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MarQuita Kilgore April 18, 2011

The danger within: mitochondria-driven, injury-related immunity and transplantation in an evolutionary context

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Anthropology

2011

Abstract

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Human survival is dependent upon healthy functioning of the immune system and its ability to raise an appropriately measured inflammatory response. Inflammation promotes survival, and the fundamental threat to survival is injury. This injury can result from trauma, illness, or infection. Inflammation carries with it significant biological costs, and thus is highly regulated. The precise mechanistic relationship between injury and inflammation is not well known beyond the maxim that injury begets immunity. This fundamental relationship is the focus of the present project.

Recent literature suggests that cellular injury triggers the disruption of mitochondria. Mitochondrial contents, particularly mitochondrial DNA (mtDNA) and formyl peptides, serve as damage-associated molecular patterns (DAMPs), which are immunostimulatory. We hypothesized that injury, specifically mitochondrial disruption, augments immunity and can, when excessive, lead to non-adaptive immune responses to injury. We suspect that such responses play a major role in clinical phenomena. Specifically, we hypothesized that mitochondrial contents foster more vigorous alloimmune responses toward transplanted organs. In order to explore the role of mitochondria in amplifying the human alloimmune response, alloimmune mixed lymphocyte reactions in the presence of whole mitochondria versus at baseline were used. These reactions were assessed using carboxyfluorescein succinimidyl ester (CFSE)-based flow cytometry and intracellular markers to monitor lymphocyte proliferation or activation. Cell surface markers were used to help categorize cells of interest. Self-mitochondria, originating from the muscle tissue of the recipient, were found to heighten the alloimmune response via a proliferative effect on T cells.

These studies relate mitochondrial DAMPs (MTDs) and evolutionarily conserved pathogen-associated molecular patterns to the process of transplanted organ acceptance. These processes are related to the general biological cognizance of injury and its subsequent triggering of immunity. More specifically, the involvement of mitochondria in human responses to injury strongly correlates with the evolutionary theory of mitochondria as endosymbionts and the clinical manifestation of systemic inflammatory response syndrome (SIRS) in the absence of documented infection. Given the roles that mitochondria play in vital processes in the human body coupled with itsnewfound involvement in immunity, our efforts hold the potential to provide insight into some of the fundamental determinants of human survival.

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Acknowledgements

First and foremost, I would like to thank God for the strength and support that made this journey possible. My honors committee has been incredibly helpful and supportive throughout this process. To my adviser, Dr. Michelle Lampl, thank you for inspiring my interest in immunology and working around the clock to help me produce my best work. You have been there to guide, encourage, calm, and inspire me throughout this project. Thank you. To my research mentor, Dr. Allan Kirk, thank you for believing and investing in my potential as a scientist. Working in the Emory Transplant Lab, especially under your superb guidance, has been a life-changing experience. Thank you. To my committee member, Dr. Pat Marsteller, thank you for all of the unwavering support that you have given me since the day I stepped foot on this campus. You have always managed to go out of your way to support my growth as a scientist, student, and woman. Thank you. To my committee member and academic adviser, Dr. Melvin Konner, thank you for your insightful input during the course of this project and throughout my undergraduate career. The passion you convey continues to challenge and inspire me. Thank you.

In terms of technical support for this project, I would like to thank the members and affiliates of the Emory Transplant Center and Lab. They have joined in on various levels to make this project possible. Thank you. I would especially like to thank the lab manager, Mr. Brad Harten, for easing my transition into the lab and continually helping me in times of need. Also, thank you to Mrs. Sam Hanna, for your support and valued lessons in the lab. I greatly appreciate this. Thank you to Ms. Linda Stempora for support with data analyses. I would also like to thank members of the biorepository for their help with securing samples for this project. Thank you to Drs. John Shires, Karnail Singh, and Denise Lo for investing time in helping me to improve the protocols for this project. Last and certainly not least, I would like to thank Mrs. Pamela Turner, who has served as a phenomenal beacon for technical, intellectual, and even emotional support.

Thank you to Dr. Sarah Gouzoules for your guidance throughout this process. To my dear family and friends, thank you for all of your encouragement throughout this process and every other challenge I face. I would especially like to thank my mother, Ms. Kathy Carter, and sisters, Ms. Katrina Carter and Ms. Patrisha Carter, as well as my roommate and dear friend, Ms. Ciara Johnson. I have been blessed with a tremendous support team. I thank each and every one of you.

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Introduction

"First, do no harm (p. 301, Matzinger, 2002)." Polly Matzinger (2002) cleverly introduced a new immunological paradigm using this excerpt from the Hippocratic oath. She proposed the Danger model on the basis that the immune system responds to threats of damage whether from self or non-self sources. The traditional method of thinking about the way in which the human immune system operates involved toleration of self and attacks against non-self. However, Matzinger (2002) claims that this Self-Nonself (SNS) model failed to explain how the immune system deals with life changes that alter the definition of self, such as puberty, pregnancy, and aging. In search of a more intuitive understanding, She acknowledged parallels between the Danger model and the updated SNS way of thinking about immune challenge, known as the Infectious-Nonself (INS) model, since both rely heavily on the ability of antigen-presenting cells (APCs) to be activated by their environment and to initiate further immune responses. In the INS model, specifically, Charles Janeway proposed that APCs have pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) and consequently activate the APCs, allowing them to alert other immune cells to infection. On the other hand, the Danger model involves the activation of APCs by danger or alarm signals released by injured or distressed cells or presented in the form PAMPs.

> "Because cells dying by normal programmed processes are usually scavenged before they disintegrate, whereas cells that die necrotically release their contents, any intracellular product could potentially be a danger signal when released (p. 302)."

With the Danger model and this statement in particular, Matzinger unknowingly foreshadowed the very foundations of "The Danger Within."

Could mitochondria function as an enemy force within the human body? As early as the 1920's, Ivan Wallin applied the endosymbiotic theory to mitochondria, suggesting their existence as once free-living bacteria captured by eukaryotic cells in a symbiotic, or mutually beneficial, relationship (Scheffler, 2006, p. 8). Nearly four decades after extreme opposition to this theory, Lynn Margulis (then, Lynn Sagan) provided the strong evidence that substantiates Wallin's claim. Concomitant to the endosymbiotic theory, recent data provided by Carl Hauser and his team implicate mitochondria as key stimulators for the human immune system in response to injury (Zhang et al., 2010). They made these efforts largely in an attempt to elucidate the unfathomable aspects of systemic inflammatory response syndrome (SIRS), a spectrum of potentially fatal conditions involving some combination of fever, increased respiratory rate, increased circulatory rate, and elevated white blood cell counts (Talan, 2006). Roger Bone also dedicated much of his life to understanding the complexities of SIRS, setting the framework for deciphering the inflammatory reactions involved (Bone, 1996). The juxtaposition of the Matzinger, Margulis, Hauser, and Bone hypotheses cultivates novel ways of framing the relationship between humans and mitochondria. Furthermore, the "selfish gene" concept, originally hypothesized by Dawkins (1979) and further enriched by Austin Burt and Robert Trivers (2006), prompts a consideration of the co-evolutionary arms race that occurs as a result of this relationship. "The Danger Within" extends the discourses constructed by the aforementioned legendary works in an effort to inform transplantation. This construal of mitochondria as "the danger within" serves to provide further insight into the role that mitochondria play in not only immunity, but

also in human evolution and consequent survival.

On the Origins of Mitochondria

The human immune system has evolved within the context of the struggle between eukaryotes and prokaryotes. Mitochondria epitomize this long-lived, delicate balance between tension and collaboration. Although most well known for its role as the energy producing "powerhouse" of the eukaryotic cell, the mitochondrion provides interesting nuances to the understanding of evolution. In 1890, German cell biologist Richard Altmann made the earliest claims for recognizing granules in muscle (later found to be mitochondria) as autonomous, elemental living entities analogous to bacteria (Scheffler, 2008, p. 1-2). Although several scientists subsequently made similar claims, Lynn Margulis provided the earliest, most definitive case for applying the endosymbiotic theory to mitochondria (Sagan, 1967). She boldly insists that the eukaryotic cells originated and now flourish primarily due to the symbiotic relationships that thrive within them. Specifically, she hypothesized that mitochondria, along with two other organelles (photosynthetic plastids and the basal bodies of flagella), originally existed as prokaryotes. This viewpoint on the mitochondrion, coupled with the dichotomy of its alliance to humans and similarities with bacteria, gives rise to interesting evolutionary questions.

Prior to taking a side on the controversy surrounding the origin and consequent implications of the mitochondrion, one must understand the broader context of its structure and functions. Since the mid-1800s, scientists have continued to reveal intricacies in the structure and function of mitochondria. The name mitochondrion refers

to the morphology of the organelle; in Greek, *mitos* means thread and *chondros* means granules (Scheffler, 2008, p. 1, 19). Although the size and shape of mitochondria vary widely, these organelles commonly appear as "sausage-shaped" structures about 3-4 μm long and 1 μm wide (Scheffler, 2008, p. 19). The number of mitochondria in a given cell also varies greatly depending on cell type, with ranges from hundreds to a few thousand per cell (Scheffler, 2008, p. 19-20). The specific mechanisms of differentiation that permit mitochondria to cater to the needs of specific cell types remain largely unknown and thus, serve as a beacon for further research.

Mitochondria boast several unique physical qualities. The mitochondrial membrane convolutes within the interior of the organelle to form cristae, greatly increasing the membrane surface area for reactions (Scheffler, 2008). In fact, mitochondria contain a double membrane; the outer member shields the organelle from the cytoplasm of the cell, and the inner membrane forms the cristae. The matrix consists of the "structureless" space that fills the interior of mitochondria. Oxidative phosphorylation and consequent massive ATP production, the most prominent functions of mitochondria in human cells, occur in the matrix (Burt and Trivers, 2006). Cells enlist their cytoskeletons to move and strategically place mitochondria around the areas in extraordinary need of the energy source, such as near the axoneme that dictates the movement of sperm cells (Scheffler, 2008, p. 30, 32). The flexibility of mitochondrial structure and morphology to accommodate the needs of the cell exemplifies a sort of inherent alliance.

In 1960, the lens through which the world viewed mitochondria significantly changed as a result of the discovery of a mitochondrial genome (mtDNA) independent of the nuclear genome that governs the rest of the cell. Data originally associated mtDNA with specialized mitochondria, known as kinetoplasts, which are found in the flagella of

Trypanosoma mega, a type of parasitic protist (Scheffler, 2008). Three years later, scientists applied the presence of mtDNA to mitochondria in general (Nass and Nass, 1963). Each mitochondrion contains multiple mitochondrial genomes, cells typically harbor numerous mitochondria and, consequently, each cell includes thousands of mitochondrial genomes (Scheffler, 2008, p. 20). However, the mitochondrial genomes in animals only code for about 12 proteins, all of which relate directly to the electron transport chain and oxidative phosphorylation. Therefore, nuclear DNA encodes all other mitochondrial proteins (Burt and Trivers, 2006). The issue of determining mitochondrial versus nuclear genome input into mitochondrial proteins remains unresolved and enthusiastically studied (Scheffler, 2008, p.28). Nonetheless, scientists have managed to sequence and find unparalleled levels of significance in the mitochondrial genome.

The significantly high degree of variability in mtDNA, a consequence of its higher rate of mutation than nuclear DNA, proves valuable in anthropological and forensic analyses (Scheffler, 2008, p. 5). In addition, the knowledge of the entire mtDNA sequence, its abundance in the cell, and its maternal mode of inheritance also make it an ideal candidate for such analyses (Scheffler, 2008, p. 418). Pioneering mtDNA analyses in a diverse sample of people led to the hypothesis that human origins trace back to an African ancestor, nicknamed the "Mitochondrial Eve" (Cann, 1987). Given its controversial nature, this finding continues to be simultaneously supported and refuted by subsequent critiques and further research (Scheffler, 2008, p. 418-422). Furthermore, mtDNA analyses also apply to the evolution of other species, with the knowledge gained about non-human primates holding the most anthropological significance (Scheffler, 2008, p. 426-428). In human populations, people from the same ethnic group share certain patterns of mtDNA. However, each individual has a set of

"distinctions" (i.e., polymorphisms) that uniquely defines their mtDNA. This individualized nature of mtDNA has promoted its use in modern forensics (Scheffler, 2008, p. 430). Thus, mitochondria inextricably relate to the facets of human identity, both past and present.

Inherited polymorphisms in mtDNA are known as heteroplasmy. While fairly common, heteroplasmy sometimes factor into mitochondrial diseases (Scheffler, 2008, p. 419). The first definitive correlations between mtDNA mutations and disease pertained to abnormalities of the brain and muscle. Specifically, these studies related neuropathy and myopathy to certain mtDNA mutations common in those affected, linking these diseases to metabolic deficiencies (Holt et al.,1988 and Wallace et al., 1988). Since these original findings, the research interest in mitochondrial disease and medicine has continued to grow, confirming the role of mtDNA mutations in diseases ranging from diabetes mellitus to cancer (Murri, 2007). Although nuclear genome mutations contribute to mitochondrial diseases, the widespread role of the mitochondrion in optimal functioning often renders it the culprit responsible in the matter of disease (Scheffler, 2008, p. 5). For example, research implicates the involvement of mitochondria in processes outside of energetic metabolism, such as aging, apoptosis, neurodegeneration, electrochemical interactions, and the production of reactive oxygen species (Murri, 2007). Given their functional importance, mitochondria now fill numerous niches in research efforts to combat disease.

In discussing the many roles of mitochondria, the coalition between mitochondria and humans becomes evident. The question remains of whether or not it is symbiosis. Margolis claims, "... The eukaryotic cell is the result of the evolution of ancient symbioses (Sagan, 1967, p. 226)." In fact, she hypothesized the claim that

mitochondria resulted from the photosynthetic prokaryote, that she further hypothesized had been consumed by the early form of the eukaryote in order to survive in an oxygen-enriched atmosphere. Despite past controversy, the paradigm of mitochondria as bacteria in symbiosis with their human and other eukaryotic hosts has gained clout over the years. An overwhelming number of modern scientists now adhere to the endosymbiotic theory on the origin of mitochondria. One extreme case even examines the idea of classifying mitochondria as a type of bacteria with its very own taxonomic family (Pallen, 2011). Sequencing of whole mitochondrial genomes provides insight into mitochondrial evolution. "Reductive evolution," the means by which mitochondria apparently lost most of the genomic information present in its bacterial ancestor, produces complications with attempts to determine the closest prokaryotic ancestor of mitochondria (Gray, 2001). Mitochondria genomes with the least losses, such as that from the protist species *Reclinomonas americana*, provides the most promise in pursuit of mitochondrial origins given their closer relation to ancestral DNA (Gray et al., 2001). Despite the fact that specific prokaryotic origins of mitochondria remain debatable, the ability to sequence more mitochondrial genomes makes a final decision on this debate more likely in the near future.

Although mitochondria and bacteria undoubtedly differ in size, shape, and level of independence, they share several striking behavioral similarities (Scheffler, 2008, p. 19). Despite some association with cytoskeletal structures, mitochondria can also independently undergo fusion, fission, or less dramatic changes to alter their shape or size and engage in subtle movement (Scheffler, 2008, p. 30). Fusion occurs largely due to mitochondrial collisions during their movement (Scheffler, 2008, p. 42). Similar to free-living bacteria, mitochondria proliferate via fission, especially in proliferating cells

(Scheffler, 2008, p. 40-41). These changes most likely occur due to cristae restructuring. The appearance of mitochondrial cristae varies in different environments, whether based on energy fluctuations in the cell, or other conditions. This behavior mimics the ability of bacteria and other free-living, prokaryotic cells to alter themselves according to their surroundings (Scheffler, 2008, p. 28).

Mitochondria also share some specific molecular similarities of interest. Firstly, mtDNA resembles bacterial DNA (Cardon, 1994). Although genetic analyses reveal that the mitochondrial genome must have lost a significant number of genes as it evolved from the genome of a prokaryotic ancestor, striking genetic similarities remain (Gray, 2001). In addition to sharing the circular genome shape with mitochondria, bacteria typically have unmethylated regions of DNA that would have otherwise been methylated in eukaryotes (i.e. CpG DNA repeats) (Zhang et. al, 2010). Secondly, their genomes produce uniquely similar protein products. Most bacterial proteins contain a particular molecular arrangement that classifies them as N-formylated (Taanman, 1999). Although eukaryotes lack the coding for these peptides in their nuclear genomes, mitochondria begin translation with a formyl-methionine at the start of every peptide (Burt and Trivers, 2006). The presence of such bacteria-like components within eukaryotes, although baffling, further suggests the cooperation characteristic of symbiosis. However, it also foreshadows inevitable tension that threatens the tenets of cooperation.

Injury, Mitochondria, and Inflammation

Recent findings in immunological research suggest that mitochondria are key players in the link between injury and inflammation. In fact, mitochondria contain

damage-associated molecular patterns (DAMPs), which are essentially a type of the danger signals to which Matzinger (2002) alluded in her danger model proposal. Specifically, mitochondrial DAMPs (MTDs) include mtDNA and formyl peptides. Most likely, several other kinds of MTDs remain undiscovered (Zhang et al., 2010). Mitochondria degradation products (MDPs) serve as an alternative name for MTDs (Raoof et al., 2010). Regardless of the cumbersome naming devices, a simple sentence encompasses the major themes surrounding the body of research connecting injury, mitochondria, and inflammation: in the event of injury, mitochondria submit a danger signal to aid the immune system in mounting a response with the goal of survival. Given this fundamental fact, the signal, initial immune response, and ultimate consequences can be discussed.

The Signal - Mitochondrial DAMPs (mtDNA and formyl peptides)

The Context: Once traumatic injury breaks open cells and their mitochondria, the intracellular components of the mitochondria roam freely (Zhang et al., 2010). As previously mentioned in drawing the parallels between bacteria and mitochondria, mitochondria contain mtDNA and formyl peptides, which are both bacteria-like products. Therefore, their ability to stimulate the human immune system should not come as a total shock (no pun intended). Like the unmethylated CpG DNA repeats of bacteria, mtDNA bind immune cells through toll-like receptor 7 (TLR 7), a type of pathogen-recognition receptor (PRR) involved in innate immunity. Like the formyl peptides of bacteria, mitochondrial formyl peptides bind two formyl peptide receptors, known as FPR1 and FPRL-1 (Zhang et al., 2010). These formyl peptide receptors are chemokine receptors, which means that they guide the migration of immune cells according to the binding of ligands and the corresponding signals. Thus, formyl peptide is a chemoattractant.

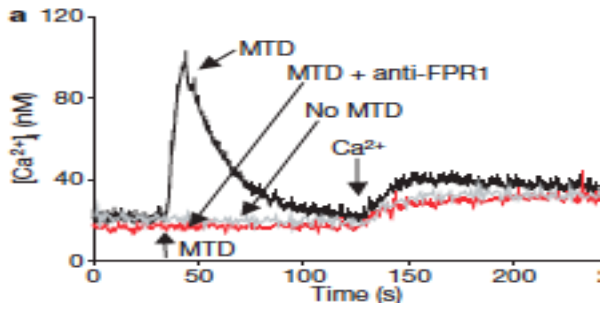
The Evidence: Trauma patients exhibit significantly higher levels of blood plasma mtDNA than presumably healthy volunteers. These data illustrate one phenomenon of interest: injury triggers the liberation of MTDs into circulation (Zhang et al., 2010).

Initial response to the signal - Neutrophil Activation

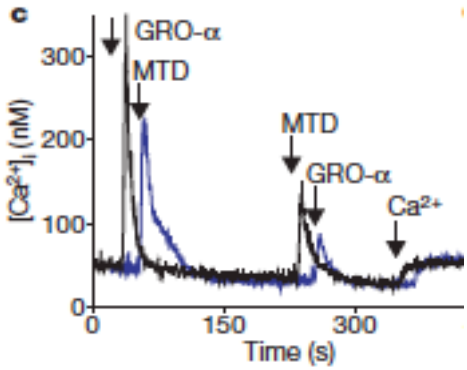
The Context: The three events indicative of PMN activation include: (1) fluxes in intracellular calcium, (2) desensitization of chemokine receptors, and (3) activation of mitogen-activated protein kinases (MAP K) (Zhang et al., 2010). MAPKs serve as the driving factor for a multitude of chemical cascade events crucial to survival, including but not limited to initiating many of the signals that allow an individual to mount an inflammatory response.

The Evidence:

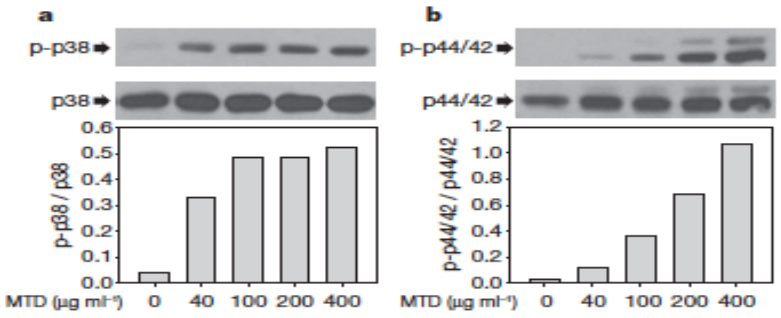
(1) Zhang et al. (2010, p104) published the graph below comparing intracellular calcium flux in neutrophils according to the following conditions: MTDs (the highest peak), MTDs and a blocking antibody to the formyl peptide receptor, and No MTDs. Evidently, MTDs cause a significant calcium flux, leading to neutrophil activation.



(2) Zhang et al. (2010, p. 104) published the graph below showing that the calcium flux in neutrophils produced by MTD stimulation as well as by stimulation with GRO-alpha (a type of chemokine, specifically CXCL1) decreases over time. MTDs have this densensitization effect on neutrophils, signifying their activation of neutrophils.



(3) Zhang et al. (2010, p. 105) published the graph below showing the result of skeletal muscle MTDs on PMN p38 and p44/42 MAPKs. The resulting phosphorylation of the neutrophil MAPKs signifies neutrophil activation by MTDs.



The Resulting Action - Inflammation

The Context: The inflammatory state of neutrophils involves increased levels of metalloproteinase (MMP) and Interleukin (IL)-8 (Zhang et al., 2010). MMP is an enzyme that cuts through collagen, allowing neutrophils to be recruited and migrate into tissues.

IL-8 is a cytokine that stimulates chemotaxis, activation, and secondary IL-8 release in neutrophils. Thus, IL-8 reinforces the activity of MMPs.

The Evidence: Zhang et al. (2010) showed that increased levels of MMP and IL-8 in neutrophils following stimulation by MTDs, signifying that MTDs cause neutrophil-mediated inflammation.

Implications of Mitochondria-driven Immunity

MTDs mobilize neutrophils to move into and impact tissues and organs in dramatic ways, even leading to inflammatory injury and disease. They accomplish this by serving as chemoattractants. In other words, PMNs migrate towards MTDs, as demonstrated by work based on assessments of clinical femur fractures (Zhang et al., 2010). PMNs infiltrate tissues in response to MTD deposits. For example, the excessive inflammatory condition, neutrophilic peritonitis, develops in response to clinical concentrations of MTDs deposited into the mouse peritoneum, the membrane lining the abdominal and pelvic cavities (Zhang et al., 2010). MTDs cause neutrophil-mediated organ injury, as demonstrated by the development of inflammatory lung injury as a result of MTD injections. Thus, the presence of MTDs in the body poses a change and potential threat to immune responses, most evident via the collective ramifications revealed in neutrophils. Although much remains unknown, the unveiling of these mechanisms, particular to MTD-driven neutrophil immunity, foreshadows the role of mitochondria and its contents in other facets of immunology and human health.

Trajectories of Human Evolution and Consequent Survival

Given the apparent autonomy of the mitochondrial genome, one would expect the evolutionary interests of, and consequent selection on mtDNA to differ from, and possibly even conflict with that of nuclear DNA. Dawkins (1979) described some instances of co-evolution between a host and its endosymbiont, or predator and prey, as an "arms race," implying conflicts between their evolutionary interests that cause their adaptations to directly counter each other. Similarly, Burt and Trivers (2006) described mtDNA as "selfish" genetic elements, characterized by their ruthless "drive" towards their own evolutionary interests despite contributing to the demise of their host. They further discussed the arms race that appears to play out between mitochondria and their human or other eukaryotic hosts. Evolutionarily recent medical advances and consequent illnesses call for attention in the discussion of this particular arms race. In particular, this section discusses two questions that help to frame human evolution and survival in the context of human relations with mitochondria: (1) Are mitochondria losing ancient battles in the arms race against humans? (2) What might sterile systemic inflammatory response syndrome further reveal about the dynamics relationship between humans and mitochondria?

Are Mitochondria Losing Ancient Battles in the Arms Race against Humans?

The pioneering evolutionary arguments surrounding work aimed at tracing human origins insist that selection does not occur on mtDNA, an assumption that is important in the timing estimations for the evolutionary molecular clock (Cann et al., 1987). However, the "selfish gene" and co-evolutionary claims by Burt and Trivers (2006) suggest otherwise. They propose that modern "reactionary" adaptations of mitochondria for

combating the selection on nuclear DNA to dictate mitochondria function seem inadequate or even completely absent in some cases (Burt and Trivers, 2006). In their evolutionary arsenal, mitochondria possess several means for outwitting their human hosts in the arms race. According to Burt and Trivers (2006), some include: “relaxed” genomes, gender bias, ATP production, and apoptosis. “Relaxed” genomes allow multiple mitochondria genomes per cell and unrestricted replications per genome. Thus, mitochondria have the potential to overpower, or at least weaken the reign of nuclear DNA by excessive replication of their own DNA. However, given the extreme loss of genetic material in mtDNA to nuclear DNA, nuclear DNA seems to outwit the possibility of mtDNA overreplication by selecting for smaller mitochondrial genomes. In a rebellious attempt, mitochondrial genomes in the female germline tend to pack tightly in order to ensure their transmission without stepping outside of the size limitations set by nuclear DNA. Maternal inheritance of mitochondria also helps to counteract mitochondrial selection within an individual by preventing overreplication attempts of male versus female mitochondria to outcompete each other.

Uniparental inheritance of mitochondria involves several interesting evolutionary implications. In addition to eggs of the animal kingdom containing more mitochondria than sperm, nuclear DNA tags male mitochondrial membrane proteins with ubiquitin in order to facilitate the degradation of male mitochondria and ensure maternal inheritance (Burt and Trivers, 2006). In order to succumb to this nuclear-driven mode of inheritance, one might expect female mitochondria to have adapted a means to help humans kill their male mitochondria. Also, one might expect male mitochondria to have adapted a means to prevent their own degradation. Research has uncovered no evidence that either phenomenon occurs. Burt and Trivers (2006) hypothesize that mtDNA has

evolved to mutate more in male mitochondria, resulting in lower quality male mitochondria. In fact, a man who accidentally inherited the mitochondria of his father in his muscles suffered from an inability to exercise (Schwartz, 2002). This lower quality in male mitochondria could contribute to maternal inheritance, especially as a means for nuclear DNA to limit mtDNA replication and mitochondrial selection (Burt and Trivers, 2006). However, maternal inheritance eventually selects for mtDNA that favors females, whereas the best interests of nuclear DNA involve maintaining both genders and reaping the benefits of meiotic diversification. Thus, uniparental inheritance encompasses multiple levels of conflict between mtDNA and nuclear DNA.

Human dependency on mitochondria for important cellular functions represents another critical avenue in which the arms race takes place. Nuclear DNA dictates mitochondrial selection by only allowing increased rates of mitochondrial replication in areas with extraordinary energy need and limiting mitochondria replication otherwise (Burt and Trivers, 2006). Burt and Trivers (2006) suggest that this particular ATP-dependent mitochondrial selection could have contradictory effects. In order to replicate freely without the constraints of ATP production, mitochondria will eventually become less efficient. This would allow more mtDNA replications to occur without exceeding the nuclear-enforced quotas for ATP production in any particular area (Burt and Trivers, 2006). Inefficient mitochondria, characterized by heteroplasmy that affects their energetic capabilities, can contribute to human metabolic diseases. Ironically, this could mean that the very thing that nuclear DNA attempted to prevent by limiting mitochondria replication and associated mutations in the first place might ultimately still occur. This theoretical example hypothesizes that the attempts of nuclear DNA to outwit

mitochondria could give rise to potentially detrimental and often contradictory side effects.

The Burt and Trivers (2006) argument assumes that mitochondria boasted more bacteria-like characteristics and efficient methods for maintaining their own autonomy near the beginning of their symbiotic relationship with humans. Overtime, however, their influence has waned, causing them to fall prey to the strong selection on nuclear DNA to antagonize and control mitochondria. The extreme loss of genetic material in mtDNA to nuclear DNA suggests that nuclear DNA has redirected the methods derived by mitochondria to exert their autonomy in a way that benefits the human host. Apoptosis serves as an example of such (Burt and Trivers, 2006). Mitochondria play a major role in activating apoptosis by harboring several proteins critical to the pathway, such as apoptosis-inducing factor and caspases. Mitochondria also heavily participate in producing the signs of cell death, such as allowing ions to easily leak through their mitochondrial membrane and swelling themselves with water until the membrane ruptures. Perhaps, mitochondria once utilized apoptosis to kill human cells that did not contain mtDNA, but nuclear DNA evolved to co-opt this ability to the benefit of humans. This perspective portrays mitochondria as initial champions, but subsequently long-term losers in the arms race co-evolutionary battle between humans and mitochondria. This challengingly bold hypothesis prompts consideration of the co-evolutionary "motives" or mechanisms that might give rise to mitochondria-derived, injury-related immunity.

What might sterile systemic inflammatory response syndrome further reveal about the dynamics of the relationship between humans and mitochondria?

Carl Hauser and his team connect their recent findings of mitochondria as immune stimulators in response to injury to the previously unexplained phenomenon of

sterile, or noninfectious, SIRS (Zhang et al., 2010). Sterile SIRS resembles sepsis, which involves widespread and commonly fatal inflammation in response to a pathogenic infection. Sterile SIRS also causes inflammatory complications such as multiple organ dysfunction syndrome (MODS), adult respiratory distress syndrome (ARDS), acute lung injury (ALI), and circulatory failure as well as death (Raoof et al., 2010). However, infection remains undocumented in cases of sterile SIRS. Hauser (2010) and his team hypothesize that the release of mitochondrial danger signals in response to injury acts analogously to PAMPs that activate the immune response during invasion by pathogens. The comparisons between mitochondria and bacteria support such a claim. Thus, they propose that SIRS as a consequence of injury instead of infection occurs due to the interactions between the human immune system and mitochondrial components. The development of "fat embolism syndrome," a type of ALI/ARDS that develops outside of the context of SIRS, following injury-inducing fractures also supports this hypothesis (Hauser et al., 2010). The same innate cells that respond to infection also respond to injury in order to clear the tissue damage. Therefore, SIRS could potentially occur due to widespread mitochondrial-driven local innate immunity that wreaks havoc systemically.

Over ten years ago, Roger Bone (1996) outlined the immunological intricacies supposedly involved in SIRS, coining the term immunologic dissonance. Immunologic dissonance refers to the devastating effects of SIRS that result from an imbalance of proinflammatory and anti-inflammatory mediators. Bone argues that local immune responses typically maintain a balance between these opposing forces, but in the event of a severe infection or injury, systemic inflammation results from the loss of balance. Although he could not definitively explain the mechanisms that determine this balance, Bone, like Hauser, recognized the similarity between the responses of the

immune system to infection versus injury. Systemic inflammatory medical incidences, such as SIRS, have been known to occur only in the past fifty years or less. Bone attributed this fact to the advances of modern medicine that permit people to survive once fatal infections or injuries. Given the Hauser and Bone hypotheses, mitochondria-driven immunity potentially serves a purpose beneficial to human survival following local injury; however, in the cases of severe and widespread injury, it threatens human survival via uncontrolled, systemic inflammatory responses, such as seen with SIRS. Again, the relationship between humans and mitochondria reveal a delicate balance between cooperation and conflict. The endosymbiotic theory provides a framework for cooperation, while the danger model and arms race evolution depict apparent conflict.

A Novel Direction: Injury, Mitochondria, and Alloimmunity

Although aforementioned immunological research collectively demonstrates the role of mitochondria in invoking inflammation as an innate response to injury, no previous studies have applied this discourse of mitochondria-driven immunity in the context of transplantation. Transplantation provides presumably healthy organs or tissues to patients in need. Recipients naturally react to any donated tissue from another individual via an alloimmune response. In order to encourage successful transplantation and recovery, the recipient must be immunosuppressed, commonly blocking the direct interactions and co-stimulatory factors that help them to mount an alloreactive immune response against transplanted tissue (Lechler, 2005). This treatment typically involves a lifelong commitment to the administration of immunosuppressive drugs, which also puts the patient at risk of infection and other diseases. Thus, the field of transplant immunology functions to uncover the triggers behind the alloimmune response.

Ultimately, these findings potentially transform into the means by which to alleviate the burden on transplant patients.

As a surgical procedure derived to repair a physiologically inadequate recipient at the expense of a donor, transplantation involves injury on multiple levels, whether in the recipient, donor, or both parties. Ideal transplant pairs tend to be matched according to the major histocompatibility complex (MHC) found on APCs that coordinates their interactions with the ultimate effectors of the immune system, lymphocytes. However, kidneys transplanted from mismatched living donors promote longer graft survival than those from matched deceased donors, namely cadavers (D'Alessandro et al., 1998). Matzinger utilized this line of evidence to establish the validity of her Danger model proposal (Matzinger, 2002). In addition, kidneys transplanted from brain-dead donors undergo systemic inflammation and result in a lower graft survival rate than kidneys from living donors (Nijober, 2004). Deceased or injured donors presumably cause the recipient to encounter an elevated level of danger signals, most likely caused by the release of mitochondrial DAMPs following injury. This contributes to stronger graft rejection than healthy living donors. Furthermore, injury encountered by the recipient prior to and during transplantation must be taken into account.

Concomitant with the idea of mitochondria-driven immunity, data suggest that the immune responses to injury greatly impact the success of transplantation. However, the mechanisms involved in this particular path towards graft injury remain obscure. Mitochondria might provide some much needed insight into the relationships between injury and the alloimmune response that largely governs transplantation outcomes. **The hypothesis of "The Danger Within" is that injury, specifically mitochondrial disruption, augments immunity and can, when excessive, lead to non-**

adaptive immune responses to severe injury. Specifically, we hypothesize that mitochondrial contents foster more vigorous alloimmune responses toward transplanted organs. This hypothesis was tested by comparing the effects of mitochondria on in vitro transplant models known as mixed lymphocyte reactions (MLRs). We suspect that evolutionary selections on the seemingly symbiotic relationship between humans and their mitochondria play a role in the phenomena of mitochondria-driven immunity, especially as it relates to the tensions between human survival and mitochondrial autonomy.

Methods

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

Background:

Peripheral blood mononuclear cells (PBMCs) include lymphocytes and monocytes. T cells, B cells, and natural killer (NK) cells comprise the group of lymphocytes. Monocytes serve as the precursor to macrophages. The innate immune response is the immune system's primary line of defense against infection or similarly threatening danger signals. During the innate response, monocytes migrate into tissues, differentiating into macrophages. Macrophages function as antigen presenting cells (APCs), engulfing and degrading the threatening components and alerting other immune cells. Also as part of the innate response, NK cells, as their name suggests, neutralize or "kill" the threats. During the subsequent adaptive immune response, T and B cells eliminate threats via cell-mediated and antibody-mediated immunity, respectively (Janeway, 2008).

BD Vacutainer® CPT™ Cell Preparation Tubes (CPTs) with Sodium Citrate provide a convenient system for isolating PBMCs from whole blood (Fotino, 1971). The tubes contain citrate as an anticoagulant, preventing the blood from clotting. They also contain Hypaque™ density gradient fluid, which ultimately aids in segregating unwanted blood cells, such as erythrocytes (red blood cells) and neutrophils. A polyester gel serves as an aide for segregation by separating the blood and citrate from the density gradient fluid. Upon centrifugation, high density blood cells, such as granulocytes and erythrocytes fall under the polyster gel. Blood plasma containing only low density cells, including PBMCs and platelets, remain on top of the gel. Each CPT tube of blood yields approximately 10 million PBMCs. Given the fact that platelets have a lower density than PBMCs, subsequent washes at low spin speeds help to select against platelets in the final cell pellet.

Required Items	Notes:
Human Whole Blood Samples	Use MLR Protocol to predict volume
BD Vacutainer® CPTTM Preparation Tubes with Sodium Citrate	Expect about 10 million cells per tube of blood
1X Dulbeco's Phosphate Buffer Solution	Abbreviated in protocol as DPBS
50-ml Falcon conical tubes	1-3 CPTs worth of cells in one tube
High-Yield Lysing Buffer	
IEC Centra GP8R refrigerated centrifuge	

Protocol:

Following the collection of blood samples into CPTs, the standard method used in the Emory Transplant Lab to isolate PBMCs was applied:

1. Cell Isolation: Spin CPTs at 1600 RCF for 30 minutes at 25°C.

2. Discard plasma about 2 cm above the cell layer (i.e., just above the blue band on the CPT label).

3. Pipette the cell layer off and place in a 50 ml conical tube.

4. Wash CPT tube with 4 ml of sterile DPBS, pipette up and down, and place in same 50 mL tube. (Note: 3 CPTs's worth of cells can be placed in one conical tube.)

5. Bring volume to 45 ml with DPBS in each 50 ml tube.

6. Spin at 1600 (change to 1200 in order to exclude platelets) RPM for 10 min at 25°C.

7. Gently discard supernatant into waste, break up pellet, and repeat previous spin (i.e., steps #5-6).

8. Add 3 ml lysing buffer to the tube, pipette vigorously to break up pellet, and let sit for 5-7 min.

9. Again, bring volume to approximately 45 ml with PBS and repeat previous spin, discarding waste afterwards.

10. Resuspend pellet in 20 ml PBS.

11. Proceed to count cells.

Analysis:

PBMCs were quantified using either or both (if counts appeared unusual) of the following options:

1. Hemacytometer - Mix 10 µl of cell suspension and 90 µl trypan blue. Pipette mix into well of hemacytometer. Under the Nikon Diaphot-TMD inverted microscope microscope, count (by eye) the number of cells in the 4 outer squares of the counting chamber grid. Average and multiply by a dilution factor of 10^5 to determine the final cell count. Since this is not an automated, objective method for counting, the next step was used to verify cell counts.

2. Beckman CoulterTM Z2 Particle Count and Size Analyzer – Invert 20 μl of cell suspension in 10 mL of isotonic fluid. Set machine to count on a 4-14 μm scale. On the Z2 software, use the following settings: curve display, diameter x-axis, groups of seven smoothing, and number per μm graph. Select for size range of 6-10 μm. The software reports concentration in number of cells per mL. (Note: This concentration must be multiplied by the 20mL volume of PBS in which the cells were resuspended.) The windows-based software used to analyze on the Beckman Coulter is called AccuComp® software. The purpose of this software is to calculate statistics on the samples used in the Coulter. This software

accomplishes this by presenting information in the form of graphs, tables, and other reports.

Isolation of Whole Mitochondria

Background:

In line with the tendency of mitochondria to populate areas in which energy remains in high demand, muscle tissue serves as the chosen source of mitochondria for this particular protocol (Scheffler, 2008). The protocol utilized originated from a procedure that allowed Wake Forest University School of Medicine scientists (Rajapakse, et. al, 2001) to successfully isolate and analyze whole mitochondria from neonatal rat brain tissue. However, differences in ultracentrifuge equipment, volumes, and samples, required pilot studies to determine what changes were necessary to accommodate these differences. The mitochondrial isolation protocol used in this study involves centrifugation that results in three distinct bands (in order from top to bottom of the centrifuge tube): (1) Non-mitochondrial cell components, (2) mitochondria, and (3) dead cells. The bands are named according to the component found within each in greatest concentration. Thus, this fractionation or banding pattern largely dictates mitochondria extraction.

Preparation:

Required Items	Notes:
Human muscle tissue samples	400-500mg per isolation
1X Mitochondrial isolation buffer	Components: 0.25 M Sucrose, 0.5 mM K+-EDTA, 10 mM Tris-HCl
Percoll	
5X Mitochondrial isolation buffer (MIB)	1.25M Sucrose, 2.5mM K+-EDTA, 50mM Tris-HCl
80% Percoll solution	Use 5X MIB to dilute Percoll
Percoll solutions: 12%, 26%, 40%	Use 1X MIB to dilute 80% Percoll
Ice	Keep in large enough container to store reagents and samples.
Beckman Ultracentrifuge – SW28 Type Rotor	
Dounce homogenizer	At least 5-mL volume and 0.06 mm clearance
Bovine serum albumin (BSA) solution	10 mg/ml
1X MIB with 10% 10 mg/ml BSA	

Protocol:

The following method was used to isolate mitochondria (Rajapske et al., 2001):

1. Make it a priority to keep all reagents and instruments on ice.
2. Weigh specimen jar containing human muscle tissue and MIB. Record mass.
3. Layer 10.5 ml of 26% Percoll on top of 10.5 ml of 40% Percoll.
4. Homogenize tissue in 11.4 ml of 12% Percoll using dounce homogenizers with glass pestle. Use at least 10 strokes with pestle or preferably, until tissue chunks no longer visible.
5. Add 9 ml of homogenate on top of the previously layered density gradient.
6. Using the ultracentrifuge Beckman SW28 rotor, centrifuge gradient at 13,000 RPM (30,000Xg) for 5 min.
7. With a 200 µl gel loading tip, remove the second clearly distinct, light creamy colored fraction (containing mitochondria). If desired, store other fractions for comparative processing.
8. Dilute mitochondria fraction 1:4 in cold MIB.
9. Centrifuge at 9,000 RPM (15,000Xg) for 10 min.
10. Discard supernatant. Resuspend pellet in 20 ml of MIB and centrifuge at 9,000 RPM (15,000Xg).
11. Discard supernatant.
12. Storage:
    a. If submitting for electron microscopy processing, immediately immerse a visible section of pellet in fix (2.5% glutomaldehyde in 0.1 Mcacodylate buffer, pH = 7.4) and store this at 4°C until submission.
    b. Otherwise, store final pellet in 200 µl of MIB with 10% 10mg/ml BSA solution at -80 °C.

Analysis:

Following isolation of mitochondria and administration of fix to pellet sample, the Emory University Robert P. Apkarian Integrated Electron Microscopy Core verified whole mitochondria in the sample under the direction of the technical director, Dr. Hong Yi. The electron microscopy protocol performed was as follows:

Samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 °C. Samples were then washed twice with the same buffer, post-fixed with 1% buffered osmium tetroxide at the room temperature for 1 hour. Following 2 more buffer washes, samples were dehydrated through a graded ethanol series up to 100%, infiltrated with Eponate 12 resin (Ted Pella), and then embedded in the same resin. Ultrathin sections of the samples were cut at 60-70 nm and counterstained using uranyl acetate and lead citrate. The examination of the ultrathin sections was carried out on a Hitachi H-7500 transmission electron microscope equipped with a Gatan BioScan CCD camera.

Assaying Proliferation: CFSE Allo-MLR

Background

Proliferation Allo-MLR results were assessed using carboxyfluorescein succinimidyl ester-based (CFSE) flow cytometry. The intensity of the green-colored CFSE correlates with the number of cell divisions, thus serving as a measurement of the aggressiveness of the immune response (Lyons, 1999). Cell differentiation (CD) markers on the surface of

immune cells identify their type and function. The surface markers used in this assay and their relevance are as follows:

CD Marker	Majority Presence	Relevant Function	Common Aliases
3	T Cells	Signal transduction of T Cell Receptor	--
4	Helper T Cells (Some Monocytes)	Glycoprotein (HIV receptor); binds MHC Class II	--
8	Killer T Cells (Some NK cells)	Surface receptor; binds MHC Class I	--
28	T Cells	Cell-adhesion, co-stimulatory ligand	--
45RA	T/B Cells Monocytes	CD45+ Naïve T Cells	--
197	Peripheral T/B Cells; Mature Dendritic Cells	Chemokine receptor; Lymphocyte trafficking	CCR7

Preparation:

Required Items	**Notes:**
IEC Centra GP8R refrigerated centrifuge	
RPMI-1640	Minimal media
R10	Supplemented RPMI-1640

FACS Buffer	Facilitated assorting cell sorting buffer: 1L DPBS, 50 mL Fetal Bovine serum,
SEB	
Mouse Anti-Human Antibodies	See #11 in protocol below for details.
96-well, round-bottomed plate	
Ice	Be sure to have a container in which the plates can fit plates during their incubation.
15-ml conical tubes	These will be used to sort out responders versus stimulators.

Note: The PBMCs and mitochondria previously isolated in the above protocols will be used here as well.

Protocol(s):

The following protocol was followed for the CFSE Allo-MLR:

1. Following PBMC Isolation protocol and analysis (see above), designate responders versus stimulators depending on cell counts and conditions in MLR. Remove volume appropriate for stimulators and place in 15-mL conical, including "self-stimulators." Leave responders in original 50-ml conical used in PBMC isolation.
2. Centrifuge responders (1200rpm x 10 minutes) and pipette off supernatant completely. Top conical with RPMI and centrifuge again, again discarding supernatant.
3. Irradiate stimulators for approximately 12 minutes. Then, wash twice with R10 using centrifuge spins of1200rpm x 10 minutes. Keep on ice until ready to count both responders and stimulators.
4. Resuspend responders at 10 million cells per mL with RPMI.

5. Add 1μL of 10mM CFSE per 1 mL of responders. Vortex and incubate at 37°C in CO_2 incubator for 10 minutes in the dark.
6. Centrifuge at 1200rpm x 10 minutes at 4°C, decant, and resuspend in 1 mL R10.
7. Count both responders and stimulators.
8. Add R10 to responders to get a concentration of 2 million cells per mL. Plate 200,000 stimulators (100 μL) per well according to experimental design.
9. Add R10 to stimulators to get a concentration of 4 million cells per mL. Plate 400,000 stimulators (100 μL) per well according to experimental design.
10. Place plate in 37°C CO_2 incubator for 5 days.
11. **On the fifth day of incubation**, in preparation for antibody staining, prepare a volume antibody cocktail that is appropriate for the number of wells used given the fact that each well should contain the following:

Fluorescence - Antibody	*Volume (Dilution, if applicable)*
APC - CD8	5μL
PerCP Cy5.5 - CD28	10μL
V450 - CD4	5μL
PECy7 - CD197	1μL
Qdot655 - CD45RA	1μL (1:10)
Alexa700 - CD3	10μL (1:8)

12. Collect cells after 5-day incubation and wash once with FACS Buffer. (Note: Always centrifuge at 1500rpm x 3 minutes.)

13. Dilute cells with 100μL FACS Buffer and then add 32μL of antibody cocktail per well.
14. Incubate on ice in the dark for 15 minutes.
15. Wash cells twice with FACS Buffer.
16. Add 200μL FACS Buffer and proceed to flow analysis.

Analysis

Please see the Flow Cytometry protocol below.

Assaying Cytokine Release and Degranulation: ICS Allo-MLR

Background:

Naïve cells proliferate upon activation. However, memory cells more commonly show signs of intracellular activation, typically via cytokine release, rather than proliferation. Therefore, an Intracellular Cytokine Staining (ICS) Allo-MLR assay was dedicated to intracellular activation. The intracellular and degranulation markers used in this assay and their relevance follows:

Marker	Purpose
CD107a	Activated cells express this Lysosomal-associated membrane protein (LAMP) upon degranulation.
Granzyme B	Cytotoxic T lymphocytes (CTLs) use this serine protease to promote apoptotic cell death.
Interferon (IFNγ)	Activated CD4+ or CD8+ T cells and NK cells produce this cytokine, which regulates a multitude of inflammatory processes and monocyte differentiation.
Tumor necrosis factor (TNFα)	Monocytes, macrophages, neutrophils, NK cells, and CD4+ T cells produce this cytokine, which acts against tumor cells and exerts immune regulation.

Preparation:

Required Items	Notes:
IEC Centra GP8R refrigerated centrifuge	
GFixation and Permeabilization Solution Kit with BD GolgiPlug™	Contains Fix/Perm and Perm/Wash used in protocol (Catalog No. 555028)
R10	
FACS Buffer	
SEB	
Mouse Anti-Human Antibodies	See #11 in protocol below.
96-well, round-bottomed plate	
Ice	Be sure to have a container in which the plates can fit plates during their incubation.
15-ml conical tubes	These will be used to sort out responders versus stimulators.

Note: The PBMCs and mitochondria previously isolated in the above protocols will be used here as well.

Protocol:

The following protocol was followed for the Intracellular Cytokinge Staining and CD107a Degranulation Allo-MLR:

1. Following PBMC Isolation protocol and analysis (see above), designate responders versus stimulators depending on cell counts and conditions in MLR. Remove volume appropriate for stimulators and place in 15-mL conical, including "self-stimulators." Leave responders in original 50-ml conical used in PBMC isolation.
2. Irradiate stimulators for approximately 12 minutes. Then, wash twice with R10 using centrifuge spins of1200rpm x 10 minutes. Resuspend in 5mL or less of R10 to count cells.
3. Centrifuge responders at1200rpm x 10 minutes, decanting supernatant afterwards. Resuspend in 5mL or less of R10 to count cells.
4. Count both responders and stimulators.
5. Add R10 to responders to get a concentration of 2 million cells per mL. Add 4μL of GolgiPlug per 1mL of responders. Vortex and allow to sit at room temperature.
6. Add R10 to stimulators to get a concentration of 4 million cells per mL. Plate 400,000 stimulators (100 μL) per well according to experimental design.
7. Plate 200,000 stimulators (100 μL) per well according to experimental design.
8. Add 3μL of CD107a to appropriate wells (i.e., wells designated for surface marker antibody panel).
9. Place plate in 37°C CO_2 incubator for 6 hours.

10. In preparation for antibody staining, prepare volumes of two separate antibody cocktails that are appropriate for the number of wells used given the fact that each well should contain the following:

ANTIBODY COCKTAIL 1 (SURFACE MARKER ANTIBODY PANEL)

Fluorescence - Antibody	*Volume (Dilution, if applicable)*
APC - CD8	5μL
PerCP Cy5.5 - CD28	10μL
V450 - CD4	5μL
PECy7 - CD197	1μL
Qdot655 - CD45RA	1μL (1:10)

Note: CD107a previously added to the wells designated to the surface marker antibody panel.

ANTIBODY COCKTAIL 2 (INTRACELLULAR MARKER ANTIBODY PANEL):

Fluorescence - Antibody	*Volume (Dilution, if applicable)*
APC Cy7 - CD8	5μL
PerCP Cy5.5 - CD28	10μL
V450 - CD4	5μL
PECy7 - CD197	1μL
Qdot655 - CD45RA	1μL (1:10)

Note: Intracellular antibodies will be added later to the wells designated to the intracellular marker antibody panel.

11. Collect cells after 6-hour incubation and spin down plate. (Note: Always centrifuge at 1500rpm x 3 minutes.)
12. Dilute cells with 150μL FACS Buffer and then add 32μL of antibody cocktail per well, using either antibody cocktail 1 or 2 depending on well designation.
13. Incubate on ice in the dark for 15 minutes.
14. Wash cells twice with FACS Buffer.
15. Resuspend wells designated under surface marker antibody panel in 80μL FACS Buffer and place in FACS tubes. Keep these tubes chilled and covered with foil.
16. Add 100μL Fix/Perm to remaining cells (i.e., those designated under intracellular marker antibody panel). Incubate on ice for 20 minutes.
17. Add 100μL Perm/Wash prior to washing cells.
18. Add 200μL Perm/Wash for a second wash.
19. Resuspend cells in 150μL Perm/Wash. Add the following intracellular antibodies to each well:

Fluorescence - Antibody	*Volume (Dilution, if applicable)*
FITC - IFNγ	10μL
APC - TNFα	5μL

20. Incubate on ice for 15 minutes.
21. Add the following intracellular antibody to each well:

Fluorescence - Antibody	*Volume (Dilution, if applicable)*
PE – Granzyme B	2μL

22. Incubate on ice for another 15 minutes.
23. Spin and wash again with 200μL Perm/Wash.
24. Resuspend cells in 80μL Perm/Wash, place in FACS tubes, and proceed to flow analysis.

Analysis:

Please see the Flow Cytometry protocol below.

Flow Cytometry

Flow cytometry counts and classifies cells and other small particles via electronic detection. This is accomplished by suspending samples in liquid (FACS buffer or permeabilization wash) and allowing this liquid to flow through the flow cytometer. The flow cytometer machine detects scattered and fluorescent light. The former is used to report cell size and complexity; the latter is used to detect the fluorescent tags, such as antibodies, added to the samples. In this investigation, fluorescent antibodies were used to classify T cell populations (CD3), helper T cells (CD4), and killer T cells (CD8). Fluorescent antibodies were also used to detect intracellular markers (TNFα, IFNγ, and Granzyme B). Futhermore, the flow cytometer was able to detect the intensity of CFSE, which is essentially a green-colored fluorescent dye.

Following antibody staining, all allo-MLR samples were compensated and ran on the BD Bisociences LSR II using BD Diva software. Approximately 50,000 events were collected from each sample. The results were analyzed using FlowJo Software. Using this software, fluorescence-activated cell sorting (FACS) plots were used to compare and assay proliferation, cytokine release, and degranulation according to the aforementioned markers.

Results

Mitochondria Isolation – Electron Microscopy

The mitochondria isolation protocol results in three fractions of material separated into distinct bands via centrifugation in a Percoll gradient. These are illustrated in Figures 1-4 as shown by electron microscopy from the present study. It was found that only the middle fraction yielded a high concentration of intact mitochondria outside of cells.

Figure 1: Top Fraction

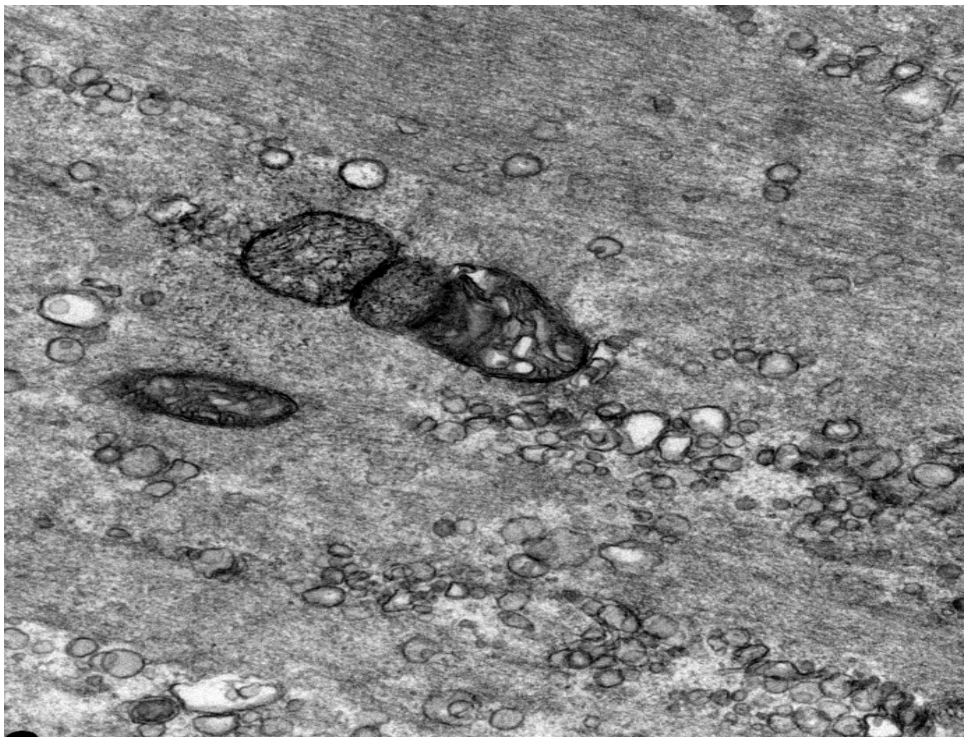


Figure 1 is a micrograph taken from the top fraction in the banding pattern. It depicts a thin slice of muscle tissue, complete with mitochondria still within cells. It was taken at the same magnification as Figure 2.

Figure 2: Mitochondrial Fraction A

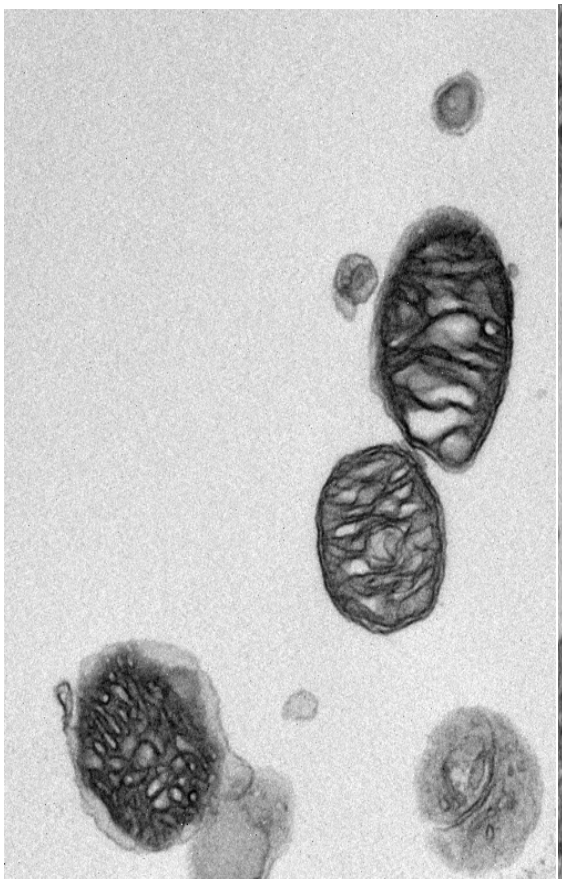


Figure 3: Mitochondrial Fraction B

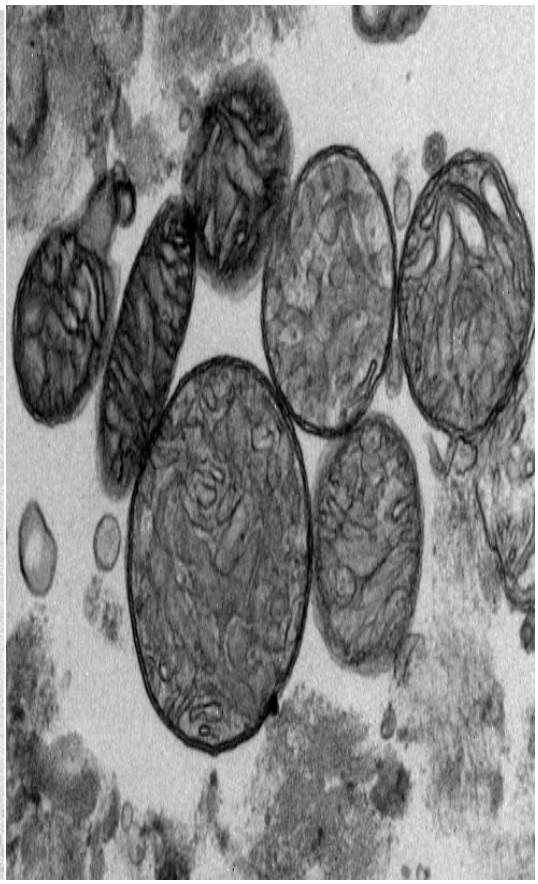


Figure 2 is a micrograph taken from the mitochondria (middle) fraction in the banding pattern. It depicts three whole mitochondria present in the fractionated mitochondria sample. As a reference of scale, the middle mitochondrion has a length of 0.5μm. The magnification of this micrograph is two times higher than the one in Figure 2. **Figure 3** is another micrograph taken from the mitochondria (middle) fraction in the banding pattern. It depicts seven whole mitochondria present in the fractionated mitochondria sample. As a reference of scale, the bottommost mitochondrion has a length of 1μm.

Figure 4: Bottom Fraction

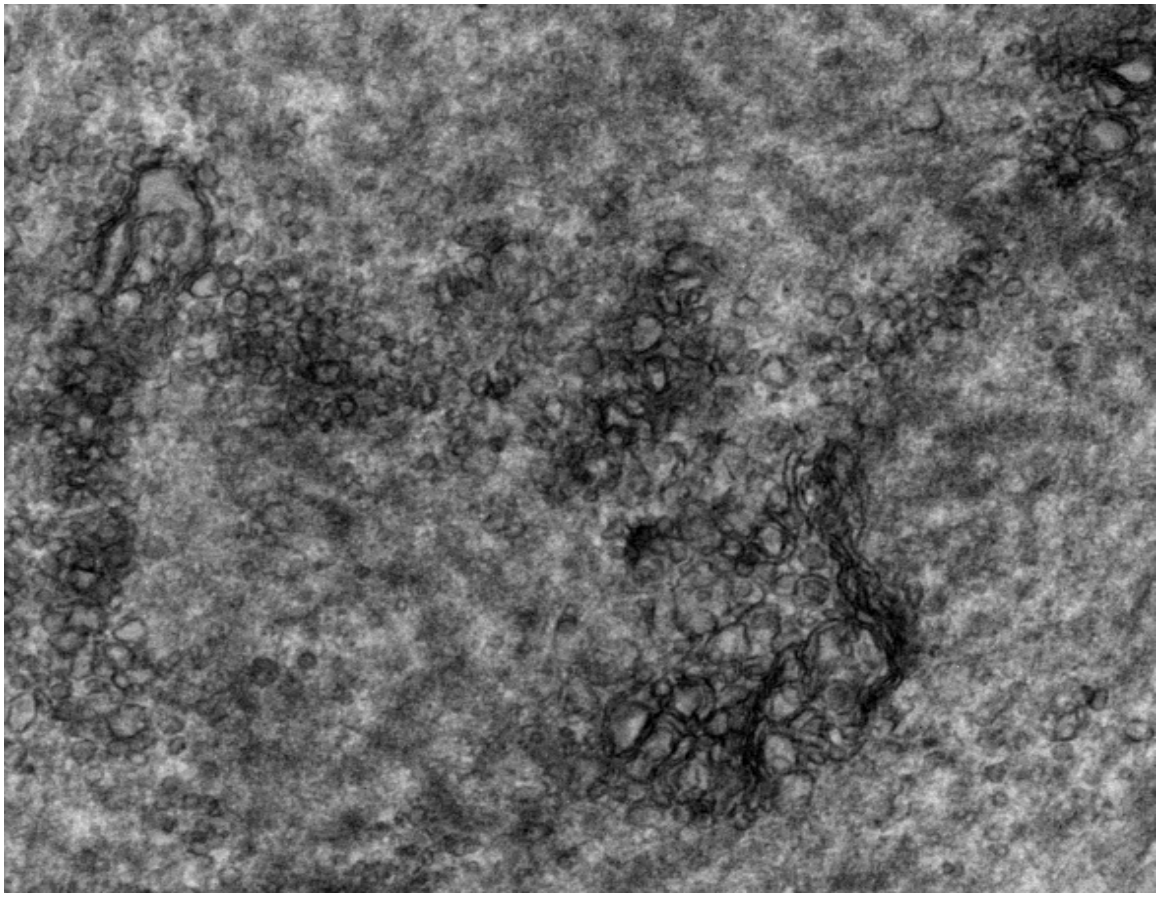


Figure 4 is a micrograph taken from the bottom fraction in the banding pattern. It depicts masses of muscle tissue, complete with mitochondria still within cells. It was taken at the same magnification as Figure 2.

Proliferation Assay: CFSE Allo-MLR Results

Immune cell proliferation in the responder (recipient) can be assayed by CFSE. The results include histograms of CFSE-stained responder T cells. With increasing divisions in the T cell population, the CFSE dye should appear less intensely. Therefore, proliferating cells appear at the left (low intensity) end of the CFSE axis and non-proliferating cells appear at the right (high intensity) end of the CFSE axis. In the proliferation assay, the donor cells are irradiated peripheral blood mononuclear cells (PBMCs). The results from two patients (A and B) are reported separately. The color code for the histogram plots remains the same for both patient reports:

- Black: Standard MLR (without addition of mitochondria)
- Blue: MLR plus addition of recipient mitochondria (i.e., self mitochondria)
- Red: MLR plus addition of donor mitochondria

The reference line than spans the space within the histogram plot allows comparison between the proliferation of the standard MLR condition in each graph and the baseline (recipient alone) proliferation. Again, the number on the left of this line represents the percentage of dividing cells, while the number on the right represents the percentage of non-diving cells.

Recipient - Patient A

Figure 5: Alone

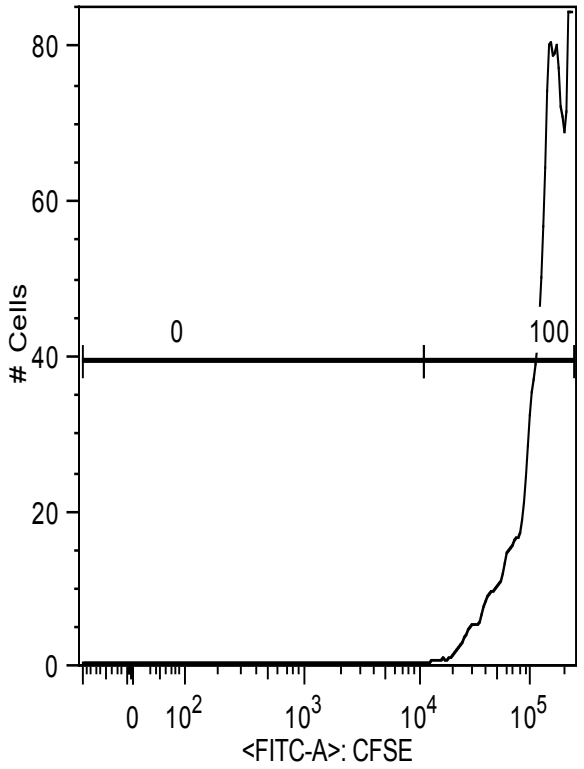


Figure 6: Self-stimulated

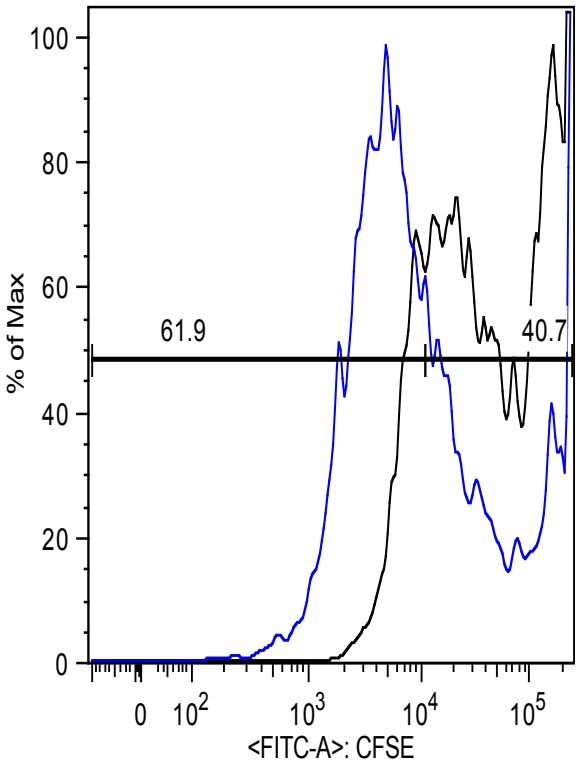


Figure 5 represents the T cell population of Patient A by itself. Since the peak is at the far right, this indicates that none of the cells are actively dividing. This result of no proliferation is expected since they are not receiving any stimulation. **Figure 6** represents the T cell population of Patient A self-stimulated by irradiated Patient A PBMCs. Since both distributions represent only Patient B cells, the standard MLR (black) would be expected to appear more similar to the distribution in Figure 1, but it (blue) shows more proliferation. Nevertheless, the addition of self-mitochondria (blue) results in more proliferation than the standard MLR.

Figure 7: B-stimulated

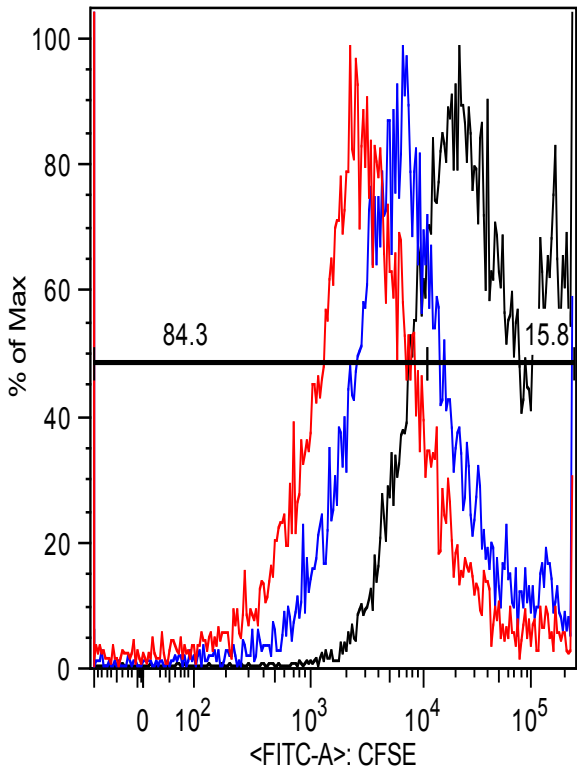


Figure 8: Third-party (E)-stimulated

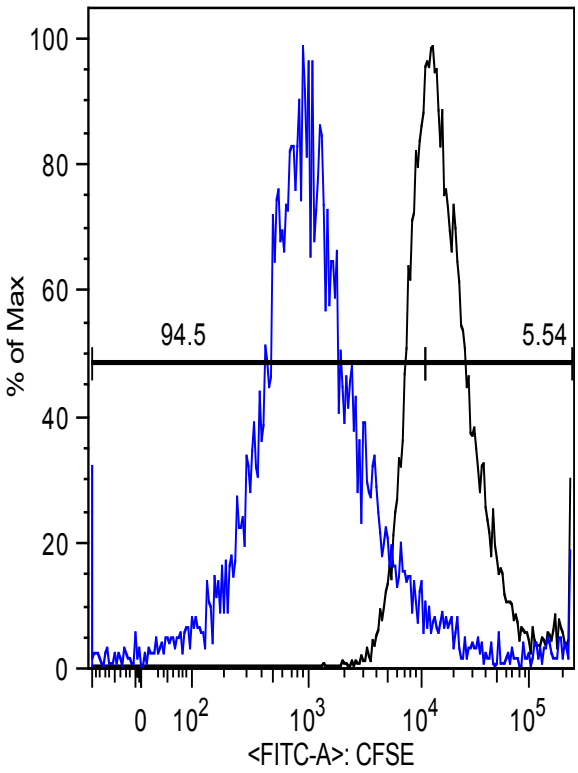


Figure 7 represents the T cell population of Patient A stimulated by irradiated Patient B PBMCs. The standard MLR (black) results in less proliferation than either of the assays involving mitochondria additions (blue and red). However, the addition of Patient B mitochondria (red) resulted in more proliferation than the addition of self-mitochondria. **Figure 8** represents the T cell population of Patient A stimulated by the irradiated PBMCs of a third party, E. The addition of self-mitochondria (blue) results in more proliferation than the standard MLR (black).

Recipient - Patient B

Figure 9: Alone

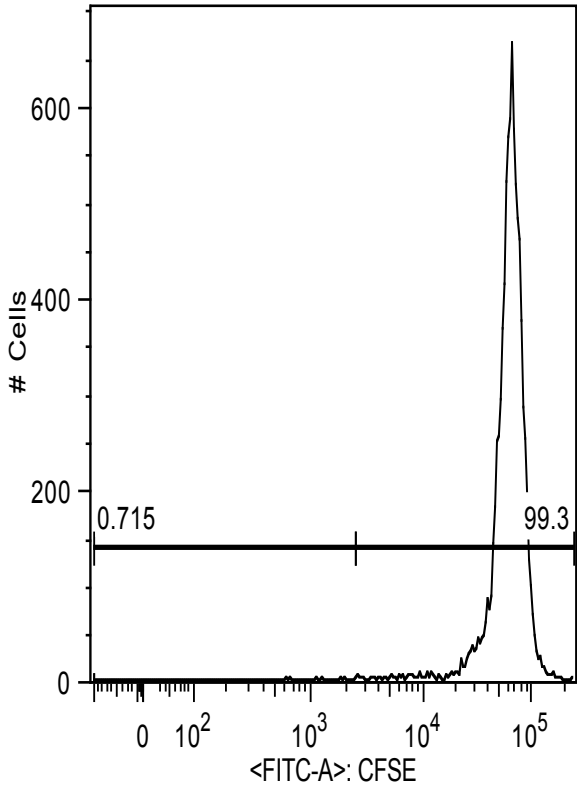


Figure 10: Self-stimulated

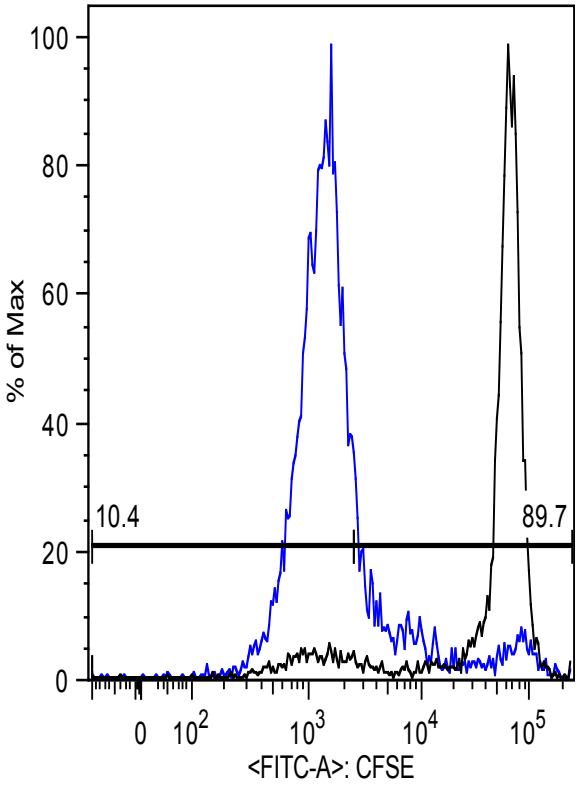


Figure 9 represents the T cell population of Patient B by itself. Since the peak is at the far right, this indicates that none of the cells are actively dividing. This result of no proliferation is expected since they are not receiving any stimulation. **Figure 10** represents the T cell population of Patient B self-stimulated by irradiated Patient B PBMCs. As expected, since both distributions represent only Patient B cells, the standard MLR (black) appears similar to the distribution in Figure 1. The addition of self-mitochondria (blue) results in more proliferation than the standard MLR.

Figure 11: A-stimulated

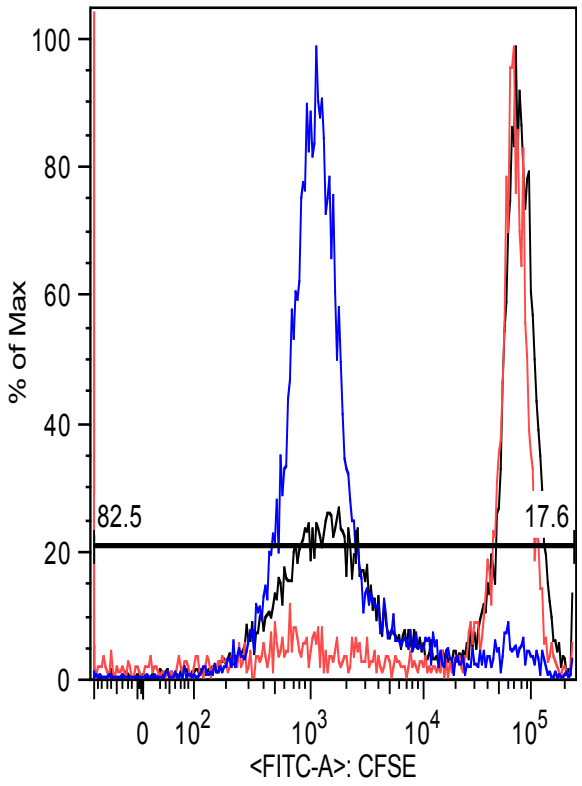


Figure 12: Third party (E)-stimulated

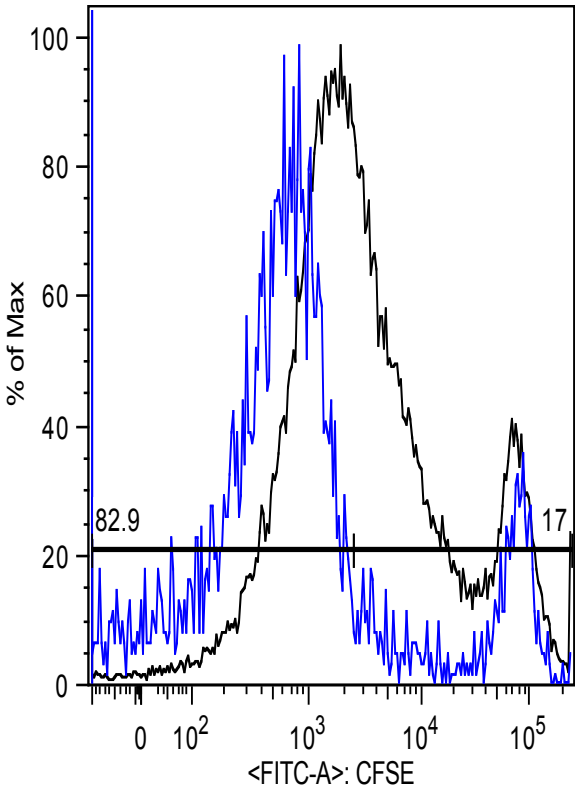


Figure 11 represents the T cell population of Patient B stimulated by irradiated Patient A PBMCs. The standard MLR (black) results in less proliferation than only of the addition of self-mitochondria (blue). The addition of Patient A mitochondria (red) resulted in less proliferation than both the addition of self-mitochondria (blue) and the standard MLR (black). **Figure 12** represents the T cell population of Patient B stimulated by the irradiated PBMCs of a third party, E. The addition of self-mitochondria (blue) results in more proliferation than the standard MLR (black).

Intracellular Cytokine/Granzyme Assay: ICS Allo-MLR Results

The purpose of the intracellular cytokine mixed lymphocyte reactions was to assay activation of T cells as characterized by cytokine release or in the case of CD8+ T cells, granzyme B release. Proliferation assays do not accurately predict activation in memory cells, which unlike that of naïve cells, does not commonly result in cell division. Increase in cytokine or Granzyme B (GB) release signifies activation of immune cells, whether naïve or memory cells. These results include pseudo-color density plots assaying intracellular markers. An increase in the density of cells is represented by color changes. In the order of increasing density, these colors include: blue, green, yellow, orange, and red. The pink quadrants are fixed for each population based on the baseline activation of the responder cells alone. The numbers in each quadrant give the percentage of the total cells in that graph that falls under each quadrant. We expect activation to show an increased density of cells moving towards quadrant I (upper left) if there is an increase in only the marker on the y-axis, quadrant IV (lower right) if there is an increase in only the marker on the x-axis, or quadrant II (upper right) if there is an increase in both markers. The results from two patients (C and D) are reported. Each patient report has been divided into two categories according to population of interest and markers tested (given in parentheses):

CD4+ T Cell Activation (TNFα and IFNγ)

Quadrant I (TNFα +and IFNγ-) Quadrant II (TNFα+ and IFNγ+)

Quadrant III (TNFα- and IFNγ-) Quadrant IV (TNFα- and IFNγ+)

CD8+ T Cell Activation (Granzyme B and IFNγ)

Quadrant I (GB+ and IFNγ-)	Quadrant II (GB+ and IFNγ+)
Quadrant III (GB- and IFNγ-)	Quadrant IV (GB- and IFNγ+)

In this section, the same color code used in the proliferation section now applies to the caption listing the conditions (i.e., Black: Standard MLR, Blue: Self-mitochondria added to MLR, Red: Donor mitochondria added to MLR).

Recipient - Patient C

CD4+ T Cell Activation (TNFα and IFNγ)

Figure 13: Alone

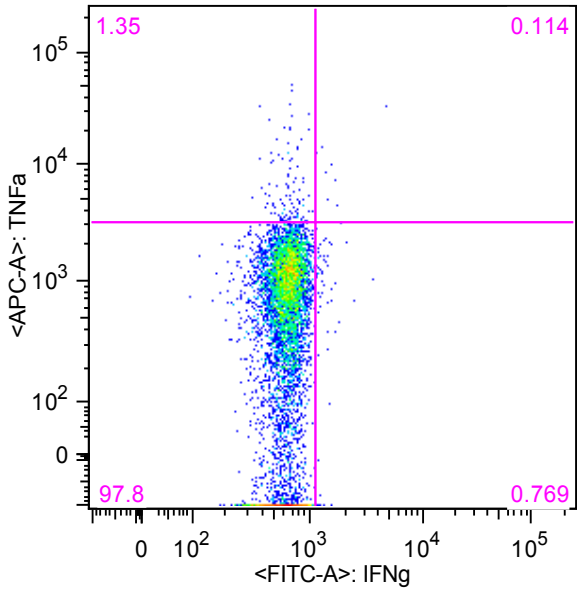


Figure 13 represents the baseline (i.e., in the absence of stimulation) level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C. This shows that majority of the population is minimally secreting TNFα and IFNγ (e.g., most are considered TNFα-IFNγ- cells). However, there are a small percentage of cells with increased levels of TNFα release.

Figure 14: Self-stimulated

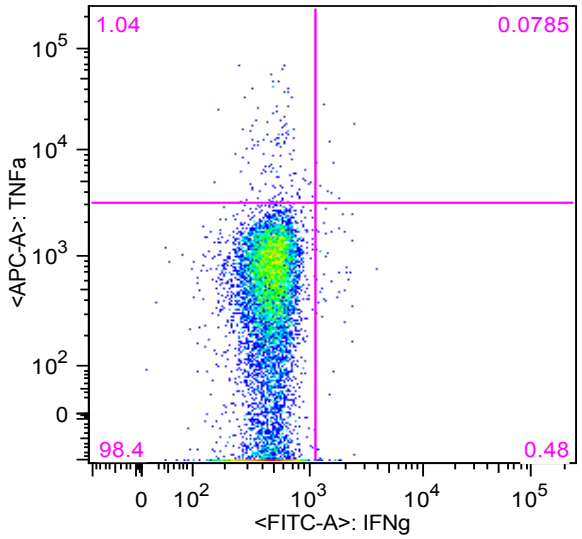


Figure 15 - Self-stimulated

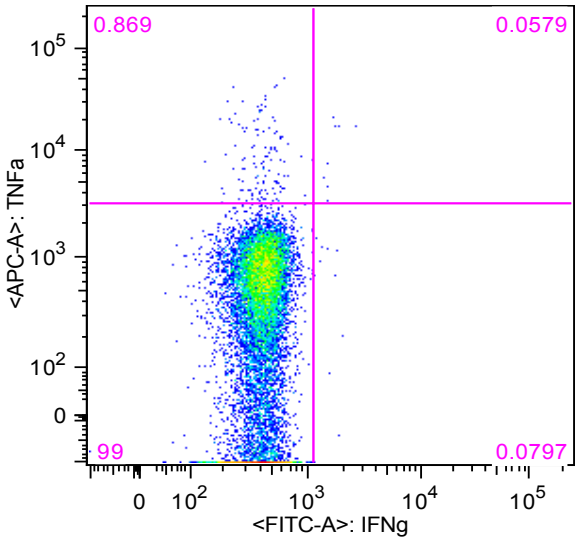


Figure 14 represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C upon self-stimulation. **Figure 15** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C upon self-stimulation plus addition of self-mitochondria. There appears to be even less release of TNFα and IFNγ in comparison to standard self-stimulation (Figure 14). **Therefore, self-mitochondria do not appear to have an activating intracellular cytokine effect on the release of TNFα or IFNγ CD4+ T cell population of Patient C upon self-stimulation, but potentially pose a proliferative effect.**

Figure 16: D-stimulated

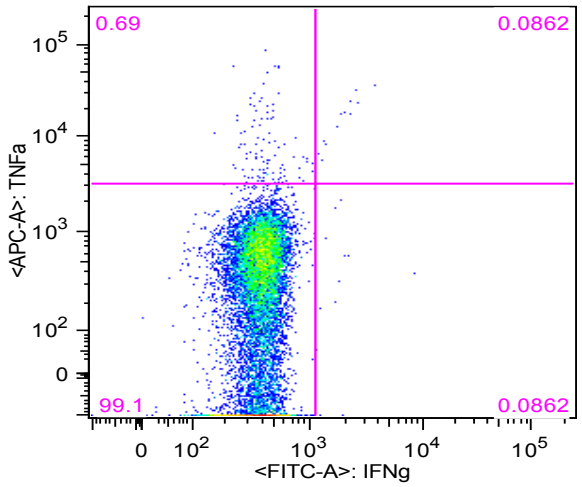


Figure 17: D-stimulated

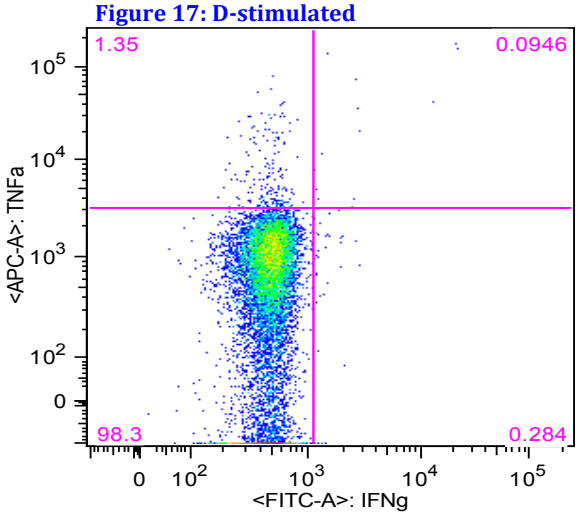


Figure 18: D-stimulated

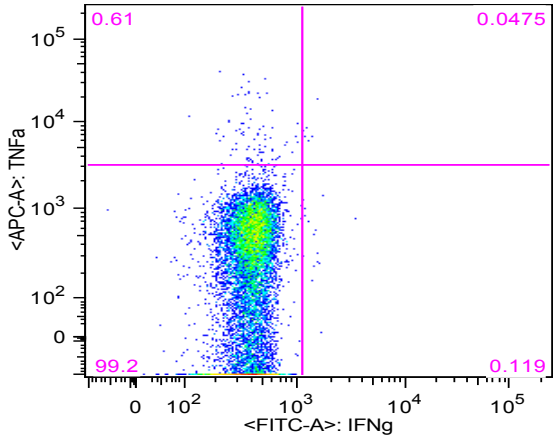


Figure 16 represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C upon standard stimulation by Patient D. **Figure 17** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C upon stimulation by Patient D with the addition of self-mitochondria. Relative to Figure 16, there appears to be an increase in the TNFα+ IFNγ- population. Although activation of TNFα release is suggested, there does not seem to be an apparent increase in IFNγ. **Therefore, self-mitochondria appear to increase TNFα release from CD4+ T cells in an alloimmune response of Patient C to Patient D. Figure 18** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient C upon stimulation by Patient D with the addition of Patient D mitochondria. Relative to Figure 16, the density of the total cell population seems a bit decreased. However, cytokine activation seems similar. **Therefore, mitochondria from Patient D does not seem to have an activating effect on the intracellular cytokine release of TNFα or IFNγ from CD4+ T cells in an alloimmune response of Patient C to Patient D.**

Figure 19: SEB

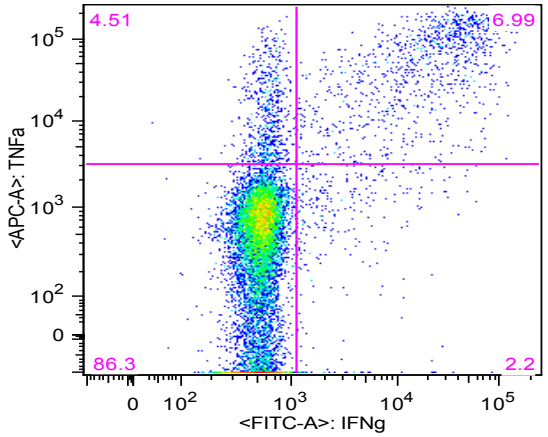


Figure 19 represents the level of cytokine release of the CD4+ T cell population in Patient C upon stimulation by SEB. Clearly, SEB strongly activates intracellular cytokine releases, resulting in a significantly larger TNFα+ IFNγ+ relative to baseline (Figure 13). The TNFα+IFNγ- population is also increased, even more so than the TNFα-IFNγ+ population. These increases are accompanied by a decrease in the TNFα-IFNγ- population. **Therefore, SEB serves as a strong positive control with the ability to incite intracellular activation of TNFα and IFNγ release in the CD4+ T cells of Patient C relative to baseline. This confirms that the assay is indeed functioning properly.**

CD8+ T Cell Activation (Granzyme B and IFNγ)

Figure 20: Alone

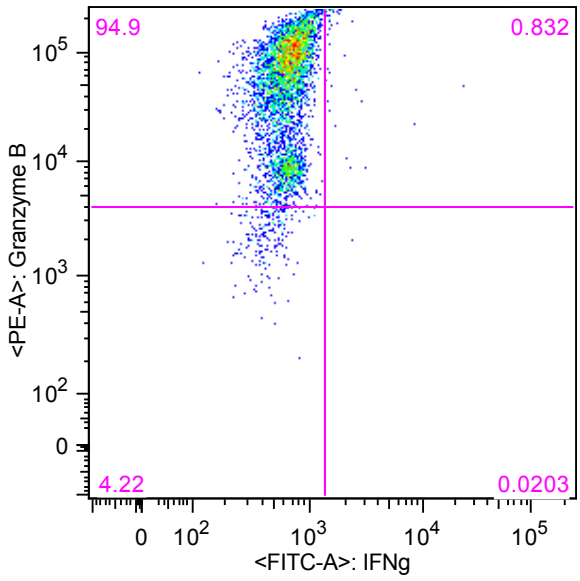


Figure 20 represents the baseline level (i.e., in the absence of stimulation) of Granzyme B and IFNγ release of the CD8+ T cell population in Patient C. The greater majority of the cell population is GB+IFNγ-. A small population of cells is GB- IFNγ-. The other cell populations, GB+IFNγ+ and GB-IFNγ+ are rare.

Figure 21: Self-stimulated

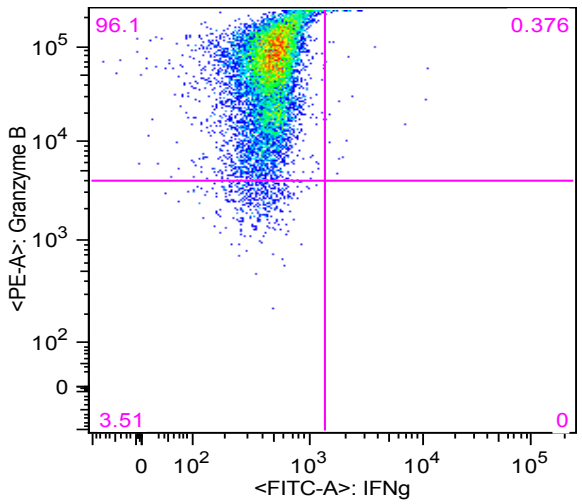


Figure 22: Self-stimulated

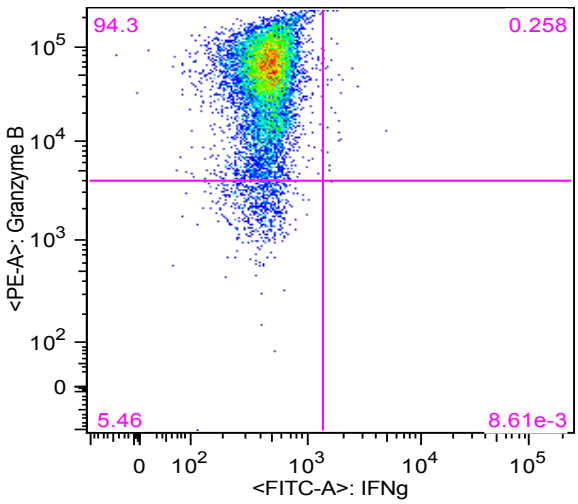


Figure 21 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon self-stimulation. There is a strong GB+ IFNγ- population and a small GB-IFNγ- population. **Figure 22** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon self-stimulation with the addition of self-mitochondria. There appears to be a slight increase in the GB-IFNγ- population relative to Figure 21. However, the remaining populations appear unchanged. **Therefore, self-mitochondria do not appear to have an activating effect on the release of Granzyme B and IFNγ by the CD8+ T cell population of Patient C upon self-stimulation.**

Figure 23: D-stimulated

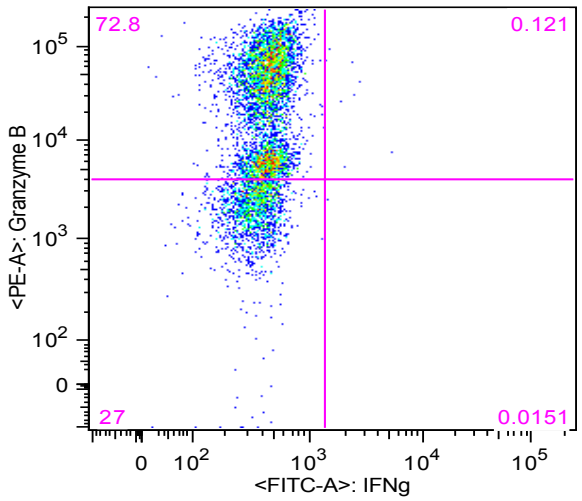


Figure 24: D-stimulated

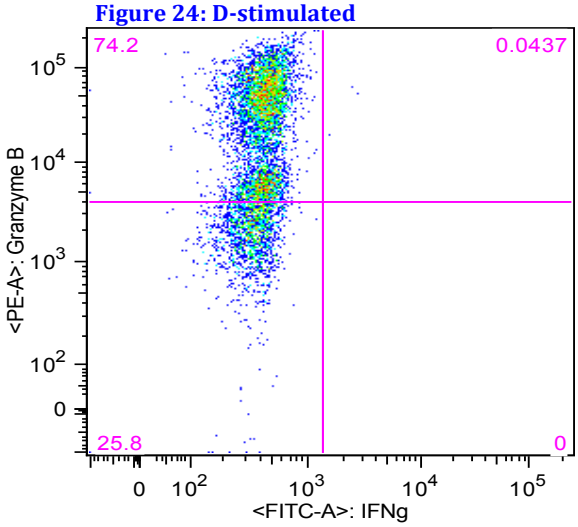


Figure 25: D-stimulated

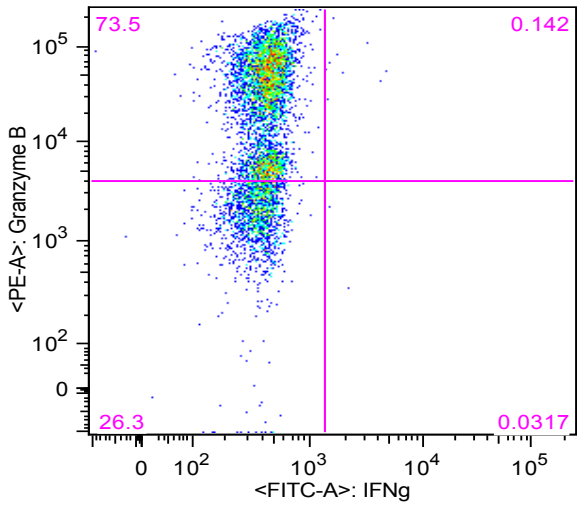


Figure 23 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon stimulation by Patient D. There appears to be two distinct populations. The more prominent one is GB+IFNγ-, and the other is borderline between GB+IFNγ- and GB-IFNγ-. **Figure 24** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon stimulation by Patient D with the addition of self-mitochondria. The distribution of cells does not seem to vary from Figure 23. **Therefore, self-mitochondria do not appear to have an activating effect on the release of Granzyme B and IFNγ by the CD8+ T cell population of Patient C upon stimulation by Patient D. Figure 25** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon stimulation by Patient D with the addition of mitochondria from Patient D. Like Figure 24, the distribution of cells does not seem to vary from Figure 23. **Therefore, mitochondria from Patient D also do not appear to have an activating**

effect on the release of Granzyme B and IFNγ by the CD8+ T cell population of Patient C upon stimulation by Patient D.

Figure 26: SEB-stimulated

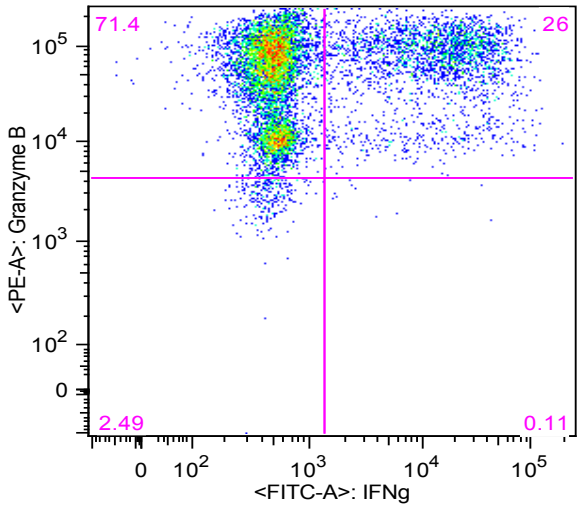


Figure 26 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient C upon stimulation by SEB. Clearly, SEB causes a large increase in the GB+IFNγ+ population that was initially rare at baseline (Figure 20). **Therefore, SEB serves as a strong positive control with the ability to incite Granzyme B and IFNγ release in the CD8+ T cells of Patient C relative to baseline. This confirms that the assay is indeed functioning properly.**

Recipient - Patient D

CD4+ T Cell Activation (TNFα and IFNγ)

Figure 27: Alone

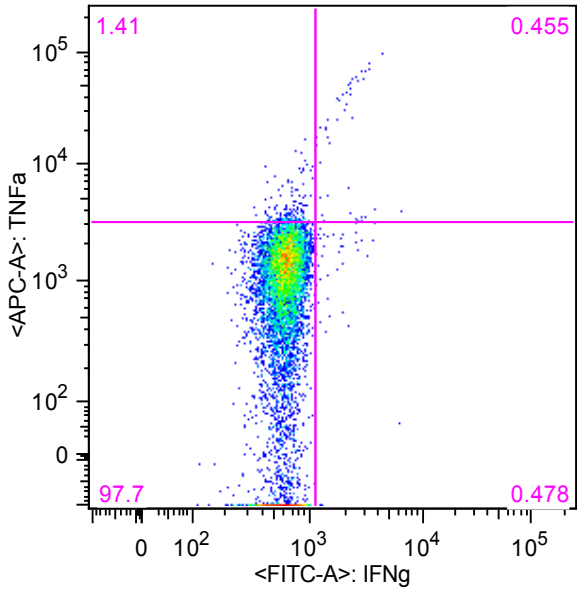


Figure 27 represents the baseline (i.e., in the absence of stimulation) level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D. Quadrant III contains the great majority of the population, which can be classified as TNFα-IFNγ- cells according to this quadrant. As shown by the low density of cells in quadrants I and IV, there appears to be minimally heightened activity in TNFα and IFNγ release, respectively, at baseline. Quadrant II shows a small population of TNFα+IFNγ+ cells are at baseline.

Figure 28: Self-stimulated

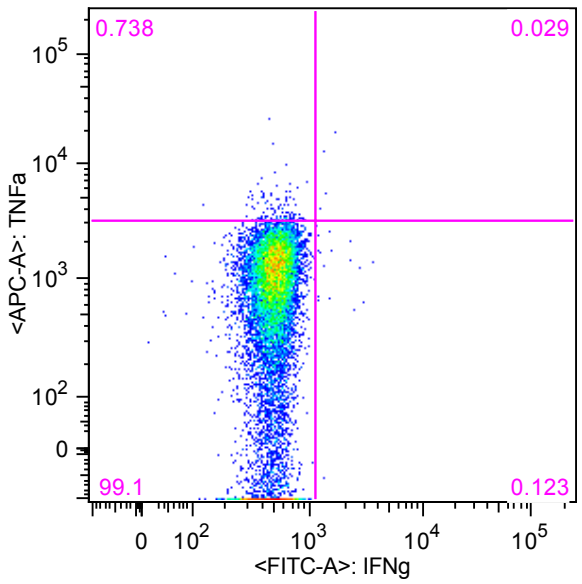


Figure 29: Self-stimulated

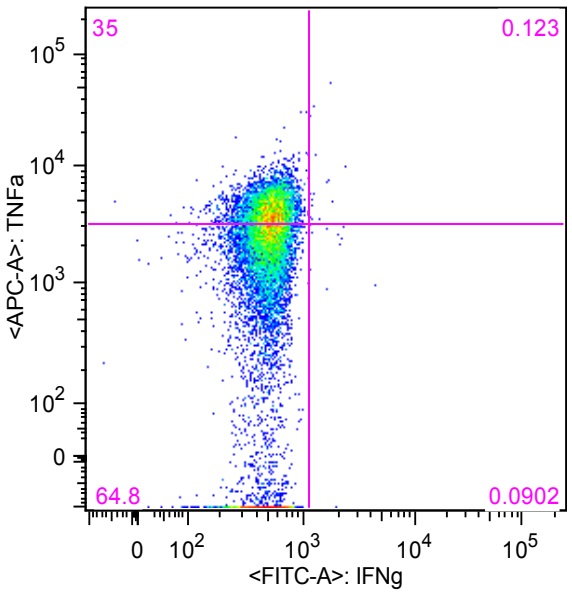


Figure 28 represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D upon self-stimulation. **Figure 29** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D upon self-stimulation plus addition of self-mitochondria. There is a significant increase in the TNFα+ IFNγ- population and decrease in the TNFα-IFNγ- relative to the distribution that resulted from the standard self-stimulation (Figure 28). **Therefore, self-mitochondria indeed appear to have an activating intracellular cytokine effect via the increased release of TNFα by the CD4+ T cell population of Patient D upon self-stimulation.**

Figure 30: C-stimulated

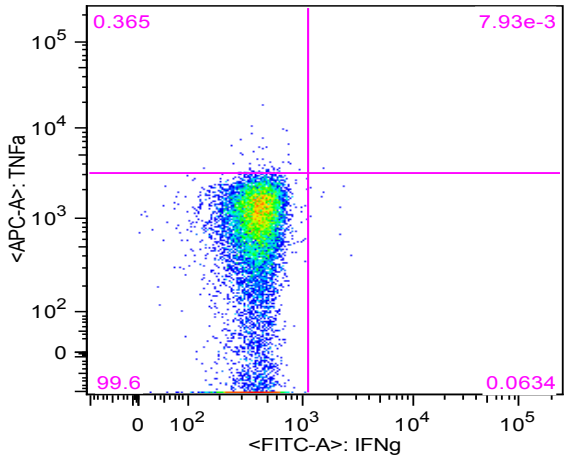


Figure 31: C-stimulated

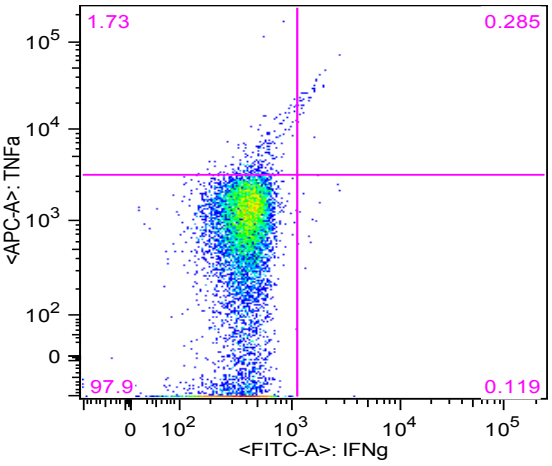


Figure 32: C-stimulated

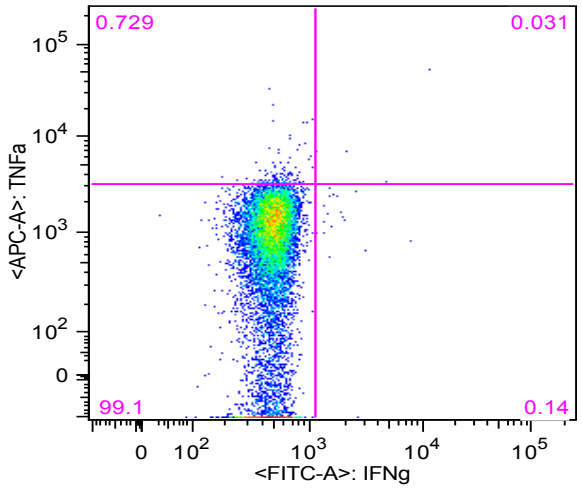


Figure 30 represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D upon standard stimulation by Patient C. **Figure 31** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D upon stimulation by Patient C with the addition of self-mitochondria. Relative to Figure 30, there appears to be an increase in the TNFα+ IFNγ- population that carries over into a TNFα+ IFNγ- population as well. However, the diagonal trajectory of this potential activation resembles the pattern resultant of interference from dead cells. **Therefore, self-mitochondria cannot be definitively considered as an activator of TNFα release from CD4+ T cells in an alloimmune response of Patient D to Patient C. Figure 32** represents the level of TNFα and IFNγ cytokine release of the CD4+ T cell population in Patient D upon stimulation by Patient C with the addition of Patient D mitochondria. Relative to Figure 30, the density of the TNFα+ IFNγ- cell population seems slightly decreased. However, all other quadrants appear similar. **Therefore, mitochondria from Patient C does not seem to have an activating effect on the intracellular cytokine release of TNFα or IFNγ from CD4+ T cells in an alloimmune response of Patient D to Patient C.**

Figure 33: SEB-stimulated

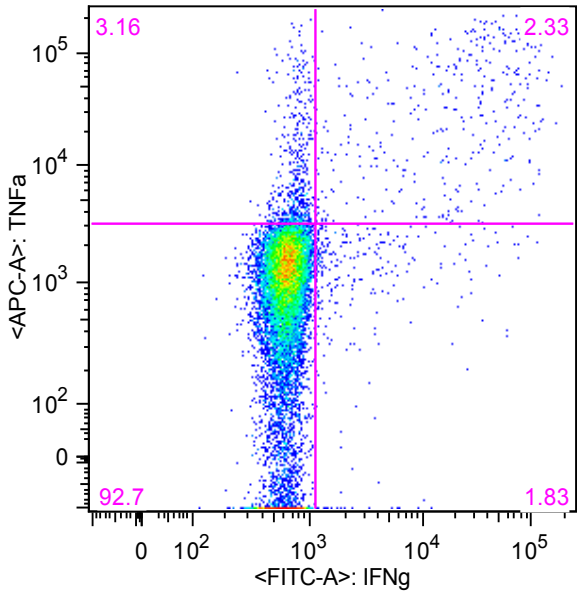


Figure 33 represents the level of cytokine release of the CD4+ T cell population in Patient D upon stimulation by SEB. Clearly, SEB strongly activates intracellular cytokine releases, resulting in significantly larger TNFα+IFNγ- relative to baseline (Figure 27). The TNFα+IFNγ+ population is also increased, even more so than the TNFα-IFNγ+ population. These increases are accompanied by a decrease in the TNFα-IFNγ- population. **Therefore, SEB serves as a strong positive control with the ability to incite intracellular activation of TNFα and IFNγ release in the CD4+ T cells of Patient D relative to baseline. This confirms that the assay is indeed functioning properly.**

CD8+ T Cell Activation (Granzyme B and IFNγ)

Figure 34: Alone

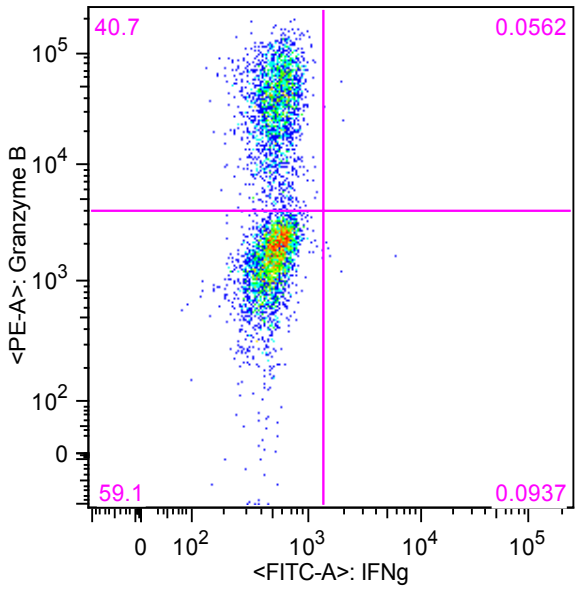


Figure 34 represents the baseline level (i.e., in the absence of stimulation) of Granzyme B and IFNγ release of the CD8+ T cell population in Patient D. The most prominent cell population is GB-IFNγ-. However, the GB+IFNγ- is also rather large. The other cell populations, GB+IFNγ+ and GB-IFNγ+ are rare.

Figure 35: Self-stimulated

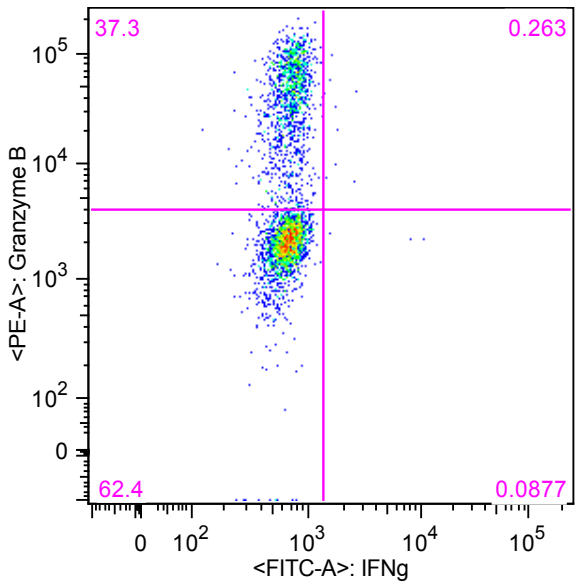


Figure 36: Self-stimulated

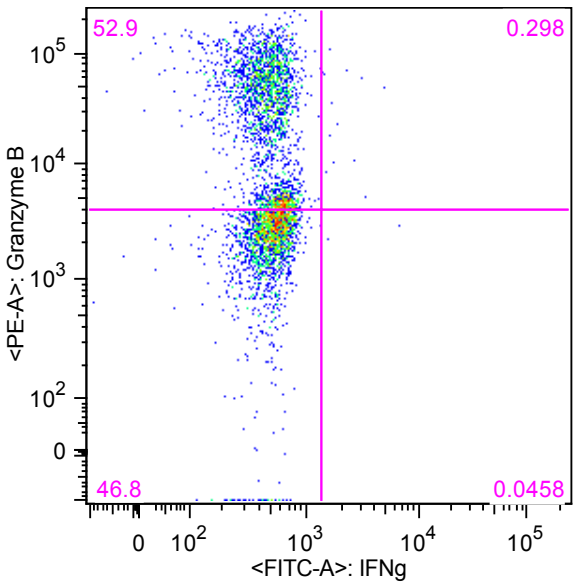


Figure 35 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon self-stimulation. There is a strong GB-IFNγ- population and a smaller GB+IFNγ- population. **Figure 36** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon self-stimulation with the addition of self-mitochondria. There appears to be a significant increase in the GB+IFNγ- population relative to Figure 35. However, the remaining populations appear unchanged. **Therefore, self-mitochondria appear to have an activating effect on the release of Granzyme B but not IFNγ by the CD8+ T cell population of Patient D upon self-stimulation.**

Figure 37: C-stimulated

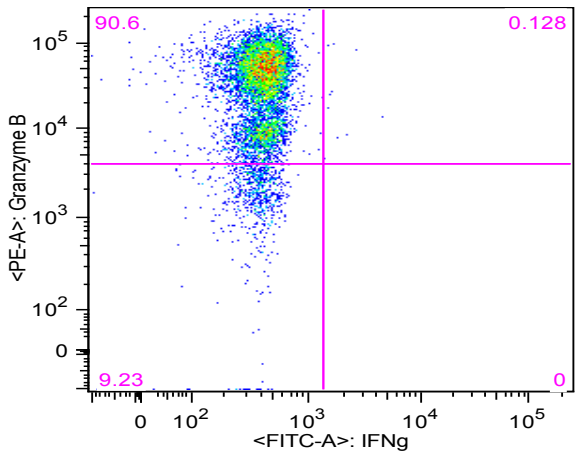


Figure 38: C-stimulated

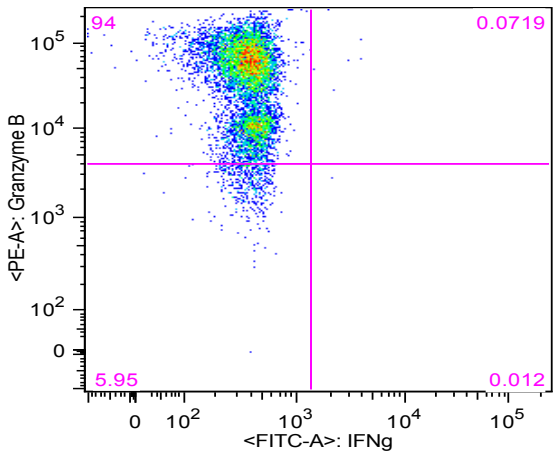


Figure 39: C-stimulated

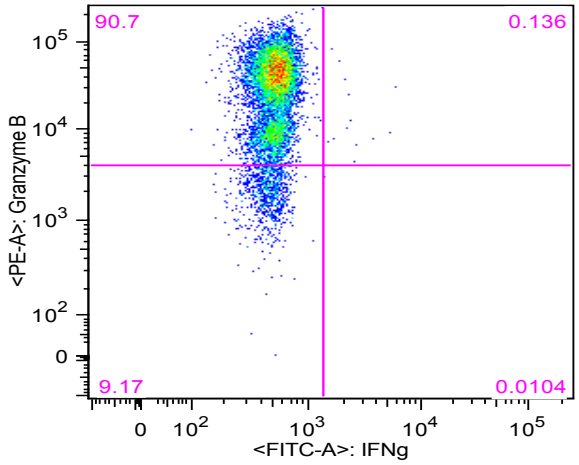


Figure 37 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon stimulation by Patient C. The majority of the total cell population is GB+IFNγ-. There is a small population of GB-IFNγ-. However, the GB+IFNγ+ and GB- FNγ+ populations are rare. **Figure 38** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon stimulation by Patient C with the addition of self-mitochondria. There is a slight increase in GB+IFNγ and similar decrease in GB-IFNγ- relative to Figure 37. However, the other quadrants appear similar. **Therefore, self-mitochondria appear to have a subtle activating effect on the release of Granzyme B but not IFNγ by the CD8+ T cell population of Patient D upon stimulation by Patient C. Figure 39** represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon stimulation by Patient C with the addition of mitochondria from Patient C. Unlike Figure 38, the distribution of cells does not seem to vary significantly from Figure 37. **Therefore, mitochondria from Patient C do not appear to have an activating effect on the release of Granzyme B and IFNγ by the CD8+ T cell population of Patient D upon stimulation by Patient C.**

Figure 40: SEB-stimulated

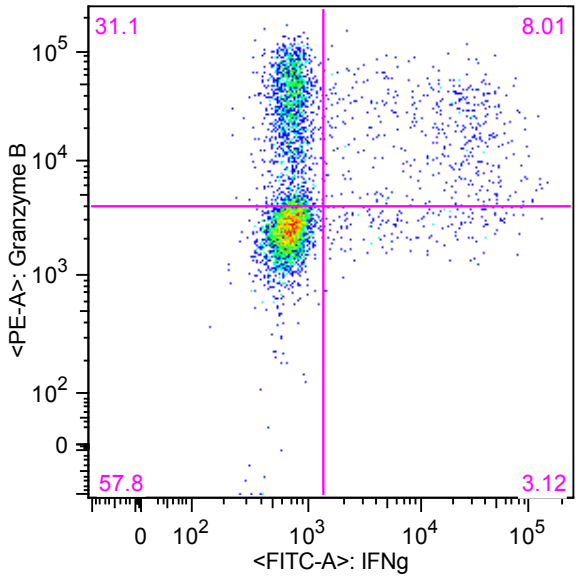


Figure 40 represents the Granzyme B and IFNγ release of the CD8+ T cell population in Patient D upon stimulation by SEB. Clearly, SEB causes a large increase in the GB+IFNγ+ population that was initially rare at baseline (Figure 34). **Therefore, SEB serves as a strong positive control with the ability to incite Granzyme B and IFNγ release in the CD8+ T cells of Patient D relative to baseline. This confirms that the assay is indeed functioning properly.**

Intracellular Degranulation Assay: ICS Allo-MLR Results

Cytotoxic cells that release granules, such as CD8+ T cells, express increasing levels of CD107a with increasing degranulation. Furthermore, T cells tend to lose CD3 upon activation. These results include pseudo-color density plots assaying CD107a and CD3 expression in T Cells. The results from two patients (C and D) are reported. Again, the same color code used in the proliferation section now applies to the caption listing the conditions (i.e., Black: Standard MLR, Blue: Self-mitochondria added to MLR, Red: Donor mitochondria added to MLR).

Recipient - Patient C

Figure 41: Alone

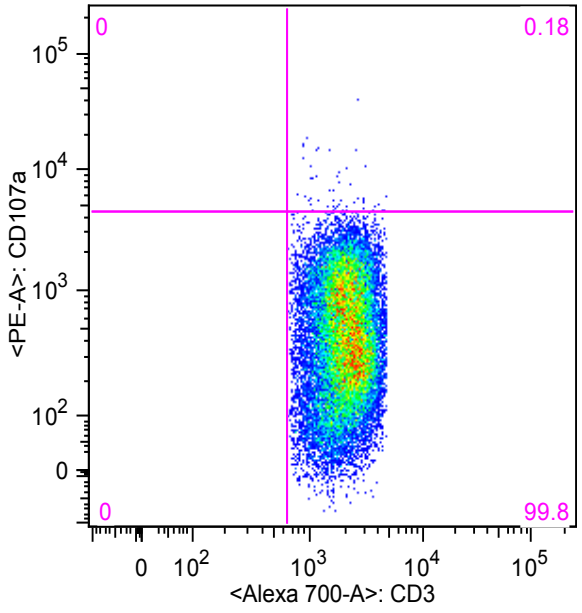


Figure 41 represents the baseline level (i.e., in the absence of stimulation) of CD107a and CD3 expression in the T cell population of Patient C. All T cells express CD3+. Only a tiny fraction of cells express high levels of CD107a as part of the CD107a+CD3+ population. The CD107a-CD3- and CD107a+CD3- populations are nonexistent.

Figure 42: Self-stimulated

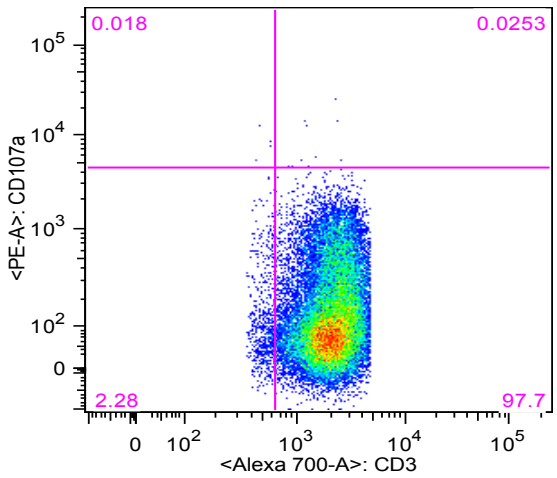


Figure 43: Self-stimulated

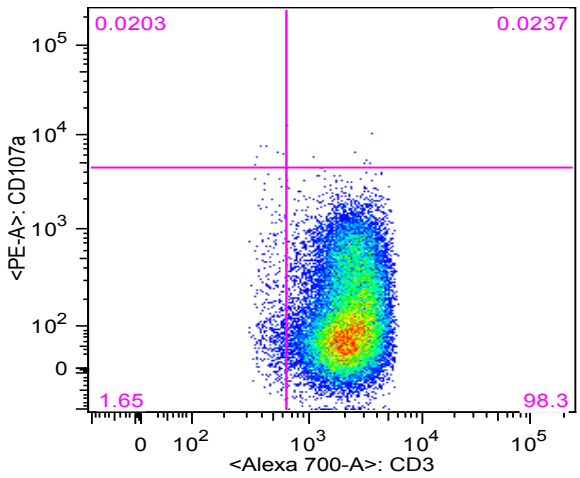


Figure 42 represents the expression of CD107a and CD3 in the T cell population of Patient C upon self-stimulation. The majority of the cells are CD107a-CD3+. However, a small subset of cells is CD107a-CD3-. The remaining populations, CD107a+CD3- and CD107a+CD3+ are rare. **Figure 43** represents the expression of CD107a and CD3 in the T cell population of Patient C upon self-stimulation with the addition of self-mitochondria. The distribution of cells appears fairly similar to Figure 42. **Therefore, self-mitochondria do not appear to cause activation as characterized by increased CD107a expression or decreased CD3 expression in the T cell population of Patient C upon self-stimulation.**

Figure 44: D-stimulated

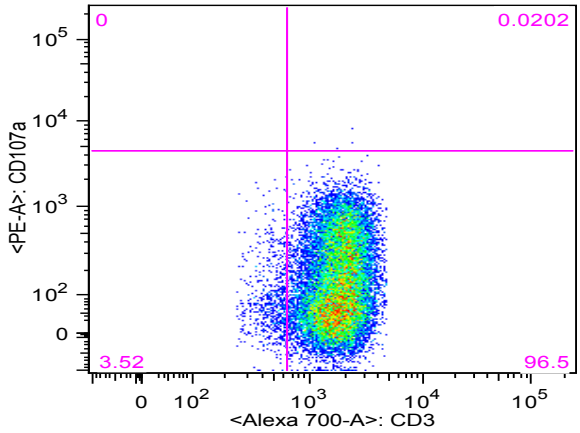


Figure 45: D-stimulated

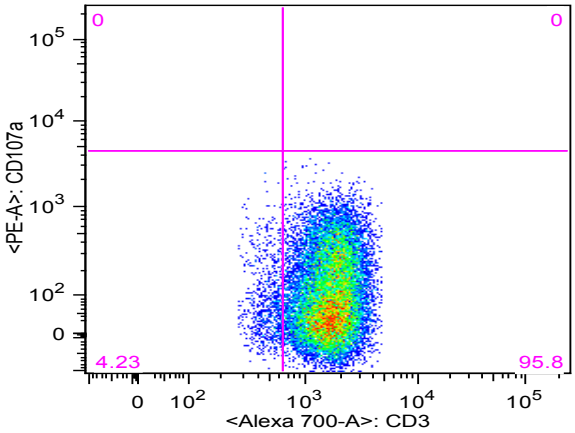


Figure 46: D-stimulated

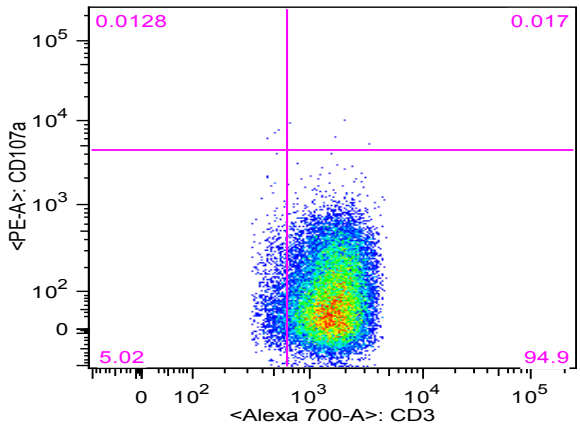


Figure 44 represents the expression of CD107a and CD3 in the T cell population of Patient C upon stimulation by Patient D. The majority of the cells are CD107a-CD3+. However, a small subset of cells is CD107a-CD3-. The CD107a+CD3+ population is rare. The CD107a+CD3- population is nonexistent. **Figure 45** represents the expression of CD107a and CD3 in the T cell population of Patient C upon stimulation by Patient D with the addition of self-mitochondria. The distribution of cells appears similar to Figure 44. **Therefore, self-mitochondria do not appear to cause activation as characterized by increased CD107a expression or decreased CD3 expression in the T cell population of Patient C upon stimulation by Patient D. Figure 46** represents the expression of CD107a and CD3 in the T cell population of Patient C upon stimulation by Patient D with the addition of mitochondria from Patient D. The distribution of cells appears fairly similar to Figure 44, with the exception of an increase in the CD107a-CD3- population and a rare rather than nonexistent CD107a+CD3- population. **Therefore, mitochondria from Patient D do appear to cause very subtle activation as characterized by increased CD107a expression or decreased CD3 expression in the T cell population of Patient C upon stimulation by Patient D.**

Figure 47: SEB-stimulated

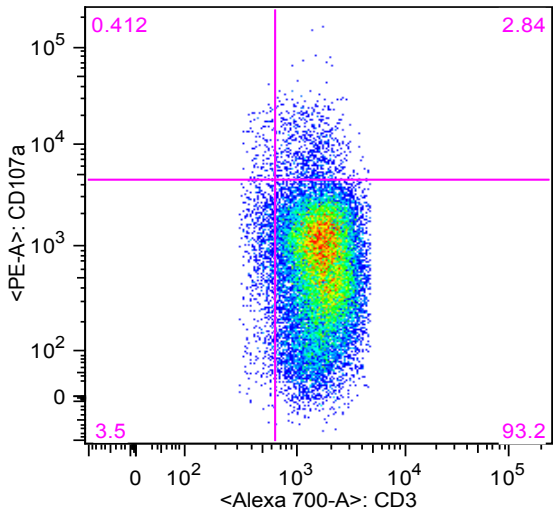


Figure 47 represents the expression of CD107a and CD3 in the T cell population of Patient C upon stimulation by SEB. Clearly, SEB causes a substantial increase in the CD107a-CD3- and CD107a+CD3- populations that were initially nonexistent at baseline (Figure 41). Additionally, the CD107a+CD3+ population that was rare at baseline is rather significant. **Therefore, SEB serves as a strong positive control with the ability to incite activation characterized by increased CD107a expression and decreased CD3 expression in the T cell population of Patient C. This confirms that the assay is indeed functioning properly.**

Recipient - Patient D

Figure 48: Alone

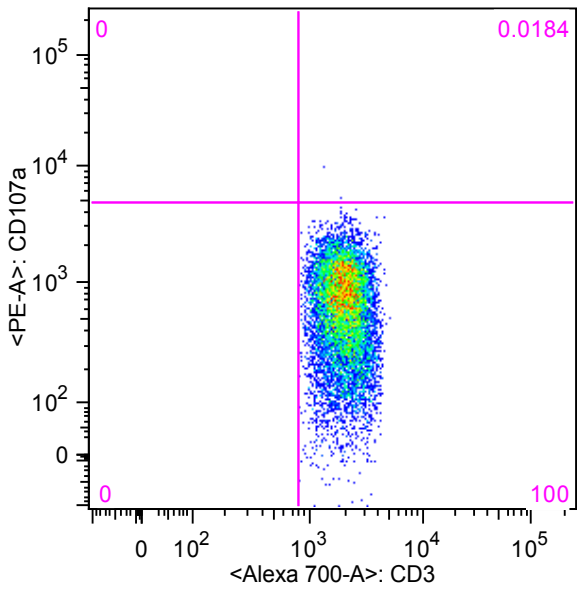


Figure 48 represents the baseline level (i.e., in the absence of stimulation) of CD107a and CD3 expression in the T cell population of Patient D. All T cells express CD3+. Only rarely do cells express high levels of CD107a as part of the CD107a+CD3+ population. The CD107a-CD3- and CD107a+CD3- populations are nonexistent.

Figure 49: Self-stimulated

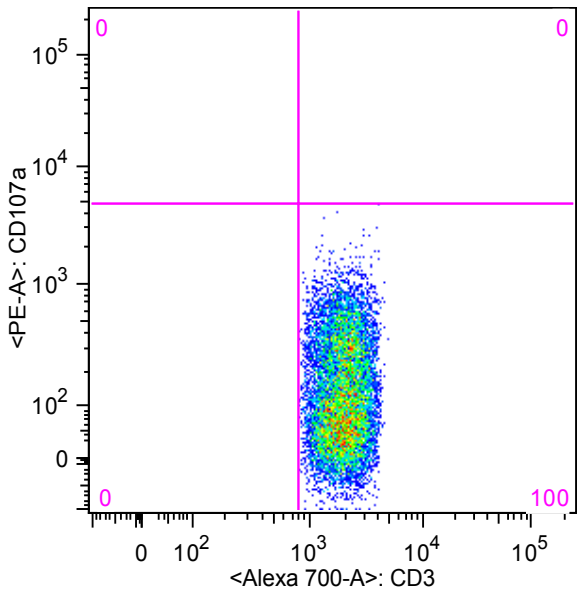


Figure 50: Self-stimulated

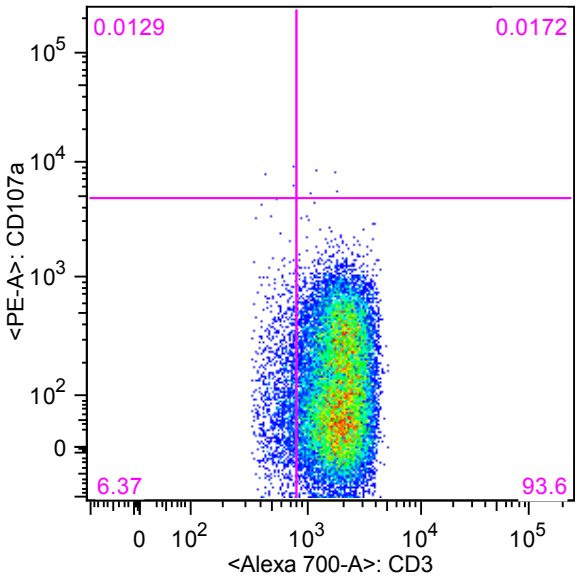


Figure 49 represents the expression of CD107a and CD3 in the T cell population of Patient D upon self-stimulation. All cells are CD107a-CD3+. **Figure 50** represents the expression of CD107a and CD3 in the T cell population of Patient D upon self-stimulation with the addition of self-mitochondria. There is a significant increase in the CD107a-CD3- population relative to Figure 49. Additionally, the CD107a+CD3- and CD107a+CD3+ populations are only rare instead of nonexistent. **Therefore, self-mitochondria do indeed appear to cause activation as characterized by significantly decreased CD3 expression and subtle increases in CD107a expression in the T cell population of Patient D upon self-stimulation.**

Figure 51: C-stimulated

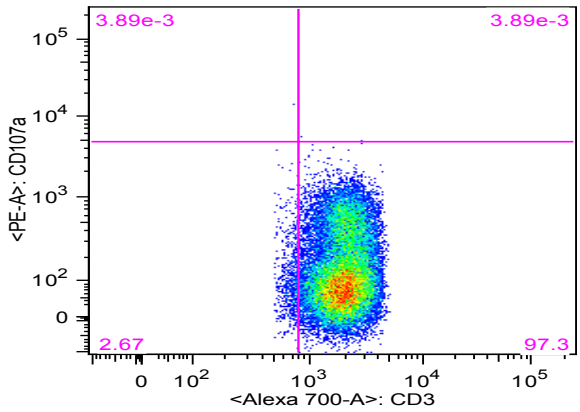


Figure 52: C-stimulated

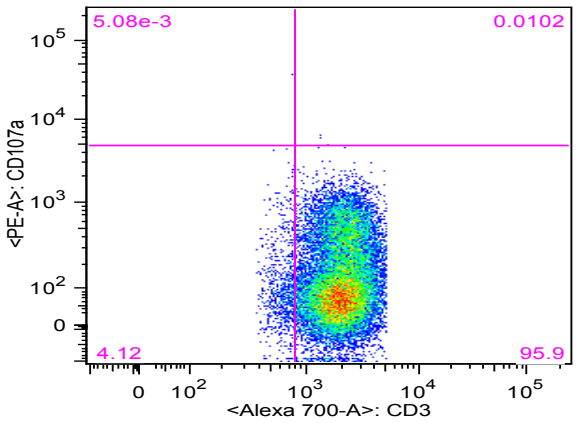


Figure 53: C-stimulated

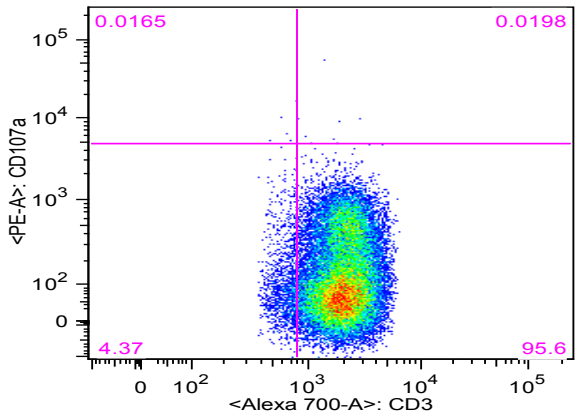


Figure 51 represents the expression of CD107a and CD3 in the T cell population of Patient D upon stimulation by Patient C. The majority of the cells are CD107a-CD3+. However, a small subset of cells is CD107a-CD3-. The CD107a+CD3+ and CD107a+CD3- populations are rare. **Figure 52** represents the expression of CD107a and CD3 in the T cell population of Patient D upon stimulation by Patient C with the addition of self-mitochondria. The distribution of cells appears similar to Figure 51, with the exception of what appears to be an increase in total population density. **Therefore, self-mitochondria do not appear to cause activation as characterized by increased CD107a expression or decreased CD3 expression in the T cell population of Patient D upon stimulation by Patient C. Figure 53** represents the expression of CD107a and CD3 in the T cell population of Patient D upon stimulation by Patient C with the addition of mitochondria from Patient C. The distribution of cells appears fairly similar to Figure 51, with the exception of what appears to be an increase in total population density. **Therefore, mitochondria from Patient C also do not appear to cause activation as characterized by increased CD107a expression or decreased CD3 expression in the T cell population of Patient D upon stimulation by Patient C; however, they might cause a subtle proliferative effect.**

Figure 54: SEB-stimulated

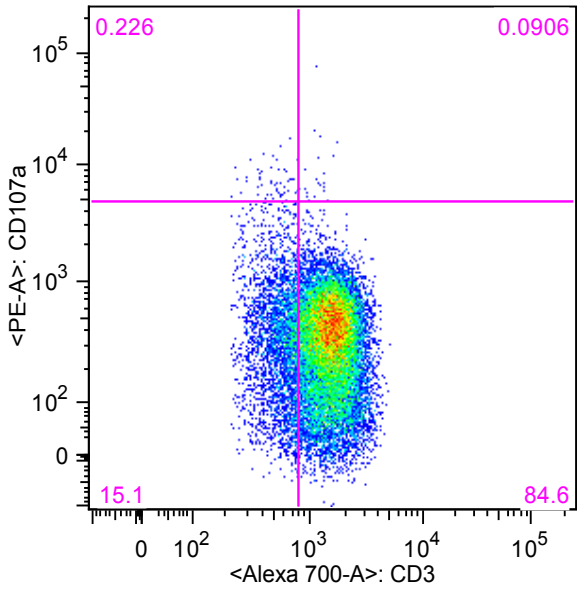


Figure 54 represents the expression of CD107a and CD3 in the T cell population of Patient D upon stimulation by SEB. Clearly, SEB causes substantial increases in the CD107a-CD3- and CD107a+3- population that were initially nonexistent at baseline (Figure 48). However, the CD107a+CD3+ population remains rare. **Therefore, SEB serves as a strong positive control with the ability to incite activation characterized by increased CD107a expression and decreased CD3 expression in the T cell population of Patient D. Although the increases in CD107a were not as substantial as the decreases in CD3, the positive result still suggests that the assay is indeed functioning properly.**

Conclusion

Discussion of Results

This investigation used the addition of fractionated whole mitochondria, an otherwise intracellular product, into in vitro assays as a model of human transplantation. Electron microscopy was used to confirm that only the mitochondria (middle) fraction of the centrifugation isolation procedure yielded numerous intact mitochondria, released from cells during the homogenization and centrifugation processes (Figures 2 and 3). The other two remaining fractions (top and bottom) only contain mitochondria still bound within the cells that compose fragments of muscle tissue (Figures 1 and 4). The addition of the mitochondrial fraction models the post-injury condition of freely circulating whole mitochondria.

If mitochondria incite T cell activation, then this activation could manifest in several possible ways. Firstly, initial activation of T cells has two requirements: (1) binding of the T-Cell receptor on T cells to the major histocompatibility complex on antigen-presenting cells (APCs) for the primary signal and (2) binding of the CD28 surface marker on T cells to its B7 ligand on APCs for the co-stimulatory signal (Janeway et al., 2008). Naïve T cells typically respond to activation via proliferator, while some other types of T cells, such as memory T cells, produce cytokines (Jameson and Masopaust, 2009). Highly activated T cells also show decreased expression of the CD3 surface marker, which complexes with the T-cell receptor and serves as a defining marker for the T cell population. Helper, or CD4+, T cells interact with peptides presented by MHC Class II. Their subsequent activation commonly manifests as increased production of IFNγ and TNFα. In addition to influencing further helper T cell activation and specialization, these cytokines allow helper T cells to “help” activate other immune cells.

Killer, or CD8+, T cells interact with peptides presented by MHC Class I. Their subsequent activation commonly manifests as increased production of IFNγ and granzyme B. Granzyme B release involves degranulation directed towards apoptosis in infected, damaged, or otherwise incompetent cells. Thus, this degranulation can result in an increased expression of CD107a, the intracellular marker that positively correlates with degranulation (Alter et al., 2004).

The results of the intracellular cytokine staining alloimmune mixed lymphocyte reactions assays reveal inconsistent patterns for the effects of self-mitochondria on intracellular activation of T cells. In Patient C assays, the addition of self-mitochondria only increased the alloimmune response against Patient D as characterized by intracellular activation of CD4+ T cells (Figure 17). In Patient D assays, the addition of self-mitochondria accomplished more incidences of increased intracellular activation in terms of CD4+ cytokine release (Figure 29), CD8+ cytokine release (Figure 36), CD8+ T cell granzyme release (Figures 36 and 38), and T cell degranulation (Figure 50). However, a clear trend in terms of the effects of self-mitochondria on the intracellular activation of T cells does not emerge.

The addition of donor mitochondria into a recipient, whether patient C or D, did not lead to significant intracellular cytokine or granzyme activation in the alloimmune response of CD4+ (Figure 18 and 32) or CD8+ T cells (Figure 25 and 39). Donor mitochondria additions also failed to show significant increase in degranulation or loss of CD3 in the T cells of both patients (Figures 46 and 53). Thus, under no conditions did donor mitochondria prove themselves as extraordinary immune stimulants in the intracellular activation assays.

The proliferation assays in both patients A and B both suggest that self-mitochondria heighten the alloimmune response. In Patient A proliferation assays, the addition of self-mitochondria to self-stimulation (Figure 6), stimulation by Patient B (Figure 7), and stimulation by a third party, E, (Figure 8) all result in a significant increase in the number of dividing cells compared with the standard MLR for each condition. Also, in patient B proliferation assays, the addition of self-mitochondria to self-stimulation (Figure 10), stimulation by patient A (Figure 11), and stimulation by a third party, E, (Figure 12) also all result in a significant increase in the number of diving cells compared with the standard MLR for each condition. However, like the additions of self-mitochondria in the intracellular activation assays, the addition of donor mitochondria manifested inconsistency results in the proliferation assays of the Patients A (Figure 7) and B (Figure 11).

In summary, results suggest that self-mitochondria activate allogeneic and self-reactive immune responses in a way conducive to T cell proliferation. However, the effects of self-mitochondria on the intracellular activation of T cells remain inconclusive. Donor mitochondria appears largely inadequate at inducing an increased intracellular activation response in both self-reactive and alloimmune contexts. Given this trend of inadequacy manifested, their inconsistency in terms of proliferative effects on the alloimmune response could be due to chance. Such issues remain unresolved until further work reveals more information. Nevertheless, mitochondria-driven immunity clearly seems to elicit a proliferative effect on T cells via interactions with self-mitochondria.

The Greater Significance

Mitochondria-driven immunity resonates with a longstanding framework for understanding the human immune system: prokaryotes (non-self) versus eukaryotes (self). Lynn Margulis hypothesized that mitochondria and prokaryotes are essentially the same. Later established similarities between bacteria and mitochondria, especially as stimulants to the immune system, could also plausibly support this association. The primitive origins of this model might foreshadow the potential role of mitochondria in immune processes outside the realm of injury-related inflammation and alloimmunity. However, there are more prokaryotes populating the human body than there are human cells without constantly alarming the immune system (Mackie et al., 1999)

Based on the present results, the Matzinger danger model of the human-mitochondria relationship is compelling. Indeed, mitochondria-driven immunity fits logically into the context of the danger model, in which "any intracellular product could potentially be a danger signal when released" (Matzinger, 2002, p. 302). The human immune system serves primarily to protect the human body from dangerous external threats against survival, such as pathogenic infection. Traumatic injury that bursts cells, causing them to release their intracellular products, also poses a significant threat. Therefore, it is logical that the immune system has derived methods for responding to these misplaced intracellular products, including, but most likely not limited to mitochondria. Through the paradigm of the danger model, mitochondria can function as a gateway into the knowledge barriers that currently restrict our understanding of the human body's capacity for and response to injury.

The consistent demonstration of the capacity of self-mitochondria to incite a proliferative response from immune cells embodies crucial evolutionary considerations. Over the course of evolution, the human immune system might have adapted to adequately respond to self-mitochondria as a danger signal in order to efficiently recuperate from injurious events. The ability of the immune cells of one individual to specifically and efficiently respond to the mitochondria of another individual, however, would most likely require priming of those cells via exposure to foreign mitochondria. The context for this exposure over evolutionary time seems unlikely. Thus, it might be expected that any immune reaction to donor, or foreign, mitochondria would be either inadequate in comparison to self-mitochondria or adequate simply by chance. The data from this investigation suggest that this immune adaptation might entail a proliferative effect in response to self-mitochondria.

This is an important framework within which to consider the present challenges in clinical findings related to transplantation and systemic conditions such as SIRS. The enigmatic health sequelae of transplantations that preclude graft survival have eluded our present understanding of pathophysiology. The novel perspective that these patterns may reflect previously unconsidered provocations from intracellular sources has much to offer in the search for treatments. Indeed, biological mechanisms underlying injury, especially in the context of transplant immunology, remain largely unknown.

The burden of injury on patients unyieldingly subsists. Given its association with injury-related immunity, the mitochondrion has the potential to prove itself valuable in translational and clinical research. The newly acclaimed proliferative effect of self-mitochondria on the alloimmune response produces novel considerations for transplant immunologists. Mitochondria surface as potential research targets in efforts to shield

future transplant recipients from the potentially negative or even devastating effects of injury on transplantation outcomes. For example, knowledge gained on the mechanisms of mitochondria-driven, injury-related could potentially inform immunosuppression drug therapy, novel therapy concepts for transplant patients, the methodology of the transplantation procedure, or any other levels of intervention possibly related to injury in the recipient.

The association of mitochondria with SIRS suggests an additional arena of potential application: understanding how ancient, evolutionarily derived mechanisms of local immunity function in the context of modern medicine and technology to induce systemic immunity and have an impact on human survival. If mitochondria-driven, injury-related immunity can be considered a "primitive fix" now counter-adaptive in severe incidences of once fatal injury, an understanding of its underlying mechanisms becomes even more crucial to clinical research and human survival. In light of the present results, we hypothesize that self-mitochondria produce an adaptive response in association with local and hence, survivable injury. However, as the magnitude of this injury increases, we further hypothesize that the degree of immune activation evoked becomes counter adaptive, manifesting as systemic inflammatory conditions such as SIRS.

Future Directions

This project has been an incredibly challenging and rewarding journey thus far. We have every intention of continuing this work in the immediate future. We aspire to eventually begin to approach transplant-specific issues in the context of mitochondria-derived immunity. However, we want to be sure to first solidify the basics and grasp the

larger framework. We will continue to employ the same methods previously discussed in order to gain more information. The following improvements will be researched and applied:

Purified Mitochondria – Although the mitochondria isolation fractions include a dense concentration of mitochondria, there was a small amount of undesired debris. The addition of buffer from other fractions containing similar debris without mitochondria served as controls in this instance. However, we will be in search of a protocol that produces a more purified sample of fractionated mitochondria.

T Cell Selection – In the results of the Intracellular Cytokine Staining Mixed Lymphoycte Reactions, it is difficult to distinguish responder and stimulator cells without selecting the stimulator T cell population. Therefore, we will select for the stimulator T Cells prior to this assay.

In terms of systematically assaying and quantifying mitochondria outside of electron microscopy, we intend to utilize flow cytometry to its full potential. Although no documented flow analyses protocols to quantify mitochondria occur in the literature, we will attempt to use antibodies to mitochondrial proteins to stain and subsequently count the mitochondria in our sample using a flow cytometer. Once in vitro assays are functioning optimally, we will proceed to in vivo assays involving murine heart transplants. In order to expand the experimental design according to the Hauser findings on mtDNA and formyl peptides as stimulants to the immune system, we will include each of these entities (in addition to whole mitochondria) in the mixed lymphocyte reactions as well as in vivo assays. Following mtDNA isolation, quantitative polymerase chain

reaction (qPCR) analyses will be applied. Primers from Human Cytochrome B gene on mtDNA will be used to tag and quantify mtDNA relative to whole DNA samples. The synthetic formyl peptide, f-MLF, will be used. Literature searches and titrations will be conducted in order to determine the appropriate volumes for each addition.

Since the assays utilized in this investigation contain monocytes and macrophages, the involvement of innate stimulation could be predicted. Although this investigation measured activation in T cell populations, the cause of such responses could be due to mitochondrial effects on any of the cell types within the well of these assays. Thus, the consideration and incorporation of markers to better categorize these effects will be another important step. Each well-planned experiment brings us closer to unlocking the mysteries behind mitochondria-driven, injury-related immunity.

In Closing

It is no coincidence that this discussion frequently refers to the potential of mitochondria. After all, the sole purpose of the framework set forth throughout the course of this project here was to highlight the potential significances of mitochondria, especially as it relates to injury-related immunity. Although mostly theoretical or speculative until more support can be discovered, the implications surrounding mitochondria-derived, injury-related immunity are fascinatingly diverse. In the investigative spirit cultivated throughout this project, I have some thoughts and open questions related to some potential implications not directly addressed in our original interests. As suggested by Burt and Trivers (2006), cooperation and tension between the selection on the mitochondrial genome and the selection on the human nuclear

genome remains a worthwhile consideration. In what ways, if any, might the co-evolutionary arms race between humans and mitochondria impact the phenomenon of mitochondria-derived immunity? Given the maternal inheritance of mitochondria, how might gender differences affect the intensity or other qualities of mitochondria-driven immunity? Since immunity involves highly organized and thus energetically costly processes, its particular interaction with energy-producing mitochondria creates a uniquely interesting evolutionary scaffold through which to understand the checks and balances involved in human survival. How can we interpret the role of energetics in the context of mitochondria-driven immunity? Mitochondria are increasingly presented as figures inextricable from both optimal and abnormal human functioning. What comparisons and contrasts can be drawn to understand more about the role of mitochondria in normal human function versus disease and injury? Although many questions encompassing mitochondria-driven, injury-related immunity remain unanswered and their implications are largely unsubstantiated, all stem from a simple paradox: mitochondria uplift human survival as the sole energy providers involved in numerous vital processes, yet simultaneously threaten it as “the danger within.”

References

Alter, G., Malenfant J.M., & Altfeld, M. (2004) CD107a as a functional marker for the identification of natural killer cell activity. Journal of Immunological Methods, 294(1-2), 15-22.

Bone, R.C. (1996) Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS). Annals of Internal Medicine, 125 (8), 680-687.

Burt, A. & Trivers, R. (2006) Genes in conflict: the biology of selfish genetic elements. Cambridge: The Belknap Press of Harvard University Press.

Cann, R.L., Stoneking M., & Wilson A.C. (1987) Mitochondrial-DNA and Human-Evolution. Nature, 325(6099), 31-36.

Cardon, L.R., Burge, C., Clayton, D.A., & Karlin, S. (1994) Pervasive CpG suppression in animal mitochondrial genomes. Proc. Natl Acad. Sci. USA 91, 3799-3803.

Dawkins, R., & Krebs, J.R. (1979) Arms Races between and within species. Proceedings of the Royal Society of London. Series B, biological Sciences 205(1161), The Evolution of Adaptation by Natural Selection (Sep. 21, 1979), 489-511.

D'Alessandro, A.M., Pirsch, J.D., Knechtle, S.J., Odorico, J.S., Van der Werf, W.J., Collins, B.H., Becker, Y.T., Kalayoglu, M., Armbrust, M.J., Sollinger, H.W. (1998) Living unrelated renal donation: The University of Wisconsin experience. Surgery. 124(4): 604-6011.

Fotino, M., Merson, E.J., and Allen, F.H. (1971) Micromethod for rapid separation of lymphocytes from peripheral blood. Ann. Clin. Lab Sci. 1, 131-133.

Gray, M.W., Burger, G., and Lang, B.F. (2001) The origin and evolution of mitochondria. Genome Biol, 2(6): 1018.1.

Hauser, C., Sursal, T., Rodriguez, E.K., Appleton, P.T., Zhang, Q., & Itagaki, K. (2010) Mitochondrial damage associated molecular patterns from femoral reamings activate neutrophils through formyl peptide receptors and P44/42 MAP Kinase. Journal of Orthopedic Trauma, 24 (9), 534-538.

Holt, I.J., Harding, A.E., and Morgan Hughes, J.A. (1988) Deletions of muscle mitochondrial-DNA in patients with mitochondrial myopathies. Nature 331(6158): 717-719.

Jameson, S.C. & Masopaust, D. (2009) Diversity in T cell memory: an embarrassment of riches. Immunity. 31: 859-871.

Janeway, Charles (2008) Janeway's immunobiology. – 7th ed./Kenneth Murphy, Paul Travers, Mark Walport. Garland Science: New York, NY.

Lechler, R.I,. Sykes, M., Thomson, A.W., & Turka, LA. (2005) Organ transplantation-how much of the promise has been realized. Nat Med, 11, 605-613.

Lyons, A.B. (1999) Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester Immunol. Cell Biol. 77(6), 509-515.

Mackie, R.I., Sghir, A., Gaskins, H.R. (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. 69(5), 1035S-1045S.

Matzinger, P. (2002) The danger model: a renewed sense of self. Science, 296 (5566), 301-305.

Murri, L. (2007) Foreword: Mitochondrial medicine. Bioscience Reports 27:1-3.

Nass, S., & Nass, M.M.K. (1963) Intramitochondrial fibers with DNA characteristics. J. Cell. Biol, 19, 593-629.

Nijboer W.N., Schuurs T.A., van der Hoeven J.A.B., Fekken, S., Wiersema-Buist, J., Leuvenink, H.G.D., Hofker, S., Homan van der Heide, J.J., van Son, W.J., & Ploeg, R.J. (2004) Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation. 78(7): 978-986.

Pallen, Mark J. (2011) Time to recognize that mitochondria are bacteria? Trends in Microbiology 19 (2): 58-64.

Raoof, M., Zhang, Q., Itagaki, K., & Hauser, C.J. (2010). Mitochondrial peptides are potent immune activators that activate human neutrophils via FPR-1. The Journal of Trauma, 68 (6), 1328-1334.

Rajapakse, N., Shimizu, K., Payne, M., and Busija, D. 2001. Isolation and characterization of intact mitochondria from neonatal rat brain. Brain Research Protocols 8: 176-183.

Sagan, L. (1967) On the origin of mitosing cells. J Theoret Biol, 14, 225-274.

Scheffler, I.E. (2008) Mitochondria. New Jersey: Wiley-Liss.

Schwartz, M. and Vissing, J. (2002) Paternal inheritance of mitochondrial DNA. New Engl. J. Med. 347, 576–580.

Taanman, J.W. (1999) The mitochondrial genome: structure, transcription, translation, and replication. Biochim. Biophys. Acta 1410, 103-123.

Talan, D. (2006) Dear SIRS: It's time to return to sepsis as we have known it. Ann Emerg Med, 48, 591-592.

Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M.S., Elsas, L.J., II, and Nikoskelainen, E.K. (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. Science 242(4884): 1427-1430.

Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal,, T., Junger, W., Brohi, K., Itagaki, K., Hauser, C. (2010) Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature, 464(7285), 104-107.