INFECTION SUSCEPTIBILITY AND ALTERNATIVE APPROACHES TO COMBATING DRUG-RESISTANT INFECTIONS

By

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DEDICATION

This dissertation is dedicated to my parents, Tan Pham and Hong Le. I am forever thankful for your endless love, support, and sacrifices.

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LIST OF ABBREVIATIONS

CDC- Centers for disease control and prevention

LPS- Lipopolysaccharides

PBP- Penicillin-binding protein

Omp- outer membrane protein

OmpA- outer membrane protein A

Omp36- outer membrane protein 36

OMVs- outer membrane vesicles

TFP- Type IV pili

PRR- Pattern recognition receptors

MAPK- mitogen-activated protein kinases

WT- Wild type

hpi- hours post infection

ROS- reactive oxygen species

NETs- neutrophil extracellular traps

Ag- Antigen

T_{RM}- tissue-resident memory T cells

T_{CM}- central memory T cells

T_{EM}- effector memory T cells

MHC- major histocompatibility complex

HSV- herpes simplex virus

HIV- human immunodeficiency virus

RSV- respiratory syncytial virus

VACV- vaccinia virus

APC- antigen presenting cells

DC- dendritic cells

NKT- natural killer T cells

αGC- α-galactosylceramide

TCR- T cell receptor

CPT1a- carnitine-palmitoyl transferase 1a

LCFA- long-chain fatty acid

FAO- fatty acid oxidation

PheWAS- phenome-wide association study

CFU- colony forming units

FAO- fatty acid oxidation

CBC- complete blood count

I. INTRODUCTION

OVERVIEW

Throughout history, infectious diseases have ranked among the leading challenges to human health, development, and progress. This intricate relationship will continue to define the 21st century as exploding population numbers and globalization pave new avenues for the emergence and re-emergence of disease-causing microorganisms. Advances in antimicrobial agents and vaccines provide a strong defense against widespread population devastation that has plagued human history. However, unrestricted movement of people, animals, and goods coupled with the remarkable adaptability of microorganisms necessitate constant vigilance to maintain this narrow lead. The fragile nature of this balance is demonstrated by the alarming rise in multi-drug resistant infections. Of critical importance are increased efforts in basic, applied, and translational research to improve our understanding of the human immune system and microbial defenses to advance countermeasures that would prevent outbreaks and pandemics from becoming a recurring theme.

The development of antibiotics is among the most significant medical advances to date. Nevertheless, infectious diseases remain a major leading cause of death worldwide (Morens et al. 2004; Khabbaz et al. 2015; Bloom and Cadarette, 2019). It is estimated that over a quarter of the 57 million annually reported deaths globally are attributed to infectious diseases, with respiratory infections accounting for approximately 4 million of these deaths (Michuad et al. 2009; WHO). In developed countries, lower respiratory tract infections rank as the leading cause of death due to infection and the 4th leading cause of death overall (Michuad et al.; Khabbaz et al.; WHO). Beyond the absolute numbers of deaths, the

years of life lost due to respiratory tract infections exceed the proportion of years of life lost due to ischemic heart disease and cerebrovascular disease (Michuad et. al., 2009). Compounding an already precarious situation, microbial agents are also the cause of, or contributing factors to, many chronic diseases (Michuad et al., 2009; Khabbaz et al.). The sustained prevalence of infectious diseases in developed countries, and worldwide, is, in part, due to the alarming rate by which disease causing microorganisms acquire antimicrobial resistance (CDC, Dadgostar, 2019; Bloom and Cadarette, 2019).

Antimicrobial resistance is regarded as one of the greatest threats to human health worldwide (CDC, Dadgostar, 2019; Bloom and Cadarette, 2019). In the United States alone, antimicrobial resistant infections account for 2.8 million infections and 35,000 deaths as a result (CDC). Each year antimicrobial resistant infections cost the U.S. healthcare system over $20 billion, with healthcare-associated infections accounting for roughly $4.6 billion (Dadgostar, 2019). The majority of nosocomial infections are caused by opportunistic bacterial pathogens, of which, *Acinetobacter baumannii* has emerged as an important nosocomial pathogen associated with significant morbidity and mortality (Jain et al., 2004; Koulenti et al., 2017; Wong et al., 2017). Isolates of *A. baumannii* resistant to all commercially available antibiotics have been identified, limiting the therapeutic approaches to treat these infections, and signifies an urgent need for the development of antibiotic-independent therapies (Fig. 1) (Looveren et al., 2004; Abbo et al., 2005; Cai et al., 2012; WHO). The CDC currently categorizes carbapenem-resistant *A. baumannii* as an urgent threat to human health in desperate need of novel therapeutic approaches (CDC, Higgins et al., 2010). A greater understanding of the innate and adaptive immune system would facilitate the development of effective preventative measures and therapies aimed at enhancing

immune defenses against infections and serves as an optimal solution to combating antimicrobial resistance.

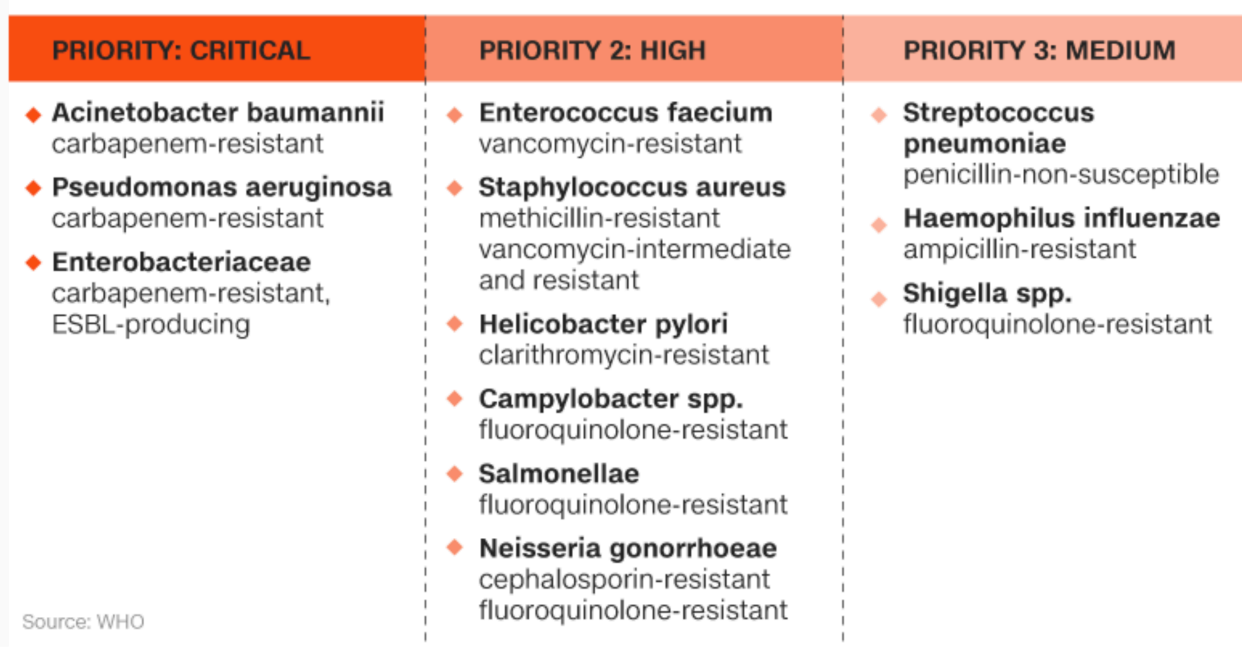


Figure 1. WHO priority list of critical pathogens in need of novel therapeutic options. Image source: WHO

ACINETOBACTER BAUMANNII, THE EMERGENCE OF A SUCCESSFUL PATHOGEN

Bacteria belonging to the genus *Acinetobacter* are identified as gram-negative, catalase-positive, oxidase-negative, nonfermenting, strictly aerobic coccobacilli (Jain et al., 2004; Looveren et al., 2004; Peleg et al., 2008; Alsan et al., 2010; Howard et al., 2012; Wong et al., 2017; Vincent et al., 2009). Species of *Acinetobacter* are ubiquitous in nature and exist as part of the human flora (Looveren et al., 2004; Peleg et al., 2008). Epidemiology studies revealed that up to 43% of healthy individuals are colonized by at least one species of *Acinetobacter,* isolates are generally found on the skin and mucous membranes (Peleg et al., 2008). Although most *Acinetobacter* spp. are avirulent, outbreaks of nosocomial infections, particular amongst patients within the ICU, are commonly associated with *A. baumannii* (Lee et al., 2017; Wong et al., 2017; Vincent et al., 2009). Infections caused by *A. baumannii* account for up to 20% of infections in intensive care units worldwide (Gaynes et al., 2005). The most prevalent form of *A. baumannii* infection is ventilator-associated pneumonia but sepsis, urinary tract infections, meningitis, endocarditis, and skin and soft tissue infections are also common (Alsan et al., 2010; Koulenti et al., 2017; Munoz-Price et al., 2008; Scott et al., 2007; Wong et al., 2017). Hospital-acquired infections caused by *A. baumannii* are associated with significant morbidity and have a mortality rate that approaches 100% in certain clinical settings (Jain et al., 2004; Srivastava et al., 2007; Vincent et al., 2009). *A. baumannii* infections are especially difficult to treat due to the intrinsic antimicrobial resistance of the organism as well as its natural ability to acquire antimicrobial resistance determinants (Gordon et al., 2010). Additionally, *A. baumannii* is extremely resistant to environmental stressors and desiccation, allowing it to persist on abiotic surfaces for extended periods of time, which contributes to its ability to become endemic within healthcare settings (Peleg et al., 2008).

MECHANISMS OF ANTIMICROBIAL RESISTANCE

Clinical concerns over *A. baumannii* infections have intensified due to the pathogen's endless abilities to develop antibiotic resistance (Fig. 2). Known isolates of *A. baumannii* have developed resistance to almost all currently available antimicrobial agents, including aminoglycosides, quinolones, and broad-spectrum β-lactams (Looveren et al., 2004). The prevalence of carbapenem-resistant *A. baumannii* further limits therapeutic options for already critically ill patients and increases the threat of spreading drug resistance to other bacteria (CDC; Higgins et al., 2010; Peleg et al., 2008; Qureshi et al., 2015). The vast array of antimicrobial resistant mechanisms possessed by *A. baumannii* includes β-lactamases, aminoglycoside-modifying enzymes, changes to outer membrane proteins, multidrug efflux pumps, and modifications of target sites (Asif et al., 2018; Gordon et al., 2010; Lee et al., 2017; Peleg et al., 2008). The expression of all four molecular classes of β-lactamases have been identified in isolates of *A. baumannii,* essentially eliminating β-lactams as a treatment option in many clinical settings (Asif et al., 2018). Class A, C and D β-lactamases are serine-dependent β-lactamases, whereas class B are metallo-β-lactamases that require zinc or other metals to exert that enzymatic activity (Asif et al., 2018). Class A β-lactamases include narrow and extended spectrum β-lactamases, that are capable of hydrolyzing penicillin and cephalosporins, and have some activity against carbapenems, whereas class B β-lactamases confer resistance to almost all β-lactams, except monobactams. Expression of class C is particularly problematic, as it confers resistance to cephamycins, penicillin, cephalosporins, and β-lactamase inhibitors. Over expression of an intrinsic AmpC, resulting in increased production of AmpC beta-lactamases, is the primary mechanism of resistance to extended spectrum

cephalosporins (Asif et al., 2018). Class D, also known as oxacillinases, hydrolyzes isoxazolylpenicillin oxacillin and benzylpenicillin. Over 400 OXA-type enzymes have been detected in *A. baumannii* isolates, many of which possess carbapenemase activity (Asif et al., 2018; Gordon et al., 2010; Lee et al., 2017). Another enzymatic mode of resistance found in *A. baumannii* is expression of aminoglycoside-modifying enzymes. These enzymes are classified as acetyltransferases, adenyltransferases, or phosphotransferases and are typically found on transposable elements. Many multi-drug resistant *A. baumannii* isolates express one or more combinations of aminoglycoside-modifying enzymes (Asif et al., 2018; Gordon et al., 2010; Lee et al., 2017).

A. baumannii also possesses an arsenal of non-enzymatic mechanisms of resistance to antimicrobial agents. Resistance to quinolones is attributed to mutations in the genes encoding DNA gyrase or topoisomerase IV, affecting the binding affinity of these drugs to the DNA-enzyme complex. Active efflux pumps that facilitate the removal of drugs and reduced expression of porins affecting permeability of the outer membrane contribute significantly to multi-drug resistance in *A. baumannii.* Down-regulation of penicillin-binding-protein (PBP) is another mechanism of resistance to β-lactams that does not involve enzymatic degradation of the drug (Asif et al., 2018; Gordon et al., 2010; Lee et al., 2017; Peleg et al., 2008). Expression of one or more combinations of these resistance mechanisms has been identified in isolates of *A. baumannii* worldwide, conferring varying degrees of drug resistance that contribute to ongoing outbreaks, while limiting therapeutic options in clinics across the globe.

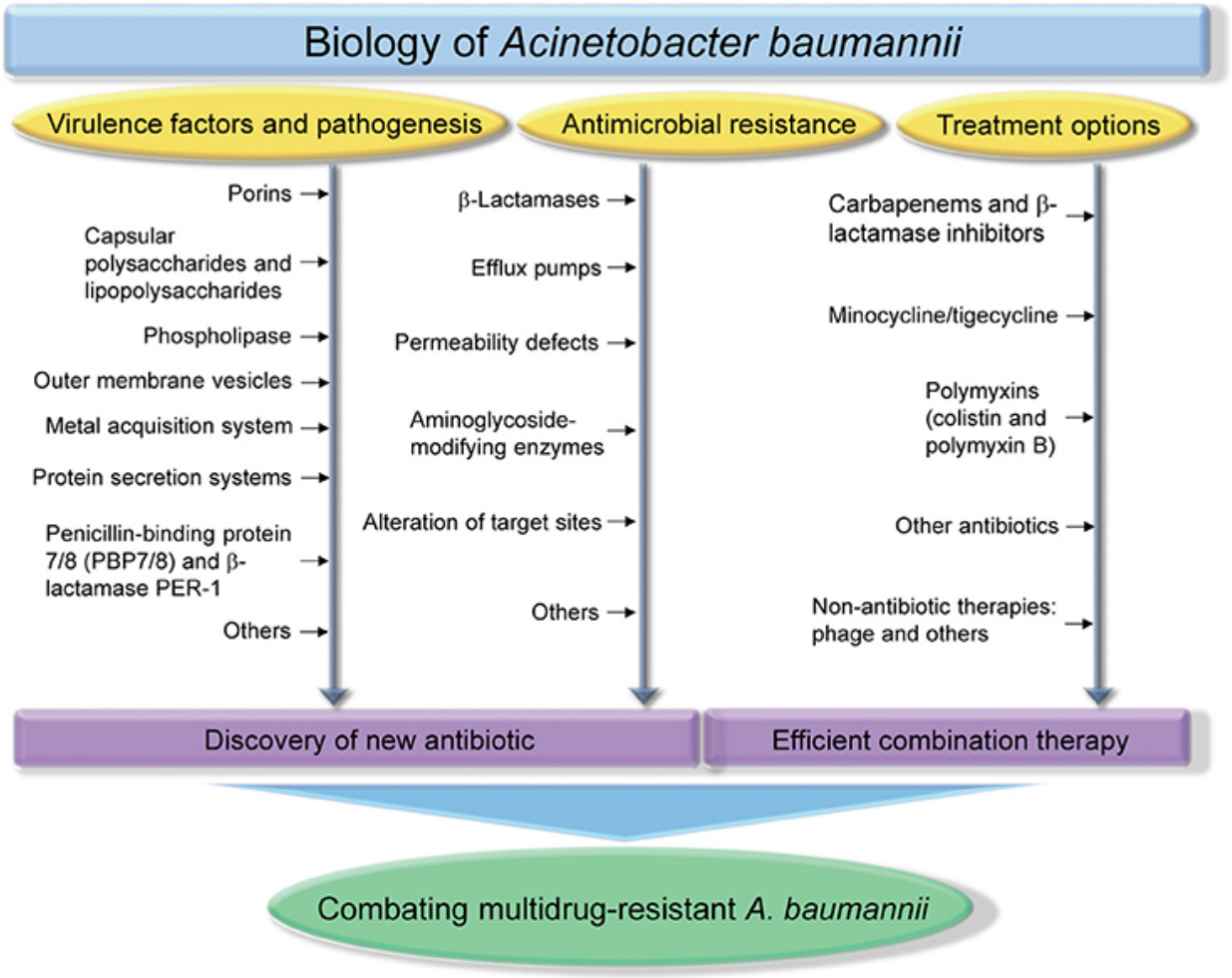


Figure 2. Biology of *Acinetobacter baumannii*. Studies of virulence factors, pathogenesis, antimicrobial resistance, and treatment options of *A. baumannii* will provide an important aid for discovering new antibiotics and determining efficient combination therapy, which are essential strategies for combating multidrug-resistant *A. baumannii* infections (Lee et al., 2017). Image source: frontiers in Cellular and Infection Microbiology).

MECHANISMS OF PATHOGENESIS AND PERSISTENCE

The mechanisms that contribute to the success of *A. baumannii* as a human pathogen remain poorly understood. However, significant progress has been made towards identifying and assigning potential roles to virulence factors possessed by this bacterium (Mea et al., 2021). Factors associated with pathogenicity include capsular polysaccharides and lipopolysaccharide (LPS), metal acquisition systems, and PBP that contribute to the *in vivo* survival, immune evasion, and antibiotic resistance of this pathogen (Fig. 2) (Lee et al., 2017; Mea et al., 2021). Outer membrane protein A (OmpA), phospholipases, and outer membrane vesicles (OMV) allow *A. baumannii* to target, adhere, and persist during an infection (Fig. 2) (Choi et al., 2005; Jacobs et al., 2015; Jin et al., 2011). The ability of *A. baumannii* to form biofilms enables persistence on medical devices and resistance to antimicrobials, which are likely to contribute to *A. baumannii* pathogenesis (Peleg et al., 2008; Vidal et al., 1996). Additionally, *A. baumannii* expresses pili that allow for natural competence, twitching motility, and adherence to epithelial cells *in vitro* (Eijkelkamp et al., 2011; Mea et al., 2021).

Rodent models of systemic infection demonstrate a requirement for capsular polysaccharides and LPS for *A. baumannii* to persist and progress to lethal infection (Wang, et al., 2014). Under stress, hyperproduction of capsular polysaccharides increases resistance to antimicrobial agents and killing by host complement (Geisinger et al., 2015). Defects in LPS formation result in decreased resistance to human serum, enhanced opsonophagocytic killing, and reduced inflammation (Luke et al., 2017; McConnell et al., 2013). Additionally, mutations in the construction of these outer membrane structures lead to significantly decreased survival in sites of soft tissue infection and attenuated lethality

(Luke et al., 2017; Lin et al., 2012; McQueary et al., 2012; Hood et al., 2013). To further combat the host defense mechanisms, the expression of PBP provides antimicrobial resistance, which was shown to be critical for survival in human serum and in rodent soft tissue infections (Russo et al., 2009). *A. baumannii* also expresses several metal-acquisition systems to overcome nutritional limitations and to acquire the necessary trace elements to establish infection.

In addition to the mechanisms employed by *A. baumannii* to ensure survival within the host, several virulence factors allow for direct targeting and killing of host cells. Porins located on the outer membrane of *A. baumannii* modulate cellular permeability and water passage. However, within a host, these proteins contribute to adherence and cytotoxicity. In *in vitro* studies, purified OmpA binds human epithelial cells and induces apoptosis (Choi et al., 2008). OmpA is able to induce apoptosis by targeting the mitochondria and releasing proapoptotic molecules or by translocating to the nucleus and inducing cell death (Lee et al., 2010). OmpA contributes to immune evasion by binding to factor H in human serum, allowing *A. baumannii* to avoid complement-mediated killing (Kim et al., 2009). Analyses of clinical isolates associated with high mortality rate showed a staggering 92% of *A. baumannii* strains displayed some level of serum resistance (Alcantar-Curiel et al., 2019). OmpA also contributes to antimicrobial resistance by removing antibiotics from the periplasmic space (Smani et al., 2014). In murine sepsis studies, the Omp33-36 is associated with adherence and invasion of epithelial cells, cytotoxicity, and establishing lethal infections (Smani et al., 2013). Other proteins involved in resistance to human serum, cytotoxicity, and virulence include the lipolytic enzyme, phospholipase. Phospholipase C and D have been identified in *A. baumannii;* these enzymes facilitate cleavage of the head

group from phospholipids, resulting in host cell destabilization and interference with proper cell signaling (Camarena et al., 2010; Jacobs et al., 2010; Stahl et al., 2015).

Gram-negative pathogens are capable of secreting OMV composed of outer membrane, LPS, periplasmic proteins, phospholipids, and nucleic acids (Kulp and Kuehn, 2010; Ellis and Kuehn, 2010). These OMV are used to deliver a wide variety of bacterial effectors. Many *A. baumannii* strains are capable of delivering OmpA, proteases, and phospholipases to host cells in secreted OMV, inducing a pro-inflammatory response and cytotoxicity (Kwon et al., 2009). OMV also play a role in the spread of antibiotic resistance (Rumbo et al., 2011).

Species of *Acinetobacter* do not express flagella and are classified as nonmotile bacteria. However, two clinically relevant *Acinetobacter* species, including *A. baumannii,* possess surface-associated and twitching motility (Eijkelkamp et al., 2011). Surface-associated motility is characterized as swarming motility and is thought to be associated with LPS expression (Barker and Maxted, 1975; McQueary et al., 2012). Twitching motility is a form of jerky motion on wet surface, attributed to functional type IV pili (TFP) cycling through rounds of extension and retraction that allows the bacterium to pull forward and attach to host cells (Corral et al., 2020; Jarrell and McBride, 2008; Mattick et al., 2002; Tala et al., 2019). The loss of motility significantly reduces the virulence of *A. baumannii* (Skiebe et al., 2012). The uptake of exogenous DNA by the TFP has also been implicated in the spread of antimicrobial resistance (Proft and Baker, 2009). In harsh environments, *A. baumannii* will downregulate motility and upregulate genes associated with biofilm formation. Expression of chaperone-usher pili enables attachment to abiotic surfaces, allowing *A. baumannii* to adhere to and persist on medical devices (Mea et al., 2021). The formation of biofilm shields

A. baumannii from nutritional deprivation, desiccation, and various antimicrobial agents, and is thus considered a major virulence factor (Vidal et al. 1996).

HOST IMMUNE RESPONSE TO *ACINETOBACTER BAUMANNII*

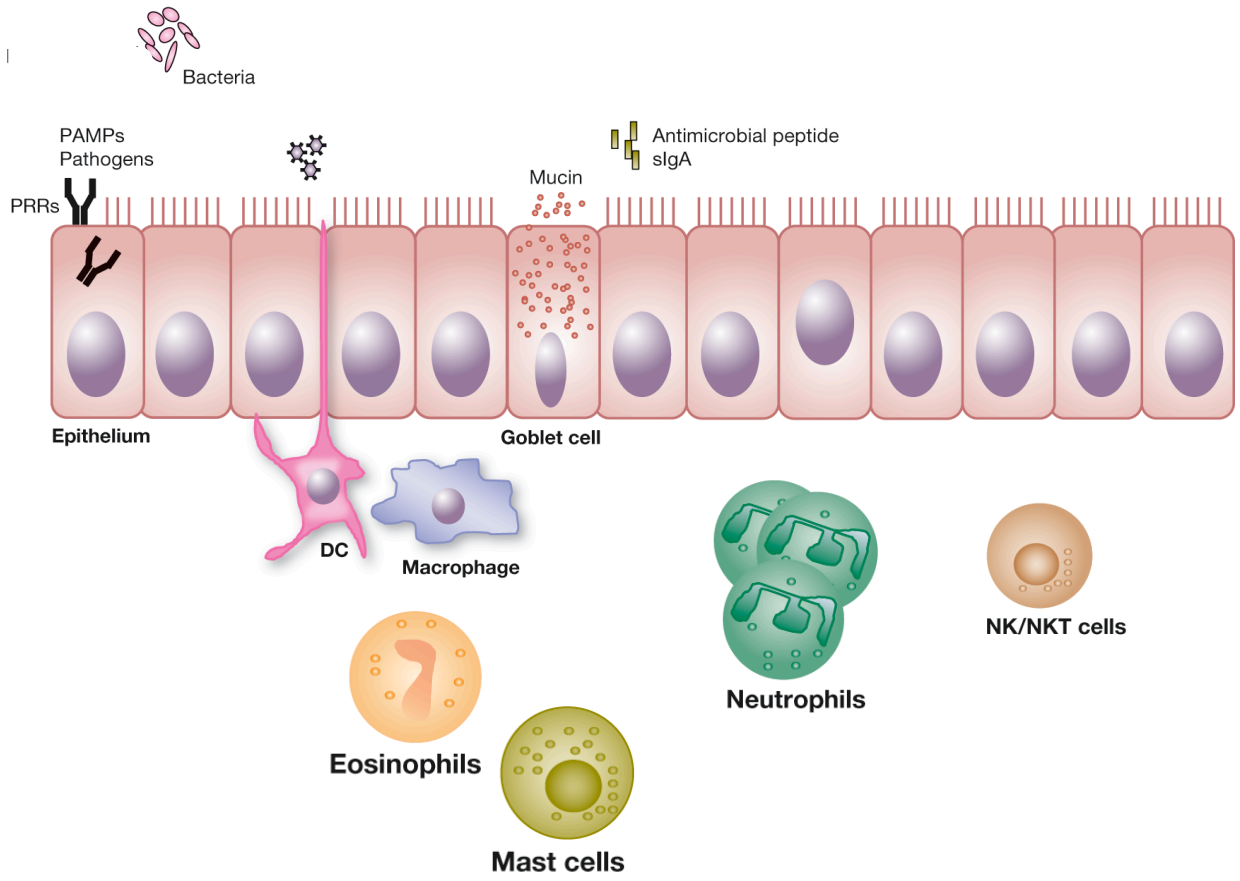


Figure 3. Host immune defenses to *A. baumannii* (pulmonary diseases and disorders) Image source: McGraw-Hill Education).

Despite the immense threat *A. baumannii* poses to global public health, few studies have examined the host innate immune response and implications of this response on the adaptive immune system following infection. Current studies into *A. baumannii* infections are focused on molecular epidemiology, contribution of specific virulence factors, distribution of antimicrobial resistance, and defined components of the host immune response. However, the available clinical and experimental data support a pivotal role for the host innate immune defenses against infections caused by *A. baumannii* (Fig. 3). Individuals susceptible to *A. baumannii* infections are generally immunocompromised or patients within the intensive care unit (Weiner et al. 2016; Wong et al., 2017). Individuals with polymorphisms in genes linked to immune regulation demonstrate higher susceptibility and worse clinical outcomes following infection (Smelaya et al., 2016; Rautanen et al., 2015). In rodent models of infection, immunocompromised mice had significantly increased susceptibility and severity of infection when compared to normal mice (Joly-Guillou et al., 1997).

PATTERN RECOGNITION RECEPTORS

Activation of the host innate immune defenses is largely regulated through pattern recognition receptors (PRR). PRR are proteins expressed on cells of the innate immune system that recognize pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). Recognition of PAMP expressed on *A. baumannii* is mediated through the membrane-bound and cytoplasmic PRR, the Toll-like receptors (TLR) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Chen, 2020; Garcia-Patino et al., 2017). *A. baumannii* engages the host innate immune cells through TLR2 and TLR4, along with the co-receptor CD14 (Knapp et al., 2006). Activation of

TLR2 or TLR4 by PAMP of *A. baumannii* leads to activation of mitogen-activated protein kinases (MAPK) and NF-κB, production of proinflammatory mediators, including MIP-2, MCP-1, IL-8, IL-6, IL-12, and TNF-α, resulting in effector cell recruitment to contain and clear the infection (Erridge et al., 2007; Knapp et al., 2006). While TLR4 is largely activated by the lipid A moiety of LPS, TLR2 can interact with a wide range of PAMP, including bacterial lipoproteins, peptidoglycan, and mycobacterial lipoarabinomannan (Chen, 2020). Intracellular detection of *A. baumannii* is mediated through TLR9, NOD1/2, and NLRP3. In a rodent model of *A. baumannii* infection, animals deficient in these PPR were more susceptible and had more severe manifestations of disease (Chen, 2020).

MACROPHAGES

Following engagement of host PRR by PAMP expressed on *A. baumannii,* innate immune cells are activated and recruited to the site of infection, coordinated by the release of proinflammatory cytokines and chemokines. *In vivo* and *in vitro* studies demonstrate an essential role for macrophages in the control and clearance of bacteria, and initiation of a robust immune response that culminates in the recruitment of effector cells. In the early stages of infection, macrophages phagocytose and kill *A. baumannii* before the arrival of other innate immune effector cells, controlling bacterial dissemination. Macrophages are capable of killing up to 80% of the phagocytosed bacteria within the first 24h, through the production of nitric oxide (Qiu et al., 2012). Depletion studies further establish the importance of macrophages, as mice depleted of macrophages had increased susceptibility and infection severity, higher bacterial burdens, increased tissue damage associated with enhanced neutrophil recruitment, and increased systemic bacterial dissemination compared to WT mice (van Faassen et al., 2007).

NEUTROPHILS

Neutrophils are the most potent innate immune effector cells against *A. baumannii* infections (van Faassen et al., 2007; Qiu et al., 2009a; Breslow et al., 2011; Grguric-Smith et al., 2015). Clinical observation of the prevalence and severity of *A. baumannii* infection amongst neutropenic patients was an initial indicator of the crucial role of neutrophils (Karim et al. 1991). In rodent models, animals depleted of neutrophils had delayed production of proinflammatory mediators involved in the recruitment of effector cells and increased bacterial burdens, dissemination, and lethality. Furthermore, sublethal infections with *A. baumannii* were converted to lethal infections in neutrophil deficient animals, a progression not observed in macrophage deficient animals. Neutrophils are recruited to sites of infection as early as 4-hpi, peaking at 24-hpi, and display more effective killing capacity than macrophages (van Faassen et al., 2007). Mechanisms utilized by neutrophils to control and clear *A. baumannii* include the release of ROS, myeloperoxidase, β-defensins, and the formation of neutrophil extracellular traps (NETs) (Qiu et al., 2009b; Kamoshida et al., 2015; Remijsen et al., 2011). The importance of neutrophils in limiting *A. baumannii* infection has been evaluated in models of pneumonia, septicemia, and skin infections (Bruhn et al., 2015; Breslow et al., 2011; Grguric-Smith et al., 2015).

OTHER INNATE IMMUNE CELLS

Natural killer (NK) cells, dendritic cells (DC), mast cells, and mucosal epithelial cells contribute to host defense against *A. baumannii* infections but their roles are relatively less defined. NK cells play an indirect role in combating *A. baumannii* infection. In depletion models, NK cell deficiency resulted in impaired chemokine production and neutrophil recruitment (Tsuchiya et al., 2012). The roles of DC have mainly been evaluated

in vitro. OmpA and LPS induced the production of IL-12, IL-6, and TNF-α, a T helper 1 (Th1) biased response (Lee et al., 2007). The mucosal epithelial and mast cells act as sentinels for the innate immune system, constantly monitoring the immediate environment (Feng et al., 2013). Detection of foreign bodies induces the production of cytokines, reactive oxygen species, antimicrobial molecules, and the recruitment of phagocytic cells to combat the invading threat. In addition to the coordinated efforts of the innate immune effector cells, the complement system contributes to defenses against *A. baumannii* through lysis or opsonization. Isolates of *A. baumannii* sensitive to serum were positive for C3 deposition (Bruhn et al., 2015; Garcia-Patino et al., 2017).

The diverse functions carried out by the host innate immune defenses to ward off infections and maintain tissue homeostasis are bioenergetically expensive and require precise regulation of metabolic pathways. Upon sensing danger signals within their microenvironment, immune cells adopt specific metabolic programs, increasing the uptake of glucose, amino acids, and fatty acids to carry out the necessary effector functions. The metabolic program adopted by immune cells also specifies the distinct fate of the cell (Ganeshan and Chawla, 2014). Tissue resident macrophages, at the frontlines of immune defenses, are highly heterogenous in their gene expression profiles. Depending on the pathogen-derived cues, macrophages will either fuel their functions by glycolysis and glutaminolysis or by fatty acid oxidation (Kempner et al., 1939). Classically activated macrophages adopt glycolysis and glutaminolysis to fuel their pro-inflammatory, bactericidal functions (Newsholme et al., 1986). Similarly, neutrophils, the first responders to sites of infection, primarily rely on glycolysis and glutaminolysis to enact their anti-microbial effector functions (Sbarra et al., 1959; Valentine et al., 1951). Consistent with this,

neutrophils maintain few mitochondria, and while not involved in generating oxidative burst, maintenance of the mitochondrial potential has been shown to be essential for cell survival (Maianski et al., 2004). However, in macrophages, activation of TLR 1, 2, and 4 results in the recruitment of mitochondria to the phagosome and increases ROS production (West et al., 2011). Therefore, genetic perturbations to immune cell metabolism can result in severe consequences to cellular activation and function in host immune defenses.

ROLE OF THE ADAPTIVE IMMUNE SYSTEM

The effectiveness of this coordinated effort is evident in the rarity of *A. baumannii* infections amongst healthy individuals. However, for those with defects in one or more components of their innate immune defenses the risk of infection significantly increases. As these infections are associated with high mortality rates, the interplay between the innate and adaptive immune system remains unclear. To date, few studies have evaluated the protective capacity of adaptive immunity in *A. baumannii* infections. The current clinical and experimental data support a role for B cells and antibody-mediated responses. However, a role for T cells, outside of the helper function, remains largely unknown. Patients infected with *A. baumannii* develop high levels of serum specific antibody titers and rodent models display partial to complete protection following transfer of immune sera (Islam et al., 2011). Vaccinating animals with OmpA or OMV also provided protection against clinically relevant *A. baumannii* isolates (Fajardo Bonin et al., 2014; Hassan et al., 2016; McConnell et al., 2011). In a model of reinfection, animals that recovered from an *A. baumannii* infection were marginally protected against re-exposure, suggesting that the innate immune system has the capacity to induce some level of protective immunity

(Qiu et al., 2016). The culmination of clinical observations, rodent models, and *in vitro* cellular experiments have provided encouraging advances towards a better understanding of the interaction between *A. baumannii* and host defenses but considerable gaps in knowledge remain. An increased understanding of the mechanisms regulating the innate immune defenses and appropriate induction of the adaptive immune system will aide in the development of preventative measures and alternative therapies to decrease susceptibility and severity amongst the at-risk population.

ALTERNATIVE APPROACHES TO COMBATING THE THREAT OF *ACINETOBACTER BAUMANNII*

Research into antibiotic-resistant *A. baumannii* infections have centered on the role of specific innate immune effector cells, proinflammatory mediators, mechanisms of pathogenesis, and development of novel antimicrobials. Few studies have rigorously assessed preventative measures that establish protection against *A. baumannii* infections. Individuals infected with *A. baumannii* are typically immunocompromised, unable to combat the infection, and face bleak outcomes. However, production of serum specific antibodies by these patients suggests vaccines could be a feasible approach to reducing the risk caused by these infections (Islam et al., 2011). Furthermore, in vaccine models, mice immunized with OmpA or OMV had lower bacterial burdens, decreased inflammation, and higher survival rates compared to control animals (Fajardo Bonin et al., 2014; Hassan et al., 2016; McConnell et al., 2011). Together, this suggests that further insight into the development of preventative measures has the potential benefit of providing protection to at-risk patients, reducing the necessity for harsh therapeutic interventions, and improving survival outcomes.

Immune memory is the central feature of the adaptive immune system that allows vertebrates the ability to rapidly respond to recurring infections with defined specificity. Recognition of the protective capacity of immune memory dates back hundreds of years, with the observation that individuals who contracted and survived a disease rarely succumb to the same infection (Sallusto et al., 2010). This observation has since lead to the current practice of vaccination, allowing individuals to develop protective immunity to infectious diseases while limiting the risk of morbidity and mortality. Although vaccines have revolutionized modern medicine, the development of novel vaccines that elicit an effective Ag-specific immune response resulting in long lasting protective immunity continues to be a significant challenge (Pulendran and Ahmed, 2011; Sharma et al., 2019; Zepp et al., 2010). The major challenge being a full understanding of correlates of protective immunity and ability to measure cell-mediated immunity. Thus, defining mechanisms of protective immunity is critical to combat antimicrobial resistant infections and emerging infectious diseases.

A. baumannii is an extracellular pathogen with the capacity to invade mammalian epithelial cells and transiently reside and replicate inside the host cells (Choi et al., 2008; Qin et al., 2020). The development of a vaccine against *A. baumannii* would have the potential to generate protective immunity by two mechanisms: a humoral response which generates antibodies, and a CD4+ T cell mediated, cellular response which generates memory lymphocytes to induce rapid recall responses. Memory CD4+ T cells have been demonstrated to be involved in the adaptive immune response to *A. baumanni*. However, due to the larger body of knowledge about the role and mechanisms of memory CD8+ T

cells, the work described and studied here uses CD8+ T cells as a model for the generation of T cell memory (Chen et al., 2020).

The vast majority of vaccines successfully allow the immune system to generate affinity-matured class-switched antibodies, facilitating the neutralization and clearance of the invading pathogen (Sarkander et al., 2016). Thus, antigen (Ag)-specific antibody titers have become the gold standard to determine the efficacy of a vaccine (Sarkander et al., 2016). However, recent studies have demonstrated a critical role for non-circulating tissue resident Ag-specific memory CD8+ T cells (T_{RM}) in the protective immune response against many viral and intracellular bacterial pathogens (Seder et al., 2008; Walch et al., 2014). Furthermore, installation of CD8+ T_{RM} at vulnerable mucosal sites frequently exposed to pathogens allow for a rapid response against infections (Ariotti et al., 2012; Bivas-Benita et al., 2013; Gilchuk et al., 2016; Mueller et al., 2016; Schenkel et al., 2014; Ariotti et al., 2014). Thus, initiatives to combat the immense threat of *A. baumannii* would benefit from the identification of immunogenic CD4+ T cell epitopes that result in long-lived protective cellular immunity.

MEMORY T CELL SUBSETS AND FUNCTIONS

Memory T cells are critical for enhanced immune surveillance and rapid recall following reinfection. Immunological memory was first characterized in the peripheral circulation with the identification of long-lived antibodies and circulating memory T cells, known as central memory T cells (T_{CM}) (Szabo et al., 2019). This soon led to the discovery of a heterogenous subset of memory cells delineated by expression of the lymph node homing receptor CCR7; $CCR7^{hi}$ and $CCR7^{lo}$ defined T_{CM} and effector memory T cells (T_{EM}), respectively (Sallusto et al., 1999). T_{CM} are restimulated in the draining lymphoid

organs, whereas T_{EM} are recruited to inflamed tissues upon re-exposure to a pathogen. Activation of T_{CM} and T_{EM} cells results in enhanced immune responses to re-exposure. However, the recall and recruitment process can cause delays that allow the spread and initiation of disease (Mueller et al., 2016). Subsequent studies have since identified subsets of CD4+ and CD8+ T_{EM} that remain resident in previously infected tissues (Wakim et al., 2008; Gebhardt et al., 2009; Masopust et al., 2010; Teijaro et al., 2011). Non-circulating T_{RM} are broadly characterized by the expression of the activation and tissue retention marker CD69, and the αE integrin CD103 for CD8+ T_{RM} at mucosal and barrier sites (Mackay et al., 2013; Wakim et al, 2012).

Activated Ag-specific CD8+ T cells are capable of killing malignant and infected cells by secretion of cytokines, production and release of cytotoxic granules, and through Fas/FasL interactions (Mueller et al., 2016). Within non-lymphoid peripheral tissue, Ag-specific CD8+ T_{RM} are capable of rapidly triggering innate and adaptive immune responses upon antigen restimulation and controlling the spread of the disease prior to the recruitment of circulating and effector memory T cells (Schenkel et al., 2014; Ariotti et al., 2014). This protective capacity has been implicated in several studies of viral and bacterial infections (Muruganandah et al., 2018). In models of herpes simplex virus-1 (HSV-1) infection, CD8+ T_{RM} were localized to sites of acute infection within the skin and latently infected sensory ganglia (Gebhardt et al., 2009). Requirement for the CXCL10/CXCR3 chemokine pathway is essential for generation of T_{RM}, as mice deficient in either are unable to control HSV-1 reactivation challenge (Srivastava et al., 2017). Influenza-specific CD8+ T_{RM} were found in human lung samples that displayed diverse TCR repertoire, high proliferative capacity, and were polyfunctional (Purwar et al., 2011; Pizzolla et al., 2018). Additionally, rhesus

monkeys infected with influenza A virus generated large numbers of T_{RM} within the lungs. In the context of human immunodeficiency virus (HIV), evidence shows a role for CD8+ T_{RM} in early control of infection, and individuals able to control HIV infections had T_{RM} capable of polyfunctional immune responses compared to individuals that are not able to control the infection (Kiniry et al., 2017; Gibbs et al., 2017; Moylan et al., 2017; Damouche et al., 2017). Experimental infection of humans with respiratory syncytial virus (RSV) demonstrates that individuals with higher RSV-specific CD8+ T_{RM} had reduced viral load and developed less severe respiratory disease (Jozwik et al., 2015). Immunity to vaccina virus (VACV) confers protection against smallpox, leading to the eradication of the disease. However, due to the threat of smallpox being used as a biological weapon there is maintained interest in studying immunity to VACV. In mouse infection models, T_{RM} have been demonstrated to play a significant role in mediating protection. In the pulmonary models of infection, higher numbers of T_{RM} correlated with better protection. Depletion of CD8+ T_{RM} increased susceptibility to VACV infections (Gilchuk et al., 2016). Other viral infections in which T_{RM} are implicated in the protective immune response include cytomegalovirus, lymphocytic choriomeningitis virus, varicella zoster virus, human papillomavirus, viral hepatitis, Epstein-Barr virus, and vesicular stomatitis virus to name a few (Muruganandah et al., 2018).

Severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) has infected over 120 million people worldwide, causing disease pathology that ranges from mild to severe, with extreme cases resulting in death (Huang et al., 2020). In response to the SARS-CoV-2 pandemic, research laboratories and pharmaceutical companies have intensified efforts to produce effective vaccines that generate neutralizing antibodies to the SARS-COV-2 spike (S) protein. However, studies into disease pathogenesis indicate an

involvement of T cell mediated immune responses to infections caused by SARS-COV-2, with distinct T cell mediated responses in patients experiencing mild versus those with severe symptoms (Ferretti et al., 2020; Grifoni et al., 2020; Le Bert et al., 2020; Mateus et al., 2020; Nelde et al., 2021; Peng et al., 2020; Schulien et al., 2021). Emerging evidence suggests individuals with T cell specificities against conserved cross-reactive human coronavirus epitopes experienced milder symptoms (Mallajosyula et al., 2021). Collectively, studies into critical human diseases highlight the importance of Ag-specific T cell mediated protective immunity and the need for enhanced efforts towards vaccine development that elicits an effective humoral and cellular response.

Fewer studies have explored the role of T_{RM} in bacterial infections. However, current evidence suggests a noteworthy role in the protective immune response. In studies of bacterial pneumonia, clearance of *Bordetella pertussis* and *Streptococcus pneumoniae* correlated with the presence and expansion of bacteria specific CD4+ T_{RM} (Wilk et al., 2017). IFNγ producing CD4+ T cells are essential for enhanced macrophage killing of *Mycobacterium tuberculosis* (Mtb). However, emerging evidence suggests a role for CD8+ TRM in controlling Mtb infection as well (O'Garra et al., 2013). Intranasal administration of the Mtb vaccine, BCG, led to generation of polyfunctional CD8+ T_{RM}. Adoptive transfer of CD8+ T_{RM} from vaccinated mice provided protection to naïve mice against Mtb challenge (Perdomo et al., 2016; Hu et al., 2017). Studies of bacterial infection of the urogenital tract demonstrate that spontaneous clearance and optimal protection of correlates with the presence of IFNγ producing T cells and the presence of T_{RM} (Stary et al., 2015; O'Meara et al., 2016). Similarly, studies focused on infections of the gastrointestinal tract highlight roles for CD8+ TRM in protection against *Listeria monocytogenes* and

Yersinia pseudotuberculosis (Stary et al., 2015). There is little evidence that CD8+ T cells play a role in the protective immune response against *A. baumannii*, however, studies suggest a critical role for CD4+ T cells (Chen et al., 2020). Nevertheless, the studies of memory CD8+ T cells are better developed with more advanced experimental systems, therefore, I have focused my studies on memory CD8+ T cells, employing a vaccinia virus infection model, as detailed below.

T LYMPHOCYTE SPECIFICITY AND ACTIVATION

T cells are unable to directly recognize antigens. The process of CD4+ and CD8+ T cell activation requires antigen presentation in the form of short peptides presented on major histocompatibility complex (MHC) molecules. MHC class I molecules are expressed by all nucleated cells and present peptides to CD8+ T cells. MHC class II molecules are primarily expressed on antigen presenting cells (APC) and present their peptides to CD4+ T cells. Genes that encode the MHC class I and MHC class II molecules, as well as those associated with antigen processing and presentation are up regulated by inflammatory stimuli on a variety of cell types (Gobin et al., 1999; Choi et al., 2011). The antigen binding groove of the MHC class I molecule has a closed configuration and accommodates peptides of 9 to 10 amino acid residues in length. In contrast, the antigen binding groove of the MHC class II molecule has an open configuration and commonly accommodates peptides of 13 to 16 residues in length (Wieczorek et al., 2017). Extensive efforts have been dedicated to predicting distinct peptide-MHC interactions for both class I and class II molecules. However, class I-associated peptides are better characterized, and the developed predictive algorithms are more accurate compared to class II

molecules. This difference in accurate prediction may be a result of the open configuration of the peptide binding groove, allowing class II-associated peptides to interact with the pocket at multiple sites.

Activation of T cells requires recognition of peptides presented on MHC molecules in combination with a co-stimulatory factor. This requirement for dual recognition of antigens to mount a robust immune response is a safeguard against autoimmunity. Many pathogens are equipped with a plethora of immune stimulating features, thus providing the necessary primary and secondary signal to elicit an appropriate immune response (Janeway et al., 2002; Gordon et al., 2002). However, in response to noninflammatory immunogens or immune evading pathogens, activation of helper CD4+ T cell is required for both a robust antibody and cellular response (Bennett et al., 1998; Schoenberger et al., 1998). Help-dependent generation of CD8+ T cell immunity is mediated by cognate CD4+ T cell licensing of dendritic cells (DC) to cross-prime naïve CD8+ T cells (Ridge et al., 1998; Schoenberger et al., 1998; Smith et al., 2004). Mice lacking CD4+ T cell help are unable to elicit a robust primary and secondary CD8+ T cell response and have poor survival and expansion of CD8+ T cell in the primary and secondary response (Shedlock et al., 2003; Sun et al., 2003; Janssen et al., 2003; Novy et al., 2007). This compounds the issue of developing T cell targeted vaccines due to the requirement that identified antigens must be presented by both MHC class I and class II molecules.

MHC POLYMORPHISMS AND THE MHC "SUPERTYPE" HYPOTHESIS

The genes encoding the human MHC molecules display extensive polymorphism, with over 15,000 different alleles encoding the classical MHC class I and class II molecules identified (Robinson et al., 2019; IPD-IMGT/HLA Database). Polymorphisms within the human leukocyte antigen (HLA) genes diversify the antigen-binding cleft and, thereby, dictate the peptide-binding motif and consequently the peptide repertoire displayed by MHC molecules (Rammensee and Stevanovic, 1998). It is estimated that the APC of an individual heterozygous at the six class I and six class II loci could, in theory, present up to 10^{12} peptides. This level of diversity of peptides presented by an individual ensures that a good portion of the population is capable of recognizing and fending off numerous pathogens including emerging and re-emerging pathogens (Wieczorek et al., 2017). Despite the incredible number of alleles encoding MHC molecules that exist within the human population, evidence suggests that MHC molecules can be clustered into "supertypes" based on the physicochemical features of the peptide binding cleft (Fig. 4). The supertype hypothesis postulates that MHC molecules within the same supertype are capable of binding overlapping peptide repertoires (Lund et al., 2004; Sette and Sidney, 1998; Guercio et al., 1995; Sette and Sidney, 1999; Bertoni et al., 1998). Thus, identifying peptide(s) that are presented by multiple members of a

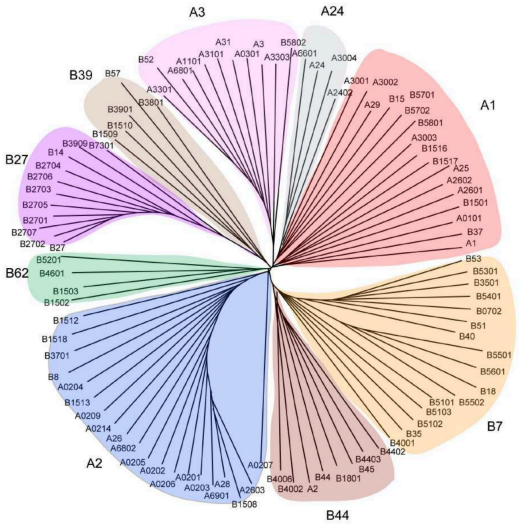


Figure 4. Grouping of HLA class I molecules into families known as 'Supertypes', based on the physio-chemistry and their B and F binding pockets (Hertz et al., 2007) Image source: Bioinformatics.

supertype is a plausible approach to developing Ag-specific CD8+ T cell targeted vaccines with the desired population coverage.

GLYCOLIPID-BASED VACCINE ADJUVANTS

Coupling the identification of promiscuous immunogenic CD8+ T cell epitopes with an adjuvant capable of circumventing the requirement of CD4+ T cell help in eliciting a robust primary and secondary CD8+ T cell response would further advance effective vaccine development. To this end, there is increased interest in natural killer T (NKT) cells and their role in regulating immune-associated diseases and enhancing protective responses. NKT cells are a distinct subset of innate-like T lymphocytes that express a semi-invariant $\alpha\beta$ T cell receptor (TCR) that recognizes foreign and endogenous glycolipid antigens presented on the MHC-like molecule, CD1d, expressed on APC (Kumar et al., 2017). NKT cells activated with the prototypical glycolipid, α-galactosylceramide (αGC), a marine-sponge derivative, have been shown to license DC for cross-priming of CD8+ T cells in a distinct mechanism that can act synergistically with the CD4+ T cell pathway to enhance the CD8+ T cell response (Hermans et al., 2003; Stober et al., 2003; Semmling et al., 2010). Activation of NKT cells following the recognition of αGC leads to the production of a variety of cytokines and chemokines, DC licensing for cross-priming of CD8+ T cells, and transactivation of cells in the innate and adaptive immune systems, including T cells (Singh et al., 1999; Carnaud et al., 1999; Vincent et al., 2002; Fujii et al., 2003; Brigl et al., 2003; Bezbradica et al., 2005; Galli et al., 2007; Leadbetter et al.; 2008; Gottschalk et al., 2015). Furthermore, CD1d is a highly conserved glycolipid presenting molecule recognized by NKT cells across species, including humans. Thus, activating NKT cells with the agonist,

αGC, can overcome the limitations posed by the highly variable nature of MHC class II molecules and the diversity of ligands needed to activate CD4+ T cells, making CD1d an appealing system for vaccine design. However, it is not fully known whether NKT cell help fully substitutes the requirement for CD4+ T cell help.

SUMMARY OF PROJECTS

Advances in preventative care, therapeutic approaches, and early predictors of disease risk factors have significantly improved human health and success. However, in an interconnected world, fueled by unrestricted movement of people, animals, and goods, it is of no surprise that an infectious disease outbreak in one small region of the world can quickly spread and affect the lives of every person living on it. This is exemplified by the pace at which SARS-CoV-2 became pandemic. As the world rallied together to combat this threat, it is clear that we are prepared for the increased frequency of emerging and re-emerging infectious disease outbreaks (Bloom and Cadarette, 2019). Thus, critical insight into mechanisms regulating the host immune defenses will aide in the development of novel therapeutic approaches and preventative measures to ensure society does not revert back to centuries past when infectious disease outbreaks were frequent.

The work presented here began in Chapter III by exploring the association between expression of a host carnitine palmitoyl transferase 1a gene (*Cpt1a*) allelic variant and susceptibility to infectious disease. This work culminates in the description of an unrecognized metabolic profile utilized by neutrophils to fuel purinergic signaling that results in signal amplification and chemotaxis to infection by microorganisms such as *A. baumannii*. With the alarming rise in antimicrobial resistant infections, novel therapeutic approaches are in dire need. Chapter IV investigates alternative therapeutic applications for aminoglycoside antibiotics in combating bacterial pneumonia caused by Gram-negative bacteria such as *A. baumannii*. Finally, Chapter V describes initial work to test the hypothesis of the HLA class I supertypes to T cell targeted vaccine development.

II. MATERIALS AND METHODS

MATERIALS AND METHODS FOR CPT1A STUDY

Mouse infection model

The murine pneumonia model was performed as previously described (Broulette et al., 2013; Jain et al., 2015). Briefly, female C57BL/6 mice in this study were purchased from Jackson Laboratories. Mice were infected intranasally with 1×10^8 colony forming units (CFU) of *A. baumannii* ATCC 17978 in 30 µl of sterile PBS for mortality studies or $5x10^6$ CFU for other outcomes. Mice were euthanized at the indicated times and organs and blood were harvested. Mice were treated with 12.5 mg/kg of etomoxir (Sigma) in PBS or an equal volume of PBS control intraperitoneally 24 hours prior to and at the time of infection. For G-CSF neutrophil mobilization experiments, mice were treated with 5 µg G-CSF (Peprotech) in 200 µL PBS or PBS control intraperitoneally daily for 5 days. Mice were treated with etomoxir or PBS control daily for 6 days starting the day prior to the first G-CSF treatment. Mice were euthanized 24 hours following the final treatment and organs and blood were harvested. Mice were randomized to treatment groups using the GraphPad QuickCalcs, an online randomization tool available at (https://www.graphpad.com/quickcalcs/randomize1.cfm).

Complete blood count and serum chemistry

Complete blood counts and chemistry analysis were performed by the Translational Pathology Shared Resource at Vanderbilt University. At the indicated time points, mouse blood was collected through a cardiac puncture and incubated on ice or in

EDTA treated vesicles. Non-EDTA-treated blood was incubated at room temperature for 15 minutes, centrifuged at 10,000 x*g* for 10 minutes, and the serum was collected for chemistry analysis. Five-part differentials were performed on EDTA-treated whole mouse blood.

Bone marrow-derived macrophage (BMDM) isolation and infection

BMDMs were isolated as previously described (Broulette et al., 2013; Scott et al., 2007; Smelaya et al., 2016; Wong et al., 2017). The hind limb bones of C57BL/6 mice were removed and placed into 1.5 mL microcentrifuge tube that contained a clipped 200 μL pipette tip and 150 μL of sterile PBS. Bone marrow was flushed from the bone via 15 second centrifugation at >10,000 x*g* inside the 1.5 mL microcentrifuge tube. Bone marrow was washed, red blood cells were lysed with ACK lysis buffer, and the bone marrow suspension was passed through a 40 μm cell strainer prior to counting. The bone marrow cell suspension was plated at a density of 3×10^6 cells/mL in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 20% L929 conditioned medium (BMDM medium) into 12-well tissue culture plates and incubated at 37°C with 5% CO_2. On day three, 5 ml of RPMI medium supplemented with 10% FBS and 20% LC medium was added to each plate. After six days, 10 mL of the media was collected from each plate, pelleted, and replaced with 10 mL of fresh RPMI containing 10% FBS and 20% LC medium. On day seven, cells were collected and plated overnight for the infection assay. BMDMs were infected with the indicated strains at a multiplicity of infection of 15 and infected cells were incubated for four hours. Following incubation, cell culture supernatants were removed, and cytokines were quantified.

Bone marrow neutrophil isolation

Bone marrow harvest and initial processing were performed as for BMDM isolation. Following RBC lysis, neutrophils were enriched using the MACS Mouse Neutrophil Isolation Kit and LC columns (Miltenyi Biotec) according to the manufacturer's protocol. Neutrophils were treated in RPMI containing the following treatment: untreated (control), treated with etomoxir (10 μM), octanoic acid (100 μM) with etomoxir (10 μM) or octanoic acid (100 μM) alone for 2 hrs at 37°C. Cells were pelleted and washed once with PBS and used for specific assays.

Flow cytometric analyses

Single-cell suspensions of bone marrow were prepared as described above and mouse blood was collected in EDTA-tubes, subjected to RBC lysis, and passed through a 40 μm cell strainer. Cells were stained with myeloid, or bone marrow antibody panels (Table 1) as described previously (Broulette et al., 2013; Rautanen et al., 2015). Analyses were carried out on a BD 5-laser LSR II (BD Biosciences) at the Vanderbilt Flow Cytometry Shared Resource, and analyses were performed using FlowJo software (Treestar Inc).

Cytokine quantification

Determination of lung cytokine levels was performed on whole lungs harvested 12 hours post-infection and homogenized in PBS. Samples were normalized to total protein prior to analysis. Determination of systemic cytokine levels was performed on serum isolated from mice 12 hours post-infection. Bone marrow-derived macrophage cytokines

were quantified from cell culture supernatant at 18 hours post-infection. Cytokines were quantified using the MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore) on a Luminex Flexmap 3D platform (Luminex) according to the manufacturer's recommended protocol or by Quantikine ELISA (R&D Systems) according to the manufacturer's protocol.

Trans well chemotaxis assay

Bone marrow neutrophils were resuspended in RPMI supplemented with 10% heat-inactivated FBS at a density of 2 x 10^6 cells in 50 μL and added to the top well of a 96-well 3 μm polycarbonate filter plate (Millipore Multiscreen). To the bottom well of the chamber, 150 μL of RPMI with 10% FBS containing PBS control or 10 nM fMLF. Plates were incubated for 1 hour at 37°C with 5% CO_2 and cells were enumerated from the bottom well using an automated cell counter (BioRad). Prior to assaying, cells were treated with 10 μM etomoxir, 100 μM octanoic acid (Sigma), or 100 μM ATP (Sigma) for 2 hours at 37°C with 5% CO_2 in RPMI with 10% FBS.

RT-PCR

Cpt1a expression: Bone marrow neutrophils were isolated as described above. Neutrophil RNA was isolated as follows: 200 μl of Trizol was added to the cells and incubated at room temperature for 5 minutes prior to the addition of chloroform at a ratio of 0.2 mL per mL of Trizol, vortexed for 15 seconds, incubated at room temperature for 5 minutes, then centrifuged at 12,000 rpm for 15 minutes. The aqueous portion was collected, added to equal volume of isopropanol, mixed by inverting the tube, and

incubated on ice for 15 minutes. RNA was pelleted at max speed for 15 minutes, washed with 75% ethanol, and resuspended in molecular grade water. RT-PCR was performed using SYBR green master mix and cpt1a primers, (5'-GGCATAAACGCAGAGCATTCCTG, 5'-CAGTGTCCATCCTCTGAGTAGC) for 35 cycles, and GAPDH was used to normalize expression. Mouse liver and muscle tissues were used as positive and negative control, respectively. MPO and MMP-9 expression: Bone marrow cells were sorted into four populations. Following cell sorting, RNA was isolated from each population as described above. RT-PCR was performed using SYBR green master mix with the forward and reverse primers (MPO 5'-TCCCACTCAGCAAGGTCTT, 5'- TAAGAGCAGGCAAATCCAG; MMP-9 5'-ATAGAGGAAGCCCATTACAGG, 5'- GTGTACACCCACATTTGACG) for 35 cycles. GAPDH was used as a reference to normalize expression.

Western blotting

Cpt1a expression: Bone marrow neutrophils were isolated as described above. Cell pellets were lysed with 1X RIPA lysis buffer (Millipore) with protease and phosphatase inhibitors (Sigma) and rotated for 30 min at 4°C and centrifuged for 10 min at 12,000 rpm. Protein was quantitated in the supernatants using a BCA assay (Thermo Scientific) and 30 μg of total protein was separated on 10% SDS gels (BioRad) and transferred to nitrocellulose membrane using a transblot turbo (BioRad). Membranes were blocked with Odyssey blocker for 1 hr at RT and probed with an anti-CPT1a antibody overnight at 4°C. Equal loading of protein was tested using GAPDH antibodies. Mouse liver and muscle tissues were used as positive and negative control, respectively.

Proteins were detected using IR labeled secondary antibodies (LiCor Biosciences) and scanned on an Odyssey scanner (LiCor Biosciences) and relative percent phosphorylation induced by fMLF was calculated.

MATERIALS AND METHODS FOR AMINOGLYCOSIDE STUDY

Mouse infection model

Wildtype, female, eight-week-old C57BL/6 mice were purchased from Jackson Laboratories. The murine model of *A. baumannii* pneumonia was performed as previously described (Hood et al., 2012). Briefly, *A. baumannii* strain ATCC 17978 was backdiluted 1:1000 from overnight culture and grown in lysogeny broth (LB) for 3.5 hours at 37℃ with shaking, washed twice with cold PBS, and resuspended in PBS at an appropriate cell density for infection. Mice were infected intranasally with $3X10^8$ colony forming units *A. baumannii* in 30 μl PBS. At 36 hours post infection mice were euthanized and organs and blood were harvested. For co-infection experiments, individual strains were prepared as above, equal volumes of each strain were mixed (or AB17978 was mixed with an equal volume of PBS to ensure comparable numbers of AB17978 in each group), and 30 μl of the mixed inoculum was used to challenge mice intranasally. Following the 3.5-hour outgrowth, *A. baumannii* was chemically killed by adding an equal volume of ice-cold acetone:ethanol to the culture, incubating on ice for 5 minutes, pelleting cells, resuspending in ice-cold acetone:ethanol, pelleting cells, and washing twice with PBS prior to resuspending at the appropriate cell density for infection. *P. aeruginosa* pneumonia was performed as above with an inoculum of 1 to $5x10^7$ CFU. Mice were treated with 200 μg of anakinra (SOBI) intraperitoneally 48 hours prior to and at the time of infection with *A. baumannii*. Lungs, livers, and spleens were homogenized, serially diluted in PBS, and plated onto LBA for bacterial enumeration. Mice were randomized to

treatment groups using the GraphPad QuickCalcs, an online randomization tool available at (https://www.graphpad.com/quickcalcs/randomize1.cfm).

Cytokine quantification

Determination of lung cytokine levels was performed on homogenized whole lungs from infected animals harvested four- and 12-hours post-infection and homogenized in PBS. Samples were normalized to total protein prior to analysis. Determination of systemic cytokine levels was performed on serum isolated from infected mice at the indicated time points. Bone marrow-derived macrophage cytokines were quantified from cell culture supernatant at four hours post-infection. All cytokines were quantified using the MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore) on a Luminex Flexmap 3D platform (Luminex) according to the manufacturer's recommended protocol.

NFκB activation

NFκB activation in response to *A. baumannii* was assessed by Western blotting using anti-mouse NF-κB p65 antibodies (Abcam). RAW264.7 cells were infected with Ab17978/pMU368 propagated in LB or LB supplemented with kanamycin at a multiplicity of infection of 100 for 2 hours. Cells were collected and fractionated following the REAP method described by Suzuki, et al. (Suzuki et al., 2010). The nuclear and cytosolic fractions were separated, normalized to total protein by bicinchoninic acid assay, run on an SDS-PAGE gel, and transferred to nitrocellulose membranes. Membranes were incubated with anti-histone 3 antibody (Cell Signaling) as a nuclear marker, anti-β-actin antibody (Abcam) as a loading control, as well as anti-NF-κB p65 antibody. Blots were imaged with

an Odyssey Imaging System (LI-COR Biotechnology). Total NF-κB in the nuclear and cytosolic fraction were quantified by densitometry using ImageJ.

Immune cell recruitment

Flow cytometric analyses were performed with total erythrocyte-free lung cells isolated at the indicated times post-infection from individual mice infected with Ab17978 or co-infected as described. Lungs were minced, digested with collagenase and DNase for 30 minutes, and passed through a 70 μm cell strainer prior to erythrocyte lysis. Cells were stained with a myeloid panel that included antibodies against CD45 (clone 104, FITC, eBioscience), CD103 (clone 2E7, PerCP-Cy5.5, BioLegend), CD64 (clone X54-5/7.1, PE, BioLegend), CD11c (clone HL-3, PE-Cy7, BD Pharmingen), Siglec F (clone E50-2440, Horizon PE-CF594, BD Pharmingen), CD11b (clone M1/70, eFluor-450, eBioscience), MHCII (clone M5/114.15.2, BV605, BD Pharmingen), CD24 (clone M1/69, APC, eBioscience), Ly6C (clone AL-21, APC-Cy7, BD Pharmingen), and Ly6G (clone 1A8, Alexa Fluor 700, BD Pharmingen). Analyses were carried out on the 4-laser BD LSR Fortessa (BD Biosciences) at the Vanderbilt Flow Cytometry Shared Resource, and analyses were performed using FlowJo software (Treestar Inc). Myeloid populations were gated according to the strategy of Yu and colleagues (Yu et al., 2016).

MATERIALS AND METHODS FOR HLA CLASS I SUPERTYPE HYPOTHESIS STUDY

Mice

B6-*K^{0}D^{0}; B*07:02^{tg}* transgenic mice were a generous gift from Drs. Jack Bennink and Jonathan W. Yewdell at the NIAID/NIH and previously described (Alexander et al. 2001). The B6-*K^{0}D^{0}; B*07:02^{tg}* transgenic mice were crossed with C57BL/6 in order to generate B6-*K^{b}D^{b}; B*07:02^{tg}* (B7.2^{tg}) mice. B6-*K^{b}D^{b}; B*35:01^{tg}* (B35.1^{tg}) mouse were developed in a C57BL/6 background at the Vanderbilt Genome Editing Resource using a genomic construct of B35.1 transgene (Boucherma et al., 2013). The monochain construct consists of HLA-A*02:01 exon 1 encoding the leader segment, the human β2m exon 2 in the mature form, the HLA-B*35:01 exons 2 and 3 encoding its α1 and α2 domains, and the H-2D^{b} exon 4-8 encoding the α3, transmembrane, and cytosolic domains (Fig. 5). In addition to littermates, C57BL/6 wild-type mice were also used as control in experiments. All mouse crosses and experiments complied with the V1900038-00 protocols approved in accordance with relevant guidelines and regulations of Vanderbilt University Institutional Animal Care and Use Committee.

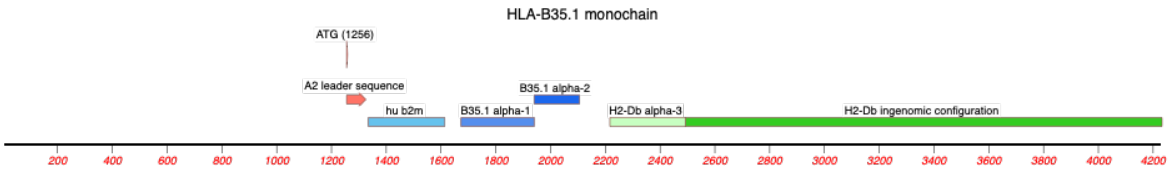


Figure 5. Linear schematic representation of the B35.1 monochain proteins adapted from Boucherma et al., 2013.

Genotyping transgenic mice

Flow cytometry analysis was used to determine cell-surface expression of transgene-encoded HLA molecules. Heparinized capillaries were used to collect tail vein blood from mice at 3-weeks old. Following red blood cell lysis, the cells were stained with APC-conjugated anti-human HLA-B7, PE-conjugated anti-human β2m, and FITC-conjugated anti-mouse H2-K^bD^b in PBS with 1% FBS (Table 1). Analyses were carried out on the 4-laser BD LSR Fortessa (BD Biosciences) at the Vanderbilt Flow Cytometry Shared Resource, and analyses were performed using FlowJo software (Treestar Inc). The B35.1tg mice were also genotyped by PCR using forward and reverse primers pairs (HLA-B35.1 F 5' TGAGCTCCTGGACCGCGGCGGACACC; H2Db R 5' GCATATGTACATGAATGTATTCACTTCAT). DNA was isolated according to Promega's protocol.

Immune cell characterization

The lungs and spleen from C57BL/6, $B7.2^{tg}$, $B35.1^{tg}$, and $B35.1^{tg}$ negative littermate control mice were harvested at 6-weeks old. The lungs were minced, digested with collagenase and DNase for 30 minutes, and passed through a 40 μm cell strainer prior to erythrocyte lysis. Cells were stained with a myeloid panel that included antibodies against Ly6G, CD24, Ly6C, MHCII, CD11b, CD45, CD64, CD11c, Siglec F, and CD103 in PBS with 1% FBS (Table 1). The spleen was smashed through a 40 μm cell strainer, cell suspension was filtered through another 40 μm cell strainer, followed by erythrocyte lysis. The lungs and spleen were stained with antibodies against CD4, B220, CD19, CD8,

CD3, NK1.1 FoxP3, CD25 PBS with 1% FBS to discern the major adaptive immune cells, T-regs, and NK cells (Table 1). Myeloid populations were gated according to the strategy of Misharin and colleagues (Misharin et al., 2013). Analyses were carried out on the 4-laser BD LSR Fortessa (BD Biosciences) at the Vanderbilt Flow Cytometry Shared Resource, and analyses were performed using FlowJo software (Treestar Inc).

Preparation of vaccinia virus stock

An aliquot of VACV was gifted to the lab from Dr. James Crowe at VUMC and propagated as previously described (Moss et al., 2017). Briefly, BSC-40 cells were cultured in complete MEM (10% FBS and 1% P/S) and infected when the cell density reached 80% confluency. The VACV stock was diluted in 2 mL of MEM with 2.5% FBS and incubated in a 37°C, 5% CO_2 humidified incubator, for 2 hours with intermittent rocking by hand at 30-min intervals. The cells were then overlayed with 10 mL of MEM with 2.5% FBS and place in a 37°C, 5% CO_2 humidified incubator. After day 3, the infected cells were collected and centrifuged for 5 min at 1,800 x*g* at 5°C. The cell pellet was resuspended in MEM with 2.5% FBS and Dounced to release virus from infected cells. Cell debris was cleared by centrifuging for 5 min at 1,800 x*g* at 5°C and collect the supernatant. To titrate VACV stock, BSC-40 cells were plated into 6-well plate at $5X10^5$ cells/ well. Cells were infected with 1 mL of VACV diluted in MEM with 2.5% FBS and place in a 37°C, 5% CO_2 humidified incubator for 2 hours. After the incubation period, media was aspirated, cells were overlayed with 2 mL of MEM with 2.5% FBS containing 0.5% methylcellulose, and incubated for two days. After that the media was removed and 0.5 mL of 0.1% crystal violet in 20% ETOH was added and incubated for 20-min at room

temperature. The crystal violet was rinsed with dH_2O, allowed to dry and plaques were counted.

Vaccinia virus LD_{50} for infection study

Wildtype, C57BL/6 mice at 8-weeks old were infected intranasally with increasing doses of VACV, starting at a minimum dose of $1X10^5$ pfu and ending at a maximum $1X10^7$ pfu diluted in 50 μL of sterile PBS. Weight loss, morbidity, and survival were assessed over the course of the infection. The pfu at which animals gradually lost weight, without signs of recovery within the first was selected as the lethal dose. Study end point was set at a loss of 30% of initial body weight accompanied by severe morbidity. A sublethal dose was determined to be $1X10^5$ pfu, while a lethal dose was determined to be $5X10^5$ pfu.

Mouse immunization and infection model

Ketamine-xylazine-anesthetized, 6- to 8-week-old C57BL/6 $B7.2^{tg}$, $B35.1^{tg}$, and $B35.1^{tg}$ negative littermate control mice were inoculated intranasally twice with 50 μg of recombinant OVA-3 (rOVA) protein, in which the original cryptic, OT-I, and OT-II epitopes were replaced with $C4R_{70—78}$, $B8R_{70—78}$ and $D1R_{808—817}$ epitopes, respectively, and formulated with 1 μg αGC (Funakoshi) in 60 μL PBS, two weeks apart (Fig. 6) (Kumar et al. 2020). To challenge, anesthetized mice were inoculated with $5X10^5$ PFU VACV in 50 μL PBS. Mice were monitored daily for weight loss, morbidity, and survival. Mice that have lost 30% of their initial body weight were euthanized per IACUC regulations. The spleen from surviving animals were processed for IFNγ ELISpot analysis. For sublethal infections

to define functional response to the cognate peptide panels, mice were infected with $1X10^5$ pfu through an intraperitoneal injection.

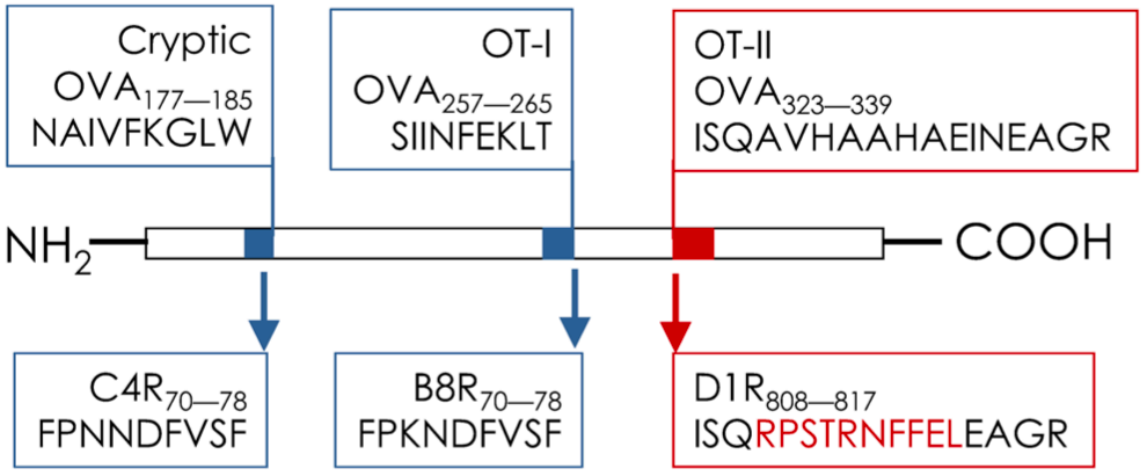


Figure 6. Schematics of OVA (rOVA-3) construct in which the original cryptic, OT-I and OT-II epitopes were replaced with $C4R_{70–78}$, $B8R_{70–78}$ and $D1R_{808–817}$ epitopes, respectively (Kumar et al., 2020) Image source: nature.

Peptides

Large scale peptide syntheses and RPC purification were performed by the manufacturer (Schafer-N, Copenhagen, Denmark). Sequences of synthetic peptides were confirmed by mass spectrometry (Gilchuk et al. 2013). Peptides were reconstituted in 100% DMSO at a concentration of 10mM and used at 10μM for ELISpot assays

ELISpot assay

ELISpot (MilliporeSigma) wells were activated with 50 μL of 100% methanol for 30 sec, washed and coated with 100 μL/well of capture antibody, 2 μg/mL of LEAF purified anti-mouse IFN-γ (clone AN-18, BioLegend) in sterile PBS. Plates were incubated

overnight at 4˚C. The following day the plates were washed with PBS and blocked with 10% FBS in RPMI and incubated for 2hr at room temperature. The spleens were harvested from immunized animals and processed to single cell suspensions in complete RPMI. Cells were reconstituted in complete media and plated at desired density into each well. Peptides were added at a concentration of 10 μM to each well, in 200 μL final volume of complete media. Plates were incubated for 48 hours in a 37°C, 5% CO_2 humidified incubator. Plates were washed with ELISpot buffer (1%FBS, 0.05% Tween in PBS) and coated with 100 μL of detection Ab, 2 μg/mL biotin anti-mouse IFNγ (clone R4-6A2, BioLegend) in ELISpot buffer and incubated for 2 hours at room temperature. Plates were washed with ELISpot buffer and 1 μg/mL of Av-HRP conjugate in 100 μL of ELISpot buffer was added to each well and incubated at room temperature for 1 hour. Plates were washed with ELISpot buffer followed by PBS, and AEC solution was added for 10 min at room temperature. Reaction was stopped with tap water and plates were allowed to dry overnight. IFNγ production was quantified using an ImmunoSpot Analyzer (Cellular Technology, Ltd).

Quantification and statistical analysis

Statistical analyses were performed using GraphPad Prism version 6. Mean comparisons were performed using unpaired Welch's *t*-test or one-way ANOVA adjusted for multiple comparisons. Median survival times were compared using a Kaplan-Meier analysis and log-rank test. P values less than 0.05 were considered statistically significant. Statistical details of experiments can be found in the figure legends.

Tables

Table 1. List of antibodies

Antigen	Fluorochrome	Clone	Source
β2-microglobulin	PE		BD Biosciences
B220	APC	RA3-6B2	BioLegend
CD103	PerCP Cy5.5	2E7	BioLegend
CD103	PerCP-Cy5.5	2.00E+07	BioLegend
CD11b	eFluor 450	M1/70	eBiosciences
CD11b	Pac Blue	M1/70	BioLegend
CD11c	PE Cy7	HL3	BD Biosciences
CD16/32	purified	2.4G2	Tonbo
CD19	FITC	1D3	Tonbo
CD19	APC	6D5	BioLegend
CD24	APC	M1/69	eBiosciences
CD25	PE Cy7	PC61	BD Biosciences
CD25	Pac Blue	PC61	BioLegend
CD34	PE	SA376A4	BioLegend
CD3e	PE Cy7	145-2C11	BioLegend
CD3e	PE Cy5	145-2C11	BD Biosciences
CD4	APC-Cy7	GK1.5	BioLegend
CD4	APC	RM4-5	BD Biosciences
CD4	PerCP Cy5.5	RM4-5	BioLegend
CD4	APC	Gk1.5	BioLegend
CD45	FITC	104	BioLegend
CD45R/B220	APC Cy7	RA3-6B2	BioLegend
CD5	APC	53-7.3	BioLegend
CD64	PE	X54-5/71	BioLegend
CD8a	PE	53-6.7	BioLegend
CD8a	Pac Blue	53-6.7	BD Biosciences
CD8a	APC	53-6.7	BioLegend
ckit	perCP Cy5.5	2B8	BioLegend
FoxP3	APC	FJK-16s	eBiosciences
FoxP4	PerCP Cy5.5	FJK-16s	Thermofisher
Ghost	BV510	viability dye	Tonbo
Ghost	APC Cy7	viability dye	Tonbo
H-2Kb/H-2Db	FITC	28-8-6	BioLegend
HLA-B7	APC	BB7.1	BioLegend
I-A/I-E	BV605	M5/114.15.2	BioLegend
IFNg	purified	an-18	BioLegend
IFNg	Biotin	R4-6A2	BioLegend
Ly6C	APC Cy7	AL-21	BD Biosciences
Ly6G	AF700	1A8	BD Biosciences
Ly6G	Alexa Fluor 700	1A8	BD Pharmingen
MHCII	BV605	M5/114.15.2	BD Pharmingen
NK1.1	PerCP Cy5.5	PK136	BioLegend
Siglec F	PE CF594	E50-2440	BD Biosciences
Siglec F	Horizon PE-CF594	E50-2440	BD Pharmingen

Table 2. List of HLA-B*07:02-associated VACV- and host HeLa cell-derived peptides, and ectromelia (ECTV) epitope

ORF[a]	SEQUENCE[b]	ECTV[c]	ORF[a]	SEQUENCE[b]
HLA-B*07:02-associated VACV derived peptides				
F4L$_{6-14}$	APNPNRFVI		J6R$_{303-311}$	MPAYIRNTL
B8R$_{70-78}$	FPKNDFVSF		O1L$_{335-344}$	RPMSLRSTII
E9L$_{175-183}$	FPSVFINPI		D1R$_{808-817}$	RPSTRNFFEL
E9L$_{526-534}$	FPYEGGKVF		A3L$_{192-200}$	SPSNHHILL
D1R$_{686-694}$	HPRHYATVM		B22R$_{72-80}$	TVADVRHCL
B15R$_{91-101}$	IPDEQKTIIGL		A4L$_{126-135}$	APASSLLPAL
D9R$_{26-35}$	IPRSKDTHVF		I6L$_{237-245}$	FPTNTLTSI
A24R$_{1002-1010}$	KPYASKVFF		N2L$_{104-113}$	RPNQHHTIDL
A34R$_{82-90}$	LPRPDTRHL		E2L$_{216-224}$	RPRDAIRFL
L4R$_{37-45}$	FPRSMLSIF		D5R$_{375-383}$	LPKEYSSEL
I6L$_{272-280}$	IPKKIVSLL		A11R$_{22-30}$	YPSNKNYEI
HLA-B*07:02-associated HeLa cell derived peptides				
B17L$_{181-190}$	APYPGNVLVY		hCG1980884$_{1807-1815}$	KPYFPPRIL
E1L$_{10-18}$	FPNITLKII		FASN$_{925-933}$	LPKTGTVSL
TA25R$_{954-962}$	FPSSNETSI		RACGAP1$_{465-473}$	LPQANRDTL
E9L$_{175-183f}$	FPSVFINPV		COPS8$_{163-172}$	LPRKPVAGAL
O1L$_{549-557}$	IPITDSLSF		PACSIN2$_{337-345}$	LPSKPSSTL
O1L$_{549-557f}$	IPITESLSF		FUS/BBF2H7$_{91-100}$	LPVSCTPGPL
I1L$_{53-62}$	IPVDLVKSSF		TYMS$_{274-282}$	RPFPKLRIL
C1L$_{102-111}$	KPKPAVRFAI		VEGFR3$_{1050-1058}$	RPGSSDRVL
C10L$_{41-49}$	LPMEDNSDI		UROD$_{163-171}$	RPQASHQLL
G7L$_{175-183}$	LPMIIGEPI		HECTD1$_{439-447}$	RPQVAKTLL
H1L$_{65-73}$	LPNSNINII		SIK1$_{430-447}$	RPRPVSPSSL
D11L$_{506-514}$	MPTVDEDLF		TBX1$_{442-450}$	RPSPPNPEL
B18R$_{305-313f}$	RPLDSITYL		LOC399706$_{139-145}$	SPAGSTRVL
H5R$_{89-97}$	SPSPGVGDI		SLC37A3377-386	SPNDKSINAL
I7L$_{342-350}$	TPPKSFKSL		CA9$_{397-406}$	SPRAAEPVQL
K6L$_{17-25}$	KPITYPKAL		KCDT10$_{28-36}$	SPSSKYVKL
I4L$_{498-507}$	RPIGIGVQGL		TNKS$_{142-150}$	SPSSPGSSL
TTF$_{2333-341}$	APAAPGLSL		MAP1S$_{945-954}$	SPVYLDLAYL
HNRPM$_{499-507}$	APIDRVGQTL		HRT3$_{162-170}$	TPSNTPTGPL
SAP145$_{18-27}$	APIQGNREEL		J3R$_{8-16}$	KPFMYFEEI
HK2$_{904-912}$	APKCDVSFL		A47L$_{227-236}$	KPVSDLYTSM
CUL-$_{4A5-14}$	APRKGSFSAL		G5R$_{341-349}$	LPCQLMYAL
ABCC12$_{1347-1353}$	APSAFGMLL		I4L$_{670-678}$	LPEDIKRVY
NLRP2213-220	GPSGLGKTAI		D13L$_{160-168}$	TPFDVEDTF
TRIM27$_{468-476}$	GPVRPYFSL		K1L$_{151-159}$	IPSTFDLAI
FASN$_{1169-1178}$	IPRDPSQQEL		CDCA$_{794-103}$	KPRPDVTNEL
Ectromelia virus epitope with homology to VACV B8R$_{70-78}$				
C4R$_{70-78}$	FPNNDFVSF	K72N		

[a]Open reading frames and location of epitopes defined based on Copenhagen reference strain (VACCC, ID 10,249).
[b]Peptide sequence of VACV- and HeLa cell-derived epitopes (Gilchuk et al., 2013; Spencer et al. 2015; Kumar et al., 2020)
[c]Amino acid change in orthologous ectromelia peptide

Table 3. List of VACV-derived HLA-B*35:01-associated peptides

ORF[a]	SEQUENCE[b]	ORF[a]	SEQUENCE[b]
A4L$_{83-91}$	VPTATPAPI	D1R$_{475-483}$	VPIKFIAEF
A8R$_{25-34}$	TPMIKENSGF	D4R$_{181-189}$	HPAARDRQF
A10L$_{111-119}$	NPIINTHSF	D4R$_{186-194}$	SPVTTIVGY
A10L$_{457-465}$	FPRKDKSIM	D8L$_{160-169}$	LPSKLDYFTY
A10L$_{853-862}$	RPKILSMINY	D11L$_{185-194}$	TPIVNSVQEF
A11R$_{298-306}$	SPVLNIVLF	D11L$_{506-514}$	MPTVDEDLF
A16L$_{250-259}$	YPKSNSGDKY	D12L$_{34-43}$	LPSLEYGANY
A17L$_{91-100}$	LPLTSLVITY	D13L$_{160-168}$	TPFDVEDTF
A18R$_{52-61}$	SPSVKTSLVF	E1L$_{10-18}$	FPNITLKII
A18R$_{237-245}$	TPRPANRIY	E3L$_{117-125}$	NPVTVINEY
A20R$_{4-12}$	LPVIFLPIF	E8R$_{233-241}$	DPVLMFLLF
A20R$_{162-170}$	IPKYLEIEI	E9L$_{488-496}$	LPQSMVFEY
A21L$_{99-107}$	IPGFARSCY	E9L$_{526-534}$	FPYEGGKVF
A24R$_{663-671}$	FPAEFRDGY	F1L$_{162-170}$	NPVKTIKMF
A24R$_{1002-1010}$	KPYASKVFF	F2L$_{26-35}$	SPGAAGYDLY
A32L$_{1103-1111}$	IPISDYTGY	F3L$_{435-443}$	YPRDNPELI
A37R$_{129-138}$	IPSKRLVTSF	G2R$_{1-9}$	MPFRDLILF
A37R$_{240-248}$	VPIKEQILY	G9R$_{69-77}$	GPGGLSALL
A39R$_{394-403}$	MPQMKKILKM	H1L$_{133-142}$	SPMLYFLYVY
A44L$_{326-335}$	SPIFDVDVAF	H2R$_{141-150}$	DPSAQQFCQY
A51R$_{94-102}$	TPTGVYNYF	H4L$_{636-645}$	EPTDASLKNF
A55R$_{213-221}$	SPQVIKSLY	H6R$_{156-164}$	SPDEIVIKF
B3R$_{39-48}$	IPSTVKTNLY	I1L$_{53-62}$	IPVDLVKSSF
B8R$_{70-78}$	FPKNDFVSF	I4L$_{670-678}$	LPEDIKRVY
B8R$_{104-112}$	PPTVTLTEY	I6L$_{159-167}$	IPMSIISFF
B8R$_{158-167}$	EPVTYDIDDY	I7L$_{153-161}$	NPKVVKMKI
B9R$_{10-19}$	FPSIIYSMSI	I12L$_{15-23}$	SPEDDLTDF
B12R$_{52-60}$	KPLLSEIRFI	J6R$_{177-185}$	WPLLEIHQY
B16R$_{4-12}$	LPVIFLSIF	L3L$_{291-299}$	VPKEDYYFI
B16R$_{52-61}$	NPTQSDSGIY	L4R$_{37-45}$	FPRSMLSIF
B16R$_{76-84}$	IPIDNGSNM	L5R$_{10-18}$	NPVFIEPTF
B17L$_{181-190}$	APLPGNVLVY	N2L$_{147-155}$	KPVYSYVLY
C2L$_{337-345}$	LPNLITPRY	O1L$_{4-12}$	YPEFARKAL
C9L$_{130-138}$	IPTCSNIQY	O1L$_{549-557}$	IPITDSLSF

[a]Open reading frames and location of epitopes defined based on Copenhagen reference strain (VACCC, ID 10,249).
[b]Peptide sequence of VACV- and HeLa cell-derived epitopes (Gilchuk et al., 2013; Spencer et al. 2015; Kumar et al., 2020)

Table 4. List of VACV-derived B7.2^{tg}- and B35.1^{tg}-associated peptides

ORF[a]	SEQUENCE[b]
A20R$_{162-170}$	IPKYLEIEI
A24R$_{1002-1010}$	KPYASKVFF
A34R$_{82-90}$	LPRPDTRHL
A3L$_{192-200}$	SPSNHHILL
A4L$_{83-91}$	VPTATPAPI
B17L$_{182-191}$	APLPGNVLVY
B8R$_{158-167}$	EPVTYDIDDY
B8R$_{70-78}$	FPKNDFVSF
D11L$_{506-514}$	MPTVDEDLF
D1R$_{808-817}$	GPKSNIDFKI
D5R$_{375-383}$	LPKEYSSEL
D8L$_{160-169}$	LPSKLDYFTY
E2L$_{216-224}$	RPRDAIRFL
E9L$_{526-534}$	FPYEGGKVF
F2L$_{26-35}$	SPGAAGYDLY
I6L$_{282-291}$	LPSNVEIKAI
L4R$_{37-45}$	FPRSMLSIF
N2L$_{104-113}$	RPNQHHTIDL

[a]Open reading frames and location of epitopes defined based on Copenhagen reference strain (VACCC, ID 10,249).
[b]Peptide sequence of VACV-derived epitopes (Gilchuk et al., 2013; Spencer et al. 2015; Kumar et al., 2020)

Table 5. List of VACV-derived H2-K^{b}D^b-associated peptides

ORF[a]	SEQUENCE[b]
B8R$_{20-27-Kb}$	TSYKFESV
A19L$_{47-55-Kb}$	VSLDYINTM
A47L$_{138-146-Kb}$	AAFEFINSL
A42R$_{88-96-Db}$	YAPVSPIVI
K3L$_{6-15-Db}$	YSLPNAGDVI

[a]Open reading frames and location of epitopes defined based on Copenhagen reference strain (VACCC, ID 10,249).
[b]Peptide sequence of VACV-derived epitopes (Lin et al., 2013)

III. NEUTROPHIL TRAFFICKING TO THE SITE OF INFECTION REQUIRES CPT1A-DEPENDENT FATTY ACID BETA-OXIDATION

At the time of this writing, the work outlined in this chapter was in the process of being submitted for publication. The data sets presented here reflect the work I directly contributed to, and work from collaborators will be summarized for cohesiveness.

ABSTRACT AND SIGNIFICANCE

Host genetic variability contributes to infection susceptibility and outcomes. Thus, great efforts have been made to leverage genome and phenome-wide association studies to identify genetic factors that impact infection outcome. An unbiased phenome-wide association study identified an association between polymorphism in the gene encoding carnitine-palmitoyl transferase 1a (*Cpt1a*), and an increased risk of infection. Homozygous recessive mutations in the *Cpt1a* gene are prevalent amongst the Alaskan and Canadian indigenous populations and have been associated with increased incidents of infant mortality due to bacterial infections. This study aims to identify the mechanisms by which Cpt1a contributes to infection susceptibility and outcomes. Cpt1a is a mitochondrial outer membrane protein that is required for long-chain fatty acid (LCFA) entry into the mitochondria for FA oxidation (FAO). Studies demonstrate a role for the mitochondria in regulating neutrophil activation and function by generating ATP for purinergic signaling that amplifies activating signals. Pharmacological inhibition of Cpt1a function leads to increased susceptibility to infection in mice due to impaired trafficking of neutrophils from the bone-marrow to the sites of lungs. Together these data identify Cpt1a as a host determinant for infection outcome and identify an unrecognized role for LCFA oxidation in neutrophil mobilization to sites of infection.

INTRODUCTION

Despite the major advances in vaccine development and antibiotic discovery, infectious diseases continue to be the major leading cause of death worldwide (WHO). It is estimated that 25% of reported deaths are attributed to infectious diseases, with respiratory infections accounting for approximately 4 million deaths annually (WHO). In developed countries, lower respiratory tract infections rank as the leading cause of death due to infections and the 4th leading cause of death overall. Many severe infections are caused by opportunistic pathogens, ubiquitous in nature and part of the human flora, affecting individuals with compromised health status (Jain et al., 2015; Cilloniz et al., 2016). Beyond instances of comorbidities exacerbating susceptibility, genetic predispositions and host factors are major contributing factors to infection outcomes (Smelaya et al., 2016; Rautanen et al., 2015). A greater understanding of host determinants governing innate and adaptive immunity would facilitate the development of effective preventative measures and therapies aimed at enhancing immune defenses.

The role of cellular metabolism in lymphocytes and myeloid cells extends beyond cell survival, functioning as an adaptable guide for cellular differentiation and activation (Voss et al., 2021). Insights into the regulatory role of immunometabolism is best described in macrophages and T cells. However, it is well appreciated that these interconnecting metabolic pathways influence many different cells and systems involved in health and diseases (O'Neill et al., 2016). In the most general sense, the glycolytic pathway is seen as characteristic of rapid proliferation and activation of effector functions, whereas oxidative metabolism is used for non-inflammatory, steady state functions (O'Neill et al.,

2016). With technological advances and increased interest in immunometabolism, the intricacy of metabolism and immune functions continues to emerge.

The *Cpt1a* variant in disease amongst the Artic population, in which a genetic mutation resulting in reduced enzymatic activity is prevalent, suggests an unrecognized link between FAO and host defense (Greenberg et al., 2019; Rajakumar et al., 2009; Gessner et al., 2010, 2016; Sinclair et al., 2019). Cpt1a is a mitochondrial outer membrane protein that catalyzes the initial step of LCFA oxidation (Fig. 7). Cpt1a converts fatty acyl CoAs to acylcarnitines, which are then shuttled into the mitochondria through the carnitine-acylcarnitine translocase. In the inner mitochondrial membrane, FAO is initiated once the FA is liberated from carnitine by Cpt2 (Bonnefont et al., 2004; Schulz et al., 1991). Given the disease association of Cpt1a and its essential role in LCFA oxidation, this work aims to define the function of FAO in host defense against infections.

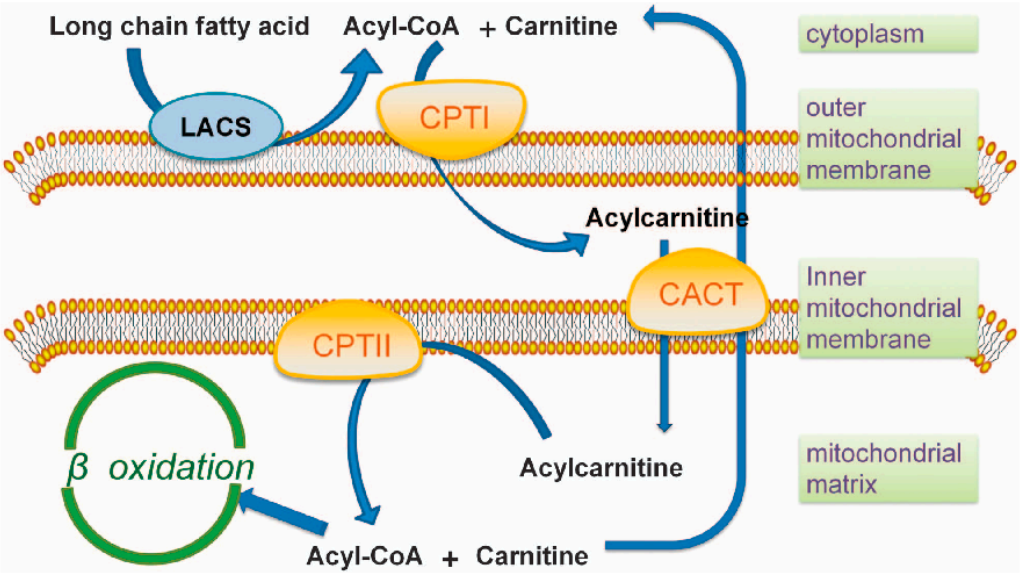


Figure 7. **Carnitine palmitoyl transferase 1a is a mitochondrial outer membrane enzyme that catalyzes the initial step of long-chain fatty acid oxidation** (Qu et al., 2016). LACS, long-chain fatty acid acyl-CoA synthetase; CPTI, carnitine palmitoyltransferase I; CACT, carnitine acylcarnitine translocase; CPTII, carnitine palmitoyltransferase II. Image source: Cell death and diseases 2016.

RESULTS

A Cpt1a polymorphism is associated with infection risk in humans.

A PheWAS identified the *Cpt1a* allelic variant, rs2229738_T, as a risk factor associated with increased susceptibility to bacterial infections. The minor allele frequency is 0.02 and carriers were more likely to be diagnosed with pneumococcal pneumonia (OR 2.7020, P=0.0066), Candidiasis (OR 2.4307, P=0.0026), cellulitis or abscesses (OR 1.5114, P=0.0296), or methicillin-resistant *Staphylococcus aureus* (MRSA) (OR 1.3857, P=0.0405). Expression of this allelic variant results in significantly reduced enzymatic activity (Gobin et al., 2003). *Cpt1a* variants are prevalent within the Artic indigenous populations, in which there are high incidents of infant mortality rate due to bacterial infections. Together, these data suggest a link between FAO and host defenses during infections.

Pharmacologic inhibition of Cpt1a-dependent FAO increases susceptibility to bacterial pneumonia in mice.

To examine whether Cpt1a is a host determinant of infection outcome, a murine model of infection was used in which animals were treated with the potent irreversible inhibitor of Cpt1a, etomoxir, or vehicle control (Kruszynska et al., 1987; Agius et al., 1991; Ratheiser et al., 1991). Due to the association between *Cpt1a* rs2229738_T allele and diagnosis of pneumococcal pneumonia, a mouse bacterial pneumonia model was used to investigate the role of Cpt1a in host defenses during infection. To account for variability in infection susceptible, mice were infected with either *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* or *Staphylococcus aureus.* Animals were treated 24 hours

prior to and at the time of intranasal infection. Results from multiple bacterial pneumonia models indicate that animals deficient in Cpt1a function exhibited increased mortality and bacterial burdens when compared to vehicle treated animals. All etomoxir treated, mock infected and vehicle treated, infected animals survived. At 24 hours post infection, 30% of the etomoxir-treated animals infected with *P. aeruginosa* survived. At 36 hours post infection, 100% of etomoxir-treated *A. baumannii* infected animals succumbed to their infection. At a reduced *A. baumannii* inoculum, animals treated with etomoxir had higher bacterial burdens in the lungs and increased dissemination of infection to the spleen. This was also observed in surviving etomoxir-treated *P. aeruginosa* infected animals. All animals infected with *S. aureus* survived but etomoxir treated animals had higher bacterial burdens and dissemination of infection. Animals treated with etomoxir and infected with *A. baumannii* had increased levels of blood urea nitrogen, aspartate aminotransferase, and alanine aminotransferase, indicative of infection-induced organ failure. These data indicate that animals treated with etomoxir to inhibit the function of Cpt1a exhibited increased mortality, bacterial burdens, and dissemination of infection when compared to vehicle treated animals.

Pharmacologic Cpt1a inhibition reduces neutrophil recruitment to the site of infection.

Increased mortality and bacterial burden in the lungs and extrapulmonary organs of mice treated with etomoxir indicate potential defects in host defense mechanisms to control infections. To investigate this, complete blood count (CBC) was performed on etomoxir, or vehicle treated mice that were either infected with a reduced inoculum of *A. baumannii* or mock infected. Total white blood cell (WBC) counts were comparable

between etomoxir and vehicle treated mice but etomoxir animals had a significant reduction in neutrophils and increase in lymphocytes (Fig. 8a-c). To determine whether the same phenomenon is observed in human, clinical collaborators performed completed blood count on carriers of the *Cpt1a* rs2229738_T allele with a diagnosis of pneumonia compared to control individuals with pneumonia. Carriers of the *Cpt1a* rs2229738_T allele, had a significant reduction in WBC and neutrophil counts. Histological analysis with H&E and MPO was performed by collaborators to determine whether the reduction in circulating neutrophils affected localization to the lungs following infection. The lungs of etomoxir treated and infected mice had a notable absence of inflammatory cells, including neutrophils, and the presence of bacteria in the alveolar space compared to control treated and infected mice. Flow cytometric analysis indicated that animals treated with etomoxir and infected had significant reduction in total CD45+ cells and approximately 50% less neutrophils in the lungs compared to control treated and infected mice (Fig. 8d-e). These data suggest a role for Cpt1a in neutrophil recruitment to sites of infection. Given the essential role of neutrophils in infection control and clearance, defects in Cpt1a function resulting in reduced neutrophil trafficking to sites of infection may be a contributing factor to the increased susceptibility to infection seen in carriers of the *Cpt1a* rs2229738_T allele (Conlan et al., 1997; van Faassen et al., 2007; Tate et al., 2011; Garvy et al., 1996; Ortega et al., 2015; Bortoletto et al., 2015).

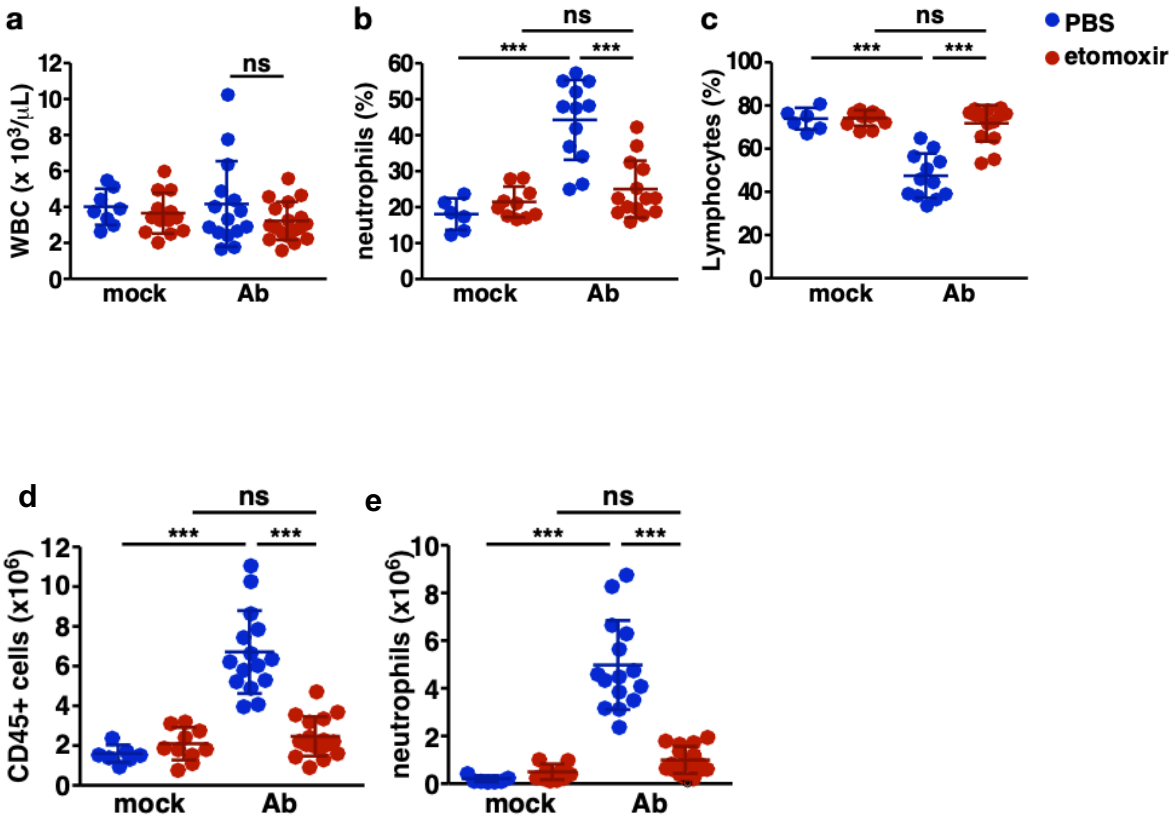


Figure 8. Pharmacologic Cpt1a inhibition reduces neutrophil recruitment to the site of infection. Mice were treated with the Cpt1a inhibitor, etomoxir, or PBS carrier and challenged intranasally with *A. baumannii* or mock infected with intranasal instillation of PBS, and blood and lungs were examined at 12 hours. Complete blood counts were performed and total WBC count (a), percentage of neutrophils (b), and lymphocytes (c) are shown. Mouse lungs from *A. baumannii*-infected mice were processed for single cell cytometric analysis following staining with a myeloid antibody panel, and the total number of CD45-positive cells (d) and neutrophils (e) are shown. Circles represent individual subjects, the horizontal line represents the mean, and error bars depict the standard deviation. Means were compared using a one-way ANOVA adjusted for multiple comparisons (a-d). WBC, white blood cell; Ab, *A. baumannii*; *, $p<0.05$; ***, $p<0.001$; ns, not significant.

Pharmacologic Cpt1a inhibition moderately alters murine neutrophil development in the bone marrow.

Neutrophil development within the bone marrow requires FAO. The observed reduction in neutrophil localization to sites of infection in animals treated with etomoxir to inhibit Cpt1a function may be due to impaired neutrophil development. An experimental protocol was first established to distinguish the developmental stages of neutrophils within the bone marrow, adopted from (Riffelmacher et al., 2017). Flow cytometric strategy to delineate developmental stages of neutrophils was based on c-kit and Ly6-G expression within the CD34-, Siglec F- cellular population. Increased Ly6-G expression is indicative of maturing neutrophils (Fig. 9a and b) (Riffelmacher et al., 2017). Quantitative analysis of MPO and matrix metalloproteinase-9 expression further distinguished immature and mature neutrophils, respectively, and cell morphology (Fig. 9c and d). To determine whether defects in Cpt1a function affect neutrophil development, mice were treated with etomoxir or vehicle control and infected with *A. baumannii* or mock infected. At 12 hours post infection, the bone marrow compartment was analyzed. Mice treated with etomoxir and infected had a modest decrease in the metamyelocytes population and a slight increase in band cells (Fig. 9e). However, these differences do not account for the significant reduction in neutrophils in the blood and at sites of infection in animals treated with etomoxir and infection.

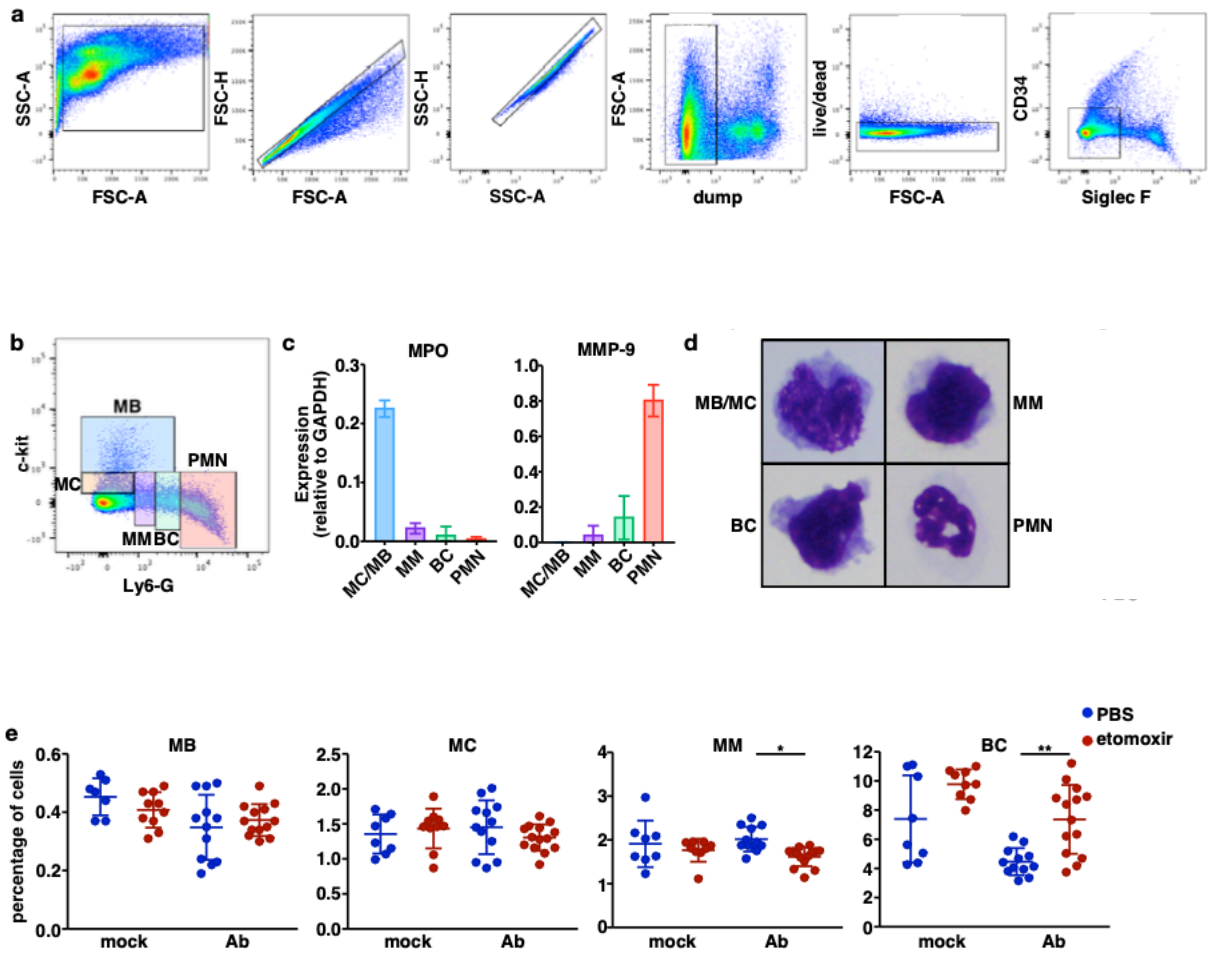


Figure 9. Pharmacologic Cpt1a inhibition does not impair neutrophil development. Bone marrow was isolated from mice and a single-cell suspension was stained with a bone marrow antibody panel for flow cytometric analyses with representative gating strategy shown in (a) and the gating of neutrophil precursors in (b). Neutrophil precursor populations were sorted, myeloperoxidase (MPO) and matrix metalloproteinase-9 (MMP-9) expression were quantified by RT-PCR (n=4 animals per group) (c), and the populations were imaged by light microscopy with representative images shown (d). Mice were treated with etomoxir or vehicle control and challenged intranasally with *A. baumannii* or mock infected, bone marrow was harvested at 12 hours and assessed by flow cytometry with the relative abundance of neutrophil precursors depicted (e). Means are depicted as columns (c) or as a horizontal line (e). Circles depict individual animals and error bars indicate the standard deviation. Means were compared using a one-way ANOVA adjusted for multiple comparisons. SSC-A, side scatter area; FSC-A, forward scatter area; MB, myeloblast; MC, myelocyte; MM, metamyelocyte; BC, band cell; PMN, neutrophil; Ab, *A. baumannii*; *, P<0.05; **, P<0.01.

Pharmacologic Cpt1a inhibition impairs neutrophil trafficking.

We considered the possibility that disruption of FAO through Cpt1a inhibition induced neutrophil apoptosis and cell death leading to the observed reduction in neutrophils within the blood and lungs following etomoxir treatment. To test this, animals were treated with etomoxir or vehicle control and infected with *A. baumannii* or mock infected. At 12 hours post infection, the bone marrow compartment and blood were assessed with annexin V staining and flow cytometric analysis. Results indicate that the reduction in neutrophils was not due to apoptosis and cell death following pharmacological inhibition of Cpt1a to disrupt FAO (Fig. 10a and b). Curiously, while mice treated with etomoxir and infected with *A. baumannii* had a significant reduction in neutrophils in the blood and lungs, the bone marrow of these animals had a higher percentage of neutrophils compared to vehicle treated and infected mice (Fig. 10c). This observation suggests that inhibiting Cpt1a function results in impairment of mechanisms facilitating neutrophil trafficking from the bone marrow following infection (Metzemaekers et al., 2020). Results from a multiplex panel demonstrate a marked reduction in proinflammatory cytokines critical for neutrophil production and recruitment in mice treated with etomoxir and infected, although interpretation of these data were confounded by the reduction in pro-inflammatory cells within the lungs (Fig. 10d). However, *in-vitro* analysis of bone-marrow derived macrophages (BMDM) treated with etomoxir and infected with *A. baumannii* indicate that inhibiting CPT1a function did not disrupt the ability of BMDM to produce proinflammatory chemoattractant factors (Fig. 10e). To further investigate defects in neutrophil mobilization following CPT1a inhibition, mice were treated with etomoxir or vehicle control and administered G-CSF daily for five days. Flow

cytometric analysis of the bone-marrow compartment suggests a subtle bottleneck in neutrophil development at the metamyelocyte stage (Fig. 10f). However, CBC revealed that animals treated with etomoxir had fewer circulating neutrophils than PBS treated animals (Fig. 10g). Administration of G-CSF is an established clinical practice used to mobilize neutrophils from the bone marrow to the blood. The failure of G-CSF administration to mice treated with etomoxir to overcome neutrophil mobilization to sites of infection suggests that Cpt1a-dependent FAO results in cell-intrinsic impairment of neutrophil trafficking (Petit et al, 2002; Basu et al., 2002; Hartmann et al., 1997). This is further supported by the observation that despite reduction in neutrophil retention factor, CXCL12, within the bone marrow and an increase in the neutrophil chemoattractant, CXCL1, animals treated with etomoxir and infected still failed to mobilize neutrophils from the bone marrow to sites of infection (Fig. 10h).

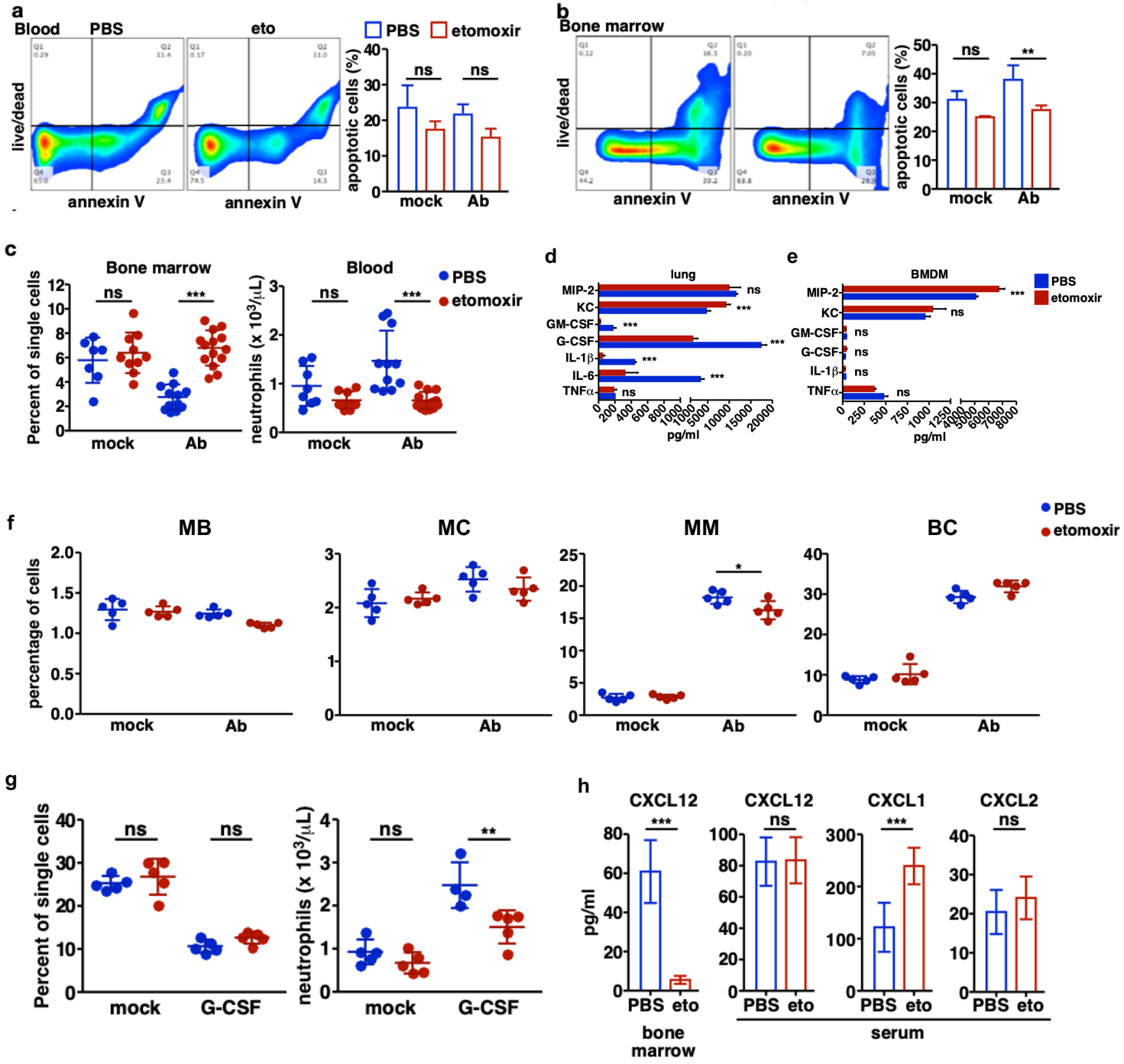


Figure 10. Pharmacologic Cpt1a inhibition impairs neutrophil trafficking. Mice were treated with etomoxir or vehicle control and infected with *A. baumannii* or mock infected. Blood (a) and bone marrow (b) were harvested at 12 hours, stained annexin V prepared, and analyzed by flow cytometry (n=4 per group). Bone marrow and blood were collected at 12 hours post infection, flow cytometric analysis of cellular population in bone marrow and blood (c). Mice were treated with etomoxir or vehicle control and challenged intranasally with *A. baumannii* and the indicated cytokines and chemokines were quantified from whole lung homogenates of mice at 12 hours following infection (n=5 animals per group)(d). The indicated cytokines and chemokines were quantified from the supernatants of bone marrow-derived macrophages that were

treated with etomoxir or vehicle control prior to infection with *A. baumannii* (n=4 replicates per group) (e). Mice were treated with etomoxir, or vehicle control and neutrophil mobilization was induced by systemic treatment with G-CSF or PBS control. Bone marrow was harvested 24 hours following treatment and subject assessed by flow cytometry, the relative abundance of neutrophil precursors depicted (f), and percentage of neutrophils in the bone marrow and blood (g) are depicted. Mice were treated with etomoxir or vehicle control and infected with *A. baumannii* or mock infected. Bone marrow and serum were collected at 12 hours and the indicated chemokines were quantified (h), (n=5 per group). Circles depict individual animals and error bars indicate the standard deviation. Means were compared using a one-way ANOVA adjusted for multiple comparisons (a-g) or Welch's t-test (h). MB, myeloblast; MC, myelocyte, MM, metamyelocytes; BC, band cell; Ab, *A. baumannii*; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant.

Mitochondrial FAO is required for neutrophil chemotaxis.

To determine whether inhibition of Cpt1a-dependent FAO results in a cell-intrinsic impairment of neutrophil trafficking to sites of infection, chemotaxis was assessed *in-vitro*. To begin these assessments, Cpt1a expression in neutrophils was confirmed at the mRNA and protein level (Fig. 11a and b). Treating bone-marrow derived neutrophils with etomoxir significantly reduced fMLF-induced chemotaxis. However, the addition of octanoic, a medium chain fatty acid that does not require Cpt1a for entry into the mitochondria for oxidation, rescued neutrophil chemotaxis (Fig. 11c). Work from collaborators go on to confirm inhibition of Cpt1a-dependent FAO impairs neutrophil chemotaxis using an alternative Cpt1a inhibitor, oxfenicine (Ma et al., 2020). Inhibiting Cpt1a-dependent FAO also impaired neutrophil chemotaxis towards CXCL2 and C5a. Additionally, lipid depletion from growth media impaired chemotaxis towards fMLF. The addition of octanoic rescued neutrophil chemotaxis towards the chemoattractant factors. Together, these data suggest a requirement for FAO in neutrophil chemotaxis.

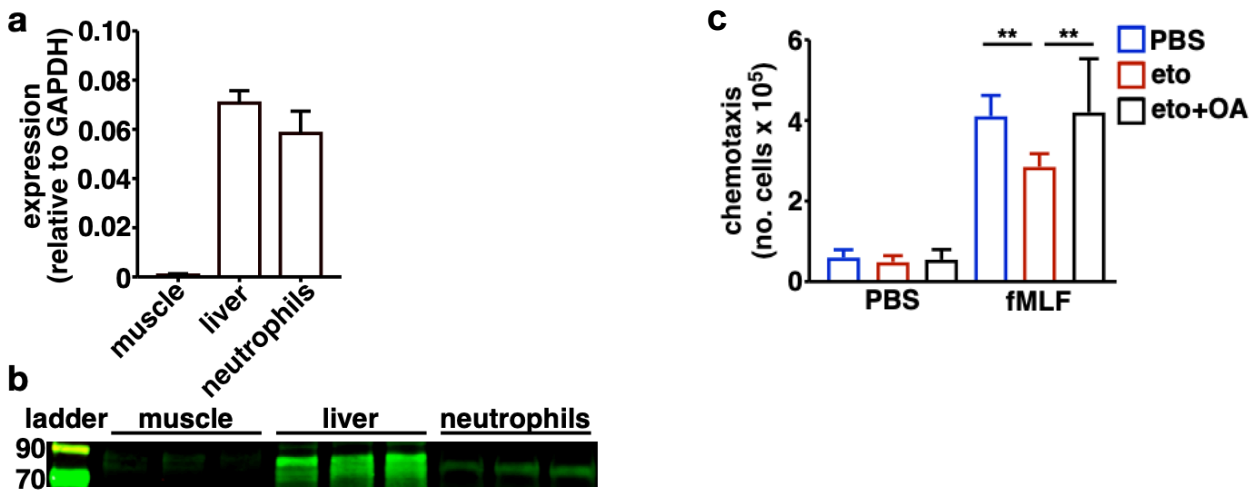


Figure 11. Mitochondrial fatty acid oxidation is required for neutrophil chemotaxis. Expression of Cpt1a was confirmed by RT-PCR (a) and western blot (b), muscle and liver tissue were used as negative and positive controls, respectively. Bone marrow neutrophils were treated in control medium, in medium containing etomoxir, or in medium containing etomoxir and octanoic acid, and fMLF-induced chemotaxis was assessed using a transwell system (c) (n=3 per group, data are representative of 4 experiments). Means were compared using a one-way ANOVA adjusted for multiple comparisons (c). eto, etomoxir; OA, octanoic acid; fMLF, N-Formylmethionine-leucyl-phenylalanine**, $p<0.01$.

Inhibition of mitochondrial FAO disrupts autocrine purinergic amplification of chemotactic signals

Activation of neutrophils requires ATP release and autocrine purinergic signaling through the P2Y2 receptors (Chen et al., 2006). The mitochondria regulate neutrophil activation and function by generating ATP to initiate purinergic signaling following recognition of an activating stimulus (Bao et al., 2013). This suggest that impaired CPT1a-dependent FAO disrupts mitochondrial mediated initiation of purinergic signaling resulting in reduced mobilization of neutrophils towards chemoattractant factors (Fossati et al., 2003; Bao et al., 2014; Chen et al., 2006; Bao et al., 2013). To determine whether inhibition of Cpt1a leads to mitochondrial dysfunction and disrupts purinergic signaling, collaborators used

differentiated HL-60 cells to assess mitochondrial potential following treatment with etomoxir or vehicle control. Treating cells with etomoxir lead to a decrease in mitochondrial membrane potential that was rescued with the addition of octanoic acid. It was further demonstrated that treating differentiated HL-60 with etomoxir reduced extracellular release of ATP, and the addition of octanoic acid rescued the etomoxir-induced reduction in ATP release. Furthermore, inhibiting Cpt1a reduces intracellular calcium levels and MAP kinase signaling. Together, this suggest that inhibiting CPT1a-dependent FAO with etomoxir disrupts mitochondrial energetics and reduces production of the ATP required for signal amplification following recognition of activation signals by neutrophils. To test this, exogenous ATP was added to etomoxir-treated neutrophils prior to assessing chemotaxis towards fMLF. The addition of exogenous ATP was able to rescue impaired neutrophil chemotaxis following etomoxir treatment (Fig. 12). Taken together, these data indicate that Cpt1a-dependent FAO supplies the ATP required for initiation of purinergic signaling, following activation of neutrophils, to amplify the activating signal that results in neutrophil function.

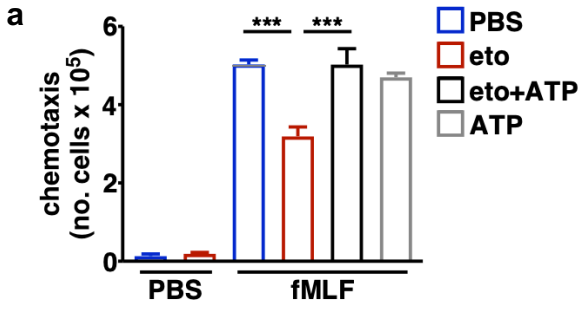


Figure 12. Inhibition of mitochondrial FAO disrupts autocrine purinergic amplification of chemotactic signals. Bone marrow neutrophils were treated in control medium, in medium containing etomoxir, medium containing ATP, or medium containing etomoxir and ATP and fMLF-induced chemotaxis was assessed using a transwell system (n=3 per group, results are representative of 3 independent experiments). Means are depicted as columns (a) and error bars indicate the standard deviation. Means were compared using a one-way ANOVA adjusted for multiple comparisons. eto, etomoxir. ***, $p<0.001$.

DISCUSSION

Homozygosity for the arctic variant of the *Cpt1a* gene is highly prevalent amongst the Alaskan and Canadian indigenous populations and is associated with increased incidence of infant mortality mediated by an increased susceptibility to bacterial infections (Greenberg et al., 2009; Rajakumar et al., 2009; Gessner et al., 2010; 2016). This study further supports the association of *Cpt1a* allelic variants and increased susceptibility to infection and defines the role of Cpt1a in host defense to bacterial infections. In an unbiased phenome-wide association study, carriers of the *Cpt1a* rs2229738_T allele were at higher risk of bacterial infections compared to non-carriers. In a murine model of bacterial pneumonia, pharmacological inhibition of Cpt1a exacerbates infections, increasing susceptibility, dissemination of infection, and mortality. Failure to contain and control infections in a host with reduced Cpt1a function is associated with impaired mobilization of neutrophils to sites of infections. Mechanistic studies demonstrate an essential role for Cpt1a-dependent FAO in neutrophil activation and function. These findings define Cpt1a as a novel host determinant for infection outcomes and identify a requirement for FAO in neutrophil biology.

The dominant metabolic program in neutrophils is glycolysis, whereby glucose is metabolized to pyruvate in the cytosol and subsequently converted to lactate (Karnovsky, 1968). The mitochondria are thought to contribute little to neutrophil energetics, but rather participate in key cellular processes such as activation, chemotaxis, and apoptosis (Borregaard et al., 1982; Sbarra and Karnovsky, 1959; Cohn et al., 1960; Reed and Tepperman, 1969; Karnovsky, 1968). Furthermore, the mitochondria contribute directly to neutrophil activation and function by releasing ATP into the extracellular space that signals in an autocrine fashion through purinergic receptors to amplify the initiating signal. This autocrine

purinergic signal amplification is required for neutrophil activation, chemotaxis, oxidative burst, degranulation, and phagocytosis (Chen et al., 2006, 2010; Bao et al., 2013, 2014, 2015).

The findings in this study provide a mechanistic framework connecting clinical observation of Cpt1a deficiency associated infection susceptibility to impaired neutrophil responsiveness. Furthermore, these data identify Cpt1a-dependent FAO as the metabolic program required for mitochondrial regulated autocrine purinergic amplification of chemoattractant signals and neutrophil chemotaxis (Fig. 13). However, caution should be taken in interpreting the infection susceptibility data as observational studies in human provide risk association and not causation. The use of a systemic pharmacological inhibitor of Cpt1a in the murine pneumonia model may exacerbate infection-related outcomes through mechanisms unrelated to the specificities of neutrophil mobilization and trafficking, as etomoxir has known off-target effects (O'Connor et al., 2018; Divakaruni et al., 2018; Raud et al., 2018). However, *in vitro* results demonstrating defects in neutrophil chemotaxis can be attributed to inhibition of Cpt1a-dependent FAO, as add-back experiments were able to reverse the defects. Treating neutrophils with etomoxir significantly reduced trafficking towards fMLF. However, addition of Cpt1a-independent medium chain fatty acid or the substrate of Cpt1a-dependent FAO, ATP, rescued neutrophil responsiveness and mobilization. Together these data support that CPT1a is critical for innate immune defenses and identify an unrecognized role for FAO in neutrophil activation and trafficking following infection. The work in this study investigates Cpt1a-dependent FAO in neutrophil chemotaxis. However, the regulatory role of purinergic signaling in other neutrophil functions warrants further investigative interest into nuanced metabolic programs affecting host defenses and infection outcomes.

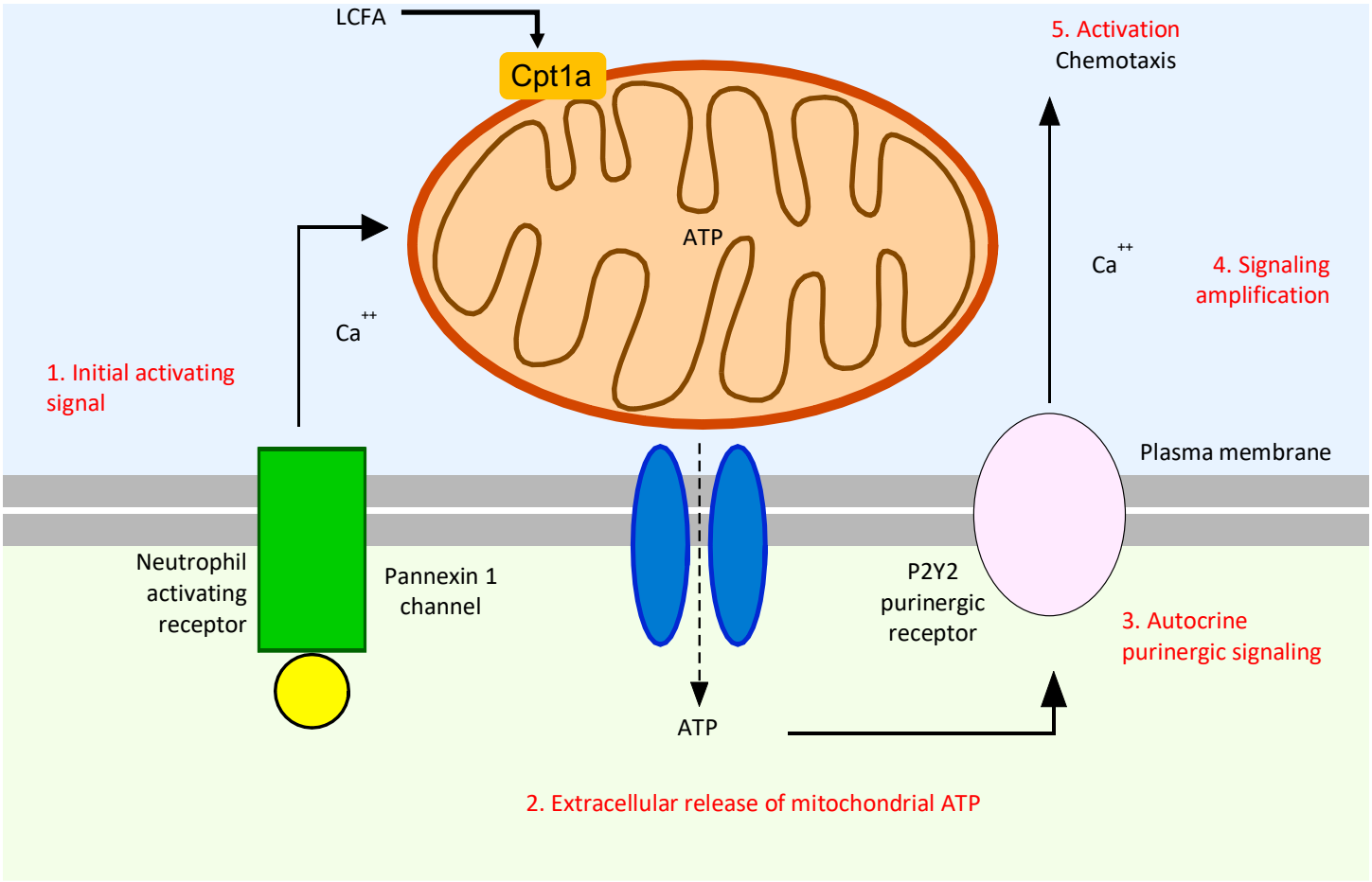


Figure 13. Schematic of autocrine purinergic signaling. The mitochondria regulate neutrophil activation by generating ATP for autocrine purinergic signaling. Following an activating signal, the mitochondria colocalizes with pannexin 1 channel and purinergic receptors to the leading edge of the cells, the mitochondria take up LCFA and produces ATP that is released into the extracellular space, this ATP signals back through the purinergic receptor, amplifying the initial activating signal, leading to activation and effector function (Bao et al., 2014).

IV. Broad-spectrum suppression of bacterial pneumonia by aminoglycoside-propagated *Acinetobacter baumannii*

This chapter is adapted from a publication in PLOS PATHOGENS by Hood-Pishchany et al., 2019. The data set presented in this chapter reflects my contribution to the publication, and contributions from co-authors will be summarized for cohesiveness.

Abstract and Significance

Acinetobacter baumannii has emerged as an important nosocomial pathogen associated with significant morbidity and mortality. The threat of *A. baumannii* to public health is compounded by its ability to develop resistance to a broad range of antibiotics. During our investigation into *A. baumannii* pathogenesis, we discovered that propagation of the human pathogen *Acinetobacter baumannii* in aminoglycoside antibiotics results in alterations to the bacterium that interact with the lung innate immune system resulting in enhanced bacterial clearance. Co-inoculation of mice with WT, non-kanamycin propagated, *A. baumannii* and a kanamycin-propagated strain induces enhanced clearance of the WT, non-kanamycin propagated strain. Co-inoculation with chemically killed, kanamycin-propagated strain replicates the enhanced bacterial clearance, indicating a role for innate immune defenses. Recognition of kanamycin-propagated *A. baumannii* alters infections, resulting in differential innate immune cell recruitment and production of pro- and anti-inflammatory cytokines. Furthermore, the enhanced bacterial clearance induced by kanamycin-propagated *A. baumannii* extends to multiple clinically relevant Gram-negative pathogens. Taken together, these findings suggest cooperation between antibiotics and the host immune system that affords protection against multiple antibiotic-resistant bacterial pathogens, highlighting the potential of innate immune modulators to combat antibiotic resistant bacterial infections.

INTRODUCTION

The development of antibiotics has revolutionized therapeutic treatments of infections, significantly improving medical care and human health. However, overuse and misuse of antibiotics coupled with the natural ability of many pathogens to develop resistance has resulted in an alarming rise in multi- and pan-resistant infections. Antimicrobial resistance is a major threat to global health and development (WHO). The rise in antimicrobial resistance not only intensifies the burden to human health but creates significant increases in health care costs (Dadgostar, 2019; CDC). This compounds the issue of antimicrobial resistance and creates a dire situation, especially in many developing countries that struggle with the availability of clean water and sanitation, preventative care, and therapeutic options.

A. baumannii is a Gram-negative, opportunistic pathogen that has emerged as a major cause of nosocomial and community-acquired infections associated with significant morbidity and mortality (Jain et al., 2004; Koulenti et al, 2017; Wong et al., 2017). Nosocomial pneumonia constitutes the most prevalent clinical manifestation of *A. baumannii* infection, although sepsis, urinary tract infections, meningitis, endocarditis, and skin and soft tissue infections are also common (Alsan et al., 2010; Koulenti et al., 2017; Munoz-Price et al., 2008; Scott et al., 2007; Wong et al., 2017). The clinical success of *A. baumannii* is attributed to the pathogen's ability to resist environmental stressors, colonize abiotic surfaces, form biofilms, and develop antibiotic resistance (Peleg et al., 2008). Known isolates of *A. baumannii* have developed resistance to all available antibiotics, prompting the World Health Organization to list *A. baumannii* as the antibiotic-resistant pathogen that poses the greatest threat to global public health (WHO).

The increase and spread of antibiotic resistance coupled to a dearth in antibiotic development has severely limited therapeutic options. Many prevalent antibiotic resistant pathogens, however, are typically opportunistic, causing disease only in persons with immune defects (Jain et al., 2015; Cilloniz et al., 2016). This suggests that therapeutic approaches targeting host immunity, rather than the pathogen, to treat bacterial infections is a promising avenue that will have the added benefit of curbing selective pressure for resistance to antibiotics (Ulevitch et al., 2004). Several studies in mouse models have demonstrated that pretreatment or concurrent treatment with a TLR agonist offered protection against an infectious challenge (Hennessy et al., 2010). A caveat to this method of immune modulation is the complexity of host-pathogen interactions, as stimulation can lead to hyperactivation of the host immune response resulting in deleterious rather than protective outcomes. Strategies using a TLR3 ligand for the treatment of chronic Mtb infection or monoclonal antibody blockade of proinflammatory cytokines in sepsis have been unsuccessful (Antonelli et al., 2018; Hotchkiss et al, 2003). Despite the current limitations of immune modulation as an alternative antibacterial therapeutic approach, furthering our understanding of mechanisms regulating the activation and coordinated response to bacterial infections may inform the development of novel therapeutic approaches.

Among the discoveries that have resulted from the research into antimicrobial resistances and mechanisms to combat this threat, are the effects of antimicrobials on the physiology of pathogenic bacteria. The expression of antimicrobial genes comes at a fitness cost to the bacterium, altering growth dynamics (Marciano et al., 2007; Albarracin et al., 2011; Gutierrez et al., 2012). Studies have also demonstrated structural modifications that alter the interactions between the bacterium and its host. Furthermore, expression of

components attributed to *A. baumannii* pathogenesis are altered in response to antimicrobial exposure (Geisinger et al., 2015). These findings highlight the complex nature of antimicrobial resistance and host-pathogen interactions. The work presented here furthers the observation that antimicrobial exposure leads to changes in host-pathogen interactions. Here, the findings demonstrate that *A. baumannii* propagated in an aminoglycoside antibiotic containing medium results in modification to the bacterium that facilitates enhanced clearance of multiple Gram-negative bacterial pathogens following co-infection in a murine model of pneumonia.

RESULTS

A. baumannii Tn5A7 induces host-mediated enhanced clearance of WT A. baumannii in a murine pneumonia model

Lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria is a highly immunogenic structure that induces an inflammatory response. Studies have demonstrated a critical role for LPS in *A. baumannii* pathogenesis. Defects in LPS formation have been shown to reduce resistance to human serum, enhanced phagocytic killing, and reduced inflammation (Luke et al., 2017; McConnell et al., 2013). Additionally, mutations in the construction of LPS leads to significantly decreased survival in sites of soft tissue infection and attenuated lethality (Luke et al., 2017; Lin et al., 2012; McQueary et al., 2012; Hood et al., 2013). Given the importance of LPS, the Tn5 mutant, Tn5A7, with a disruption in the *lpsB* gene was selected for further investigation using a murine model of bacterial pneumonia. The *lpsB* gene encodes a glycosyltransferase critical for construction of the core component of LPS.

Consistent with previous findings, Tn5A7 exhibited significantly reduced virulence compared to the wild-type (WT) strain, Ab 17978. At 36 hours post infection, animals infected with Tn5A7 had significantly reduced bacterial burdens within the lungs and reduced tissue damage of the lungs (Hood et al., 2013). As some degree of inflammation is beneficial to the pathogen during an infection, animals were co-infected with an equal inoculum of *Ab 17978* and Tn5A7. Surprisingly rather than rescuing the virulence of Tn5A7, the co-infection resulted in significant reductions in bacterial burdens and damages to the lungs, similar to outcomes in Tn5A7 mono-infections. Further investigation determined that attenuation of the WT strain during a co-infection was not the result of interbacterial antagonism, dynamics of bacterial interactions during an infection, and Tn5A7 does not need to be viable to attenuate WT infections. Clearance of the infecting bacteria occurs as early as four hours post infection. Similar results were not seen when animals were co-infected with chemically killed WT *A. baumannii.* Together, these findings suggest the presence of Tn5A7 induces host defense mechanisms that result in rapid clearance of the WT infection.

Tn5A7-mediated clearance of WT bacteria from the lung is dependent upon aminoglycoside exposure and independent of the gene disrupted by transposon insertion

Initial investigation into the mechanisms of Tn5A7-mediated enhanced clearance of *A. baumannii* infection focused on disruption of the *lpsB* gene and antibiotic selection used to propagate Tn5A7 (Hood et al., 2013). To determine whether disruption of *lpsB* gene is responsible for the attenuated virulence and clearance of WT infection, allelic exchange was used to replace the *lpsB* gene with a kanamycin-resistance cassette in the WT strain. Co-infection with WT∆*lpsB* led to clearance of WT infection, similar to the phenomenon

observed in co-infection with Tn5A7 (Fig. 14a). However, complementing *lpsB* back into Tn5A7 did not reverse the observed enhanced clearance of WT infection. To determine the involvement of the Tn5 insertion, the insertion was replaced with *tetA* in Tn5A7 (Tn5A7Δ*lpsB).* Inoculating mice with WT mixed with an equal inoculum of chemically killed Tn5a7Δ*lpsB* did not lead to the observed enhanced bacteria clearance, indicating a requirement for the Tn5 insertion for the observed enhanced clearance mediated by Tn5A7 (Fig. 14a). The Tn5 insertion contains a kanamycin-resistance cassette. To determine the involvement of kanamycin exposure in the Tn5A7-mediated enhanced clearance of WT infection, the plasmid, pMU368, containing a kanamycin determinant was introduced into Tn5a7Δ*lpsB.* Co-infection with Tn5a7Δ*lpsB*/pMU368 resulted in enhanced clearance of WT infection, suggesting exposure to kanamycin is responsible for the Tn5A7-mediated enhanced clearance of WT infection (Fig. 14a). Propagation of Tn5A7 in kanamycin is necessary for the enhanced clearance of WT infection, as Tn5A7 propagated in the absence of kanamycin did not exhibit the protective capacity (Fig. 14b). Furthermore, co-infection with WT/pMU368 and seven additional Tn5 mutants, all with intact *lpsB,* propagated in kanamycin resulted in enhanced clearance of the WT infection (Fig. 14c). Together, these findings indicate that exposure to kanamycin is responsible for the enhanced clearance of WT infection observed during a co-infection with Tn5A7.

Kanamycin exposure induces structural changes to *A. baumannii* that result in altered interactions with the host defense mechanisms culminating in enhanced clearance of WT infection. To define the specificity of this interaction, a derivative of Ab 17978 that is resistant to multiple antibiotics, Tn5A7Δ*pilQ*/pAT02, was used to investigate whether exposure to other antibiotics resulted in the same enhanced infection clearance.

Tn5A7Δ*pilQ*/pAT02 was propagated in kanamycin, ampicillin, trimethoprim and sulfamethoxazole, or tetracycline prior to co-inoculation with WT *A. baumannii*. Only exposure to kanamycin resulted in the enhanced clearance of the WT infection (Fig. 14d). Further investigation determined that *A. baumannii* propagated in an aminoglycoside antibiotic resulted in enhanced clearance of WT infection. Additionally, resistance to aminoglycosides was not found to be necessary, as WT *A. baumannii* chemically killed and exposed to kanamycin was sufficient to induce enhanced clearance of the live strain in a co-infection. Together, these data suggest that exposure to aminoglycoside antibiotics results in modifications that alter the dynamic of *A. baumannii* and host defense interactions that culminates in enhanced clearance of WT infection in a model of bacterial pneumonia.

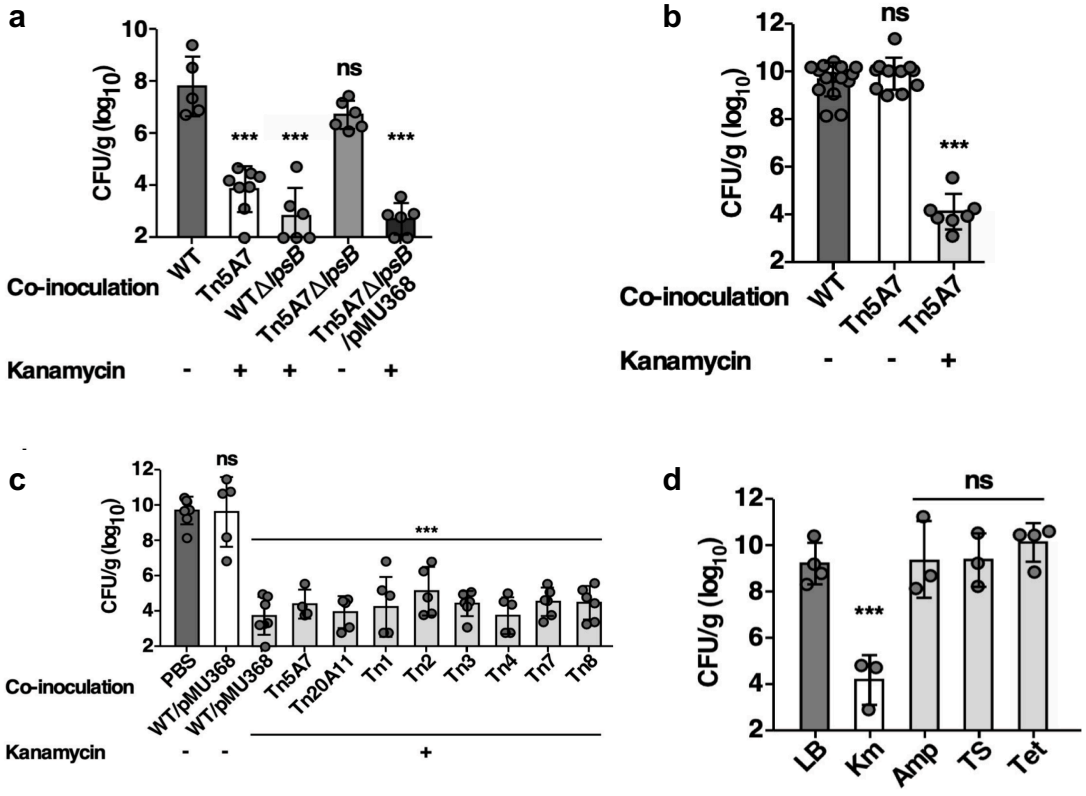


Figure 14. Propagation of *A. baumannii* in kanamycin prior to infection induces antibacterial innate immune interactions. (a) Mice were infected with WT mixed with the chemically killed strains indicated. Propagation in the presence or absence of kanamycin in the growth medium prior to infection is depicted below the graph. (b) Mice were challenged with WT mixed with an equal volume of chemically killed Tn5A7 propagated with and without kanamycin prior to infection. (c) Mice were infected with WT mixed with an equal volume of PBS or the chemically killed strains listed. Propagation in the presence or absence of kanamycin in the growth medium prior to infection is depicted below the graph. (d) Mice were infected with WT propagated in lysogeny broth prior to infection that was mixed with a chemically killed derivative of Tn5A7 that was propagated in lysogeny broth alone or containing kanamycin (Km), ampicillin (Amp), trimethoprim and sulfamethoxazole (TS), or tetracycline (Tet) prior to infection. For panels a—d mice were challenged intranasally and bacterial burdens in the lung at 36 hpi are depicted. Circles represent individual animals, columns depict the mean, and error bars show standard deviation of the mean. Means were compared with the mean of the first column using a one-way ANOVA adjusted for multiple comparisons. CFU/g, colony forming units per gram of organ homogenate *, P<0.05; **, P<0.01; ***, P<0.001; ns, not statistically significant.

Aminoglycoside exposure results in enhanced clearance of aminoglycoside-resistant A. baumannii

To investigate whether kanamycin-propagated *A. baumannii* had the capacity to enhance the clearance of infections caused by strains of *A. baumannii* resistant to kanamycin, Ab5075 was tested in this system. Ab5075 is a kanamycin-resistant strain of *A. baumannii* that exhibits enhanced virulence in a murine pneumonia model (Jacobs et al., 2014). Infection with kanamycin-propagated Ab5075 resulted in a greater than 3-log10 reduction in bacterial burdens of the lungs compared to Ab5075 propagated in the absence of kanamycin (Fig. 15a). Similarly, killed kanamycin propagated Ab5075 enhanced the clearance of live Ab5075 infections in a co-infection model (Fig. 15a).

Further investigation into the mechanism underlying the enhanced clearance of *A. baumannii* infection determined that molecules responsible for this enhanced killing of live bacteria during a co-infection are present in the supernatant collected from kanamycin propagated *A. baumannii.* However, while the addition of kanamycin-propagated *A. baumannii* supernatant resulted in enhanced clearance of the live bacterial infection, the magnitude was less robust than what was observed when cell suspensions were added. Additionally, live *A. baumannii* incubated in the supernatant from the kanamycin-propagated strain did not result in enhanced killing of the live strain *in vitro.* Co-incubation of live *A. baumannii* with the killed kanamycin-propagated strain did not increase susceptibility to the detergents or innate immune effectors. Taken together, these data demonstrate the utility of this system against aminoglycoside resistant strains and that the factors responsible are present within the supernatant. However, the mechanism leading to enhanced killing of the live strain during a co-infection pneumonia model remains unclear.

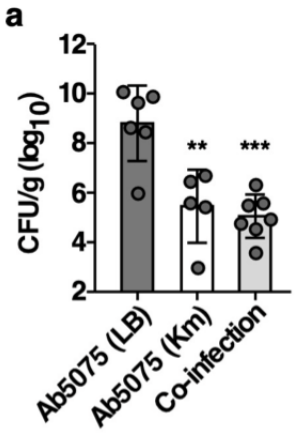


Figure 15. Aminoglycoside exposure results in enhanced clearance of aminoglycoside-resistant *A. baumannii*. (a) Mice were infected with Ab5075 propagated in lysogeny broth with and without kanamycin or co-infected with Ab5075 propagated in lysogeny broth mixed with killed Ab5075 propagated in lysogeny broth containing kanamycin. Circles represent individual animals, columns depict the mean, and error bars show standard deviation of the mean. Means were compared with the mean of the first column unless otherwise indicated using a one-way ANOVA adjusted for multiple comparisons. CFU/g, colony forming units per gram of organ homogenate; CFU/mL, colony forming units per mL of medium; **, P<0.01; ***, P<0.001.

The enhanced clearance of WT infection is MyD88-dependent but independent of TLR4, TLR9, or IL-1 receptor signaling

The enhanced clearance of live *A. baumannii* infection induced by the presence of aminoglycoside-propagated strains during a co-infection could be mediated by differential recognition of aminoglycoside induced PAMP by host PRR. The MyD88 protein is required for downstream signaling of many PRR (Medzhitov et al., 1998). Using a MyD88$^{-/-}$ knockout mouse model, it was determined that MyD88 is required for the enhanced bacterial clearance mediated by aminoglycoside-propagated *A. baumannii*, as animals deficient in MyD88 signaling did not exhibit the enhanced bacterial clearance. TLR4, TLR9, and inflammasome activation have been implicated in host response to *A. baumannii* infection (Medzhitov et al., 1998; Muzio et al., 1997; Wesche et al., 1997). Co-infection

experiments using TLR4$^{-/-}$ and TLR9$^{-/-}$ mice determined that neither TLR4 nor TLR9 are required for enhanced clearance of bacteria. To determine the role of IL-1 signaling in differential recognition of aminoglycoside-propagated *A. baumannii,* mice were treated with the IL-1R antagonist, anakinra, prior to infection with *A. baumannii* alone or *A. baumannii* with chemically killed kanamycin-propagated Tn5A7. Treatment of mice with anakinra prior to infection did not alter the enhanced bacterial clearance induced by aminoglycoside propagated Tn5A7 (Fig. 16a). Signaling down stream of MyD88 results in activation of pro-inflammatory transcription factors. To test whether differential recognition is dependent on NFκB signaling, RAW264.7 cells were infected with *A. baumannii* grown in the presence or absence of kanamycin and nuclear localization of NFκB was assessed by Western blotting. RAW364.7 cells infected with kanamycin-propagated *A. baumannii* strain did not exhibit increased NFκB activation, relative to cells infected with *A. baumannii* propagated without kanamycin (Fig. 16b). Together, these data indicate that MyD88 signaling is required for the enhanced bacterial clearance induced my aminoglycoside-propagated *A. baumannii* but differential activation of NFκB signaling is not involved.

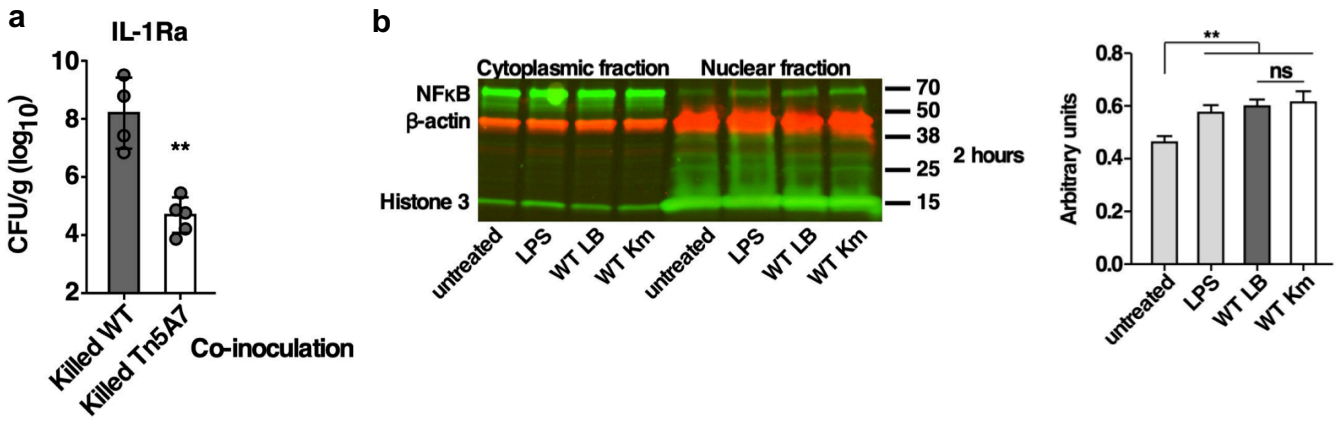


Figure 16. Kanamycin propagated *A. baumannii*-mediated enhanced clearance of WT infection is MyD88-dependent but independent of TLR4, TLR9, or IL-1 signaling. (a) Mice were treated with 200 μg of the IL-1 receptor antagonist, anakinra, two days prior and on the day of infection with WT propagated in lysogeny broth mixed with PBS or mixed with chemically killed Tn5A7 propagated in lysogeny broth containing kanamycin prior to infection. (b) RAW264.7 cells were treated with LPS or infected with *A. baumannii* grown in the presence or absence of kanamycin prior to infection and nuclear localization of NFκB was assessed by Western blotting at two hours post-infection. A representative gel image and densitometric quantification of three independent replicates are shown. Circles represent individual animals, columns depict the mean, and error bars show standard deviation of the mean. Means were compared with the mean of the first column using a one-way ANOVA adjusted for multiple comparisons (b) or Welch's t-test (a). CFU/g, colony forming units per gram of organ homogenate; β-actin is a loading control; Histone 3 is a control for nuclear localization; **, $P<0.01$; ns, not statistically significant.

Aminoglycoside-propagated A. baumannii alters cytokine and chemokine production

Recognition of aminoglycoside-propagated *A. baumannii* may alter the microenvironment within the lungs resulting in differential host response culminating in bacterial clearance. To investigate this, cytokines and chemokines from the lungs and blood of mice infected with an equal inoculum of WT, Tn5A7 propagated in kanamycin, or co-infected with both strains were quantified. BMDM were also infected with WT/pMU368 grown in the presence or absence of kanamycin for cytokine and chemokine quantification. Mice infected with kanamycin-propagated *A. baumannii* had reduced levels of IL-1β and IL-10 in the lungs and blood, and reduction of KC and MIP-2 in the blood (Fig. 17a and b). Levels of KC and MIP-2 were also reduced in BMDM infected with kanamycin-propagated *A. baumannii* (Fig. 17c). The involvement of IL-10 in the enhanced bacterial clearance was assessed by treating mice with an IL-10 neutralizing antibody or isotype control at the time of infection. The results indicated that IL-10 production is not responsible for the enhanced clearance of bacterial infection induced by aminoglycoside propagated *A. baumannii.*

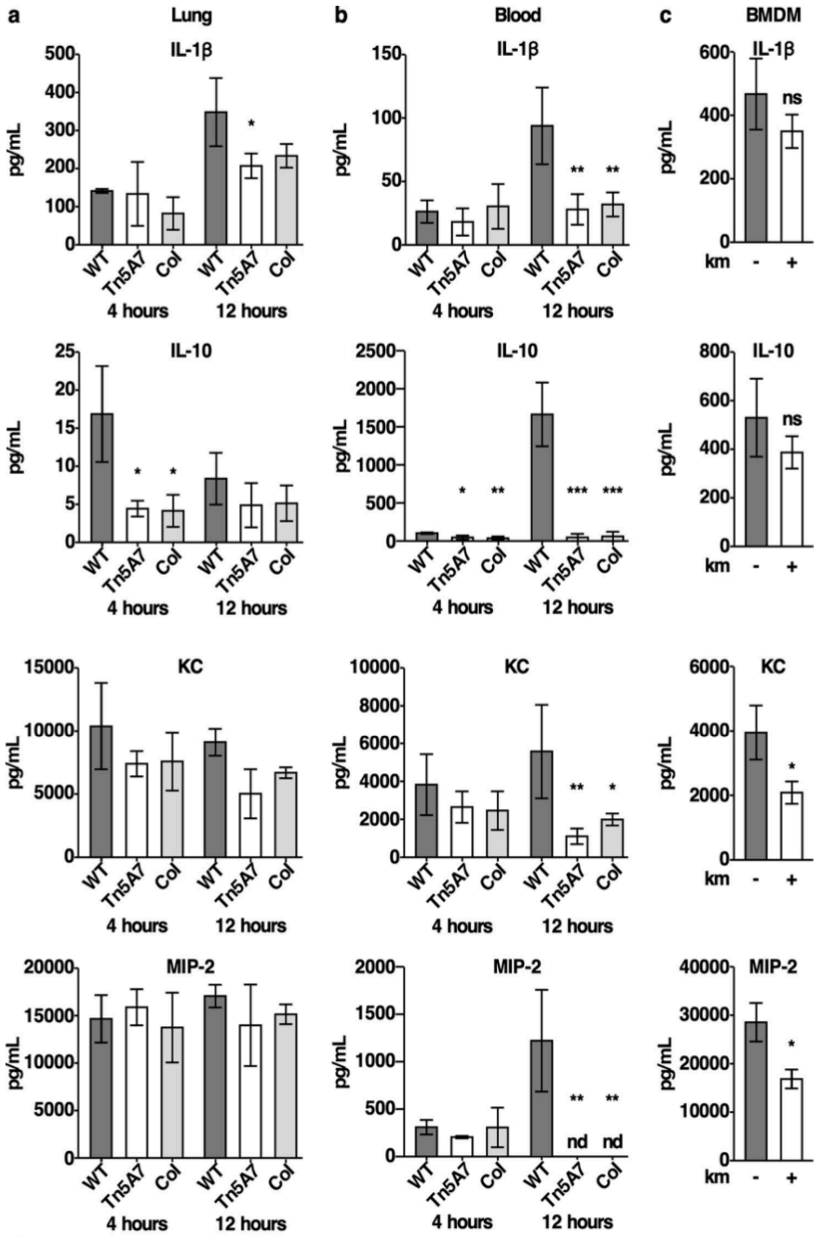


Figure 17. Infection with kanamycin propagated *A. baumannii* results in reduced cytokine and chemokine production in the lung and blood. The indicated cytokines and chemokines were quantified from whole lung homogenates (a) and blood (b) of mice at four- and 12-hours following infection with WT propagated in lysogeny broth, Tn5A7 propagated in lysogeny broth containing kanamycin, or co-infected with an equal mixture of the two strains. (c) BMDMs were infected with the indicated bacterial strains at a multiplicity of infection of 15. Infected cells were incubated for 4 hours at which time the cell culture supernatant was removed and assayed. Columns depict the mean and error bars show standard deviation of the mean. Means were compared with the mean of WT at that time using a one- way ANOVA adjusted for multiple comparisons. *, $P<0.05$; **, $P<0.01$; ns, not statistically significant.

Aminoglycoside-propagated A. baumannii reduces early neutrophilic lung inflammation

Altered cytokine and chemokine profile may affect recruitment of innate immune cells to the lungs. To assess immune cell infiltration, mice were infected with WT, Tn5A7 propagated in kanamycin, or co-infected with both strains, the lungs were harvested for flow cytometric analysis and complete blood counts were performed at 4- and 12-hours post infection. At 4-hours post infection, mice inoculated with kanamycin-propagated Tn5A7 or co-inoculated with Tn5A7 and WT exhibited significant reduction in neutrophil recruitment to the lungs, but no difference was detected in the blood (Fig. 18 a and b). A non-significant increase in macrophages and monocytes was observed in the lungs at 4-hours post infection, and no difference was detected in the blood (Fig. 18c-e). To investigate the role of neutrophils and macrophages in the enhanced bacterial clearance induced by kanamycin-propagated *A. baumannii*, mice were depleted of neutrophils or macrophages and infected with WT alone or co-infected with WT and kanamycin-propagated Tn5A7. Bacterial burdens within the lungs of mice deficient in neutrophils or macrophages were higher but depletion of these cells did not reverse the enhanced bacterial clearance in the lungs. These data indicate neutrophils and macrophages are not required for enhanced bacterial clearance from the lungs induced by aminoglycoside-propagated *A*. *baumannii*.

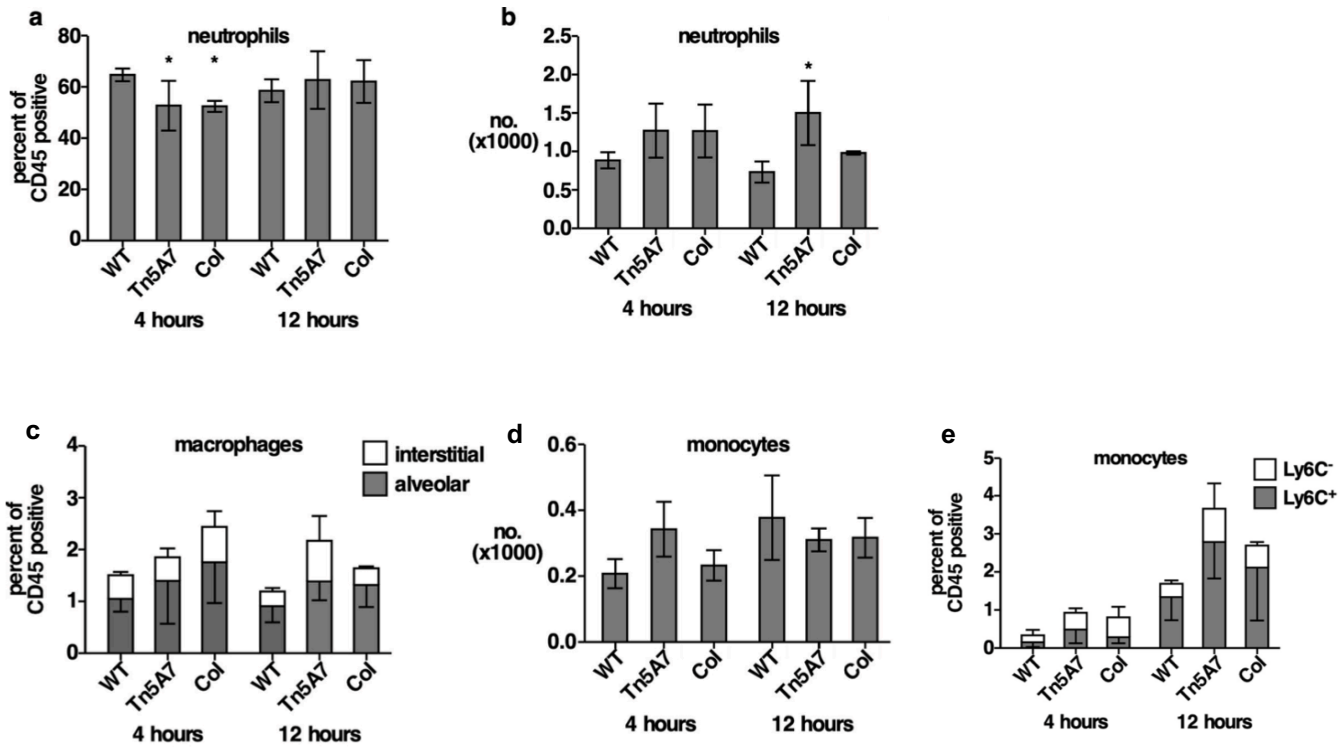


Figure 18. Kanamycin propagated *A. baumannii* alters neutrophil recruitment to the lung (a, c, e) Neutrophils, macrophages, and monocytes were quantified from the lungs of mice using flow cytometry at four- and 12-hours following infection with WT *A. baumannii* propagated in lysogeny broth, Tn5A7 propagated in lysogeny broth containing kanamycin, or co-infected with an equal mixture of the two strains. The y-axis depicts the percentage all CD45-positive cells. (b and d) Neutrophils and monocytes were quantified from the blood of mice at four- and 12-hours following infection with WT propagated in lysogeny broth, Tn5A7 propagated in lysogeny broth containing kanamycin, or co-infected with an equal mixture of the two strains. Columns depict the mean, and error bars show standard deviation of the mean. Means were compared with the mean of WT at that time using a one-way ANOVA adjusted for multiple comparisons (a-e) *, $P<0.05$.

Kanamycin propagated A. baumannii enhance the clearance of multiple Gram-negative bacterial pathogens during pneumonic infection.

To determine the protective capacity of aminoglycoside-propagated *A. baumannii*, this system was tested against other prominent pneumonia causing bacteria. Mice were infected with *Pseudomonas aeruginosa,* and mock treated with PBS or with chemically killed kanamycin-propagated Tn5A7. Co-inoculation with kanamycin-propagated Tn5A7

resulted in a 2-$\log_{10}$ reduction in bacterial burden of the lungs (Fig. 19a). Kanamycin-propagated Tn5A7 also significantly reduced bacterial burdens within the lungs of mice co-infected with *A*. *baumannii* (strain 307) and *Klebsiella pneumoniae*. However, treatment with kanamycin-propagated Tn5A7 did not change the course of a *Staphylococcus aureus* infection. In a time-course assessment of the therapeutic potential, all time points resulted in significant reduction in bacterial burdens of the lungs. However, administration of treatment at the time of infection had the most robust effect. Furthermore, administration of aminoglycoside intranasally, but not intraperitoneally, was able to reduce the bacterial burdens within the lungs of mice infected with an aminoglycoside-resistant *A. baumannii* strain. These data demonstrate that chemically killed kanamycin-propagated *A*. *baumannii* were protective against a wide range of bacterial pneumonia caused by Gram-negative bacteria and localized administration of aminoglycoside was effective against aminoglycoside-resistant infections.

Figure 19. Kanamycin propagated *A*. *baumannii* enhance the clearance of multiple Gram-negative bacterial pathogens during pneumonic infection. (a) Mice were infected with *P. aeruginosa* mixed with PBS or chemically killed Tn5A7 propagated in lysogeny broth containing kanamycin. Means were compared using unpaired Welch's *t*-test and unless otherwise noted, all comparisons were made with the first column of each graph. CFU/g, colony forming units per gram of organ homogenate; ***, P<0.001.

DISCUSSION

Infections caused by *Acinetobacter sp.* were first identified as early as 1914 (Wong et al., 2016). By the 1960s, confirmed cases of infections caused by *Acinetobacter sp.* were rapidly increasing in frequency. This increase paralleled medical advances and increasing complexity of patient care. *Acinetobacter* infections account for 1.8% of all healthcare-associated infections, with the highest density of infections occurring within the intensive care unit (Wong et al., 2016). The genus of *Acinetobacter* is highly diverse, with over 50 identified species. However, among the disease-causing species, *A. baumannii* is the most virulent and most frequently isolated within the clinical setting. It is estimated that over 45,000 cases of *Acinetobacter* infections are reported annually in the United States, and over 1 million cases globally (Wong et al., 2016). Host innate immune evasion is a prominent virulence property employed by *Acinetobacter sp.*; however, antibiotic resistance is the primary driver of infection outcomes. Given that *Acinetobacter* sp. exhibit multi- and pan-antibiotic resistance, mortality rates due to infection are extremely high, prompting a desperate need for alternative therapeutic approaches. The findings reported here demonstrate that exposure to aminoglycoside antibiotics alters *A. baumannii* physiology resulting in differential interaction with host innate immunity that enhances bacterial clearance. These findings add to the growing body of evidence that *A. baumannii* are capable of altering their phenotype in the presence of antibiotics.

The data presented here demonstrate that exposure of an aminoglycoside resistant isolate of *A. baumannii* to an aminoglycoside antibiotic results in a marked reduction in pathogenicity and essentially renders the strain avirulent in a murine model of pneumonia. This reduced pathogenicity is the result of the coordinated effects of

antibiotic exposure and differential interaction with host defense mechanisms. In co-inoculation models, the presence of aminoglycoside-propagated *A*. *baumannii* induced enhanced clearance of multiple clinically relevant Gram-negative pathogens. Furthermore, the protective effects of aminoglycoside-propagated *A. baumannii* enhance bacterial clearance while limiting inflammation and reducing neutrophilic and necrotizing bronchopneumonia. These findings suggest that the host innate defense mechanisms can be manipulated in nuanced ways to combat bacterial infection while limiting deleterious effects on the host.

While TLR4, TLR9, and IL-1 have been implicated in host defense mechanisms against *A. baumannii* infections, these signaling pathways are not required for the enhanced bacterial clearance induced by aminoglycoside-propagated *A. baumannii* strains (Muzio et al., 1997; Wesche et al., 1997). It is plausible that the differential recognition is mediated by other pattern recognition receptors, and it is also possible that exposure to aminoglycoside-propagated *A. baumannii* strain somehow sensitizes the co-infecting strain to host defense mechanisms. Alternatively, cell-associated aminoglycoside could be liberated from the aminoglycoside-propagated strain altering the course of infection by the co-infecting strain. While the mechanisms remain to be determined, this work demonstrates an unrecognized cooperation between aminoglycoside antibiotics and host innate immunity to target a prominent multidrug-resistant bacterial pathogen (Fig. 20). Furthermore, the finding that aminoglycoside-propagated *A*. *baumannii* alters host defense in the lung in a manner that enhances immune antibacterial activity while reducing inflammation supports the concept of host-mediated strategies to treat bacterial infection.

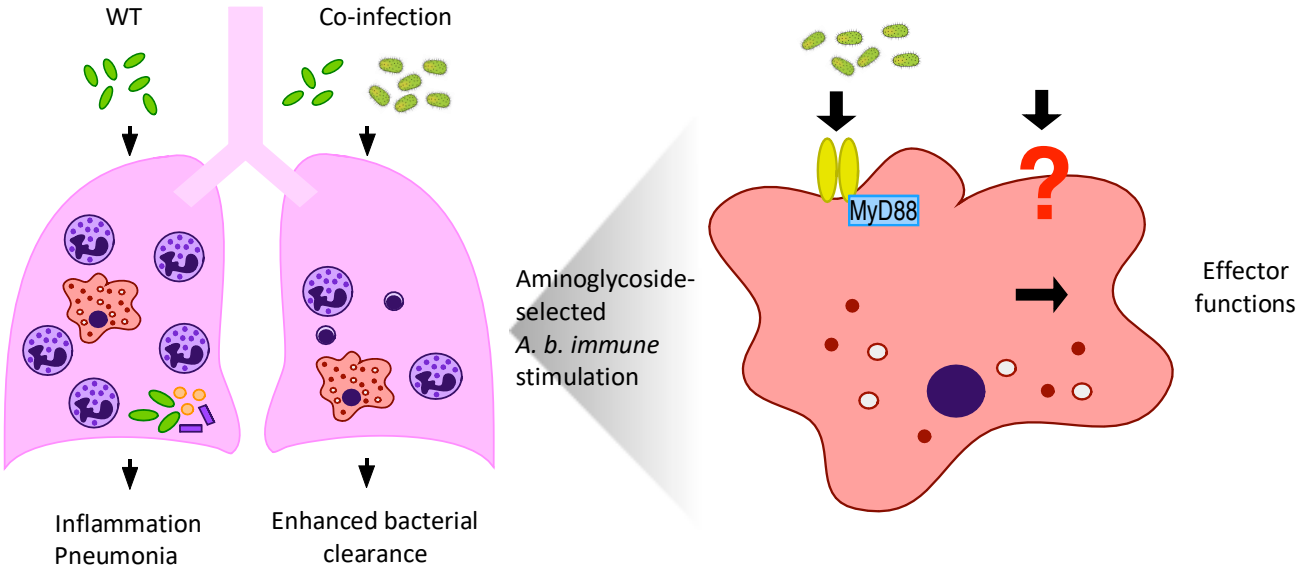


Figure 20. Schematic of enhanced-bacterial clearance mediated by aminoglycoside-propagated *A. baumannii*. Co-infection of Gram-negative pneumonia causing bacteria with aminoglycoside-propagated *A. baumannii* results in enhanced bacterial clearance from the lungs. The enhanced-bacterial clearance is dependent upon MyD88 signaling, however, the host immune effector function that contributes to the enhanced- bacterial clearance remains unknown.

V. HLA CLASS I SUPERTYPE HYPOTHESIS IN T CELL TARGETED VACCINE DESIGN

This chapter details progress made on this project.

ABSTRACT AND SIGNIFICANCE

Vaccines are among the greatest advances in modern medicine, improving the health and quality of life for people on a global scale. However, despite their positive impact we still lack fundamental knowledge regarding what constitutes an effective vaccine that generates lasting cellular and humoral immunity. Importantly, evidence suggests that effective protection against many viral and bacterial infections requires installation of a long-lived antigen (Ag)-specific memory CD8+ T cell pool. Unfortunately, current vaccines are developed empirically and generally elicit a humoral response and a weak, short lived cellular response. Movement towards rational Ag-specific vaccine design would benefit from the identification of immunogenic CD8+ T cell epitopes. However, a major obstacle is the extensive polymorphism among the HLA class I genes. In fact, over 9,300 HLA class I alleles have been identified. Interestingly, despite the extensive polymorphic nature of the HLA genes, HLA-A and HLA-B molecules can be grouped into functional supertypes based on the shared physio-chemical architecture of the antigen-binding groove, especially pockets B and F within this groove. Whether the overlapping ligand specificities of HLA class I molecules within the same supertype can be leveraged to expand the population coverage of Ag-specific vaccine design has yet to be determined. Here, I present my initial attempts towards this goal, employing a mouse model of immunization and challenge with lethal vaccinia virus (VACV) infection of the lower respiratory tract.

INTRODUCTION

Vaccines have revolutionized modern medicine. However, the development of novel vaccines has lagged behind the emergence of critical human pathogens. The origins of vaccination date back hundreds of years, beginning with the observation that individuals who contracted and survived a disease rarely succumbed to the same infection (Sallusto et al., 2010). This observation was followed by the practice of variolation to prevent smallpox infections. However, it was Edward Jenner's observation that milkmaids who contracted cowpox were protected from smallpox infections that led to the scientific basis of vaccination. Interestingly, the practice of vaccination was developed with no fundamental understanding of Immunology or Microbiology (Sallusto et al., 2010; Pulendran and Ahmed, 2011). Despite the extensive knowledge gained since, vaccines continue to be developed largely empirically. While vaccines have been tremendously successful at reducing the global burden of infectious diseases and, in the case of smallpox, have led to the eradication of a disease that once plagued the world, this empirical method of vaccine development has hindered our preparedness for many emerging human pathogens (Sharma et al., 2019).

An effective vaccine generates long-lasting cellular and humoral immunity. The goal of vaccination is to generate protective immunity by two mechanisms: a humoral response which generates antibodies and a cellular response which generates memory lymphocytes to induce rapid recall responses (Sallusto et al., 2010; Pulendran and Ahmed, 2011; Sharma et al., 2019; Zepp et al., 2010). Current vaccination strategies successfully allow the immune system to generate affinity-matured class-switched antibodies, facilitating the neutralization and clearance of the invading pathogen. Licensed vaccines are generally

formulated and administered to elicit a systemic immune response rather than a localized immune response at the sites directly exposed to invading pathogens. Several studies demonstrate the critical role of CD8+ T_{RM} stationed at barrier sites to provide frontline protective immune defenses, capable of rapidly responding to invading pathogens (Seder et al., 2008). Furthermore, CD8+ T_{RM} positioned within vulnerable mucosal sites frequently exposed to pathogens control and prevent dissemination of infection before the recruitment of effector immune cells (Ariotti et al., 2012; 2014; Bivas-benita et al., 2013; Gilchuk et al., 2016; Mueller et al., 2016; Schenkel et al., 2014). Thus, identification of immunogenic CD8+ T cell epitopes would advance the efforts of developing vaccines that result in long-lived protective cellular immunity.

The increasing prevalence of pathogen-mediated diseases highlights an urgent need to reassess current vaccine strategies. Advances in Immunology and Microbiology have greatly improved available therapies against many infectious diseases. However, the development of novel effective vaccines continues to fall short of disease prevention. Shortcomings in vaccine development are due to the lack of knowledge regarding the mechanisms that produce effective vaccines. Continued progress on the work presented here will provide fundamental insight regarding the generation of broadly functioning subunit vaccines. A movement towards subunit-based vaccines formulated with an immune enhancing adjuvant has the potential to improve efficacy and safety. However, the population coverage of subunit-based vaccines will continue to be hindered by HLA polymorphisms.

HLA class I molecules bind and present peptides of 9 to 10 amino acid residues in length. Peptide binding is defined by the physical and chemical features of the B and F

pockets within the antigen-binding cleft. The two pockets accommodate the main anchor residues found at position 2 and at the C-terminus of the peptide ligand, respectively (Falk et al., 1991; Rammensee et al., 1993; Engelhard et al., 1994). Polymorphisms within the HLA genes diversify the antigen-binding cleft and, thereby, dictate the peptide-binding repertoire of HLA molecules (Rammensee and Stevanovic, 1998). It is estimated that over 9,300 HLA class I alleles exist within the human population creating a significant challenge towards developing Ag-specific vaccines that have the desirable population coverage (Lund et al., 2004). Yet, despite the incredible number of HLA alleles that exist within the human population, HLA molecules can be clustered into "supertypes" based on the physicochemical features of the B and F pockets. This supertype hypothesis postulates that HLA allelic products within the same supertype are capable of binding overlapping peptide repertoires (Lund et al., 2004; Sette and Sidney, 1998; 1999; del Guercio et al., 1995; Bertoni et al., 1998). Thus, identifying peptide(s) that are presented by multiple members of a supertype is a plausible approach to Ag-specific vaccine development. However, whether the overlapping ligand specificities of HLA class I molecules within the same supertype can be leveraged to expand the population coverage of Ag-specific vaccine design has yet to be determined. The work presented here focuses on the B7.2 and B35.1 class I molecules, within the B7 supertype, due to their prevalence within the human population. Class I molecules within the B7-supertype share a peptide-binding specificity of proline at position 2 and a hydrophobic aliphatic or aromatic residue at the carboxyl-terminus (Sette and Sidney, 1998; 1999). Testing of the HLA supertype hypothesis will undoubtedly contribute to effective vaccine development.

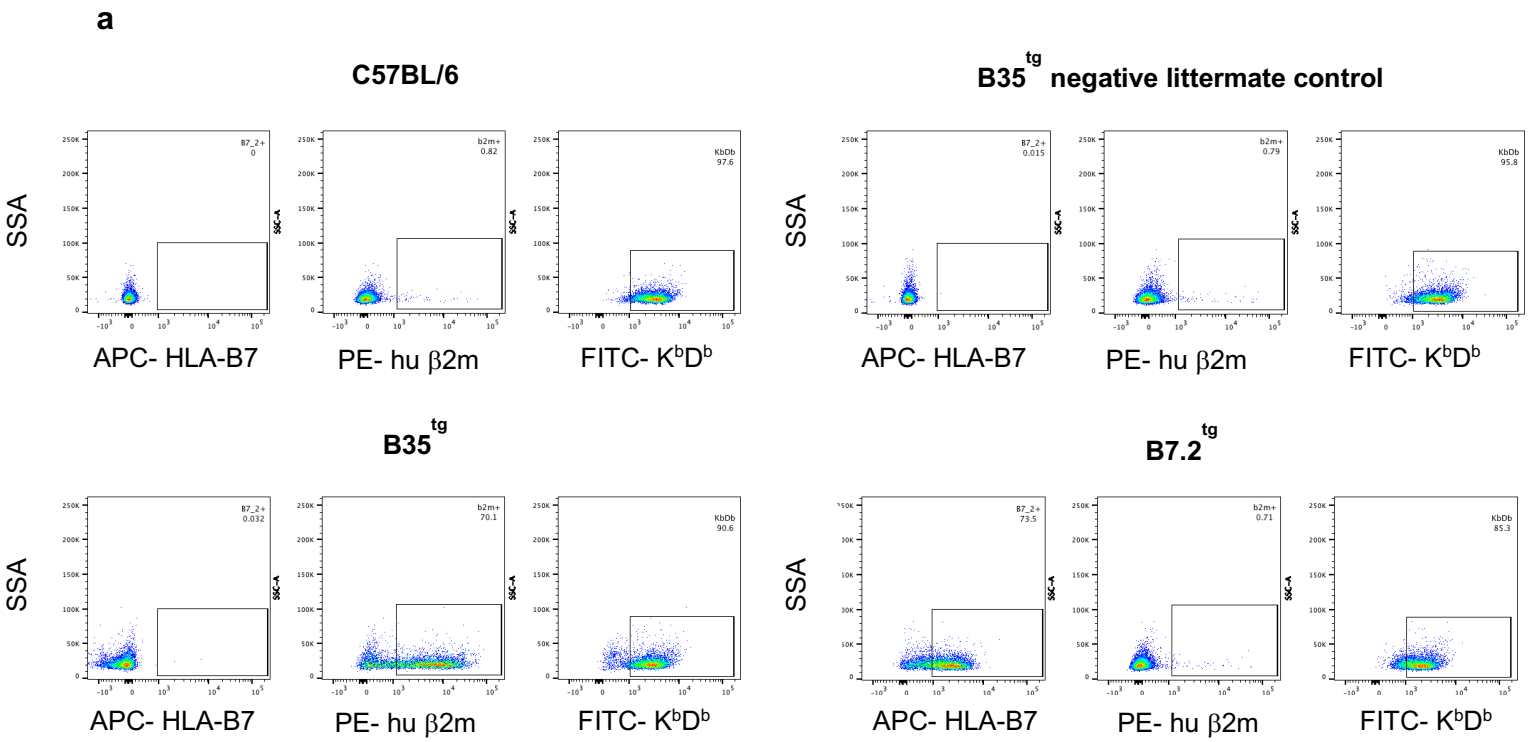
The work described here utilizes a vaccinia virus (VACV) model of viral pneumonia to study immunization and protection. VACV has become the model virus for epitope discovery and investigative work into vaccine development due to its large proteome and the ability to accommodate additional genetic material. Previous work in the Joyce lab demonstrated the ability to identify naturally processed HLA class I-restricted antigenic determinants that confer long term protective immunity against VACV infections (Gilchuk et al., 2013; 2016). Using a subunit-based intranasal vaccination strategy the lab has identified a distinct tissue resident memory CD8+ T cell population localized within the interstitial tissue of the lungs that provides critical protection at vulnerable barrier sites (Gilchuk et al., 2016). Furthermore, the proteomics approach used by the Joyce lab has identified naturally processed determinants that are presented by B35.1 and B7.2 molecules within the B7 supertype (Gilchuk et al., 2013). Whether these determinants inform on subunit-based vaccine candidates capable of population-wide coverage has yet to be tested.

RESULTS

Characterizing B7.2 and B35.1 transgenic animals

To begin the investigation into the utility of the HLA supertype hypothesis in vaccine development, HLA class I transgenic mice were assessed for surface expression of the transgenic class I molecules. Cell surface expression of the B7.2 and B35.1 molecules encoded by the transgenes was evaluated by flow cytometry analysis of splenocytes using APC-conjugated anti-human HLA-B7.2 mAb and PE-conjugated anti-human β2m mAb, respectively. Expression of the transgenes was compared to expression levels in C57BL/6 WT mice, which served as a negative control. Cell-surface

expression of HLA class I molecules was detectable in B7.2^{tg} and B35.1^{tg} mice, and expression was not detected in C57BL/6 mice (Fig. 21a). Next, the immune cell compartment was assessed by flow cytometry to ensure no defects were acquired due to the random integration of the transgene and the consequent altered expression. The lungs and spleen of C57BL/6, B7.2^{tg}, B35.1^{tg}, and B35.1^{tg} negative littermate controls mice were harvested for assessment. For the lungs, the innate and adaptive cells were assessed, and in the spleen the adaptive cells were assessed. No significant differences were detected in the major immune cell types when comparing the lung and spleens of the respective animals by flow cytometry analysis (Fig. 21b and c).



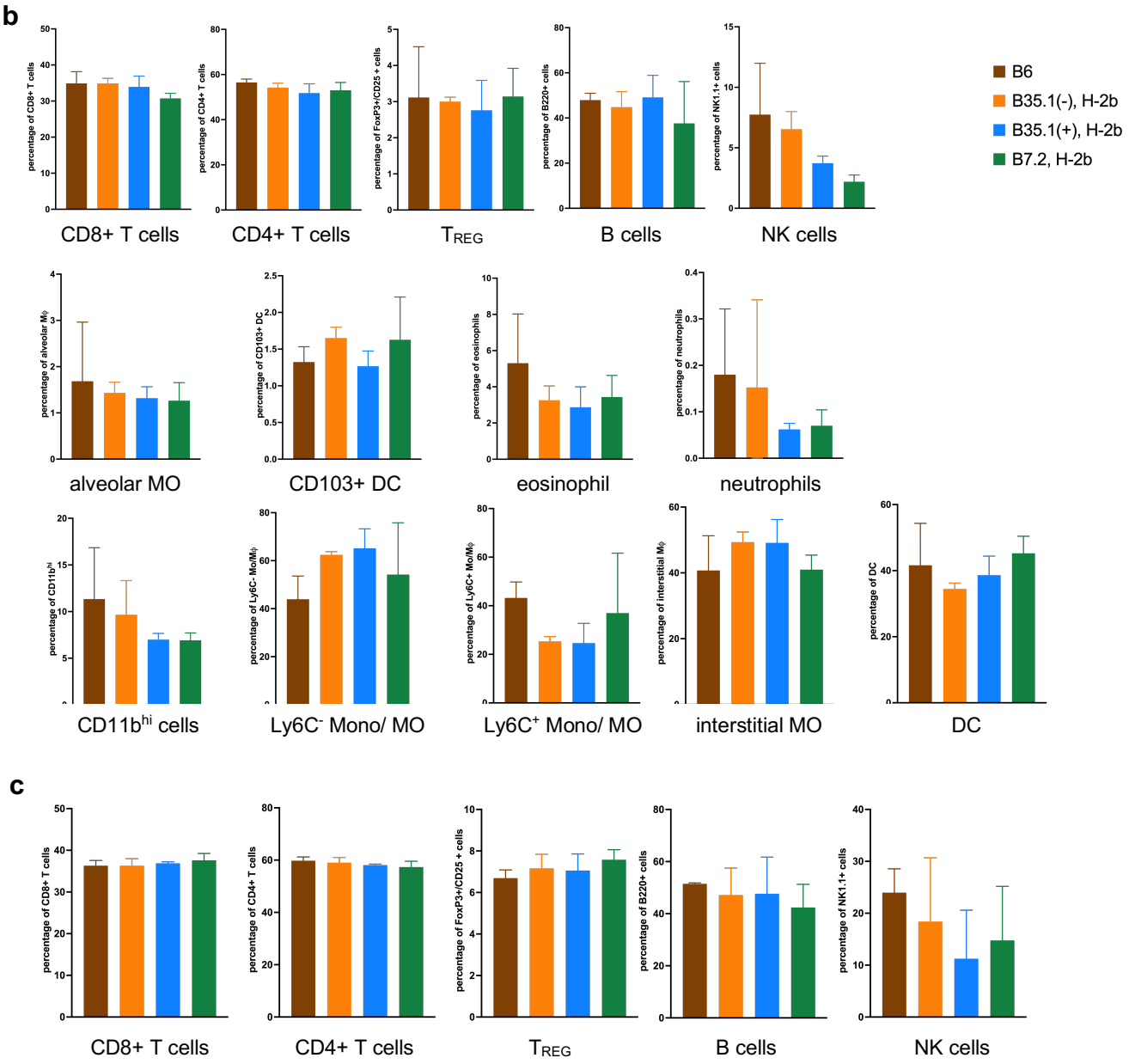


Figure 21. Characterizing B7.2 and B35.1 transgenic animals. Flow cytometry analysis of splenocytes for cell-surface expression of HLA class I molecules isolated for 8-week-old C57BL/6, B7.2^{tg}, B35.1^{tg}, or B35.1^{tg} negative littermate controls (a). Flow cytometry analysis to compare the immune cells within the lungs of 8-week-old C57BL/6, B7.2^{tg}, B35.1^{tg}, or B35.1^{tg} negative littermate controls, n=3 per group (b). Flow cytometry analysis to compare the adaptive immune cells within the spleen of 8-week-old C57BL/6, B7.2^{tg}, B35.1^{tg}, or B35.1^{tg} negative littermate controls, n=3 per group (c). Columns depict the mean, and error bars show standard deviation of the mean. Means were compared with the mean of every other column using a one-way ANOVA adjusted for multiple comparisons.

Overlapping peptide specificity elicits variable protective immune responses against lethal VACV challenge

Intranasal immunization with recombinant protein expressing the protective epitope, $B8R7_{70\text{-}78}$, formulated with αGC, conferred protection against lethal VACV challenge in $B7.2^{tg}$ mice (Gilchuk et al., 2016). As $B8R_{70\text{-}78}$ was also eluted from B35.1 molecules, this immunization and challenge strategy was exploited to determine whether the overlapping peptide specificity confers protection in $B35.1^{tg}$ mice (Gilchuk et al., 2013; Spencer et al., 2015). To do this, recombinant OVA was engineered to express the poxvirus epitopes $B8R_{70\text{-}78}$, $D1R_{808\text{-}817}$, and $C4R_{70\text{-}78}$ (rOVA-3) as previously described (Kumar et al., 2020). The epitope $B8R_{70\text{-}78}$ is processed and presented by both B7.2 and B35.1 molecules, $D1R_{808\text{-}817}$ is presented by B7.2 but not B35.1 molecules, and $C4R_{70\text{-}78}$ is an ectromelia epitope derived from an orthologous ORF not presented by either B7.2 or B35.1 molecules. C57BL/6 mice were used to determine the LD_{50} of the VACV stock. A viral dose of $2.5X10^5$ pfu was empirically determined as the LD_{50} and, at this dose, 50% of mice succumb to the VACV infection by day-8 post inoculation. Based on the LD_{50}, a viral dose of $5X10^5$ pfu was determined to be lethal, and inoculation with this viral load resulted in gradual weight loss and increases in morbidity, as all mice succumbed to the infection by day-8 (Fig. 22a-c).

C57BL/6, $B7.2^{tg}$, $B35.1^{tg}$, and $B35.1^{tg}$ negative littermate control mice were primed and boosted intranasally with rOVA-3 formulated with αGC. At thirty days post boost, animals were challenged with $5X10^5$ pfu of VACV. Weight loss, morbidity score, and survival were tracked over the course of two weeks post inoculation. Following inoculation, $B7.2^{tg}$, $B35.1^{tg}$, and $B35.1^{tg}$ negative littermate control mice lost the same amount of weight as immunized C57BL/6, and mock-immunized $B7.2^{tg}$ and $B35.1^{tg}$

animals. Beginning at day-8 post inoculation, the B7.2^{tg}, B35.1^{tg}, and B35.1^{tg} negative littermate control mice recovered and regained body mass (Fig. 22d). A similar trend was observed in terms of morbidity (Fig. 22e). Mice expressing the HLA class I transgenes and immunized with rOVA-3 also had an increased survival rate when compared to mock immunized and C57BL/6 immunized control mice. Results demonstrate that 100% of B7.2^{tg} (n=6) and 57% of B35.1^{tg} (n=7) mice survived the lethal VACV challenge. Interestingly, the B35.1^{tg} negative littermate control mice (n=3) that were immunized all survived the lethal VACV challenge. C57BL/6 immunized, B35.1^{tg} mock immunized, and B7.2^{tg} mock immunized animals all succumbed to a lethal VACV challenge (Fig. 22f). In an ELISpot assay quantifying IFN-γ production by splenocytes stimulated with the B8R$_{70-78}$, D1R$_{808-817}$, or C4R$_{70-78}$ peptides, the B7.2^{tg} mice had a significant IFN-γ response to B8R$_{70-78}$ and D1R$_{808-817}$, whereas the B35.1^{tg} mice had a significant but less robust response to B8R$_{70-78}$. Splenocytes from B35.1^{tg} mice stimulated with D1R$_{808-817}$ and C4R$_{70-78}$ produced IFNγ that trended towards significance (Fig. 22g). Furthermore, B35.1^{tg} negative littermate control mice displayed a mild IFNγ response to B8R$_{70-78}$ and D1R$_{808-817}$ that reached significance when compared to the IFNγ response of splenocytes incubated in media alone. Together, these data demonstrate variable protection conferred by immunization with processed and presented VACV epitope based on HLA class I expression. Furthermore, functional response to the D1R$_{808-817}$ and C4R$_{70-78}$ epitopes suggests the B35.1 molecule has some degree of overlap with B7.2 molecule in peptide processing and presentation. Detection of IFNγ response in B35.1^{tg} negative littermate control mice may be an indicator that low cell-surface expression of B35.1 molecules might have been missed by flow cytometry used for phenotyping this mouse line.

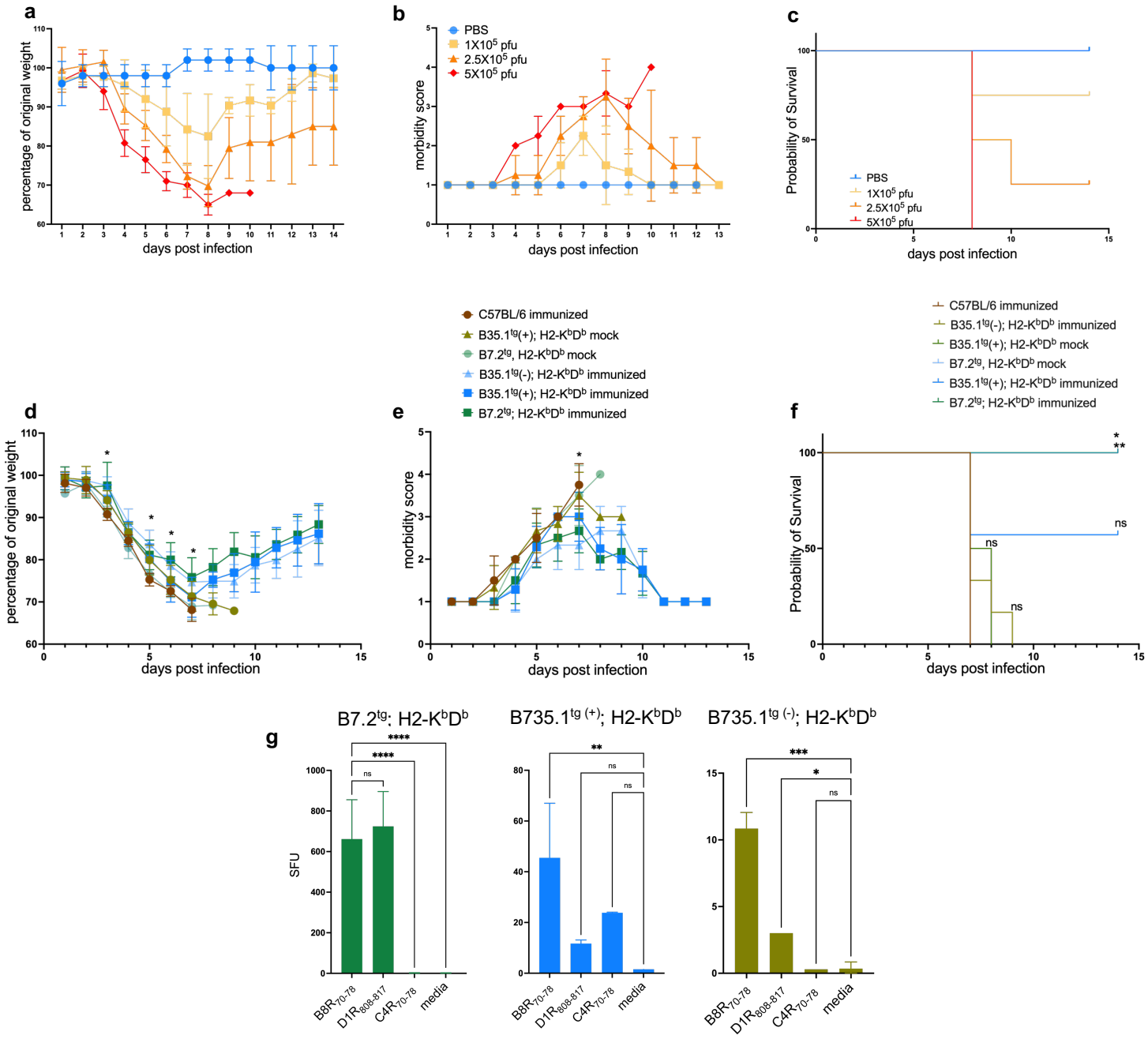
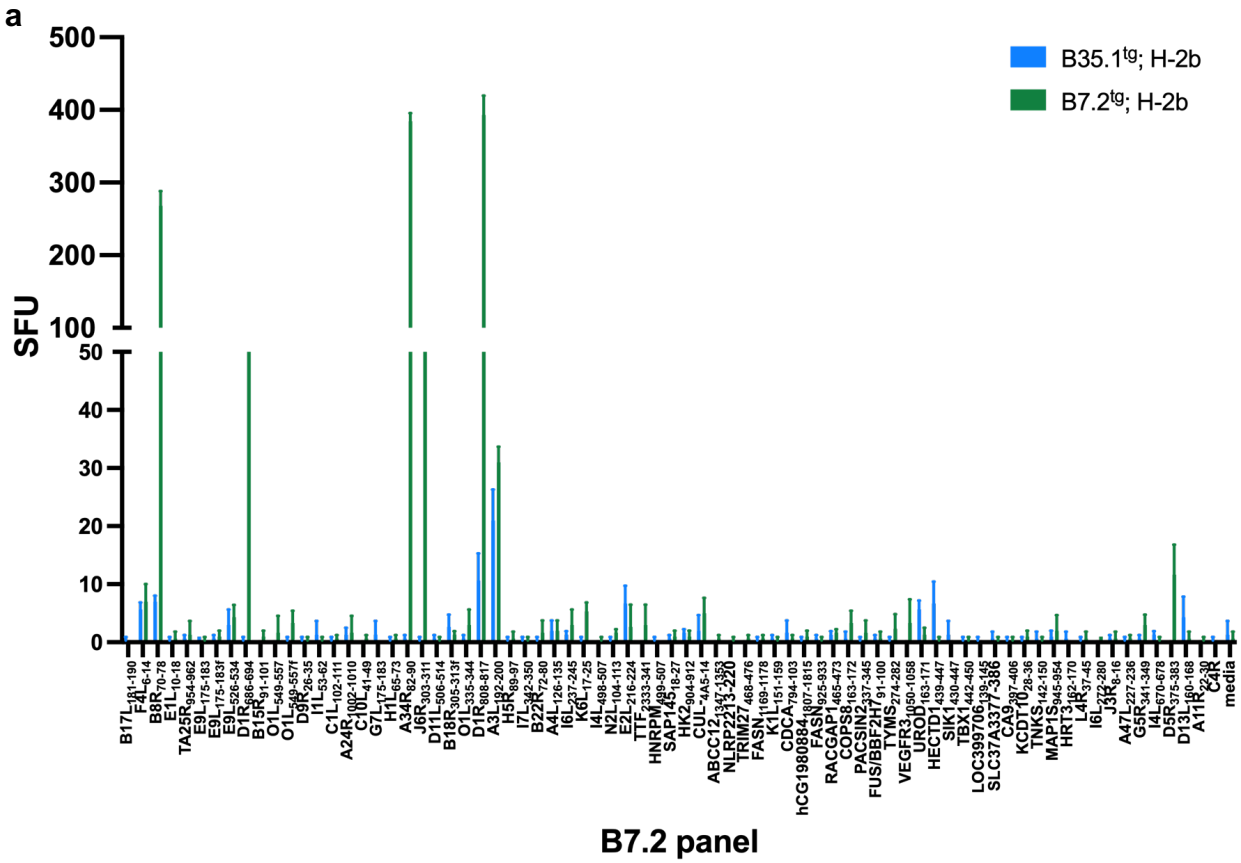


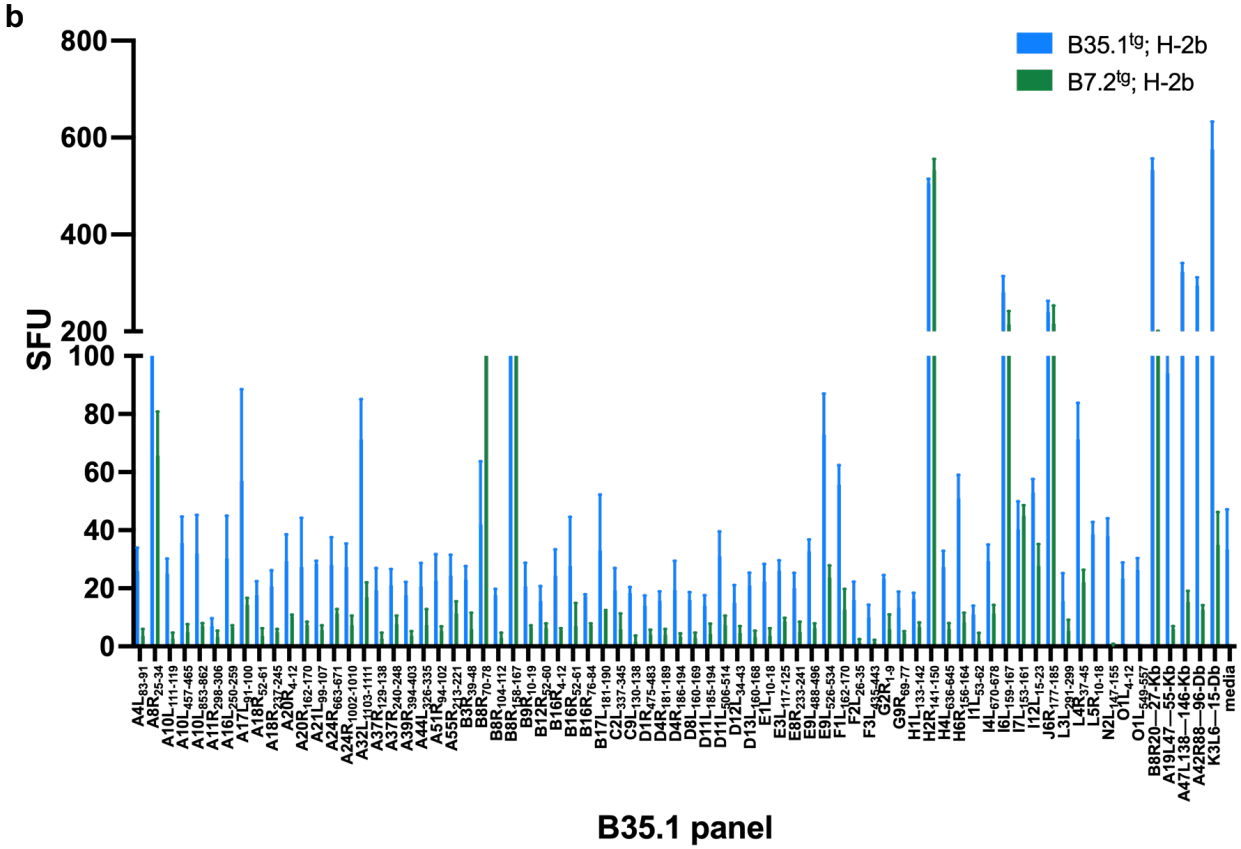
Figure 22. Overlapping peptide specificity elicits variable protective immune responses. Mice were infected with VACV intranasally, and weight loss, morbidity, and survival were monitored for the indicated length of time. C57BL/6 mice were used to determine the LD_{50} of the VACV stock (a-c; n=4 per group). B7.2^{tg} and B35.1^{tg} were primed and boosted with rOVA-3 or PBS, and at 30-days post boost, mice were infected and monitored for weight loss, morbidity and survival. C57BL/6 mice were primed and boosted with rOVA-3 as a control (d-f). ELISpot quantifying IFNγ response in surviving animals (g). Means were compared by two-way ANOVA, mixed effects analysis (a,b,d,e), Log-rank (Mantel-Cox) test (c and f), and one-way ANOVA adjusted for multiple comparisons (g). *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$; ns, not significant.

Identification of overlapping peptide determinants that elicit a functional response in HLA class I transgenic mice.

The variable protection conferred by $B8R_{70\text{-}78}$ suggests that naturally processed and presented peptides, with overlapping specificities, have the potential to provide protection to carriers of alleles that belong to a HLA class I supertype. To expand the investigation into the protective capacity of overlapping peptide specificities, qualitative and quantitative CD8+ T cell responses to eluted peptides were compared between the $B7.2^{tg}$ and $B35.1^{tg}$ mice (Table 4). Naturally processed and presented VACV determinants were previously eluted from B7.2 and B35.1 molecules (Gilchuk et al., 2013; Spencer et al., 2015). To define the functional response, spleen was harvested at seven days post inoculation from $B7.2^{tg}$ and $B35.1^{tg}$ mice acutely infected with a sublethal dose of VACV, and splenocytes were stimulated with the B7.2- or B35.1-restricted peptide panels in an IFNγ ELISpot assay, and H2-Kb and -Db derived peptides were added as controls (Table 2, 3, and 5). The functional response of splenocytes from $B7.2^{tg}$ mice to the B7.2 panel was consistent with previously published data. Splenocytes from $B35.1^{tg}$ mice displayed weak functional responses to peptides within the B7.2 peptide panel, including $B8R_{70\text{-}78}$, $D1R_{808\text{-}817}$, and $C4R_{70\text{-}78}$ (Fig. 23a) (Gilchuk et al., 2016). Functional responses from $B7.2^{tg}$ and $B35.1^{tg}$ mice to the B35.1 peptide panel were non-specific compared to the VACV K^b and D^b control peptides (Fig. 23b). To determine whether the non-specific functional responses to the B35.1 peptide panel were due to expression of mouse H2-K^bD^b, $B7.2^{tg}$ and $B35.1^{tg}$ mice in the K^bD^b null background mice were acutely infected with a sublethal dose of VACV. At seven days post inoculation, the spleen was harvested and processed for IFNγ assessment in an ELISpot assay. Results demonstrate that the $B7.2^{tg}$;

K^0D^0 and B35.1^{tg}; K^0D^0 mice elicit weak functional responses to peptides within the B35.1 panel (Fig. 23c). Taken together, these data suggest that there are weak functional responses that overlap between B7.2^{tg} and B35.1^{tg} to the VACV-derived peptide panels. The weak functional responses from the splenocytes isolated from B35.1^{tg} mice to the peptides eluted from the B35.1 molecules suggests potential issues in the construct of the transgenic mouse strain. The VACV-derived peptides used were eluted from HLA molecules that were constructed as a non-covalently linked dimer consisting of the heavy and light chain. By contrast, the B35.1^{tg} mice were generated using a construct that encodes the heavy and light chains as a covalently linked monochain (Boucherma et al., 2013). This difference in chain construction could affect peptide binding affinity and presentation. Additionally, the monochain construction could affect the dynamics of the light and heavy chain resulting in a more stable but empty HLA class I molecule. To advance the investigation into the utility of the HLA class I supertype hypothesis in vaccine development it would be necessary to generate transgenic mice with HLA constructs designed in the same manner as the ones used to elute the naturally processed and presented VACV-derived peptides.


a
SFU
B35.1tg; H-2b
B7.2tg; H-2b
media
B7.2 panel


b
SFU
B35.1tg; H-2b
B7.2tg; H-2b
media
B35.1 panel

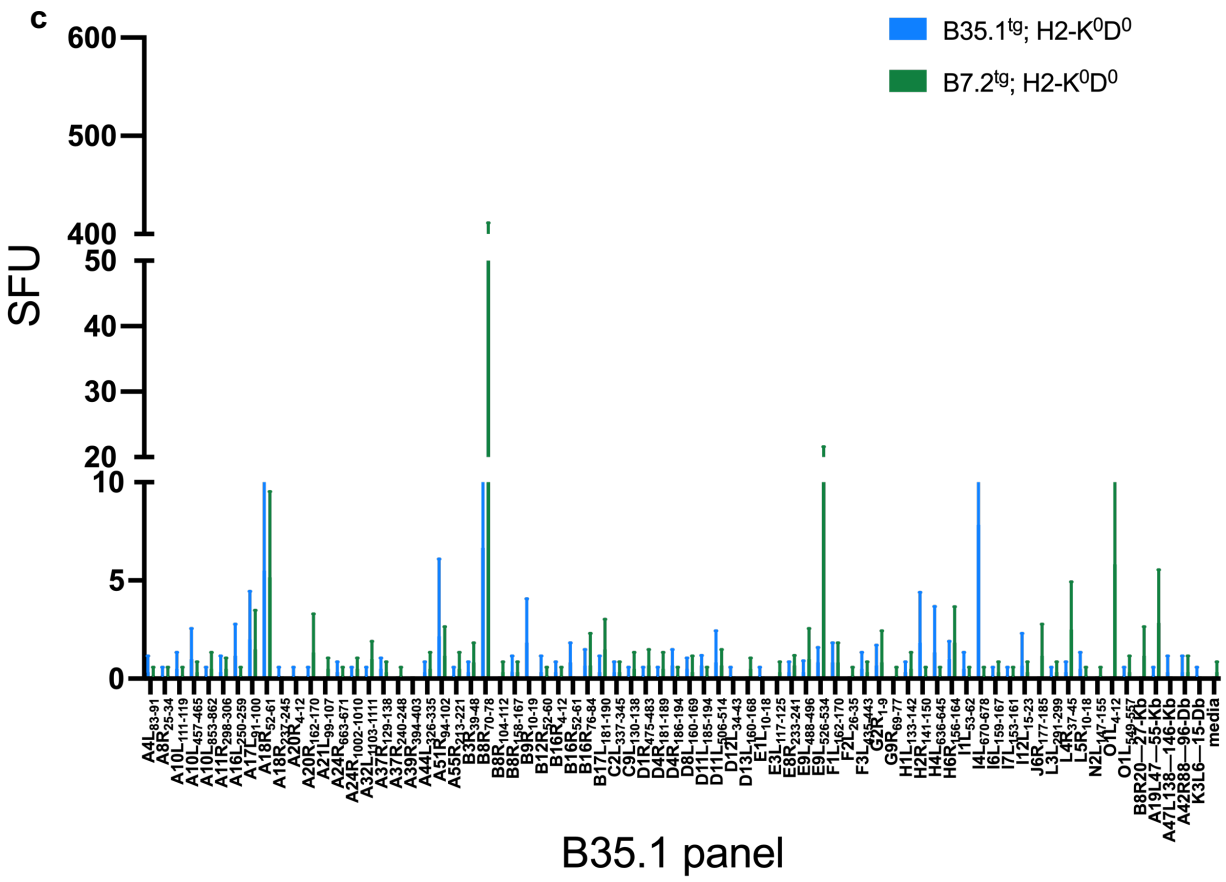


Figure 23. Identification of overlapping peptide determinants. Mice were infected with a sublethal dose of VACV, and at seven days post inoculation, the spleens were harvested and processed for IFNγ ELISpot analysis. Splenocytes from acutely inoculated B7.2^{tg} and B35.1^{tg} mice were stimulated with the B7.2 peptide panel in an IFNγ ELISpot assay (a). Splenocytes from acutely inoculated B7.2^{tg} and B35.1^{tg} mice were stimulated with the B35.1 peptide panel in an IFNγ ELISpot assay (b). Splenocytes from acutely inoculated B7.2^{tg} and B35.1^{tg} mice in the K^{b}D^{b} null background were stimulated with the B35.1 peptide panel in an IFNγ ELISpot assay (c).

DISCUSSION

The practice of vaccination has significantly improved human health and longevity. However, emerging and re-emerging infectious diseases continue to pose major threats to global health and development. The increased prevalence and virulence associated with emerging and re-emerging infections significantly burdens the quality of life for humanity, economical resources, and the infrastructure of developing nations. The ever-increasing movement of people, animals, and goods, coupled with the remarkable adaptability of microorganisms necessitate constant vigilance to ensure countermeasures are available to prevent and control outbreaks of infections. Medical care and therapeutic options have significantly improved patient outcomes. However, it is of critical importance to develop preventative measures to ensure preparedness for the next outbreak.

Current vaccine strategies successfully allow the immune system to generate neutralizing antibodies that facilitate the control and clearance of invading pathogens (Sarkander et al., 2016). However, CD8+ T_{RM} have been demonstrated to play a critical role in the protective immune response against many viral and intracellular bacterial pathogens. Furthermore, tissue-resident memory CD8+ T cells localized to mucosal sites rapidly respond to invading pathogens before the recruitment of other immune effector cells (Ariotti et al., 2012; 2014; Bivas-benita et al., 2013; Gilchuk et al., 2016; Mueller et al., 2016; Schenkel et al., 2014). Thus, initiatives to combat the immense threat of emerging and re-emerging infectious disease would benefit from insight into vaccine development that generates a long lasting protective cellular response.

Pathogen free subunit-based, T cell targeted vaccines have the potential of increasing safety and efficacy. However, the extensive diversity of the HLA class I gene

within the population creates a major barrier. Work from Sette's group and Buus' group demonstrates that many of the HLA class I molecules can be grouped into 12 supertypes based on the physicochemical properties of the B and F binding pocket (Sette and Sidney, 1999; 2008), which dictates the peptide binding motif of HLA class I molecules. This suggests that HLA class I molecules belonging to the same supertype are able to bind overlapping peptide ligands. The work outlined here begins a rigorous investigation into the utility of the HLA class I supertype hypothesis in expanding population coverage of T cell targeted vaccine design.

Here, the HLA-B7.2 and -B35.1 molecules belonging to the B7 supertype family are investigated in an immunization study against lethal VACV infections of the lower respiratory tract. Immunization of $B7.2^{tg}$ and $B35.1^{tg}$ mice with recombinant protein expressing VACV epitopes resulted in variable protection against a lethal VACV challenge. In a peptide screen to identify overlapping peptide specificities that elicit a functional response in both transgenic mouse models, the $B35.1^{tg}$ displayed a weak response to their cognate peptide and to peptides within the B7.2 panel. However, numerous peptides within both peptide panels elicited weak functional responses in both the $B7.2^{tg}$ and $B35.1^{tg}$ mice, suggesting further investigation is necessary to identify protective overlapping peptide specificities. The weak IFNγ response to peptides within the B35.1 panel by the $B35.1^{tg}$ mice could be due to the construction and expression of the HLA molecules. The B35.1 peptide panel used in this study was eluted from B35.1 molecules constructed as a non-covalently linked dimer, whereas the $B35.1^{tg}$ mice were generated using a construct that encodes a covalently linked monochain. In order to properly investigate the supertype hypothesis in a vaccination study, it would be

necessary to remake the transgenic mice. The variable protection demonstrated by the immunization experiment is consistent with the resulting IFNγ response elicited from the B7.2^{tg} and B35.1^{tg} mice. The reduced efficacy of rOVA immunization in B35.1^{tg} mice could be due to this difference in immune response generated against the VACV-derived epitopes or a difference in the LD50 between the two transgenic mouse models. As this project is still in its infancy, no claims can be made based on the current data. Nevertheless, the data presented thus far supports the need for rigorous investigation into the HLA class I supertype hypothesis for vaccine development (Fig. 24).

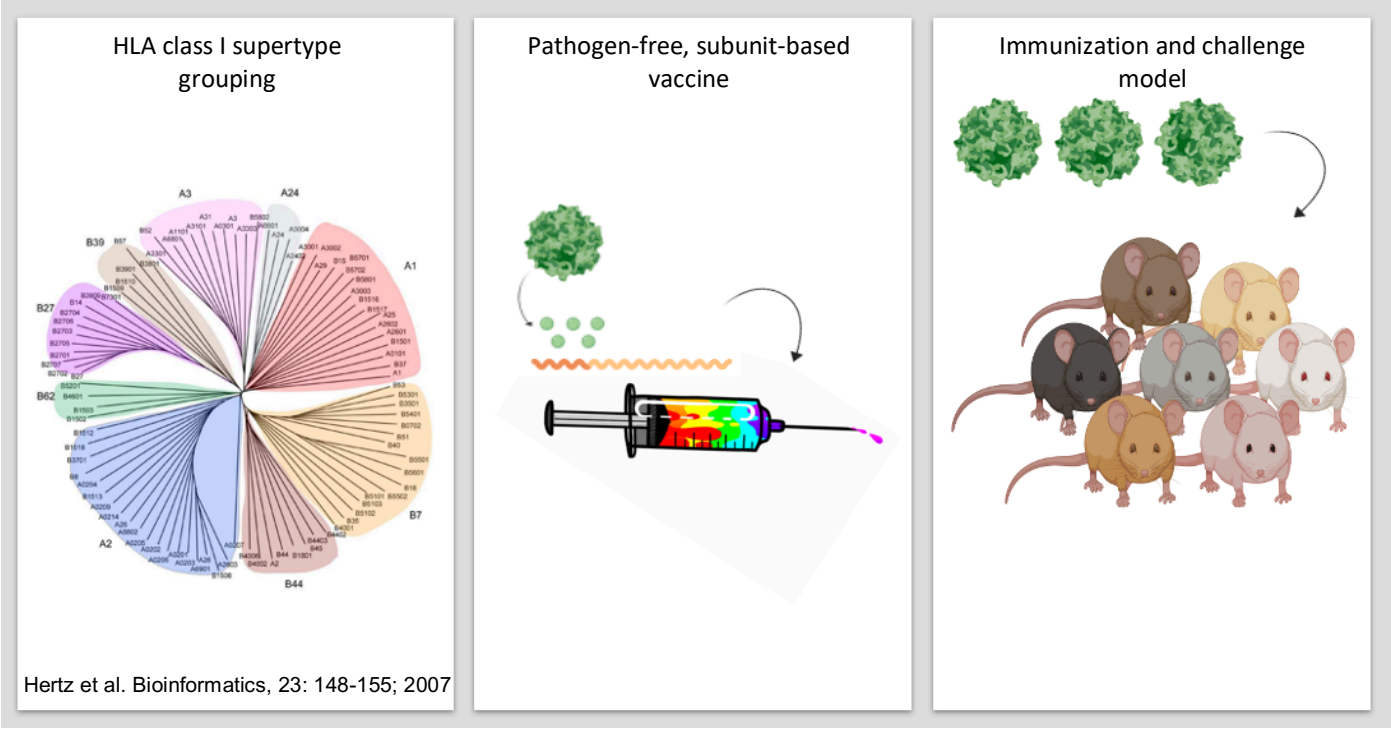


Figure 24. Schematic of the central hypothesis: can the HLA class I supertype hypothesis be utilized in a pathogen-free, subunit-based, T cell targeted vaccine that has effective population coverage? Representative HLA class I molecules will be identified from the supertype grouping. Their VACV processed and presented peptide repertoire will be elucidated to construct the recombinant protein expressing VACV epitope. Transgenic mice expressing the representative HLA class I molecules will be used in an immunization/challenge study to determine vaccine efficacy.

VI. Discussion

For centuries, infectious diseases have ranked among the greatest challenges to human survival and progress. Advances in modern medicine and patient care, particularly the development of vaccines and antimicrobial agents, have greatly shifted the odds against infectious pathogens. However, emerging and re-emerging infectious diseases continue to be a constant threat that requires vigilance to ensure outbreaks and pandemics do not become a recurring theme. Among the greatest contributors that threaten this fragile balance is the alarming rate at which disease causing microbes are acquiring antimicrobial resistance. Drug-resistant infections significantly increase patient morbidity and mortality, and in the U.S. alone, antimicrobial-resistant infections cost the healthcare system approximately $20 billion annually, and over a quarter of this cost is attributed to healthcare-associated infections. The nosocomial pathogen, *A. baumannii,* is currently categorized as an urgent threat to human health as isolates of this pathogen are resistant to all available antimicrobial drugs. This highlights a critical need for greater understanding into the innate and adaptive immune system in order to identify risk factors associated with infection susceptibility and expand preventative care and therapeutic options. The work presented here fills critical gaps in knowledge by providing a mechanistic framework associating genetic variants of host *Cpt1a* with increased bacterial infections, describes alternative therapeutic applications for aminoglycoside antibiotics, and initiates a rigorous investigation into the utility of the HLA class I Supertype hypothesis in T cell targeted vaccine development (Fig. 25).

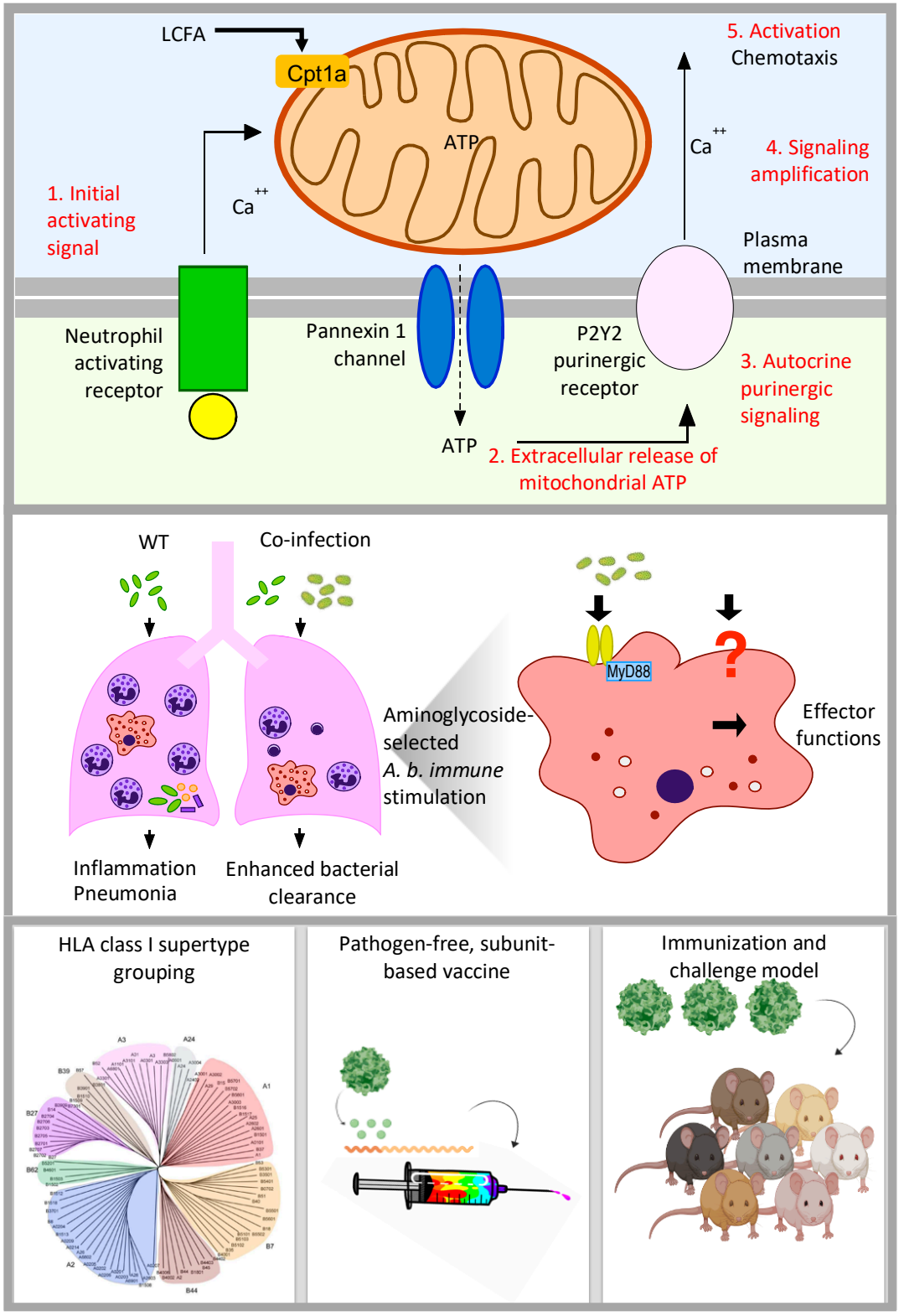


Figure 25. Schematic summarizing projects. Top panel, requirement for FAO in neutrophil activation and effector function. Middle panel, aminoglycoside propagated *A. baumannii* enhances clearance of WT bacteria following co-infection. Bottom panel, testing the HLA class I supertype hypothesis in vaccine design.

HOST GENETIC VARIANTS AND DISEASE SUSCEPTIBILITY

The coordinated efforts of the innate and adaptive immune defenses generally result in the control and clearance of many invading pathogens. The innate immune system is equipped with a vast array of receptors that allow recognition and response to foreign invaders, whereas the adaptive immune system provides memory of past encounters that results in rapid and enhanced defenses to a repeat exposure. However, expression of genetic variants could lead to inborn errors of infection control due to disruption of critical defense mechanisms. Investigation into the association of *Cpt1a* allelic variants and increased susceptibility to infection resulted in the discovery of an unrecognized metabolic profile required for neutrophil chemotaxis.

Multiple studies have demonstrated an association between homozygosity for the arctic variant of the *Cpt1a* gene and increased bacterial infection susceptibility. The work presented here further supports the association of *Cpt1a* allelic variants and increased susceptibility to infection and defines the role of Cpt1a in host immune defenses. In a murine model of bacterial pneumonia, a pharmacological inhibitor of Cpt1a increased infection susceptibility and severity. *In vivo* results suggest that the poor infection outcomes were due to defects in neutrophil trafficking from the bone marrow to the site of infection. *In-vitro* analysis demonstrates a requirement for Cpt1a in neutrophil chemotaxis. Following stimulation with an activating signal, ATP produced by the mitochondria is released into the extracellular space and signals back through the purinergic receptors to amplify the initial signal resulting in neutrophil function. Taken together, the data suggest deficient Cpt1a function disrupts neutrophil activation and mobilization to sites of infection for infection control and clearance.

Caveats to the study include the fact that human observational studies provide correlations and not causations and the use of a global small molecule inhibitor with known off-target effects may disrupt other mechanisms that exacerbate infection outcomes. Therefore, while *in vitro* data demonstrate defective chemotaxis following treatment with a Cpt1a inhibitor, it could not be ascertained that this mechanism is the cause of *in vivo* infection outcomes. To identify the requirement for Cpt1a *in vivo*, it would be necessary to use a genetic model and address any compensatory mechanisms resolving Cpt1a deficiency. However, investigation into the association between *Cpt1a* variants and infection susceptibility resulted in the discovery that neutrophils require FAO for activation and function. Expanding upon the mechanistic framework presented here, further insight into the regulatory role of the mitochondria in other neutrophil-mediated bactericidal functions would undoubtedly inform therapeutic options and advances. Beyond the control of infectious diseases, an increased understanding of mechanisms regulating neutrophil function would advance therapeutic strategies for modulating the severity of neutrophilic disorders. Furthermore, increased interest in PheWAS will provide insight into mechanisms affecting host immune defenses, allowing for early patient identification and mitigation of diseases.

ALTERNATIVE APPLICATIONS OF CURRENT ANTIMICROBIAL THERAPEUTICS

Advances in antimicrobial discovery have greatly improved treatment options and patient outcomes following infection. However, the widespread use of antibiotics has ushered us into an era defined by extreme drug resistant infections. The alarming rise in antimicrobial resistance in disease causing pathogens is regarded as a severe threat to

human health and development. Clinical isolates of the nosocomial pathogen, *A. baumannii,* have developed resistance to all available antimicrobial drugs, highlighting an urgent need for novel therapeutics against infections.

Investigation into *A. baumannii* pathogenesis led to the discovery that aminoglycoside propagated strains enhanced the clearance of Gram-negative bacterial infections in a model of murine bacterial pneumonia. The enhanced clearance of bacterial pneumonia caused by Gram-negative pathogens is a coordinated effort between aminoglycoside antibiotic propagated *A. baumannii* and host defenses within the lungs. However, the aminoglycoside antibiotic propagated strain does not need to be viable to enable bacterial clearance, suggesting modification to the outer membrane resulting in altered interactions with the host. Together, these findings suggest that current therapeutics can be re-explored in alternative applications that may render them an effective treatment.

The development of novel therapeutics continues to lag behind the rise of drug resistant infections. The rate at which many pathogens acquire antimicrobial resistance ensures that novel discoveries will lose some degree of efficacy overtime. Insight into therapeutics that enhance immune defenses is promising. However, this can lead to exaggerated responses that are detrimental. The work presented here demonstrates the feasibility of alternative approaches to current treatment options for bacterial pneumonia. In order to explore the clinical utility of this system, it would be necessary to determine the host immune factors involved, the aminoglycoside-mediated alteration to the outer membrane of *A. baumannii*, and the applicability of localized antibiotic delivery in the enhanced bacterial clearance from the lungs. Further insight into these mechanisms has

the potential for the development of a bacteria-free delivery system of immune enhancing and therapeutic agents that mimics the enhanced bacterial clearance induced by aminoglycoside-propagated *A. baumannii.*

SHIFTING THE FOCUS TO DISEASE PREVENTION

Advances in medical care have significantly improved disease treatment and patient outcomes. However, innovations in preventative therapeutics would greatly decrease the necessity for extensive treatments and hospital stays, resulting in improved quality of life and decrease the financial burden to the health care system. The threat of antimicrobial resistant infections and potential outbreaks of emerging and re-emerging infection signify a critical need for insights into mechanisms of effective vaccine development.

Current vaccine strategies are developed empirically, despite the wealth of knowledge gained in Microbiology and Immunology since the systematic study of specific 'immunity' to previous infections by Edward Jenner. The effectiveness of a licensed vaccine is gauged by the resulting neutralizing antibody produced. While antibodies effectively facilitate the neutralization and clearance of the invading pathogen, recent studies demonstrated a critical role for CD8+ T_{RM} in the protective immune response against many viral and intracellular bacterial pathogens. Furthermore, non-circulating tissue resident CD8+ T cells rapidly trigger innate and adaptive immune responses upon antigen restimulation thus controlling the spread of the disease prior to the recruitment of circulating and effector memory T cells. Thus, to ensure preparedness for the next emerging or re-emerging infectious diseases and to combat treatment-resistant

infections, insights into mechanisms that result in robust humoral and cellular responses are critical. Devising vaccine strategies that result in non-circulating tissue resident CD8+ T cells at vulnerable sites requires insight into effective population coverage. Therefore, it is necessary to rigorously test the utility of the HLA class I supertype hypothesis in vaccine development.

The preliminary data presented here demonstrate the potential of designing recombinant proteins with overlapping peptide specificities to immunize and protect transgenic mice expressing different HLA class I molecules to a lethal VACV challenge. The current data were generated using a recombinant protein expressing the VACV epitope, $B8R_{70\text{-}78}$, which is processed and presented by the HLA class I molecules B7.2 and B35.1. The immunization strategy resulted in 100% protection in $B7.2^{tg}$ mice and approximately 50% protection in $B35.1^{tg}$ mice. To expand upon this work and rigorously test the utility of the HLA class I supertype hypothesis in designing effective vaccines, it would be necessary to identify limitations within the experiments that resulted in the efficacy difference observed between the two transgenic models. In order to test the design of a vaccine with population-wide coverage, the mouse model will need to be expanded and incorporate HLA class I molecules from the other supertypes. Additionally, the use of the NKT cell activating ligand, αGC, as an adjuvant for vaccine formulation poises this project to expand into investigating the utility of NKT cell help for primary and secondary activation of CD8+ T cells in the absence of CD4+ T cell help. Thus, progressing the initial work presented here would undoubtedly inform mechanisms of rational vaccine design.

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