nM), assay medium, DPI, and 2DG.

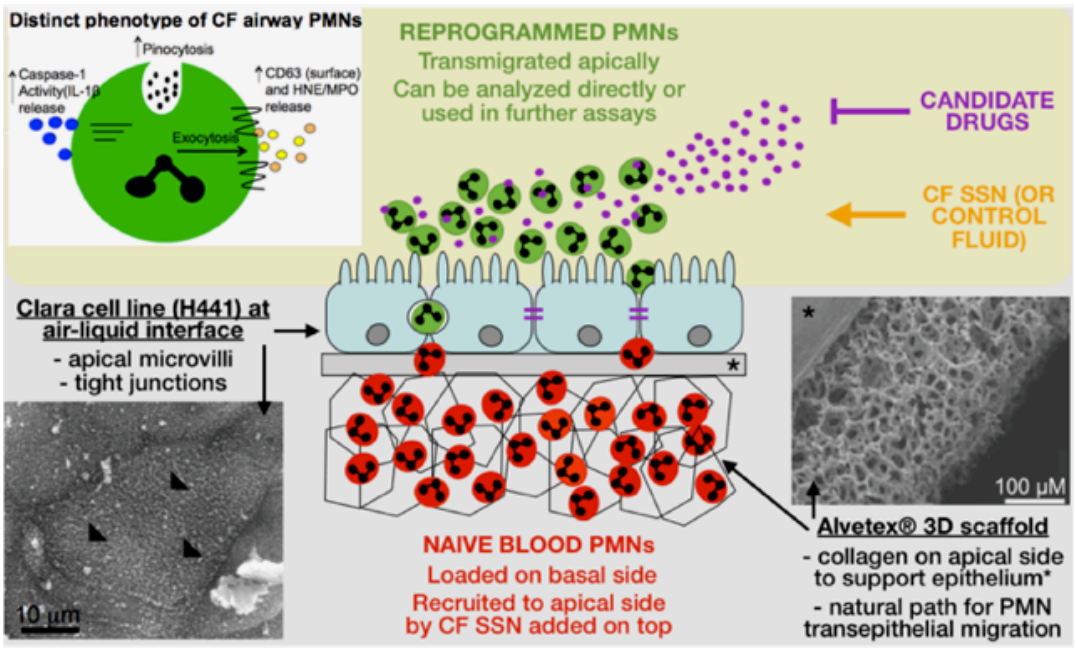


Figure 11: Diagram of transmigration model[20].

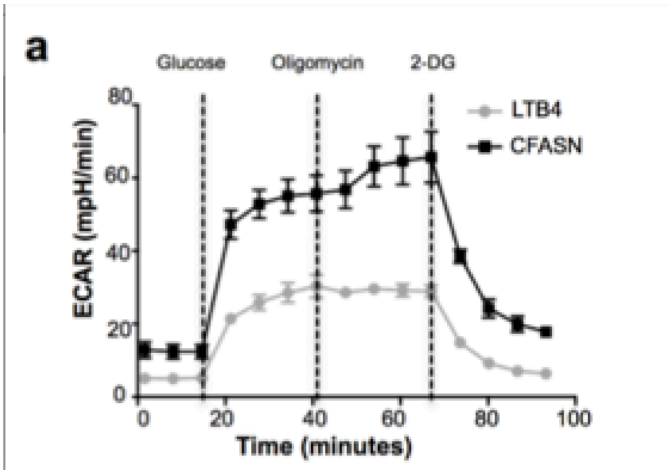


Figure 12: Transmigration to CF ASN increases glycolysis in PMNs. PMNs were transmigrated for 10 hours to LTB4 (100 nM, grey) or CF ASN (black), after which their glycolytic activity, reflected by the extracellular acidification rate (ECAR), was measured in a Seahorse XFp system upon sequential injections of glucose, oligomycin, and 2-DG, as shown in this representative tracing.

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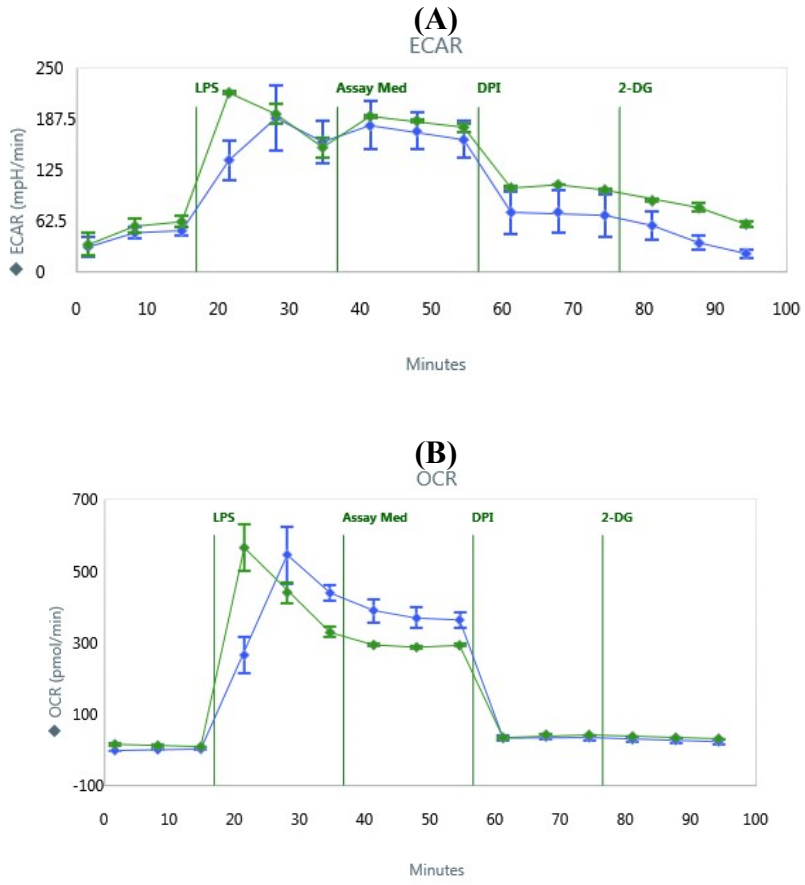

Figure 13: Priming by LTB4 prior to LPS stimulation elicits faster and more pronounced ECAR and OCR increases in transmigrated PMNs. PMNs were incubated in LTB4 (100nM) diluted in assay medium for 12 minutes at room temperature and plated in experimental wells (green), or left untreated and plated in the control wells (blue). ECAR **(A)**, and OCR **(B)**, were measured upon sequential injections of LPS (500 ng/mL), assay medium, DPI, and 2DG.

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