

## Curriculum Vitae

Name: Adrienne Renée Kambouris

Contact Information: Adrienne.kambouris@gmail.com

Degree and Date to be Conferred: Ph.D., December 2023

### **Education**

#### **University of Maryland School of Medicine**

Baltimore, Maryland

Aug 2016 – Present

Medical Scientist Training Program

- Completed the first two years of medical training and first medical licensing board exam at the University of Maryland School of Medicine
- Completed a PhD in the Cross Laboratory, Center for Vaccine Development, University of Maryland School of Medicine
  - Area of Study: The innate immune response to burns and its effect on infection susceptibility
  - Supported by NIH T32 AI095190

#### **Morgan State University**

Baltimore, Maryland

Sept 2020 – Dec 2022

Master of Arts

Major: Business Administration

#### **Augusta University (formerly Georgia Regents University)**

Augusta, GA

May 2013 – May 2016

Bachelor of Science

Major: Cell and Molecular Biology

Bachelor of Science

Major: Chemistry

## **Cochise College**

Sierra Vista, AZ

Sep 2002 – Dec 2011

Associates of Applied Science in Intelligence Operations Studies

## **Research**

**University of Maryland School of Medicine**

Jan 2021 – Present

Mentor: Alan Cross, MD

*The host innate response to non-lethal burns and the effects on post-burn infections*

For my dissertation project, I investigated the immune response to burns and subsequent susceptibility to *Pseudomonas aeruginosa* infection. Using NanoString™, the change in expression of specific host and *P. aeruginosa* genes concurrently in various tissues, including the wound site, was observed. The central hypothesis to be tested was that neutrophil dysfunction following the burn has a critical role in the observed decreased *P. aeruginosa* LD<sub>50</sub> post-burn. Cytochrome C reduction and phagocytosis assays were used to measure neutrophil function.

**University of Maryland School of Medicine**

Jun 2018 – Dec 2020

Mentor: Patrik Bavoil, PhD

*How SinC subverts host cell pathways to enhance Chlamydia pathogenesis*

This project involved investigating the novel *Chlamydia* Type III secreted protein SinC and its interactions with the host cell. I studied the acquisition of host pools of cholesterol and its effects on the *Chlamydia* inclusion membrane, the interaction of SinC with host protein MAVS, and the production of IFN  $\beta$ .

**Augusta University**

Jun 2014 – May 2016

Mentors: Jessica Reichmuth, PhD; Christopher Bates, PhD

*Genetic determination of soil microbial populations in the Satilla River Estuary using 16S rRNA sequencing*

The Satilla River Estuary has been modified over several decades to accommodate logging activities. Cuts were made in the land between tributaries to ease the transport of logs; however, they were not maintained. This resulted in altered water flow and nutrient levels. To determine the effects of cuts on the system, microbial populations were used as a proxy to assess site similarity and diversity. The populations were quantified using 16S rRNA sequencing and analyzed using Jaccard Similarity and Shannon-Weaver Diversity Indices.

**Augusta University**

Dec 2015 – May 2017

Mentor: Thomas Crute, PhD

*Determining the concentration of nicotine in the mouths of smokers using standard addition*

To develop an absorbent gum or lozenge targeted at smokers, the concentration of nicotine in the mouths of smokers must be determined. The extracted concentration was below the limit of detection of the gas chromatographer mass spectrometer (GCMS). To obtain an accurate concentration, the standard addition method was used.

### **Publications**

1. Abdi, J., Bhatt, M., Gettas, M., Harriot, A., **Kambouris, A.**, & Marks, N. (2020). *President's White Paper Project: Implementing Core Values*. Health Sciences and Human Services Library. <http://hdl.handle.net/10713/14711>

2. Brammer, J., Choi, M., Baliban, S. M., **Kambouris, A. R.**, Fiskum, G., Chao, W., Lopez, K., Miller, C., Al-Abed, Y., Vogel, S. N., Simon, R., & Cross, A. S. (2021). A nonlethal murine flame burn model leads to a transient reduction in host defenses and enhanced susceptibility to lethal *Pseudomonas aeruginosa* infection. *Infection and Immunity*, *89*(10). <https://doi.org/10.1128/iai.00091-21>
3. Brammer, J., Wolf, G., Baliban, S. M., Allen, J. C., Choi, M., **Kambouris, A. R.**, Simon, R., Fiskum, G., Chao, W., Lopez, K., Miller, C., Singh, N. J., & Cross, A. S. (2021). A nonlethal full-thickness flame burn produces a seroma beneath the forming eschar, thereby promoting *Pseudomonas aeruginosa* sepsis in mice. *Journal of Burn Care & Research*. <https://doi.org/10.1093/jbcr/irab195>
4. Landford, W. N., Stewart, T., Abousy, M., Ngaage, L. M., **Kambouris, A.**, & Slezak, S. (2020). A roadmap for Navigating Occupational Exposures for surgeons: A special consideration for the pregnant surgeon. *Plastic & Reconstructive Surgery*, *147*(2), 513–523. <https://doi.org/10.1097/prs.0000000000007581>
5. **Kambouris A.R.**, Brammer, J.A., Roussey, H., Chen, C., Cross, A. S. (2023). Combination of Burn Wound Injury and *Pseudomonas* Infection Elicits Unique Gene Expression that Enhances Bacterial Pathogenicity. *mBio*. <https://doi.org/10.1128/mbio.02454-23>
6. Ngaage, L. M., Rose, J., Pace, L., **Kambouris, A. R.**, Rada, E. M., Kligman, M. D., & Rasko, Y. M. (2019). A review of National Insurance coverage of post-bariatric Upper Body Lift. *Aesthetic Plastic Surgery*, *43*(5), 1250–1256. <https://doi.org/10.1007/s00266-019-01420-7>

7. Ngaage, L. M., Wasicek, P. J., Puthumana, J., **Kambouris, A. R.**, Tannouri, S., Rada, E. M., Kligman, M. D., & Rasko, Y. M. (2020). A cross-sectional analysis of insurance coverage of extremity contouring after massive weight loss. *Annals of Plastic Surgery*, 84(3), 253–256. <https://doi.org/10.1097/sap.0000000000002150>
8. Shetty, P. N., Araoye, E., Yesantharao, P., **Kambouris, A. R.**, Hultman, C. S., & Aliu, O. (2020). Prevalence of autoimmune disease in patients with hidradenitis suppurativa seen in ambulatory settings from 2008 to 2017. *Plastic and Reconstructive Surgery - Global Open*, 8(9S), 82–83. <https://doi.org/10.1097/01.gox.0000720720.10051.17>
9. Verhoeve, V. I., Brammer, J. A., Driscoll, T. P., **Kambouris, A. R.**, Rasko, D. A., Cross, A. S., & Gillespie, J. J. (2022). Genome sequencing of *Pseudomonas aeruginosa* strain M2 illuminates traits of an opportunistic pathogen of Burn Wounds. *G3 Genes|Genomes|Genetics*. <https://doi.org/10.1093/g3journal/jkac073>

### **Presentations**

07/2023 MD-PhD National Student Conference – Poster Presentation

04/2020 Graduate Student Research Symposium – Poster Presentation

03/2019 Chlamydia Basic Research Society Biannual Meeting – Poster Presentation

02/2019 Mid-Atlantic Microbial Pathogenesis Meeting – Poster Presentation

03/2016 Southeastern Estuary Research Society – Oral Presentation

11/2015 Coastal Estuarine Research Federation Biennial Conference – Poster Presentation

## **Work Experience**

### **University of Maryland Interprofessional Student Learning and Service Initiatives**

#### *Diversity Fellow*

Baltimore, MD

Sep 2016 – Feb 2017

Devised, advertised, and facilitated events related to diversity, equity, and inclusion for UMB.

### **Augusta University Department of Biological Sciences**

#### *BIOL 1107/1108 Tutor; CHEM 1101/1102 TA*

Augusta, GA

Aug 2015 – May 2016

Tutored students, prepared and facilitated lab sessions, and graded assignments for undergraduate biology and chemistry students.

### **Georgia Regents University Military and Veterans Services**

#### *Work-Study Program*

Augusta, GA

Jul 2014 – Aug 2015

Assisted veteran students in transitioning from military service into their educational careers.

### **United States Army**

Jun 2002 – Feb 2013

#### *Battalion SI NCOIC*

Fort Gordon, GA

May 2012 – Feb 2013

Tracked and processed all personnel, administrative actions, financial transactions, medical and dental readiness, legal processing, evaluations, awards, and official mail for a battalion of over 550 soldiers while operating outside of my MOS (formal, military trained job).

#### *Intelligence & Electronic Warfare Shop Foreman*

Fort Gordon, GA

Sep 2010 – May 2012

Supervised the maintenance shop for a consolidated battalion maintenance facility in an echelon above corps theatre intelligence operations battalion and provided technical guidance and supervision to all lower grade technicians, conducted all training and quality checks.

*Rear Detachment Operation NCO*

Fort Carson, CO

May 2009 – Sep 2010

Managed the operations, tracking, and training of 99 Rear Detachment soldiers; scheduled and tracked the training of all newly assigned soldiers and ensured successful completion and deployment. Added to the unit's deployed strength by 18%.

*Company Operations and Training NCO*

Fort Carson, CO

Jan 2008 – May 2009

Scheduled and tracked all movement of military personnel in an 88 soldier unit; ensured company combat training requirements were scheduled and tracked.

*Tactical Unmanned Aerial Vehicle (TUAV) Unit Trainer*

Camp Taji, Iraq

Oct 2006 – Dec 2007

Fort Carson, CO

Sep 2005 – Sep 2006

Camp Al Taqqadum, Iraq

Aug 2004 – Aug 2005

Camp Essayons, Korea

Jan 2004 – Jul 2004

Accountable for the maintenance and flight readiness of four Unmanned Aerial Vehicles and responsible for over \$1 million in equipment. Selected and trained all Crew Chiefs, Technical Inspectors, and Quality Assurance/Quality Control (QA/QC) NCOs.

## **Volunteer Experience**

### **Saint John Christian Community Church**

Baltimore, MD

August 2016 – Present

- Serve as one of the four members of the Executive Team, implementing the mission and vision of the pastor for St. John Christian Community Church. I am directly responsible for the administration and media aspects of ministry. I produce all visual, audio, and motion media for SJCCC and manage all social media accounts. I also manage the church calendar and all communications with the congregation. Finally, I represent the church in all external communications. I've been in this role since 2019.
- Served as assistant director of the youth department. Planned and executed all programming for the youth department, including the annual Back to School Block Party.
- Sing on the Harvest Mass Choir and participate in the dance ministry.

### **UMB CURE**

Baltimore, MD

Aug 2016 – Aug 2021

- Provide mentorship to inner-city, at-risk youth by exposing them to STEM resources, tutoring, and conducting college preparatory events.

### **La Clinica Latina**

Augusta, GA

Apr 2015 – May 2016

- Transcribe medical records using the Kareo program in a free primary care clinic for the uninsured Hispanic community of Augusta and surrounding areas.

## **Equality Clinic**

Augusta, GA

Apr 2015 – May 2016

- Transcribe medical records using the Kareo program in a free primary care clinic for the uninsured LGTBQ community of Augusta and surrounding areas.

## **New Life Church**

Augusta, GA

Sep 2013 – May 2016

- Lead Wednesday and Sunday services in the Children's Ministry, providing spiritual guidance and education to youth; participate in New Beginnings, a program designed to provide clothes, food, and furniture to Augusta's impoverished communities.

## **Media**

### **Podcasts**

The Premed Years, Episode 313: Army Vet, Mom of 3, Wanted to Quit During Her MD/PhD Journey – 21 Nov 2018

Fit to Lead, Episode 010: *Army SSG to Medical Student: Leadership Lessons from Adrienne Kambouris* – 22 Jun 2020

Surviving Medicine, Episode 90: Staff Sergeant to MD/PhD with Adrienne Kambouris MSV – 15 Jun 2020

### **Interviews**

Team 226 Huddle – 28 Sep 2023

VanguardSTEM Unbound Mentoring – April 25, 2023

PreMedCC - From US Army to Community College, Motherhood, and MD/PhD Student – 21 Jan 2022

365 Days. 365 Women – 13 Apr 2023

## **Panels**

MappdCon - Is MD/PhD Right for Me? – 08 Oct 2023

National Premed Day- Is a Dual Degree Program Right for You? – 28 May 2020

## **Academic Achievements**

Selected as a Top Poster at the 38<sup>th</sup> Annual MD/PhD Student Conference in 2023

Selected to receive a Diversity Travel Award for the 38<sup>th</sup> Annual MD/PhD Student Conference in 2023.

Selected as a Sentinel of Freedom in 2021.

Selected to receive funding under the NIH T32 AI095190 grant entitled “Signaling Pathways in Innate Immunity” in 2021.

Selected as UMB PNC (Mercantile) Scholarship winner in 2020.

Selected as 2019-2020 UMB President’s Fellow.

Mid Atlantic Microbial Pathogenesis Meeting Travel Award Recipient.

Selected as Meyerhoff Fellow at UMBC in 2018.

Received the Charles-Stewart Freshman Scholarship in 2017, awarded to the outstanding freshman member of University of Maryland School of Medicine’s SNMA Chapter.

Selected as an Army Women’s Legacy Foundation Scholarship recipient in 2017.

Won Outstanding Senior for the Department of Biological Sciences for the 2015-2016 school year.

Represented Augusta University at the Southeastern Estuarine Research Society Spring Meeting in March 2016; won best oral undergraduate presentation award and a travel award.

Member of Beta Beta Beta, the National Biological Honor Society.

Represented GRU as a highlighted veteran student during a nationwide broadcast on CBS Sports.

Member of the sixth class of Tillman Scholars.

Augusta University Dean's List Spring 2014, Summer 2014, Spring 2015, Fall 2015, Spring 2016.

Wrote a successful grant proposal to initiate a scholarship program for veteran students at Georgia Regents University.

### **Military Achievements**

Awarded Army Commendation Medal (x5), Army Commendation Medal (x4), Meritorious Unit Commendation (x2), USN Unit Commendation, Army Superior Unit Award, Army Good Conduct Medal (x3), National Defense Service Medal, Global War on Terrorism Expeditionary Medal, Global War on Terrorism Service Medal, Korean Defense Service Medal, Iraq Campaign Medal (3 Campaign Stars), NCO Professional Development Medal (x3), Overseas Service Ribbon (x3).

Was selected for promotion to Sergeant First Class in 2012 ahead of my peers.

Sponsored, mentored, and developed a soldier through multiple levels of competition to represent INSCOM and the National Capital Region in the 2012 Army Best Warrior Competition.

Earned the Iron NCO award at the Military Intelligence Senior Leader Course in Fort Huachuca, AZ through physical competitions and GPA.

Selected to represent the Army through photos, newspaper articles, and video advertisements in 2008 during the Army Strong recruiting campaign.

Completed over 5,000 accident-free flight hours during OIF 06-08.

Graduated on the Commandant's List from Warrior Leader's Course, Advance Leader Course Common Core, and the Senior Leader Course.

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## **Abstract**

Altered Gene Expression Profiles and Immune Responses in a Murine Model of a Non-lethal Flame Burn with *Pseudomonas aeruginosa* Infection

Adrienne R. Kambouris, Doctor of Philosophy, 2023

Dissertation Directed By: Alan S. Cross, Professor of Medicine, Center for Vaccine Development and Global Health, University of Maryland School of Medicine

Humanity has lived with fires for millennia, but combat, domestic use, and recent wildfires have increased the risk of burn injuries. Worldwide, over 100,000 deaths occur each year due to burns. If these patients survive the burn wound itself, the most common causes of death are multiorgan failure and sepsis, often caused by infection by *Pseudomonas aeruginosa* (PA). Utilizing a 10% total body surface area (TBSA) non-lethal flame burn model in mice, a superimposed infection of PA caused 100% mortality within 36 hours post-burn. This effect was transient, as infection 72 hours post-burn resulted in survival. The hypothesis was that this mortality could be linked to changes in gene expression that altered host-pathogen interaction. NanoString™, a system that allowed us to develop a custom panel of probes, was utilized to measure *Mus musculus* and PA gene transcripts simultaneously in each sample. Sampling from the blood, spleen, liver, and skin, gene expression in the burn and infection condition (B/I) was significantly different in each tissue when compared to mice that were burned alone, infected alone, and neither burned nor infected (Sham). The expression of the anti-inflammatory gene, *Il10* is significantly increased over time in the spleen; administering anti-IL-10 antibodies delayed mortality by one day. While *Arg1* and *Nos2* gene expression were not significantly altered, administering arginine concurrently with PA restored survival in our mouse model, likely

due to an inhibition of both PA motility and growth. We also hypothesized that burn-induced neutrophil dysfunction allowed for PA proliferation. Neutrophils isolated from the seroma of burned mice had a decreased ability to produce antibacterial reactive oxygen species (ROS) compared to neutrophils in the circulation of the same mice. Surprisingly, naïve neutrophils in the circulation of burned mice had a decreased ability to kill PA, possibly due to their premature ROS production induced by a burn-generated DAMP, HMGB1, present in the serum of burned but not Sham mice. In conclusion, a non-lethal burn injury is sufficient to induce multi-faceted changes in the murine immune system that results in an increased susceptibility to lethal PA superinfection.

Altered Gene Expression Profiles and Immune Responses in a Murine Model of a Non-lethal Flame Burn with *Pseudomonas aeruginosa* Infection

by  
Adrienne Renée Kambouris

Dissertation submitted to the Faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2023

## **Preface**

Before diving into my work, I want to talk about impossible things. When I decided to pursue an MD/PhD, it was both implied and directly said that it would be impossible. I was raised by a single mother in inner-city Baltimore, a latchkey kid since I was eight. I served in the United States Army for over 10 years with 27 months deployed in Iraq. I started undergrad as a mom of two with one on the way. I'm raising three children, currently aged 14, 12, and 10, with my husband of 15 years, Steven. I come from a family that has been in America for centuries, but I am still doing something for the first time. I am the impossible thing.

Your background and life circumstances should not limit your dreams and goals. If I can serve as motivation for anyone to take on big things, I'm happy to. We certainly shouldn't be telling people that they're not qualified or that they won't complete a program because their life looks a bit different. We should be encouraging these students and increasing the diversity of thought.

So, if you are a parent, a spouse, a veteran, come from a lower socioeconomic status, or whatever non-traditional box you may fit in, I challenge you to do impossible things. People who can't imagine themselves doing what you are will find it impossible. Don't let them stop you.

To Victoria, Blair, and Orion, my reasons for everything.

## **Acknowledgments**

In 2020, I was faced with a seemingly insurmountable obstacle: starting my thesis over. In my search for a new lab home, I reached out to Dr. Cross. Not only was he willing to accept me as a student, but he has also always believed in my success, even when I didn't. I would never have reached this goal without his support and encouragement. Thank you, Dr. Cross, for seeing me through this difficult process, even when all odds were set for me to fail. For all the training, knowledge, technical skills, answering all of my questions, and tolerating me breaking things, I have to thank Ethel, Jin, Jared, Chiguang, Kun, Sang, Zhiyong, Surekha, and Michael in the Cross Lab. I eventually learned where most of the things are stored. To my committee, Dr. Bruno, Dr. Carbonetti, Dr. Ernst, Dr. Hultman, Dr. Vogel, and Dr. Webb: thank you for keeping me straight, giving me hard truths, and holding me accountable. While I definitely tested the limits of programmatic support, thank you to the MMI, GPILS, and MSTP programs for not kicking me out or allowing me to quit. To Dr. Alison Scott, thank you for seeing my potential even when I didn't see it myself and encouraging me to advocate for myself. To Dean (my forever dean) E. Albert Reece, thank you for challenging me professionally and encouraging me to think big, aim high, stay focused, and be relentless. To Dr. Jessica Reichmuth, you put me on a boat and made me a scientist. Thank you for giving me the courage to apply to PhD programs.

When I entered this program, one of the first things I did was make an appointment at the Student Counseling Center. I have been in the care of Dr. Jenna Silverman since my first month at the University of Maryland. Dr. Silverman has been there for me at every turn, affording me the space to process my experiences, supporting change and growth,

and being an accurate mirror of myself and my life. I cannot express how valuable Dr. Silverman and her team in the SCC have been to me on this journey. Thank you for always being there. Thank you to the campus Parking and Transportation Service and Police and Public Safety, specifically Aunder Russell and Averil Coleman. The constant concern for my safety during late nights, keeping track of my progress, and motivating me to keep pushing was greatly appreciated. I would also like to thank the University of Maryland Genomics Core Facility for processing our NanoString™ samples and the Program of Veterinary Resources for the care of our mice.

I am not successful in a vacuum. I have so many people around me who helped me, motivated me, and pushed me to win. To my MSTP cohort Olivia, Louis, Gideon, Sikorski, and my day one Trevor, you all understand more than others what this took, and you made sure to prop me up as much as you could. Whether studying for quals, grabbing lunch, or sending texts of encouragement, I appreciated every moment. I'm not one to make friends easily, and some people just jump into your life and don't let go. To Jasmine and Grant, you guys are stuck with me for life.

I have wanted to be a physician since I was eight. The St. John Christian Community Church family has supported that endeavor for as long as I can remember. I served weekly in my church throughout this project, through COVID, and beyond, and the congregation of SJCCC has kept me uplifted in prayer throughout. Thank you for your continued support.

While I may be the first to earn a doctorate in my family, I know that I will not be the last. To our matriarch Alberta Johnson, to my aunts and uncles Renée, Adrienne, Charles, and Tommie, to my cousins Tommie, Chris, Charles, and Rachel, and their

families, I know that we will continue to reach new heights together. Thank you to my mother-in-law Susan for always making connections for me and supporting us throughout this work. For my father-in-law, Wayne, I'm sorry I couldn't research sickle cell like you wanted but thank you for loving me like a daughter and taking me to my interview for this program. To the people who know me best, who helped form me into the person that I am today, my mother Sharen and my brother Dorian, this is a long way from Federal Street, right? Despite everything, we're changing the trajectory of this family one day at a time.

To my circle: Monique, Jeremy, Shareese, Rashan, Romaine, and Tres thank you for constantly showing up, listening to me, taking care of me, celebrating with me, plotting and scheming with me, and laughing with me. My life is better because you all are in it.

To the Kambourii Clan, my rugrats, my loves Victoria, Blair, and Orion, know that I wore myself ragged to shield you from this process as much as possible. Know that I worked to make sure that you still got to enjoy your childhoods, participate in activities, spend time with your friends, and have memorable holidays. I was once told that I couldn't be your mom and be successful in this process. I hope I proved them wrong. I regret none of the times I chose you all over science. To my fur babies Lola, Rampage, Loki, Midnight, and Onyx, thank you for your unconditional love.

I don't even know where to start thanking my partner in life and everything, Steven. This life has definitely taken a turn from when we were teenagers. I would not have been able to do this if you were not in my corner. Back and forth we sway like branches in a storm. On to the next adventure.

**FUNDING:** This project was funded in part by a grant through NIH T32 AI095190 and from the US Air Force Research (FA8650-18-2-6H17). The views expressed are those

of the authors and do not reflect the official guidance or position of the United States Government, the Department of Defense, or the United States Air Force. I was also privately supported by the Sentinels of Freedom Foundation [1].

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## List of Abbreviations

<b>B/I</b>	burned and infected
<b>BMDM</b>	bone marrow derived macrophages
<b>BWE</b>	burn wound exudate
<b>C'</b>	Complement
<b>CF</b>	cystic fibrosis
<b>CFU</b>	colony forming unit
<b>DAMP</b>	danger-associated molecular pattern
<b>DC</b>	dendritic cell
<b>cDC</b>	conventional dendritic cell
<b>pDC</b>	plasmacytoid dendritic cell
<b>fMLP</b>	N-formyl-methionyl-leucyl-phenylalanine
<b>GCMS</b>	gas chromatograph mass spectrometer
<b>HBSS</b>	Hank's balanced salt solution
<b>hpb</b>	hours post burn
<b>IATS</b>	International Antigenic Typing Scheme
<b>ILC</b>	innate lymphoid cells
<b>LPS</b>	lipopolysaccharide
<b>MAC</b>	membrane attack complex
<b>MΦ</b>	macrophage
<b>NET</b>	neutrophil extracellular trap
<b>NK</b>	natural killer cells

<b>PA</b>	<i>Pseudomonas aeruginosa</i>
<b>PBS</b>	phosphate buffered saline
<b>PMA</b>	phorbol myristate acetate
<b>PMN</b>	polymorphonuclear cell/neutrophil
<b>PRR</b>	pattern recognition receptor
<b>ROS</b>	reactive oxygen species
<b>TBSA</b>	total body surface area
<b>TSA</b>	tryptic soy agar
<b>UTI</b>	urinary tract infection

## **Chapter 1. Introduction**

### **Pseudomonas aeruginosa**

#### **Bacteriology**

*Pseudomonas aeruginosa* (PA) is a ubiquitous bacterium with a presence in soil, water, vegetation, and human flora. It is a Gram-negative, motile, facultative aerobe that can grow in low oxygen or oxygen-depleted environments by using nitrogen as a terminal acceptor [2]. It is also able to ferment arginine [2]. PA grows at 37° C, but can survive in temperatures from 4-42° C and can use numerous organic molecules as a carbon source [3, 4]. When PA is grown, it has a characteristic blue-green tint, due to its production of pyocyanin and pyoverdine [5]. It also has a characteristic grape-like odor. PA has a single, polar, flagella which allows for swarming motility, which is an essential virulence factor [6, 7]. PA has a genome size of 5.5-7 Mbp [8].

#### **Clinical Presentations**

*Pseudomonas aeruginosa* causes a wide range of infections. From minor to severe, PA can infect a variety of tissues. While the infection of intact skin is uncommon, PA folliculitis infection is associated with the use of hot tubs, even involving skin covered by clothing [9]. PA is also able to cause malignant otitis media, frequently in diabetic patients [10, 11].

PA is an important opportunistic infection that typically occurs in specific patient populations, such as hospitalized, immunocompromised, or burned patients. It is associated with nosocomial infections such as ventilator-associated pneumonia, catheter-associated UTI, and surgical site infections [12, 13]. PA is also an important pathogen in patients with immunodeficiencies such as patients with HIV, organ transplantation, and burn wounds

[14]. Finally, PA is uniquely associated with people with cystic fibrosis (CF), where it is a common cause of chronic respiratory infection; about 30% of patients are colonized with PA by 6 months of age, and approximately 75% of adult patients are infected. It is a common cause of respiratory failure in CF patients [15-17].

### **Virulence Factors**

*Pseudomonas aeruginosa* has a variety of virulence factors that allow it to evade the immune response and cause disease in the host. As a Gram-negative bacterium, PA contains lipopolysaccharide (LPS) in its outer membrane. LPS is recognized by MD-2/TLR4, which activates an immune response. To evade this, PA can alter its lipid A structure, the innermost segment of LPS [18, 19]. Using proteins such as PagL and LpxO, PA can manipulate the structure of LPS to evade immune surveillance [20]. Also within its membrane are various peptidoglycan-associated proteins from the Opr family. A number of these are porins that confer antibacterial resistance, while others are lipoproteins, such as OprL [21]. OprL aids in cell integrity maintenance and protection against oxidative stress [22].

PA possesses further structural advantages, including unipolar flagella and pili. The flagellum allows for swimming motility, giving PA the ability to move to resource-rich environments but also disseminate within the host [7, 23]. PA has type IV pili, allowing for twitching motility, DNA binding, and surface adhesion [24, 25]. Twitching motility is flagella-independent movement, involving the extension, tethering, and retraction of pili, allowing for PA to move across moist surfaces [24, 26].

One of the most devastating virulence factors, especially in CF patients, is the formation of biofilms. A biofilm is a community of bacteria enmeshed with an extracellular

matrix usually composed of alginate and related exopolysaccharides [27]. In the biofilm, bacteria can communicate, share resources, and grow while evading host immune responses and antibiotics, and surviving in potentially hazardous environments [16, 28, 29]. In CF patients, this allows PA to establish a recurrent and chronic infection that is antibacterial resistant [17].

Finally, *Pseudomonas aeruginosa* can directly harm its host. Using a type III secretion system, PA can inject exotoxins directly into host cells [30, 31]. The Exo family of effector proteins have functions that directly inhibit host cell function or cause cytolysis. ExoS and ExoT are both able to activate GTPases and transfer ADP ribosyl moieties, however, their targets differ [21, 32]. ExoU is a phospholipase, which results in cytolytic activity [21, 32].

### **Pseudomonas in Burns**

PA is a common opportunistic pathogen found in burn wound infections and is associated with significant morbidity and mortality [33-35]. Burn wounds are particularly susceptible to PA infection due to the loss of the skin barrier and the compromised immune system of the patient [36]. Once established, PA forms biofilms on the wound surface, which protect the bacteria from the host immune response and antimicrobial agents [30, 37]. The host response to burn injury, including the release of anti-inflammatory cytokines, also contributes to the pathogenesis of PA infection [38]. These factors contribute to the dissemination of PA, sepsis, and mortality observed in burned people. Therefore, understanding the relationship between PA pathogenesis and burn wounds is critical for developing effective strategies for the prevention and treatment of burn wound infections.

## **The Innate Immune System**

### **Physiology**

The innate immune system is the host's first line of defense against microbial infection. Because pathogens can infect interstitial spaces, the blood, epithelial surfaces, and intracellularly, each compartment of the body must be protected. To do so, the innate immune system utilizes a number of cell types, physical and mechanical processes, and commensal bacteria [39-41]. One of the largest organs of the immune system is the skin. Physically, the skin's role is to protect the internal organs and systems of the body. It is a barrier that defines what is self and what is foreign. Other examples of physical barriers include the mechanical action of breathing and the beating of cilia [42]. The movement prevents the attachment of potential pathogens and has also been implicated in innate cell activation [43, 44]. In the gut, the microbiome interacts with intestinal epithelial cells to induce the production of anti-microbial peptides [39, 45]. The gut microbiome also occupies niches and prevents invasion of the mucosal lining by foreign pathogens [39, 45]. These functions prevent pathogens from interacting with internal organs and cells.

Within the body, epithelial and phagocytic cells secrete anti-microbial peptides (AMPs) into mucosal surfaces and tissues. These peptides include defensins, cathelicidins, and histatins. They can form pores within cell membranes, causing them to become leaky, or act in a detergent-like matter, disrupting the lipids that form the cell membrane [46]. There are also proposals of non-lytic AMPs, which translocate into the cell [47, 48]. Defensins are short, cationic peptides with an amphipathic structure [49]. Specifically,  $\alpha$ -defensins are stored within granules of neutrophils and aid in killing phagocytosed bacteria [49]. Cathelicidins are made as inactive propeptides by epithelial cells, neutrophils,

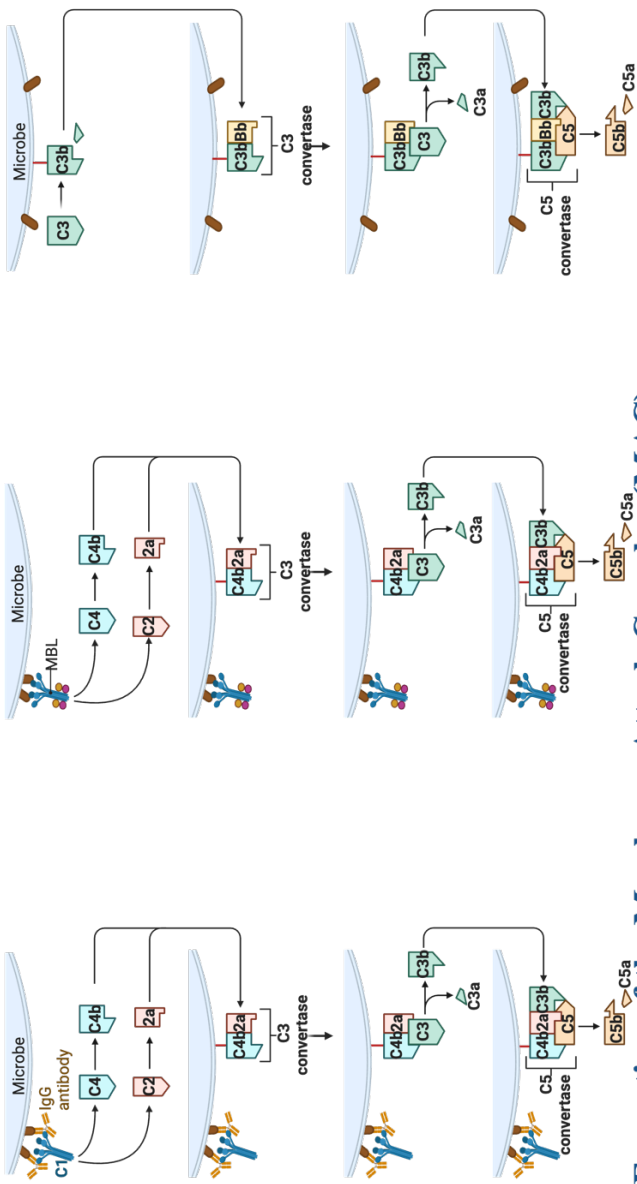
macrophages, and keratinocytes. They are cleaved by elastase, which is released by activated neutrophils, allowing for them to be activated when needed. These peptides act similarly to defensins in that they disrupt the cell membrane and initiate lysis [49]. Histatins are produced by the salivary glands. These peptides are histidine-rich and are involved in anti-fungal protection [50].

In circulation, one form of innate protection is the complement system (Figure 1-1). The complement system is a family of proteins that opsonizes bacteria by binding to their surface and serving as a binding site for innate cells and assemble to form pores in cell membranes. The complement system can be activated in three ways. Complement proteins can bind to antibodies bound to microorganisms (Classical pathway), carbohydrate structures specific to pathogens (Lectin pathway), or to the membrane of the pathogen itself (Alternative pathway) [51-54]. Once bound, they can attract immune cells to kill the pathogen, or begin a binding cascade to form pores in the cell surface (Figure 1-1) [51-54]. By-products of the assembly cascade act as chemoattractants for immune cells [51-54]. This system is important for the function of the innate immune response because of the ability to alert immune cells to danger, attract immune cells to the site of inflammation, and increase their effectiveness.

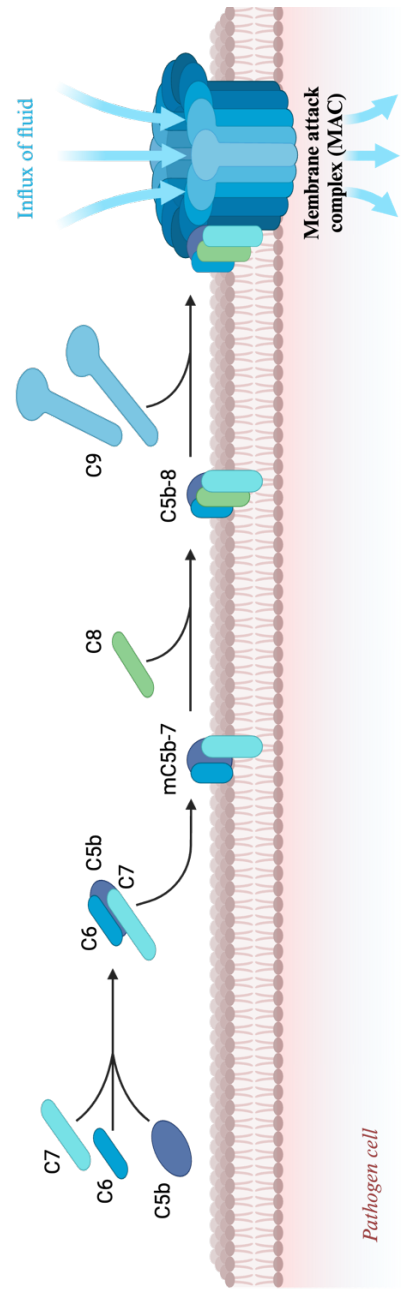
**Figure 1-1 The complement system can be activated in three different pathways, resulting in the formation of the membrane attack complex (MAC).**

The complement system is used to opsonize and kill pathogens and can be activated in multiple ways. The complement (C') proteins are constitutively expressed in circulation, able to recognize and bind specific ligands. In classical activation, C' protein C1q binds to antibodies on the surface of pathogens. Carbohydrate binding proteins such as ficolins can bind to carbohydrates on microbes and activate proteases in the C' cascade. Finally, if C3 is spontaneously hydrolyzed, it can bind directly to a microbe's surface. C' activation results in a cascade of proteins and activating proteases, whose byproducts opsonize pathogens and promote inflammation. The three pathways converge with the formation of C3 convertase, which cleaves C3 into C3a and C3b. C3b binds to C4a2b and forms the C5 convertase, cleaving C5 to form C5a and C5b. C3b also acts as an opsonin, binding to the surface of bacteria and facilitating phagocytosis. C5b activates the formation of the membrane attack complex (MAC). C5b, 6, and 7 form a complex and bind to the surface of pathogens. C8 inserts into the membrane to form an anchor. Finally, C9 proteins bind and polymerize, forming pores in the surface of pathogens.

## Complement System Activation



## Formation of the Membrane Attack Complex (MAC)

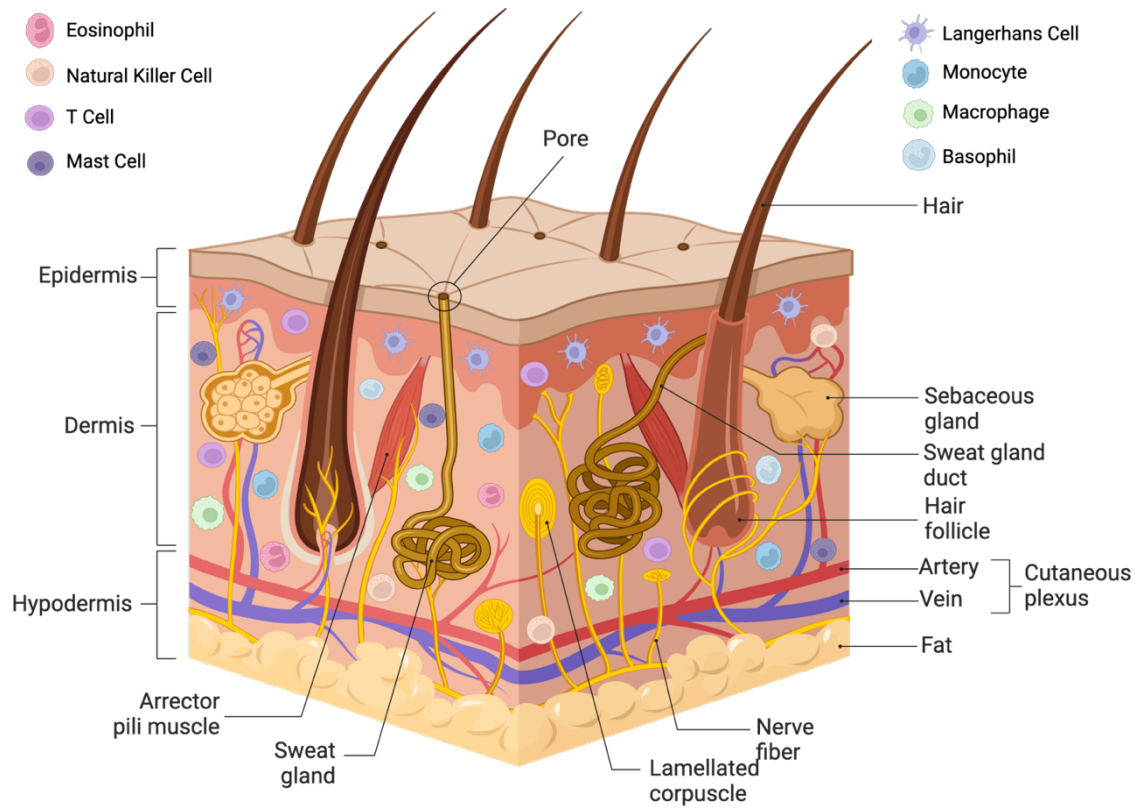


## *The Skin*

Relevant to this dissertation work, the skin has many functions in the host. Aside from immune functions, it provides protection from UV radiation, is a primary source of sensation, and is involved in temperature regulation [55]. It is critical to the body's homeostasis. The skin is comprised of two primary layers, the epidermis and dermis, and support structures (Figure 1-2). The outermost layer, the epidermis is comprised of squamous epithelial cells in five layers connected by tight junctions. The stratum basale is where basal epithelial cells are continuously produced [56]. Loss of this layer affects wound healing and protection from disease [55]. The epithelial cells move through the next three layers (stratum spinosum, stratum granulosum, and stratum lucidum), differentiating and maturing [55]. These layers are also responsible for producing cytokines and releasing vitamin D in response to UV exposure [55, 57]. The stratum corneum is the top layer, comprised primarily of dead keratinocytes. It protects from salt and water loss and secretes defensins [55].

The epidermis also contains key structures and cell types. Sebaceous glands release oils onto the skin's surface for moisturization and waterproofing. Melanocytes store melanin, which absorbs UV radiation [58]. Finally, the epidermis also contains free nerve endings that report sensory information. They can sense pain, crude and fine touch, vibration, temperature, and pressure [55, 58].

The dermis largely contains support structures for the epidermis. It is a dense organ, responsible for protecting the body from mechanical stress. It is connected to the epidermis via a basement membrane. The dermis is largely comprised of fibroblasts which synthesize collagen and elastin, giving skin its elasticity and strength [55, 58]. The vasculature and



**Figure 1-2 Anatomy of the skin.**

Human skin is comprised of a number of anatomical structures in two compartments, the epidermis and the dermis. The epidermis is the outermost layer, made of epithelial cells differentiated into keratinocytes. The dermis contains support structures, such as vasculature, nerve endings, hair follicles, and sweat glands. Within the skin, there is a host of immune cells. T cells in the figure include memory  $CD4^+$  and  $CD8^+$  cells, Th1, Th2, Th17, and Treg cells [58-60].

lymphatics for the skin reside in the dermis; these structures are key for temperature regulation. The autonomic nervous system regulates blood flow through these blood vessels to control the body's temperature. The dermis also contains apocrine glands at the base of hair follicles, which release sweat in response to high temperatures [55, 58]. There are nerve endings that sense deep pressure and vibration.

### **Activating the Innate Immune System**

Within the skin and in circulation, the innate immune response is comprised of several leukocytes: monocytes, dendritic cells, macrophages, natural killer (NK) cells, basophils, eosinophils, and neutrophils (Figure 1-2). More recent work has led to the discovery of cells such as resident memory lymphocytes, ILCs,  $\gamma\delta$  T-cells. These cells express pattern recognition receptors (PRRs), which have evolved to recognize common patterns that indicate the presence of a pathogen, pathogen-associated molecular patterns (PAMPs) (Table 1-1) [61-63]. They also recognize molecules associated with cellular damage or distress, danger-associated molecular patterns (DAMPs) [64-67]. The PRRs

<b>Pattern Recognition Receptor</b>	<b>Associated Cells</b>	<b>Recognized Ligands</b>	<b>Notes*</b>
TLR-1	NK, MΦ, PMN, monocytes, DCs, mast cells, eosinophils	triacyl lipopeptides, peptidoglycan, lipomannans, lipoteichoic acids, β-glucans, zymosan	Forms heterodimer with TLR2
TLR-2	NK, MΦ, PMN, monocytes, DCs, mast cells, eosinophils	triacyl lipopeptides, peptidoglycan, lipomannans, lipoteichoic acids, β-glucans, zymosan, defensins, histones	Forms heterodimers with TLR1 and TLR6
TLR-3	NK	dsRNA	
TLR-4	MΦ, DC, mast cells, eosinophils	LPS, lipoteichoic acid, HMGB1, HSP, S100, defensins, histones	Complexes with MD-2 and CD14
TLR-5	Intestinal epithelium	flagellin	
TLR-6	NK, MΦ, PMN, monocytes, DCs, mast cells, eosinophils	triacyl lipopeptides, peptidoglycan, lipomannans, lipoteichoic acids, β-glucans, zymosan	Forms heterodimer with TLR2
TLR-7	pDCs, NK, eosinophils, B cells	ssRNA, cathelicidins	
TLR-8	NK	ssRNA	Humans only
TLR-9	pDCs, eosinophils, basophils	Unmethylated CpG islands on DNA, mtDNA, cathelicidins	
TLR-10	pDCs, eosinophils, basophils	unknown	
TLR-11	MΦ, DC, epithelial cells of the liver, kidney, and bladder	profilin and profilin-like proteins	Mice only
RIG-I	widely expressed	ssRNA	
MDA5	widely expressed	dsRNA	
LGP2	widely expressed	ssRNA	
MAVS	widely expressed		
STING/cGAS	widely expressed	dsDNA	
NOD1	widely expressed	γ-glutamyl diaminipimelic acid	
NOD2	Monocytes, DCs, MΦ, B cells, T cells	muramyl dipeptide	
NLRP3	MΦ, monocytes, DCs	extracellular ATP, crystalline uric acid	
Dectin-1	NK	β-glucan carbohydrates	
Dectin-2	MΦ, PMN, DCs	zymosan	
MINCLE	MΦ, PMN, DCs, monocytes	glycolipids, glycoproteins, SAP-130, cholesterol crystal, β-GlcCer	
RAGE	widely expressed, type-1 pneumocytes	HMGB1, LL-37, S100, cathelicidins	
P2XR/P2YR	epithelial cells	ATP, uric acid, cathelicidin	

**Table 1-1 Pattern Recognition Receptors are expressed on specific cells to recognize PAMPs and DAMPs.**

PRRs recognize a range of molecules within a class. Activated PRRs trigger a signaling cascade and mediate an immune response. TLR: Toll-like receptor, MΦ: macrophage, NK: natural killer cell, PMN: polymorphonuclear cells/neutrophils, DCs: dendritic cells, pDCs: plasmacytoid dendritic cells [61, 63, 64, 67-70]. \*Unless otherwise noted, expressed on both human and mouse cells.

recognize a diverse array of ligands, allowing them to respond to multiple types of threats.

DAMPs are host-derived proteins or molecules that alert the immune system to cellular dysfunction. In patient trauma, they are responsible for causing an inflammatory response [68, 71, 72]. This can overwhelm the immune system, causing cytokine storm, sterile sepsis, and death [69, 73]. The circulating levels of DAMPs (cell-free DNA, mtDNA, uric acid, HMGB1, etc.) are positively correlated with increased morbidity and mortality [65, 72, 74].

High Mobility Group Box 1 (HMGB1) is a highly conserved, nuclear DNA-binding protein, with a variety of compartment-specific functions. In the nucleus, it stabilizes chromatin, regulates gene transcription, and is involved with cellular replication and DNA repair. Intracellular functions include inflammasome activation, vesicle formation, and autophagy induction. Extracellularly, HMGB1 serves as an aforementioned DAMP which signals through MD-2/TLR4, but it can also activate platelets and is pro-angiogenic [75-77].

PAMPs indicate the presence of pathogens. Because the sources of PAMPs are bacteria, viruses, parasites, or fungi, the host immune system has evolved to recognize these threats. PAMPs are typically small molecules with common structural motifs that are critical to the survival of the pathogen, such as peptidoglycan or single-stranded RNA. While PAMPs are not species-specific, in that PRRs cannot distinguish one bacterial species from another, PAMPs are patterns that are not found within the host itself. This ensures that the immune response is activated only in response to a threat.

Innate immune cells highly express pattern recognition receptors (Table 1-1). These cells can be tissue-resident cells or serve as sentinel cells in circulation. Once activated,

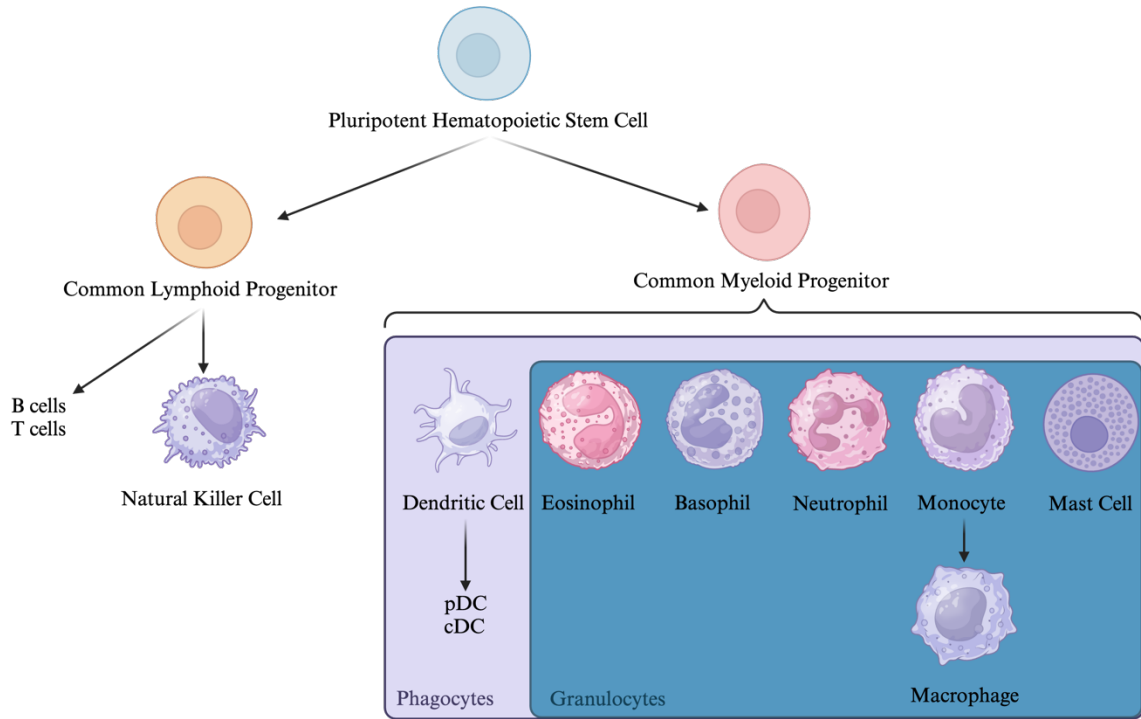
these cells produce cytokines which further recruit immune cells to the site of infection or cell damage. They also participate in removing the insult. This can include phagocytosing bacteria, triggering death in infected or damaged cells, or clearing cellular debris. With prolonged exposure to DAMPs, PAMPs, and cytokines, innate immune cells will travel to lymph nodes to activate the adaptive immune response.

### *Innate Immune Cells*

One family of innate cells is phagocytes. They can ingest and digest other cells, such as bacteria or senescent red blood cells [78]. They include monocytes and macrophages, granulocytes, and dendritic cells. Once targets are within the phagosome, it becomes acidified and generates a phagolysosome. Dendritic cells are also able to use cell-mediated endocytosis and macropinocytosis to sample the extracellular environment for PAMPs or DAMPs [79].

Resident in tissues, dendritic cells (DCs) migrate from the bone marrow and circulate to distal organs and lymphoid organs. Dendritic cells can phagocytose, but their main function is digesting the contents of the phagolysosome and alerting other cells to the presence of a threat, not necessarily killing [60]. DCs have two functional types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [60, 80]. Conventional DCs process phagocytosed bacteria to present antigens to naïve T cells to generate an adaptive immune response, while pDCs produce antiviral interferons [60, 80].

Phagocytes can be further delineated into granulocytes (Figure 1-3). They are the most abundant cells in circulation due to neutrophils, which will be discussed in detail below,



**Figure 1-3 Development of lymphocytes.**

In the bone marrow, lymphocytes begin with a common progenitor stem cell, which also serves as the stem cell for red blood cells and platelets. Through differentiation, the common progenitor cell produces two progenitor cells, for myeloid cells and lymphoid cells. The lymphoid progenitor gives rise to three cells: B cells, T cells, and NK cells. The myeloid progenitor gives rise to the phagocytes: dendritic cells and the granulocyte progenitor. Neutrophils, eosinophils, basophils, monocytes, and mast cells arise from the granulocyte lineage. Monocytes further differentiate into macrophages.

and have prepared antimicrobial proteins synthesized within granules that are released upon activation (*i.e.* degranulation). Eosinophils and basophils are involved with the innate response to parasites, which are too large to be phagocytosed. Eosinophils contain granules with arginine-rich proteins. Most eosinophils are located in the tissues, beneath mucosal surfaces, to protect against invading organisms. IgG, C3b, or IgA can cause degranulation of eosinophils [81]. They also synthesize pro-inflammatory mediators (leukotrienes, prostaglandins, and cytokines). Basophils comprise less than 1% of circulating white blood cells. They are activated by two pathways, IgE-dependent and IgE-independent. IgE activation causes degranulation and the release of cytokines (IL-4, IL-13) [82]. Basophils also release histamine and leukotriene, which cause contraction of smooth muscle and dilation of capillaries. Overreactive basophils and eosinophils are responsible for allergic responses and asthma [83, 84]. Neutrophils are also a part of this group and will be discussed in greater detail below.

Monocytes developed in the bone marrow and can differentiate when they enter tissues into macrophages or dendritic cells. In homeostasis, monocytes are involved with tissue development and monitoring. In response to tissue damage or infection, they are able to respond with a wide variety of effector functions including reactive oxygen species (ROS) production, phagocytosis, production of cytokine and chemokines, and increased adhesion and migration [85]. Monocytes are also involved with the resolution of inflammation, with abilities to release cytokines such as IL-10, phagocytose apoptotic cells, and induce negative regulation of pro-inflammatory cytokines [72, 86].

Macrophages mature from circulating monocytes (bone marrow-derived macrophages, BMDM) or reside in tissues [87, 88]. These tissue-resident macrophages

serve as sentinels within organs, sensing immediate threats without needing to be recruited. Tissue-resident macrophages can also perform non-immune functions. In the liver, Kupffer cells remove senescent red blood cells from circulation. In the bone, osteoclasts degrade bone to release calcium into the body. Alveolar macrophages clear surfactant from alveoli.

Generally, macrophages are involved in the generation and resolution of inflammation. They can exist as two phenotypes with opposing functions. M1-like macrophages have pro-inflammatory effects. They are “classically activated,” in that when exposed to pro-inflammatory mediators such as IFN- $\gamma$ , TNF- $\alpha$ , or LPS, they further produce and secrete immunostimulatory cytokines, increase antigen presentation, and promote the proliferation of other immune cells [89, 90]. “Alternatively activated” or M2-like macrophages change their phenotype in response to IL-4, IL-13, and other inducers. These cells produce anti-inflammatory mediators, such as TGF- $\beta$  and IL-10, and upregulate proteins involved in wound healing, such as *Arg1* [89-91]. Tissue macrophages share some functions with neutrophils, however, are able to act sooner because they do not need to be recruited.

Apart from the phagocytes, natural killer (NK) cells are the second type of innate immune cells. NK cells circulate in the blood and are activated in response to viral infection or malfunctioning cells. NK cells exist in an equilibrium of inhibition and activation, with intracellular signaling from multiple surface receptors dictating the phenotype of the NK cell [92, 93]. Activating stimuli include DNA damage, senescence, virus-encoded ligands, and tumor suppressors [92, 93]. They can also be spontaneously activated by cells that are not expressing MHC-I [94]. Activated NK cells can induce apoptosis, produce cytokines, and release granules containing cytolytic proteins [94].

## Neutrophils

### *Physiology*

The bone marrow is the site of development for all cells that form the blood. There is one common stem cell to all cells that make up the blood tissue, the pluripotent hematopoietic stem cell. Through cytokine signaling and active expression factors, the hematopoietic stem cell terminally differentiates into the previously described cells and the cell of focus for a portion of this thesis: the neutrophil (Figure 1-3). As neutrophils develop, they develop granules that are filled with preformed proteins that aid in various neutrophil functions [95-98].

- Primary granules: azuracidin, defensins, neutrophil elastase, and myeloperoxidase
- Secondary granules: cathelicidins, lactoferrin, lipcalin 2, metalloproteinases, and olfactomedin 4.
- Tertiary granules: arginase-1, gelatinase, and lysozyme
- Secretory vesicles: albumin, cytokines, membrane receptors, and components of NADPH oxidase complex

Approximately  $10^{11}$  mature neutrophils are released into the bloodstream daily. Neutrophils are very short-lived, with a lifespan of hours to days (in mice, up to 12.5 hours). In circulation, neutrophils have a unique morphology of a multilobed nucleus. The segmented nucleus is thought to make the cell more malleable, which aids in extravasation from blood vessels [99]. On their surface, mature neutrophils express proteins for opsonin

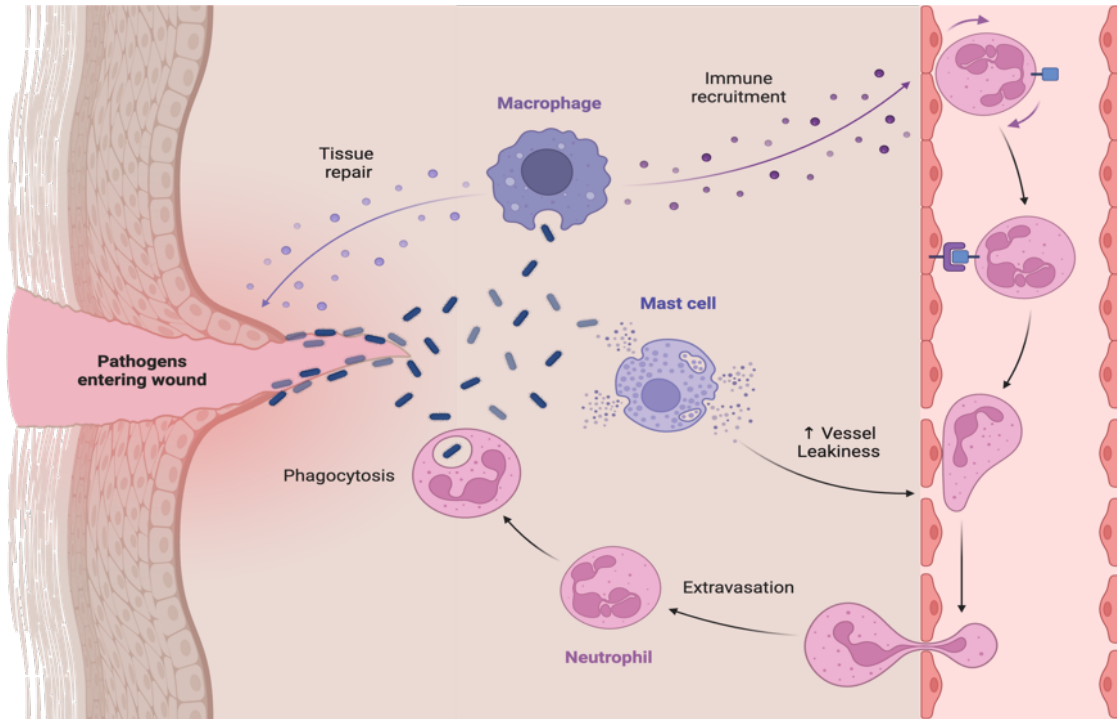
sensing such as CXCR2 and Mac-1, extravasation such as CD62L and PSGL-1, and several pattern recognition receptors (Table 1-1) [100].

### *Recruitment*

As neutrophils exist primarily in circulation, they need to be recruited to the site of infection or cell damage. At the recruitment site, sentinel immune cells produce inflammatory mediators which cause changes to the endothelial cells that line blood vessels (Figure 1-4) [101]. The endothelial cells express P-selectin and E-selectin, which allows for circulating neutrophils to become tethered to the blood vessel wall. The innate cells also release histamine and bradykinin, which cause vasodilation and further slow the circulation of neutrophils. P- and E-selectin bind to neutrophil CD62L and PSGL-1, allowing for neutrophils to tether to the vessel wall and slowly roll to decrease speed. After full arrest and firm adhesion via integrins to endothelial ligands, such as ICAM-1, neutrophils are able to extravasate either paracellularly or transcellularly into the extracellular space to the inflammatory nidus [51, 101-103]. The neutrophils then use chemotaxis to follow chemokine gradients, such as C' protein C5a or PAMPs like N-formyl-methionyl-leucyl-phenylalanine (fMLP), to locate the site of infection or damage [104, 105].

### *Function*

Neutrophils utilize multiple functions to neutralize pathogens and have a critical role in the innate immune system. While sentinels are the cells that raise the alarm, neutrophils are the first responders for bactericidal activity. Neutrophils are able to recognize a variety of DAMPs and PAMPs (Table 1-1) and respond accordingly. A main function is the production of reactive oxygen species. Using NADPH oxidase,  $O_2^-$  is formed within the



**Figure 1-4 Neutrophil recruitment, extravasation, and chemotaxis.**

Tissue resident macrophages and mast cells recognize DAMPs and PAMPs, which causes the release of cytokines and chemokines. Endothelial cells lining blood vessels respond to these mediators, expressing lectins to slow circulating neutrophils. Once stopped, neutrophils are able to extravasate and travel up a chemotaxis gradient to colocalize with the threat.

phagosome to disrupt the membrane, proteins, and genomes of pathogens resulting in bactericidal activity. NADPH has two membrane-bound components and four cytosolic components that are assembled with neutrophil activation [106, 107]. Superoxide production also results in hydroxyl radical and hydrogen peroxide, which myeloperoxidase converts to hypochlorous acid [106, 107]. Reactive oxygen species production can result in tissue damage in the host if not appropriately balanced. Patients with a deficiency in any of the components of the NADPH oxidase have a condition termed chronic granulomatous disease [108]. These patients have a greater susceptibility to bacterial and fungal infections [108]. Through prolonged activation of NF- $\kappa$ B, these infections are walled off in the host in the form of granulomas, which is a clustering of immune cells [108]. PMNs are able to ingest bacteria, acidify the phagosome, and digest the contents using ROS. Neutrophils also degranulate, releasing a host of preformed toxic proteins and enzymes from their granules and exosomes into the extracellular space [97, 98]. In the case of large pathogens, neutrophils can release extracellular traps (NETs) in either a process of slow cell death called NETosis or in a rapid, non-lytic manner [109-112]. NETs are made of DNA and granular proteins such as elastase. NETs putatively inhibit the movement of pathogens and concentrate effector proteins [109, 112, 113]. After performing these functions, the neutrophils undergo many methods of cell death, including apoptosis or necroptosis [114, 115].

### *Neutrophil Dysfunction*

The characteristic feature of sepsis is a dysregulated immune response causing cytokine storm followed by a period of pathogenic inflammation and immune suppression[116]. Dysfunctional neutrophils have been found to have a role in the

progression of sepsis and inappropriate immune responses by distributing abnormally, localizing to tissues other than the site of infection [73, 117, 118]. There has also been an “exhausted” phenotype characterized in neutrophils isolated from septic patients. These cells exhibit an increased expression of immunosuppression markers such as PD-L1, as well as adhesion molecules [119]. The TLR4-activated TICAM2/TRIF-dependent pathway has been implicated in this exhaustion phenotype [119].

## **Burns**

### **History**

As humanity evolves, the tools used evolve in parallel. Electricity, fire and steam, chemicals, radiation – implements that can be used to accomplish great goals but carry with them the danger of injury. While the first instance of fire was noted over 420 million years ago [120], man’s relationship with fire changed as technology advanced. In the 1700s, wood-burning stoves moved into homes, bringing fire indoors [121]. Today, approximately 35% of homes in the United States contain gas-burning stoves [122, 123]. Fire is also used recreationally in fireplaces and campfires. As construction prices rise, homes are less likely to be built with fireplaces. The number of fireplaces in newly built homes has been decreasing since the 1990s, with less than 40% of new single-family home constructions containing a fireplace [124]. In the US, one in five house fires are caused by fireplaces.

As fire becomes more accessible, accidents are increasingly likely to occur. The World Health Organization estimates that globally over 100,000 deaths occur due to burns each year. The United States Fire Administration reports 13 fire deaths per million people in 2021, with a 17.9% increase over 10 years [125]. The threat of fire is not just to individuals. In 2022, over 60,000 wildfires burned 7.6 million acres in the United States;

the U.S. Forest Service employs over 10,000 firefighters to combat these fires [126]. Recently, wildfires ravaged Maui and, with a death toll of over 100, it is the deadliest fire in the U.S. in over 100 years [127]. In 2023, Canada had a record-breaking fire season, with over 18.4 million hectares burned and 6 deaths [128]. Greece was also plagued by wildfires in 2023, with over 20 deaths [129]. The Iraq and Afghanistan Wars saw an increase in burn patients, with the patients presenting younger, with higher total body surface area (TBSA) percent burned, but with lower mortality [130].

### **Epidemiology**

Burns can occur as a result of a variety of insults: flame and scald, chemicals, electricity, radiation and UV, friction, and conduction [131-133]. Globally, in 2017, there were between 7,481,218 to 10,740,897 injuries due to flame, heat, or hot substances with 1.6% of those burns resulting in death [132]. The overall rate of flame burns decreased in the U.S. from 1990-2017, however, mortality rates remained stagnant [134]. Children aged 1-4 burned due to thermal sources are the most hospitalized age group in the U.S. [135]. Chemical compounds have been developed to be used in the home for housekeeping purposes, including bleach and ammonia [136]. Chemicals also pose occupational hazards, with compounds such as cement, liquid nitrogen, and lye being able to harm their users [136]. Chemical burns comprise approximately 3-10% of burn injuries and 30% of burn mortality [136, 137]. Occupational burns are a source of chemical and electrical burns, even though they are rare. A US study found that 90% of occupational burn patients were male, with the highest rate of occurrence in the manufacturing industry [133]. Of the patients surveyed, the majority of the burns were caused by thermal or scald interactions, however chemical and electrical sources made up 16% and 11%, respectively [133]. While

it is evident that all burn modalities pose a danger, this dissertation will focus on the impact of flame burns.

### **Pathophysiology**

Burn injuries are classified by depth and surface area of damage. Burns can be: superficial, only damaging the epithelium; partial thickness, which involves portions of the dermis; full thickness, where the dermis is fully destroyed; fourth-degree burns reach muscle or bone [138] (Figure 1-5). Patients with burn injuries are also assessed to measure the size of their burn defect, termed total body surface area (TBSA). There is a positive correlation between increased TBSA and mortality rate [139]. When burns occur, the structural integrity of the skin is damaged. This causes a release of DAMPs, which trigger an inflammatory response within 24 hours of the wound [138]. Macrophages and neutrophils infiltrate the wound, clearing cellular debris, attacking potential pathogens, and producing pro-inflammatory mediators. Activated platelets network to close the wound [138]. In large burns, this results in eschar formation. As endothelial cells repopulate, fibroblasts cause tissue remodeling [138].

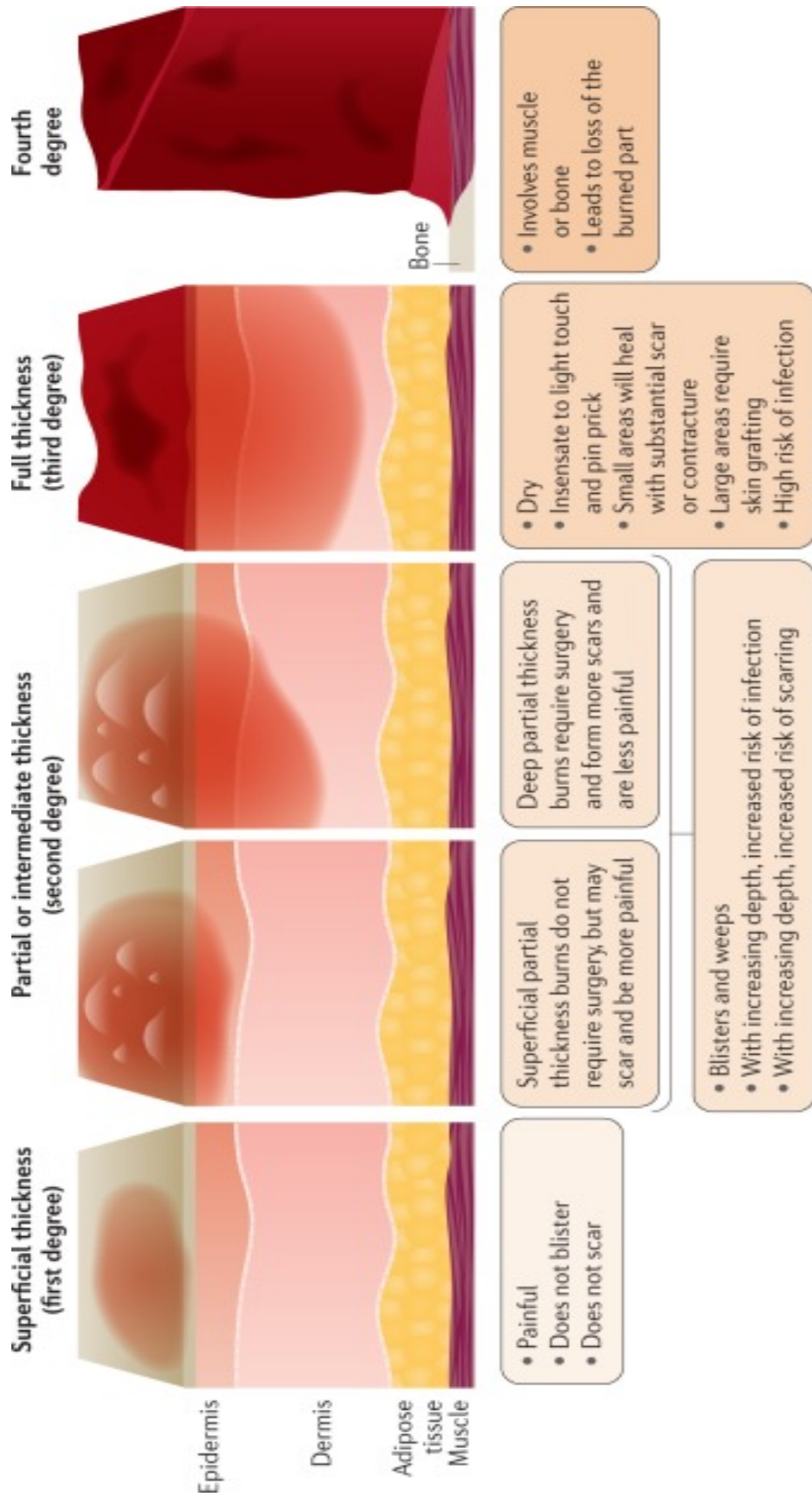
### **Burn-associated Immune Response**

Within 24 hours of burn injury, neutrophils and macrophages arrive at the burn site to remove cellular debris. They also initiate the wound-healing signaling cascade. There is a significant release of the cytokines IL-17, TNF- $\alpha$ , and IL-6 post-burn [140-142]. The cytokines and growth factors released cause the proliferation of endothelial cells and fibroblasts. Systemically, the immune response to burns can have devastating results. Patients with decreasing circulating levels of C3 were associated with increased mortality

[143]. Sustained release of cytokines can result in distributive shock and decreased cardiac function [138].

**Figure 1-5 Burn wounds are characterized by size and depth.**

The severity of a burn wound is determined by the size and depth of the defect. Superficial burn wounds only involve the outermost layers of the epidermis. They commonly involve redness, swelling, and pain, but do not blister and resolve without scarring. Partial-thickness burns damage the deep layers of the skin and can reach the dermis. These burns form blisters and may scar depending on the depth of the wound. Surgery may be required for repair. Full-thickness burns destroy the epidermis and dermis. Because the underlying nerve endings, blood vessels, and stem cells are destroyed, large, full-thickness burns require surgery to repair. Fourth-degree burns extend beyond the skin into muscle and potentially bone. These wounds are debilitating and result in the loss of burned tissues. Used with permission from Springer Nature by Copyright Clearance Center [138].



## **Burn Wound Sequelae**

Burns are accompanied by severe sequelae: hypercoagulability, hypermetabolism, multisystem organ dysfunction, and sepsis [138]. The hypercoagulable state could be because of malfunctioning, underproduced, or depleted coagulation factors. In the hypermetabolic state, patients rapidly catabolize lipids and proteins, have hyperdynamic circulation, and disordered body temperature regulation. Multisystem organ dysfunction is linked to a systemic inflammatory response post-burn. Patients with high TBSA have increased risk, which can result in the failure of several organs, including the lungs, kidneys, liver, and heart [144]. There is increased intestinal permeability, bacterial translocation, and changes to the gut microbiome [145].

One of the most common complications of thermal injury is burn wound infection and sepsis. With the first barrier to infection compromised, and an altered metabolism and immune response, patients are predisposed to infection with opportunistic pathogens, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. As the infection progresses, patients can experience fever, tachycardia, tachypnea, and hypotension. Approximately 70% of mortality following burns is due to infection.

## **Treatment**

Superficial burns do not require medical intervention. Patients can treat these wounds with first aid materials, such as aloe vera cream or antibiotic ointment. Treatment of isolated, minor burns (partial thickness < 10% TBSA in patients aged 10-50 or < 5% in patients under 10 or over 50 years of age) involves cooling the wounded area, cleansing blister treatment or debridement, and utilizing appropriate dressing to cover the wound [146]. Patients may also require pain management and prophylactic vaccination against

tetanus. Special considerations must be taken for burns on the face, hands, perineum, feet, or across major joints. These burns are considered severe.

Severe burns are wounds that are complicated by additional factors, such as inhalation injuries, or are  $\geq 20\%$  TBSA. Severe burns often occur concurrently with other trauma mechanisms, such as fractures, traumatic brain injuries, and complex soft tissue injuries. These factors, along with preexisting comorbidities, increase the risk of death. Initially, these patients require stabilization. This involves monitoring their vital signs and keeping them within normal limits, respiratory support, and fluid resuscitation. Once the patient is stable, care transitions to care of the burn wound [147-149]. This can include debridement or excision of the wound, control of hypermetabolism, and autograft or allograft placement [146, 147, 150]. These patients would also receive pain management and anticoagulation therapy. Because of the loss of a physical barrier, it is critical that these patients are treated in sterile environments to decrease the risk of nosocomial infection. These wounds must also be covered in specific dressings; nonadherent, silver-containing gauze is often supplemented with films, foams, and hydrogels to promote wound healing and minimize infection [151].

### **Patient Outcomes**

Patient mortality has been positively correlated with multiple factors. A main factor is TBSA: larger wounds have an increased rate of mortality [131, 148, 152]. Other demographics, such as age and sex also play a role [130, 148, 149]. Patients with increased levels of DAMPs, such as mtDNA, HMGB1, and S100A in circulation, are more likely to have poor outcomes [73, 74, 153]. There are also risks to mortality associated with immune dysfunction, such as failing to recover complement protein production [143] and increased

and sustained levels of IL-10 [154]. Further research into burns can determine methods to limit pathogen exposure and decrease mortality.

### **NanoString™**

Gene expression is the process by which information encoded in genes is used to make functional products, such as proteins. It is a fundamental process that occurs in all living organisms, and it is essential for the proper functioning of cells, tissues, and organs. Gene expression is regulated at various levels, including transcription, RNA processing, translation, and post-translational modifications. By studying gene expression, insights are gained as to how cells and organisms function, how diseases develop, and how they might be treated. Additionally, gene expression profiling can be used to diagnose diseases and predict the course of treatment. Overall, the study of gene expression is critical for advancing our understanding of biology and improving human health.

A technique to measure specific gene expression is made by NanoString™. Using its nCounter® analysis system, researchers can develop a custom panel of RNA probes to interrogate their samples. The probes are labeled with a fluorophore “barcode” which fluoresce when complexed with its target gene. This allows for the quantification of specific transcripts in each sample. This specificity also introduces selection bias, as genes that are not included in the panel will not be measured. This technique is best utilized for direct comparison of specific gene expression under multiple conditions [155-158]. It is also a fast, relatively inexpensive option for measuring expression in a large number of samples (96 per protocol) with high sensitivity and reproducibility [158].

## **Burn Models**

There are many modalities of burn, as detailed above. To study burn wounds, researchers use several animal models, most commonly in mice, rats, or pigs [159-162]. While animals are not a perfect substitution for humans, there are overlaps in physiological and pathological characteristics in many organ systems. Pigs have the most similarities to human skin; however, they are more expensive to use and maintain [159, 160]. Mice are cheaper, breed faster, and have firmly established phenotypes in literature. Mice, however, have denser hair that regenerates faster than humans [159, 160, 163]. Their wound-healing process also differs from humans in that mice heal primarily through contracture [159, 160, 163]. Mice have a subcutaneous muscle layer, the panniculus carnosus, which contracts to approximate wound edges after an injury [159, 160, 163]. In contrast, humans repair wounds by re-epithelialization and granulation.

The experimental burn wound can be caused through many methods. Many labs use a contact or scald model. In a contact model, a heated metal rod contacts the skin for a prescribed amount of time, whereas in a scald model the model organism has exposed skin submerged in a heated water bath [67, 164-166]. Other methods include radiation, phenol chemical burns, or inhalation models [167-171]. This project utilized a flame burn model, in which a flame-resistant polymer template was placed on the dorsal aspect of mice with their hair clipped. Ethanol (500 $\mu$ L, 100%) was administered within the template and lit; the flame was allowed to burn for 10 seconds. The model allows for consistent application of a 10% TBSA, full-thickness burn wound between animals.

**Conclusion: alterations of the innate immune response to a 10% TBSA non-lethal burn increase the propensity to bacterial infection.**

In our non-lethal flame burn model, 100% of mice succumb to sepsis with a 4-log reduction of LD<sub>50</sub> of *Pseudomonas aeruginosa* between 20- and 30-hours post-burn (hpb). In our experimental system, mice that are challenged with heat-killed PA, PA LPS, or live PA without burn have 100% survival. Further, PA has been shown to disseminate to distal organs of mice between 12- and 18-hours post-burn and infection, prior to their demise.

**Hypothesis: both the host and the pathogen alter gene expression over time, allowing for disease progression to sepsis and death.**

Additional work has shown that CD45<sup>+</sup> leukocytes are actively recruited to the burn site and are the major constituents of the seroma fluid with forms beneath the burn wound. Gram stain of the seroma found that PA was present in close proximity to, but not within, these cells in the seroma. **Hypothesis: neutrophils recruited to the burn site are “exhausted” and/or impacted by the seroma fluid and are unable to respond to the overlying *Pseudomonas aeruginosa* infection.**

### **Chapter 3 Overview**

Using NanoString™, the gene expression of both host and pathogen genes in the burn alone, infection alone, and burn and infection conditions were compared. The skin and blood, and the common sites of dissemination: liver and spleen were sampled. Based on preliminary findings, the roles of IL-10 and arginine in the observed transient mortality post-burn and infection were assessed.

### **Chapter 4 Overview**

The functional activity of neutrophils isolated from the blood of Sham, burned mice, and the seroma was compared to determine how a burn affects neutrophil function. While investigating the effect of burn and infection on neutrophil function, the role of the

DAMP HMGB1 in the host response to burns was investigated. Previous work showed that with a burn in the absence of infection, there was a small increase in HMGB1 in the circulation that returned to baseline within a day; however, with a burn and infection, the HMGB1 levels increased 20-fold and continued to increase until death [172]. Mice that were burned and infected with PA saw restored survivability when treated with P5779, a competitor of HMGB1 for its binding site on MD-2. This led us to investigate innate immune signaling and neutrophils as a source of mortality post-burn and infection.

### **Specific Aims**

**Aim 1:** Investigate simultaneous host (*Mus musculus*) and pathogen (*Pseudomonas aeruginosa*) gene expression in multiple tissues as infection progresses post-burn.

- Sample the blood, liver, spleen, and skin of burned, infected, burned and infected, and Sham mice to determine if the burn and infection condition uniquely impacts gene expression in the host or pathogen.
- Determine if any gene products can be inhibited to improve survival.

**Aim 2:** Characterize neutrophil function post-burn.

- Isolate PMNs from circulation and seroma of burned and infected mice to measure functionality, including ROS production and phagocytosis.
- Assess the functions of naïve neutrophils incubated with the sera of burned mice and burned and infected mice.
- Determine if incubation with P5779 can restore functions to PMNs from burned and infected mice.

## **Chapter 2. Methods**

### **Bacterial Preparation**

The *Pseudomonas aeruginosa* M2 isolate (IATS O5) was kindly provided by Dr. Alan Holder, formerly of the Shriners Burn Institute at the University of Cincinnati. PA glycerol stocks were streaked for single colony isolation on Tryptic Soy Agar (TSA) (Sigma-Aldrich, St. Louis, MO) plates and incubated at 37° C for 18 h. A single colony was transferred to 3.0 mL of Hy-Soy Broth, containing 0.5% sodium chloride (American Bio, Canton, MA), 0.5% HY-Yeast (Kerry Bio-Science, Norwich, NY), and 0.25% animal-free soytone (Teknova, Hollister, CA) and grown to stationary phase at 37° C in a shaking incubator at 225 rpm. Two hundred forty  $\mu$ L of overnight inoculum was added to 12 mL of Hy-Soy broth and grown at 37°C in a shaking incubator until log phase, OD<sub>600</sub> of 0.2-0.3. The bacteria were pelleted, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in PBS to the desired concentration.

### **Burn and Infection Procedure**

With the approval of the University of Maryland, Baltimore IACUC Protocol 0322001, the burn procedure was performed using the Stieritz and Holder method [173] as previously described [172, 174]. Briefly, female Crl:CD1 mice (Charles River Laboratories, MA) between 8-10 weeks old had their dorsal hair clipped 24 h before the burn procedure. The following day, mice were administered 5% isoflurane for seven minutes and a toe pinch was performed before each burn to ensure that the mice were successfully anesthetized. Mice were placed on their bellies in a chemical fume hood and a flame-resistant polymer card circumscribing roughly 10% TBSA (2.5 cm x 4.0 cm) was pressed down on the clipped area of the back. Five hundred  $\mu$ L of 100% ethanol was

deposited onto the exposed area with a glass dropper. The ethanol was ignited using a lighter and a timer was used to ensure the burn lasted exactly 10 sec. The flame was extinguished by breath and immediately post-burn, mice were provided 500  $\mu$ L of Ringer's solution i.p. for fluid resuscitation. Mice were placed in their original cages for anesthesia recovery. Sham mice received the same treatment as experimental mice (*i.e.*, clip/anesthesia) except for burn and infection. In order to compare the responses of burned to non-burned mice to a similar bacterial challenge, mice were infected with 100  $\mu$ L of  $1 \times 10^6$  CFU/mL (one log less than LD<sub>50</sub> for non-burned mice) of PA subcutaneously at the burn site directly after the burn.

#### **Tissue Sample Collection for NanoString™ Analysis**

Mice were placed under 5% isoflurane anesthesia for seven minutes. Sedation was assessed by toe pinch. Mice were placed on their backs and their thoraces were sterilized with 70% ethanol. Blood samples were collected via cardiac puncture with a 1mL syringe and 25g needle. Whole blood was collected and stored in EDTA tubes (Sarstedt, Newton, NC). Secondary euthanasia was completed by cervical dislocation. Post-euthanasia, a horizontal incision was completed on the dorsal aspect and the skin was resected at the burn margins. The same area was collected from Sham mice. A vertical incision was then made in the abdominal cavity and the liver and spleen were resected. Each sample was stored in 3 mL of RNeasy Lysis Buffer (Qiagen, Crawfordsville, IN) and stored at -80° C until downstream assays were performed. The number of mice in each group at each time point is indicated in Appendix Tables 6-4 and 6-5.

## **RNA Isolation**

RNA isolation was completed using Trizol reagent (Invitrogen, Thermo Fisher, MA), following the manufacturer's protocol. Tissue samples were sliced using surgical scissors and weighed to be between 50 and 100 mg, then homogenized through sonication. After RNA was isolated, the samples were processed using the Monarch® RNA Clean Up Kit (New England BioLabs, Ipswich, MA). RNA was quantified using ThermoFisher NanoDrop Lite.

## **NanoString™ Analysis**

NanoString™ allows for the selection of a custom panel of genes to be interrogated. Using both target and reporter probes, NanoString™ captures transcripts as small as 100 bp. Analysis was performed using the nCounter XT CodeSet Gene Expression Assay (NanoString™ Technologies) on all samples, reading the unique barcode fluorophores assigned to each gene. The samples were interrogated using a custom panel of 67 *M. musculus* and 32 PA (Tables 6-1 to 6-3). *Gapdh*, *Polr1b*, *Rpl19*, and *Tbp* were used as housekeeping genes for the host while *oprL* and *algD* were pathogen housekeeping genes and were incorporated into the NanoString™ code set. The RNA was prepared and processed according to the manufacturer's protocols. RNA (200 ng) was loaded into the codeset and hybridized for 18-20 h.

## **NanoString™ Data Analysis**

Analysis of raw mRNA data was completed using the NanoString™ Technologies nSolver analysis software version 4.0. The geometric mean of eight proprietary negative control genes were used for background subtraction. The geometric mean of six positive controls (minimum threshold of 0.3 and a maximum threshold of 3), along with the

geometric means of the housekeeping genes (minimum threshold of 0.1 and a maximum threshold of 10) [156, 158, 175], were used to normalize the counts of each sample in accordance with the manufacturer's protocol. The gene counts were transformed using  $\text{Log}_2$ , the geometric means were subtracted to calculate ratios of change between samples, then analyzed using two-way ANOVA and Tukey's multiple comparison test.  $\text{Log}_{10}$  of p-values and ratios were calculated using Excel (Microsoft Corporation, WA). The threshold of significance was  $p=0.05$ . Data were visualized in Prism 9. Venny 2.1.0 was used to categorize significant genes which is reflected in Table 3-1 [176].

### **IL-10 Neutralization**

Mice were administered one dose of 100  $\mu\text{L}$  of 500  $\mu\text{g}/\text{mL}$  of monoclonal rat anti-mouse IL-10 neutralizing antibody (JES052A5, Invitrogen, ThermoFisher, MA) i.p. 12 h after burn and infection. This timepoint was selected based on previously published measurements of IL-10 in the circulation [172], the significant expression of *Il10* in the spleen and liver at 12 h post-burn and infection, and the observed onset of clinical symptoms. Mice were monitored for survival. Significance was determined using the Log Rank Mantel-Cox test.

### **Arginine Supplementation**

Immediately following the burn, mice were infected with 100  $\mu\text{L}$  of  $10^6$  CFU/mL of PA and 200  $\mu\text{L}$  of 0.125 g/mL arginine (Life Technologies, ThermoFisher, MA) administered either subcutaneously at the burn site, superior to the burn site at the scruff of the neck, or systemically via i.p. injection. Serine (Life Technologies, ThermoFisher, MA) was used as a negative control. At the burn site, arginine (or serine) and PA were mixed immediately prior to the burn and administered simultaneously. In some

experiments, arginine was administered i.p. 12 h post-burn. These doses and inoculation concentrations are consistent with reported previous work [177]. Significance was determined using the Log Rank Mantel-Cox test.

To measure bacterial dissemination, blood, skin, liver, and spleen were harvested at 24 h after each treatment. Samples (0.25g or 100  $\mu$ L) were placed in 1 mL of PBS, homogenized, then serially diluted. The dilutions were plated on TSA agar plates, incubated at 30° C overnight, then counted and log transformed. Significance was determined using a two-way ANOVA REML mixed model with p-values corrected for multiple comparisons [178].

### **Motility Assay**

PA was grown as previously described without shaking. Solutions of arginine and serine diluted in control sera were prepared at the indicated doses. 34  $\mu$ L of the solution or sera containing FlaB antibodies was placed in each well, then 1 mL of tryptic soy agar was added. The immune and non-immune sera were generated as previously described [179]. Once solidified, a sharp tip was used to inoculate each well with PA. Plates were grown overnight at 30° C, images recorded, and diameters of growth were measured with ImageJ. Data was entered into GraphPad Prism, and one-way ANOVA test with multiple column comparison was performed.

### **Neutrophil Isolation**

After administering isofluorane, mice were placed on their backs and their thoraces were sterilized with 70% ethanol. Blood samples were collected via cardiac puncture with a 1 mL syringe and 25g needle. Whole blood was collected and stored in EDTA tubes to prevent clotting. Red blood cells were lysed using ACK Lysis Buffer [180], according to

manufacturer's protocol. For seroma isolation, the collected seroma fluid was incubated with 2 mL of 2.5 g/mL of collagenase in PBS for 2 hours at 37° C and the samples passed through a 70 µm cell strainer. After washing, the resuspended pellets were then overlaid on a Histopaque-based density gradient (1.119 g/mL and 1.077 g/mL; Sigma-Aldrich, St. Louis, MO) and centrifuged at 872xg for 30 minutes [181]. The cell layer was carefully removed and washed in HBSS<sup>-</sup> and spun at 1400 rpm for 7 minutes with no brake. Live cells were counted using trypan blue dye (>90% viable cells) and immediately used in functional assays. Each experiment was conducted with pooled blood from 5 mice. Isolated cell population was verified using flow cytometry (gating for CD11b and Ly6G) (Figure 6-1).

### **HMGB1 quantification**

Blood was collected at 18 hours post-burn and infection as previously described then centrifuged in 1 mL serum gel tubes (Sarstedt, Newton, NC) at 12,000 x g for 10 minutes. 100 µL of each sample was aliquoted for quantification, the remaining sample was snap-frozen using liquid nitrogen. Samples were quantified using a Mouse HMGB1 ELISA kit (Novus Biologicals, CO), and the assay was completed according to the manufacturer's instructions.

### **Bacterial Killing Assay**

Isolated neutrophils were incubated in 96-well plates with log phase PA at a 1:1 ratio. In seroma supplement experiments, neutrophils were resuspended in 250 µL of seroma in the 25% condition and 750 µL in the 75% condition, then diluted to 1 mL with HBSS<sup>+</sup>. Immediately after plating, a sample was taken for serial dilution to quantify the starting inoculum. The plates were placed on a shaker at 150 rpm in a 37° C incubator for

2 hours. A second sample was taken for serial dilution. The dilutions were grown on TSA plates overnight and counted. Data was visualized using Prism and statistics were analyzed by one-way ANOVA with multiple comparisons.

### **Cytochrome C Reduction Assay**

Neutrophil samples were diluted to  $1 \times 10^6$  cells/mL in each trial. In a 96-well plate, cells (15  $\mu$ L) were incubated with cytochrome C (1.5  $\mu$ L) and HBSS<sup>+</sup> (5  $\mu$ L and diluent to 31.5  $\mu$ L), and either SOD inhibitor (SODi) (5  $\mu$ L), 10  $\mu$ g/mL phorbol myristate acetate (PMA) (5  $\mu$ L), or both. For 20 minutes, the plate was shaken at 150 rpm in a 37° C incubator. Colorimetric readings were taken at 550 nm using a plate reader. In Sham neutrophil experiments, 5  $\mu$ L of sera was added in place of PMA. Data was visualized using Prism and analyzed with REML mixed model with multiple comparisons.

## **Chapter 3. Combination of Burn Wound Injury and *Pseudomonas* Infection**

### **Elicits Unique Gene Expression that Enhances Bacterial Pathogenicity**

#### **Abstract**

Burns are a leading cause of morbidity and mortality worldwide with the most common cause of death resulting from sepsis, often from *Pseudomonas aeruginosa* (PA). A non-lethal flame burn induced an altered host immune response in previous reports. Using this model, gene expression in both the murine host and PA was measured using a NanoString™ custom probe panel. Differing patterns of gene expression were observed in both host and PA in the skin, blood, liver, and spleen of mice that were burned and/or infected, compared to mice that were neither burned nor infected (*i.e.*, Sham). In mice that were both burned and infected (B/I) there were changes in gene expression in both the host and PA that were distinct from all other treatment conditions. These data suggest that the combination of the burned state and superimposed infection affects both host and pathogen gene expression, possibly to increase infection propensity. Gene expression significantly changed from 6-24 h post-B/I in each tissue. Finally, inhibiting IL-10 signaling or co-administering arginine at the time of PA infection prolonged or restored survival in an otherwise 100% fatal burn and infection model. These findings suggest that disease states such as burns may differentially alter innate immune response gene expression in both a host- and pathogen-specific manner.

#### **Importance**

The interaction between an underlying disease process and a specific pathogen may lead to the unique expression of genes that affect bacterial pathogenesis. These genes may not be observed during infection in the absence of, or with a different underlying process

or infection during the underlying process with a different pathogen. To test this hypothesis, Nanostring™ technology was used to compare gene expression in a murine burned wound infected with PA. The Nanostring™ probeset allowed the simultaneous direct comparison of immune response gene expression in both multiple host tissues and PA in conditions of burn alone, infection alone and burn with infection. Using Nanostring™, arginine and IL-10 were identified as important contributors to the lethal outcome of burned mice infected with PA. While other examples of altered gene expression are in the literature, our study suggests that a more systematic comparison of gene expression in various underlying diseases during infection with specific bacterial pathogens may lead to the identification of unique host-pathogen interactions and result in more precise therapeutic interventions.

### **Introduction**

Burns are a leading cause of injury and mortality worldwide. Globally, in 2017, there were almost 9 million new burn injuries due to flame, heat, or hot substances with ~121,000 reported deaths [132]. A study investigating burn patient outcomes identified flames as the second most common burn source, occurring in 44% of all patients surveyed [149]. Another found flame burns to be the cause of 58-66% of all burns surveyed [131]. While these patients enter treatment for burn injury, patients that succumb die from complications of infection such as shock and organ failure [182]. The most common bacterial pathogens that burn patients encounter include *Staphylococcus aureus*, *Pseudomonas aeruginosa* (PA), and *Acinetobacter baumannii* [13, 35]. PA is a ubiquitous, opportunistic pathogen that causes severe infections in certain patients, such as those with cystic fibrosis, oncology patients, or those with burns [183].

Patients become infected with an opportunistic pathogen due to an impaired or failed immune response. Many models of burn injury use different burn modalities (*e.g.*, scald, contact, chemical) or a model that results in mortality without infection. Under these conditions, it may be difficult to evaluate the effect of the burn itself on the immune response. A 10% total body surface area (TBSA) non-lethal flame burn was used to study the immune response to burns and subsequent susceptibility to infection. In our previous study [172], when burned mice were infected with  $10^6$  CFU of PA, 100% of the mice succumbed between 24 and 36 h post-burn and infection. Further, the LD<sub>50</sub> in burned mice was reduced 1000-fold from what was seen in mice that were not burned, suggesting a burn-induced defect in the immune response. Death after burn and infection was due to active infection by PA. Inoculating burned mice with heat-killed PA or PA lipopolysaccharide (LPS) resulted in no change in mortality. Consequently, the hypothesis was that there were changes in expression in both the host and PA that affected survival in burned and infected mice.

In this study, NanoString™ technology was used to simultaneously investigate gene expression in mice and PA post-burn. This technology allows for custom panels of up to 800 gene probes. Our panel consisted of a more manageable number including relevant host and pathogen genes (Appendix Table 6-1 to 6-3), selected in collaboration with experts in immunology and microbiology, which gave us the opportunity to directly observe gene changes concurrently in both *Mus musculus* and *Pseudomonas aeruginosa* in each sample. NanoString™ was chosen to investigate host immune gene expression dynamics while simultaneously observing growth, virulence, and quorum sensing genes in PA. It is sensitive enough to measure the PA genes, which would be relatively fewer in

count than *M. musculus*. Samples were collected from the blood, liver, spleen, and skin at the burn site because we previously determined that these are sites of dissemination for PA at approximately 12 h post-burn [172].

Using this probeset panel, the expression responses of Sham mice (mice that were clipped, received anesthesia and rehydration, but were neither burned nor infected) were compared to those of mice that were burned only (Burn), and mice that were infected only (Infection) as reference conditions. These conditions were used to determine if mice that were both burned and infected (B/I) had unique gene expression in response to this combined treatment. Tissues were sampled at multiple time points post-B/I to determine if and how gene expression changed over time in both host and pathogen. Significantly, unique changes in gene expression of B/I mice were identified and subsequent experiments were designed to assess the efficacy of interventions that targeted these gene products.

## **Results**

### **Gene expression was modified by each treatment individually**

Tissue samples from Burn mice and Infection mice were harvested at 12 h post-treatment, the time at which clinical changes were first observed in the B/I mice. In addition, the B/I mice had tissues harvested from 6-24 h post-treatment to assess changes over time. Volcano plots were utilized to display the magnitude and significance of gene expression changes when compared to a base condition (*i.e.*, 12 h B/I vs. Sham was the gene expression at 12 h in mice that were burned and infected over the gene expression of Sham mice). The genes whose expression was significantly altered are shown above the dotted line at  $y=1.3$  ( $-\log_{10}$  of 0.05); significance was determined using a two-way ANOVA or a mixed model test. Genes to the left of the dotted line at  $x=0$  were down-regulated when

compared to the reference condition, while genes to the right were up-regulated. *M. musculus* genes are indicated by circles and PA genes by triangles. Significantly modulated genes are indicated by open symbols.

Compared to the same tissues in the Sham mice, the four tissues differed in response to a burn injury (Figure 3-1). Overall, more genes were up-regulated in the skin (Figure 3-1a) and blood (Figure 3-1d) in response to a Burn, while the liver and spleen had more significantly down-regulated genes (Figure 3-1g, j). The skin from mice that were Burned alone had a high number of significantly up-regulated genes (Figure 3-1a). The blood was the least affected, with increased expression of only *Tlr2* and *Cxcr2* (Figure 3-1d). In the spleen and liver, the overall pattern was down-regulation of gene expression (Figure 3-1g, j).

### **B/I condition impacts each tissue differentially and significantly**

As was observed in the Burn condition, the Infection condition caused increased up-regulation of host genes the skin (Figure 3-1b) and blood (Figure 3-1e) while the liver and spleen had more down-regulation (Figure 3-1h, k). The skin and blood showed an increase in the expression of pro-inflammatory mediators and neutrophil chemokine genes in response to PA infection over the Sham condition. The liver (Figure 3-1h) and spleen (Figure 3-1k), however, were less affected. From these figures, both the Burn condition and the Infection condition impacted gene expression differently.

**Figure 3-1 Change in gene expression in the skin in Burn alone, Infection alone, or B/I conditions compared to Sham.**

Mice (n=5) were either subjected to a non-lethal 10% total body surface area (TBSA) burn (Burn alone), infected s.c. with  $1 \times 10^6$  CFU/mL of *Pseudomonas aeruginosa* strain M2 (IATS O5) in the absence of burn (Infection alone) or were both burned and infected (B/I). Sham mice had fur clipped and received anesthesia but were neither burned nor infected. All samples were collected at 12 h post-Burn or Infection and were analyzed using NanoString™. Counts were transformed to the  $\log_2$ . Ratios were calculated by subtracting the geometric mean of Sham samples from the geometric mean of Burn, Infection, or B/I 12 h samples. Significance was determined using a two-way ANOVA and Tukey's test. The  $-\log_{10}$  of the p-value is plotted on the y-axis, with the limit of significance indicated by the dotted line at  $y=1.3$  ( $p=0.05$ ). Significant host genes above this line are indicated with an open circle and PA genes are an open triangle. Genes to the left of the vertical line at  $x=0$  were down-regulated and genes to the right were up-regulated. The closed symbols below the dotted line represent host and PA genes respectively in the probeset that were not significantly altered. The pattern of gene expression when compared to Sham differed in Burn, Infection, and B/I conditions. (a-c) Skin (d-f) Blood (g-i) Liver (j-l) Spleen.



When visualizing the B/I condition, the previously observed patterns in each of the tissues of the Burn only and Infection only conditions were not maintained. There were both significantly up- and down-regulated genes at 12 h in the skin (Figure 3-1c). The PA gene, *oprL*, was only significantly up-regulated in the B/I condition (Figure 3-1c). The tissues distal to the burn site were also differentially altered by the B/I condition. There were both significant up- and down-regulation changes in the blood, spleen, and liver (Figures 3-1f, i, l).

### **The B/I condition affects unique genes in all tissues**

The Burn only and Infection only samples were collected at 12 h post-treatment, when clinical symptoms for B/I mice first appeared, which allowed for direct comparison with samples collected at 12 h post-B/I. To compare the three conditions, genes significantly up and down-regulated in relation to Sham (*e.g.*,  $p\text{-value} \leq 0.05$ ) were analyzed by the Venny 2.1.0 program. To confirm if the B/I condition impacted gene regulation differently than it did for Burn alone or Infection alone, Venny 2.1.0 analysis was used to create groups of similarly expressed genes that are identified in Table 3-1. Down-regulated genes are indicated in bold and up-regulated genes are not in bold; host genes are normal text, and the only PA gene is underlined.

	(a) Present in Infection Only	(b) Present in Burn Only	(c) Present in B/I 12 h Only	(d) Present in Both Burn Only and Infection Only	(e) Present in Both Burn Only and B/I 12 h	(f) Present in Both Infection Only and B/I 12 h	(g) Present in Burn, Infection, and B/I 12 h
<b>Blood</b>	<i>Il1b</i> , <i>Traf3</i> , <i>Myd88</i> , <i>Cd44</i> , <b><i>Fcgrt</i></b>		<b><i>Tbp</i></b> , <b><i>Traf5</i></b> , <b><i>Cd40</i></b>		<i>Cxcr2</i>	<i>Tlr4</i> , <i>Chil3</i>	<i>Tlr2</i>
<b>Liver</b>		<b><i>Cxcl10</i></b>	<i>Tlr2</i> , <i>Nos2</i> , <i>Myd88</i> , <i>Il6</i> , <i>Ccl2</i> , <b><i>Traf4</i></b> , <i>Irak3</i> , <i>Ddx58</i> , <b><i>Traf5</i></b>		<i>Irak2(1)</i> , <i>Cxcl1</i>	<b><i>Il18</i></b>	<b><i>Abl1</i></b>
<b>Spleen</b>	<b><i>Traf6</i></b> , <b><i>Nod2</i></b> , <b><i>Cxcr5</i></b>	<b><i>Il6</i></b> , <b><i>Irak3</i></b> , <b><i>C8a</i></b>	<i>Cxcl1</i> , <i>Il4</i> , <b><i>Irak2</i></b> , <b><i>Rag2</i></b> , <b><i>Irak1</i></b>	<b><i>Fcgrt</i></b>		<b><i>Abl1</i></b>	
<b>Skin</b>	<i>Il10</i> , <i>Rag2</i>	<i>Il4</i> , <i>Fcer1a</i> , <i>Myd88</i> , <i>Ccl2</i> , <i>Cd44</i> , <i>Polr1b</i> , <b><i>Traf4</i></b> , <i>Tlr2</i> , <i>Nos2</i> , <i>Cxcr5</i> , <i>Rag1</i> , <i>Cxcl10</i> , <i>Fasl</i>	<b><i>Retnla</i></b> , <b><i>Irak2</i></b> , <b><i>C3</i></b> , <b><i>Clqa</i></b> , <b><i>Pparg</i></b> , <b><i>Il18</i></b> , <b><i>Fcer1g</i></b> , <b><i>Traf3</i></b> , <b><i>Fcgr2b</i></b> , <b><i>Pecam1</i></b> , <b><i>Ddx58</i></b> , <b><i>Fcgr3</i></b> , <u><i>oprL</i></u> , <b><i>Gapdh</i></b> , <b><i>Tgfb1</i></b> , <b><i>Irak1</i></b> , <b><i>Traf2</i></b> , <b><i>Tlr4</i></b> , <b><i>Casp8</i></b>		<i>Chil3</i> , <i>Cxcr2</i> , <i>Il6</i> , <i>Ptgs2</i> , <i>Tnf</i> , <b><i>Abl1</i></b> , <b><i>Fcgrt</i></b> , <b><i>Cxcl1</i></b> , <b><i>Igf2r</i></b>	<b><i>Traf5</i></b>	<i>Fcgr4</i> , <i>Il1b</i>

**Table 3-1 Shared significant gene changes in each condition reference to Sham.**

Significant changes in gene expression from each tissue were analyzed using Venny 2.1.0. Genes that were significantly expressed in relation to Sham mice were input into the software with the groupings of Burn, Infection, or B/I 12 h. The software then searched for genes that were shared among the groups. Columns are arranged to show genes that were similar within the groups (e.g. *Fcgrt* was significantly down-regulated in both the Burn alone (column b) and Infection alone (column a) [but not in the B/I condition -column c] conditions in the spleen). Down-regulated genes are in Bold; the only PA gene is underlined in the skin-column c. Each tissue had gene expression changes in the B/I condition that were not observed in Burn or Infection conditions. In each tissue, there were also more genes significantly impacted solely by the B/I condition (c) that were not affected by Burn alone (b) or Infection alone (a).

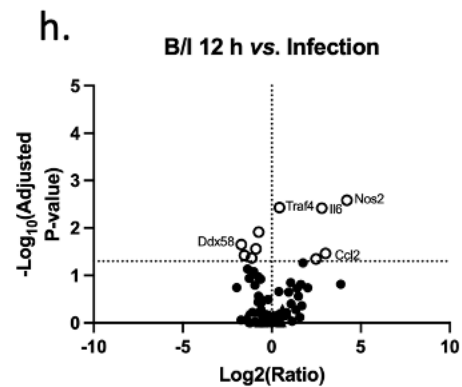
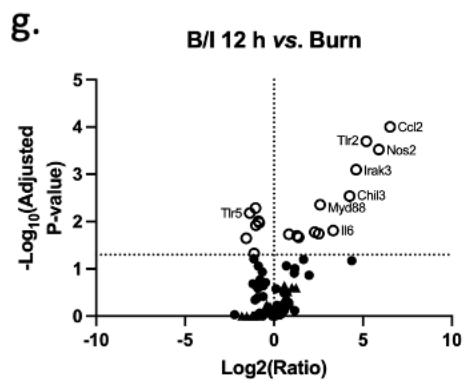
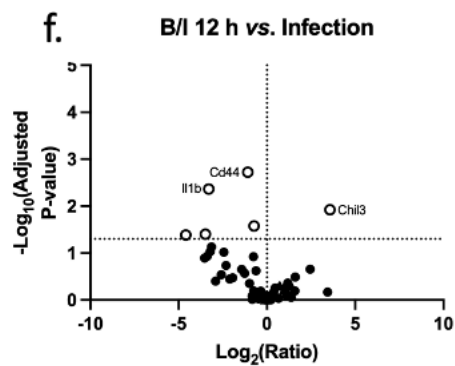
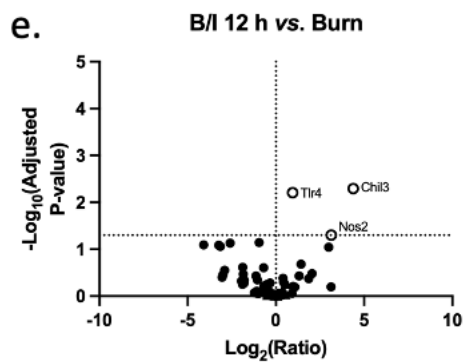
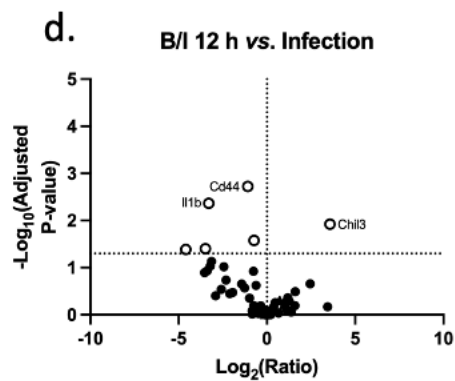
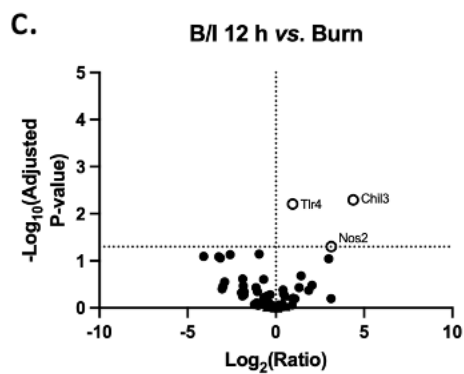
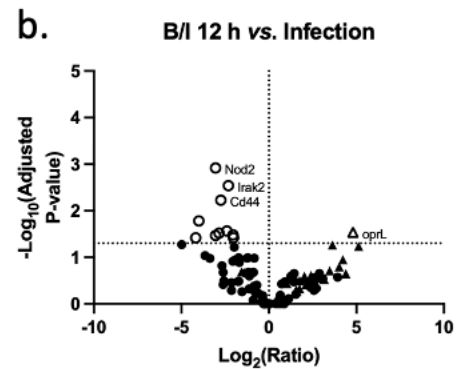
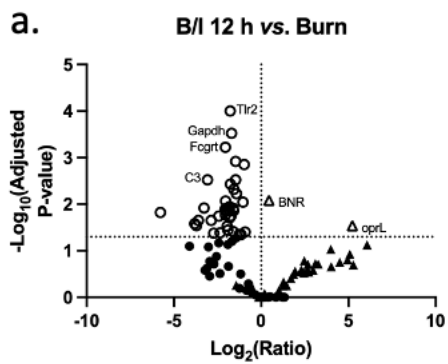
In each tissue, there were genes significantly and uniquely affected by the B/I condition (Table 3-1). While in some instances the same genes were similarly expressed in all three conditions, the overall expression patterns appeared to differ in the three conditions and in all four tissues. Specifically, in the skin there were two genes, *Fcgr4* and *Il1b*, that were commonly up-regulated under all conditions (Table 3-1g). Infection caused only two uniquely up-regulated genes, *Il10* (IL-10) and *Rag2* (Table 3-1a). There were several genes that were up-regulated and one down-regulated in response to Burn only (Table 3-1b). In contrast, the B/I condition caused one uniquely up-regulated and 18 uniquely down-regulated genes in the skin (Table 3-1c). The uniquely up-regulated gene in the B/I condition was a PA gene, *oprL*, which is a peptidoglycan-associated lipoprotein (Table 3-1c). In the blood there were three significantly down-regulated genes in the B/I condition and one gene commonly up-regulated in all three conditions, *Tlr2* (Table 3-1g). The liver and spleen followed the same pattern, with the B/I condition changing expression in the highest number of genes, nine and five, respectively (Table 3-1c).

To determine the magnitude of gene expression in relation to the Burn alone and Infection alone conditions, gene expression in the skin in the B/I condition was compared to both the Burn alone and Infection alone conditions in volcano plots. When compared to Burn alone, there were no significantly increased host genes at 12 h B/I (Figure 3-2a). There was, however, decreased expression of numerous genes in the host panel. At 12 h B/I, PA had significantly up-regulated *oprL* when compared to the Burn condition (Figure 3-2a). In reference to Infection, the B/I condition had down-regulation of several inflammatory mediator genes. In sum, both the host and pathogen had altered gene expression in the B/I condition (Figure 3-2a). When using Burn or Infection conditions as

the baseline, the B/I condition had uniquely and significantly impacted genes in all tissues in the host (Figure 3-2).

**Figure 3-2 Change in gene expression in the skin in the B/I condition compared to Burn alone or Infection alone condition at 12 h.**

When calculating the gene change ratio, either Burn or Infection alone gene counts were used as the baseline. The figures are oriented as previously described in Figure 1. In comparison to both reference conditions, the B/I condition resulted in a high number of down-regulated host genes in the skin (a-b). In fact, the only up-regulated gene in the B/I condition when compared to Burn and Infection alone are the PA genes, *oprL*. (c-d) Blood (e-f) Liver (g-h) Spleen



### **Kinetics of gene expression changes over time in the B/I condition**

In the B/I condition, the profile of gene expression in the skin changed over time, as visualized in the heat map (Figure 3-3). The heat map expresses the ratio of  $\log_2$  of gene expression over the geometric mean of the five Sham condition samples. Above the horizontal white line are *Mus musculus* genes while *Pseudomonas aeruginosa* genes are depicted below. Samples were obtained over time from 6-24 h (left to right). Each column represents one mouse. The map shows a generalized period of quiescence from 6-12 h in both the host and PA. At 12 h post-B/I, when clinical symptoms first appeared, there was a predominate down-regulation of genes in the host, while PA exhibited an increase in gene expression. This can be more clearly visualized with volcano plots (Figure 3-4).

#### **Figure 3-3 Gene ratios in the skin over time in the B/I condition.**

A heatmap illustrates how gene ratios changed over time. The ratio was calculated as the gene expression in B/I mice compared to the Sham condition. Each column on the heatmap is the sample collected from one mouse. The columns are organized increasing in time from left to right, starting at 6 h post-B/I and ending at 24 h. Each row is an interrogated gene in the Nanostring™ panel. (Table 6-1 to 6-3); Genes above the horizontal white line are from *M. musculus* and below are from PA. An increase in ratio is indicated in red and a decrease in green. The figure qualitatively suggests that after a period of relative quiescence from 6-12 h post-B/I, there is a clear divergence in expression patterns between the host and the pathogen: there is a global decrease in host gene expression and an increase in pathogen gene expression from 12-24 h.

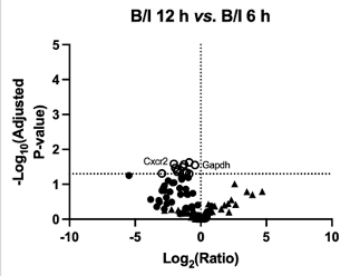


To determine the magnitude of gene change over time, gene count ratios and significance were calculated with the B/I 6 h time point as the baseline. *M. musculus* genes that were significantly modulated are indicated by an open circle while significantly changed PA genes are shown as open triangles. At B/I 12 h, several host genes significantly decreased in expression (Figure 3-4a). At 18 h, a pattern emerged, mirroring the observations in the heatmap (Figure 3-4b). The significantly up-regulated genes were solely the PA genes, including the LPS synthesis gene *lpxC* and toxin gene *exoT*; the number of host genes that were significantly down-regulated increased. This trend continued through 24 h (Figure 3-4c). The changes in gene expression in the skin under the B/I condition were significantly different as time progressed, with a decrease in host gene expression simultaneously with an increase in pathogen gene expression. In all tissues, the significant gene expression patterns differed over time (Figure 3-4).

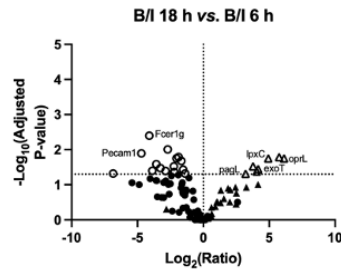
**Figure 3-4 Gene expression in the skin over time in the B/I condition using gene expression at B/I 6 h as baseline.**

Gene ratios calculated using the geometric mean at 6 h post-B/I subtracted from the geometric mean of gene expression at 12 h, 18 h, and 24 h post-B/I. Statistical significance was determined using a 2-way ANOVA. Figures are oriented as previously described. As the time of skin (a-c) harvest increased, there were more host genes down-regulated (shift to left) and more PA genes up-regulated (shift to right). (d-f) Blood, (g-i) Liver, (j-l) Spleen.

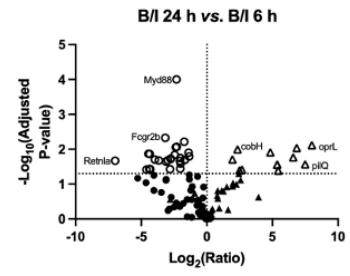
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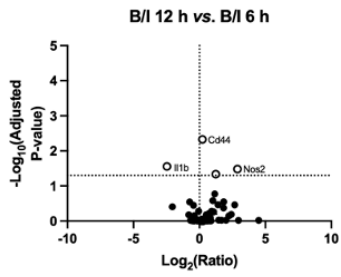
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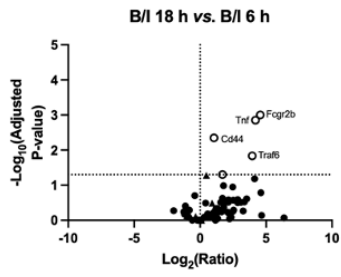
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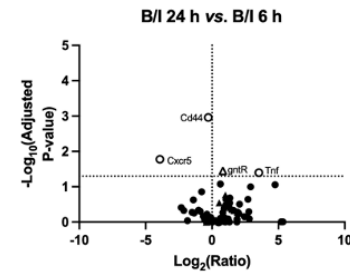
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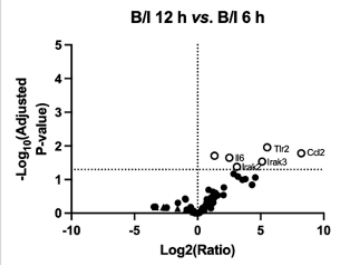
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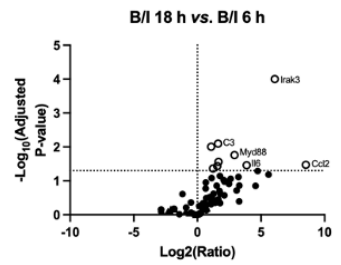
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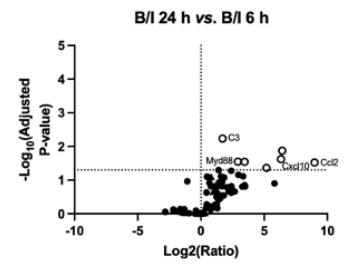
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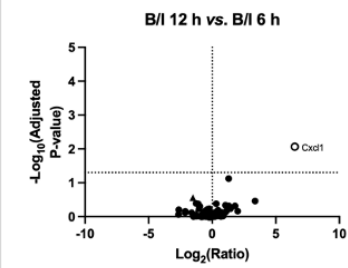
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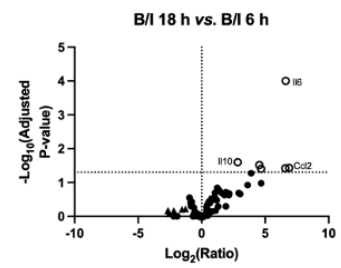
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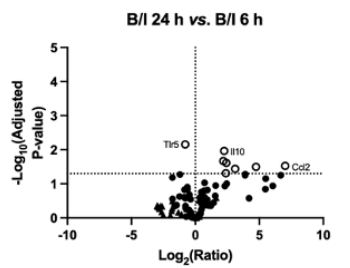
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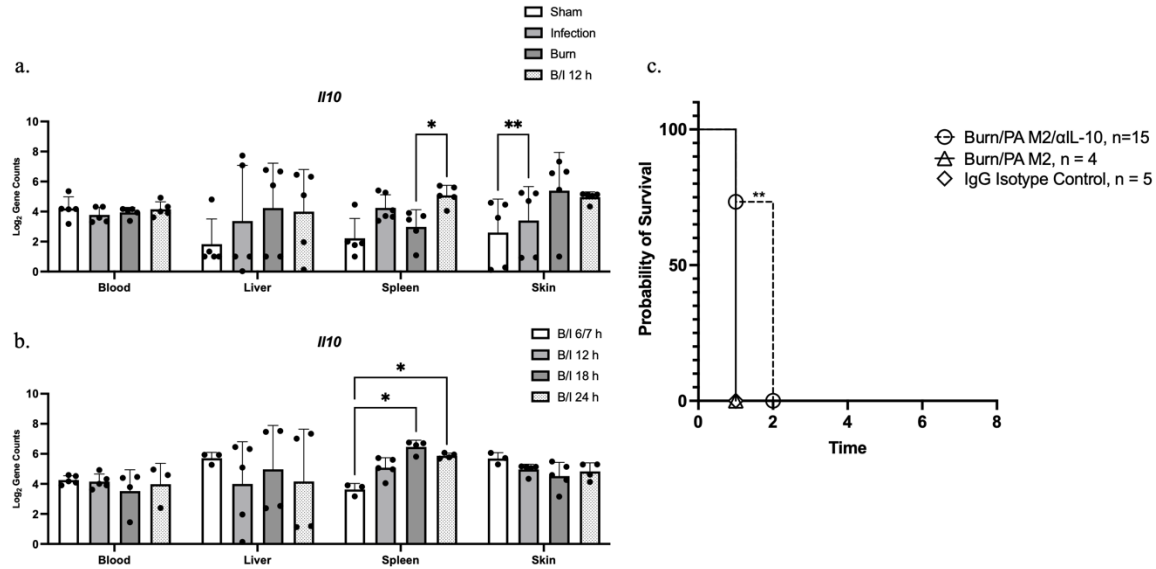
l.



## Neutralizing IL-10 prolongs survival

Significant gene expression was investigated to identify host gene transcripts that were impacted by B/I and determine if they exerted a role in the observed decrease in host defense post-burn. IL-10 is an anti-inflammatory mediator primarily produced by monocytes [86]. It inhibits cytokine production in the Th1 response [86]. Previous work in our laboratory showed that in this model, IL-10 protein was found circulating in the blood of mice at 12 h post-B/I, peaking at 18 h, and persisting until death [172]. A transient increase in infection susceptibility was also observed, with mice succumbing between 20- and 30-h post-B/I [172]. In the spleen, there was a significant increase at 12 h in expression of *Il10* in the B/I condition compared to the Burn alone condition (Figure 3-5a) and *Il10* expression significantly increased from 6-24 h (Figure 3-5b). Importantly, there was no significant change in expression in the blood, suggesting that the source of IL-10 protein previously observed was from distal tissues such as the skin or spleen.

Based on significant expression of *Il10* in the spleen, this gene was selected for inhibition to determine its impact on survival. Since the significant increase in expression began in the spleen at 12 h post-B/I, the neutralizing anti-IL-10 antibodies were delivered i.p. at 12 h post-B/I. Mortality was significantly delayed in anti-IL-10-treated mice by one day (Day 1 Survival: No Antibody 0/4, Antibody 11/15,  $p=0.0102$ , Log Rank Mantel-Cox) (Figure 3-5c). An additional dose at 24 h post-B/I, doubling the initial dose of antibody or use of an irrelevant isotype control monoclonal antibody did not enhance survival or further delay death (data not shown). It is likely that the sustained production of IL-10 by the tissues overwhelmed the antibody treatment.



**Figure 3-5 IL-10 neutralizing antibody prolonged survival.**

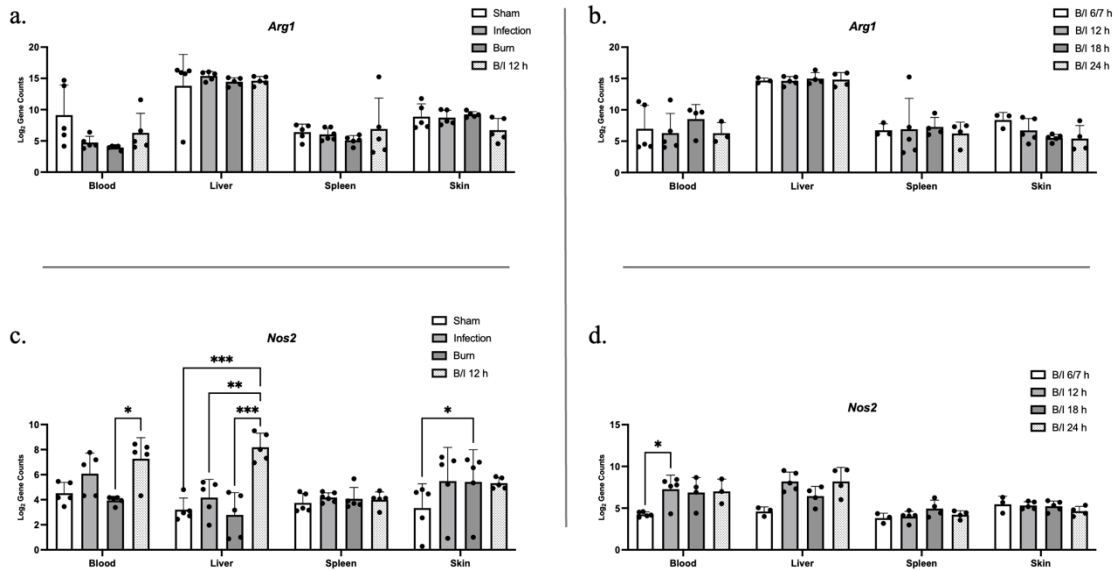
The bars represent the mean of  $\text{Log}_2$  of *Il10* gene transcript counts in each tissue and each dot represents an individual mouse. Error bars indicate standard deviation. Significance was determined using an REML mixed model [16]. (a) The Infection, Burn, and B/I gene counts are at 12 h post-treatment. There is significant expression of *Il10* at in the spleen in the B/I condition compared to the Burn condition. (b) Gene expression in each tissue over time in the B/I condition. Expression of *Il10* is significantly increased at 18 and 24 h post-B/I in the spleen. (c) Kaplan-Meier plot of survival of mice that were administered anti-IL-10 neutralizing monoclonal antibody. Significance was measured using a Log Rank Mantel-Cox test. Antibody was administered i.p. at 12 h post-B/I; mice had prolonged survival of one day (Day 1: No  $\alpha$ -IL-10 0/4 vs.  $\alpha$ -IL-10 11/15). For panels a-c: \*  $p < 0.05$ , \*\*  $p < 0.01$ .

### Site of arginine administration impacts mortality

Arginine is an amino acid utilized in the host immune response. Depending on the activating immune environment, arginine can be metabolized in two ways: either by arginase (*Arg1*) in M2 macrophages in response to a Th2 immune response, or by inducible nitric oxide synthase (iNOS; *Nos2*) produced by M1 macrophages during a Th1 response [184]. Arginase, whose expression is a marker for M2 macrophages [91, 185, 186], is a key component of the resolution of inflammatory response and tissue repair. If arginase predominates in this system, arginine is metabolized into precursors for collagen, which aids in the wound healing process [184]. In a pro-inflammatory environment, iNOS converts arginine to produce nitric oxide, which is bactericidal. Nitric oxide inhibits arginase activity and collagen precursors inhibit iNOS [184].

Arginine also plays a role in the pathogenesis of PA post-burn. It can serve as a carbon source under anaerobic conditions and PA grown on arginine-enriched plates have decreased motility *in vitro* [177]. In the same study, administering arginine s.c. at the burn site at the time of burn and infection *in vivo* increased survival [177]. If arginase or iNOS expression were high, it would diminish the amount of available arginine to inhibit the motility, and therefore promote dissemination of PA.

High expression of *Arg1* would be expected to result in depletion of available arginine and promote PA dissemination; however, arginase gene expression was not significantly increased in any tissue or conditions (Figure 3-6 a, b). *Nos2* expression, however, was significantly increased in the liver and blood of the B/I condition at 12 h (Figure 3-6c) and increased from 6 to 12 h post-B/I in the blood (Figure 3-6d). The expression in the skin in the B/I condition was not significantly impacted (Figure 3-6c, d). If arginine levels were high in the immediate burn environment, a decrease in PA dissemination would be predicted. Therefore, we hypothesized that the effect of arginine on survival in B/I mice would be due to its co-localization with PA and its effect on motility, rather than the tissue expression of *Arg1* and *Nos2*.



**Figure 3-6 Changes in gene expression of *Arg1* and *Nos2* in each condition and over time in the B/I condition.**

The bars represent the mean of the Log<sub>2</sub> of *Arg1* and *Nos2* gene transcript counts in each tissue and each dot represents an individual mouse. Error bars indicate standard deviation. Significance was determined using an REML mixed model [16]. There was no significant increased expression of *Arg1* in any treatment condition (a) or any significant changes in expression over time in the B/I condition (b) in any tissues. *Nos2* was significantly expressed in the liver and blood in the B/I condition (c), but not in the skin. Its expression increased from 6-12 h in the blood (d). \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

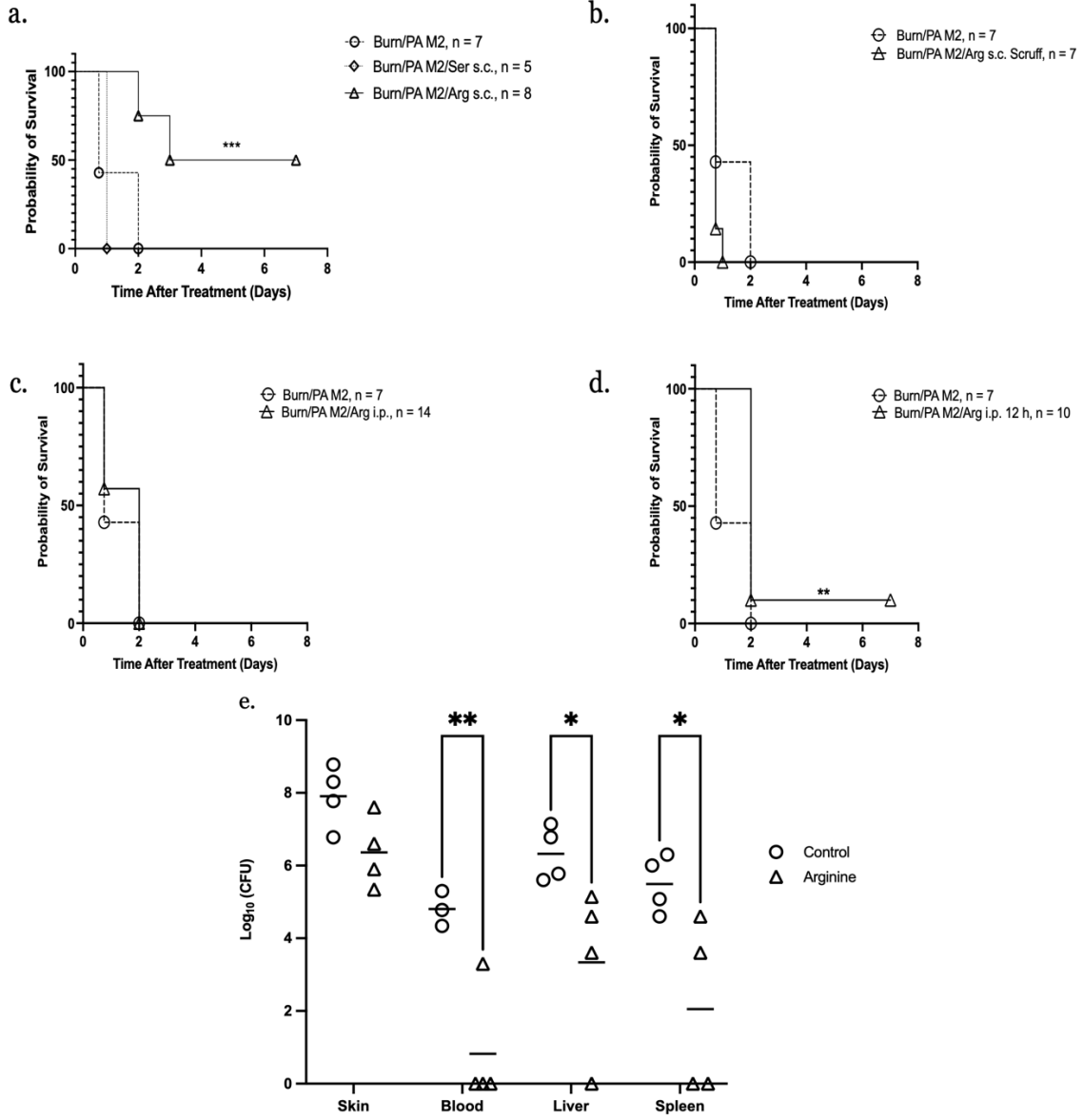
To test this hypothesis, 0.125 g/mL of arginine was administered immediately post-burn either s.c. or i.p. to determine if it affected host responses. The s.c. doses were given either concurrently with PA at the burn site or superior to the burn site, and distal to the PA, at the scruff of the neck of the mouse. The i.p. dose was to evaluate the effect of systemic arginine. These sites were chosen to determine the relationship between the location of arginine and PA dissemination. The mice were then monitored for survival. All statistics in the survival plots were calculated using the Log Rank Mantel-Cox test.

Delivering arginine s.c., concurrently with PA infection, post-burn at the burn site resulted in 50% of mice surviving when compared to B/I alone (No Arginine 0/7 vs. Arginine 4/8, p=0.0013, Log Rank Mantel-Cox) (Figure 3-7a). This appeared to be a local effect on the pathogen, as administering arginine distally in the scruff of the neck immediately following B/I caused the mice to succumb sooner (No Arginine 0/7 vs.

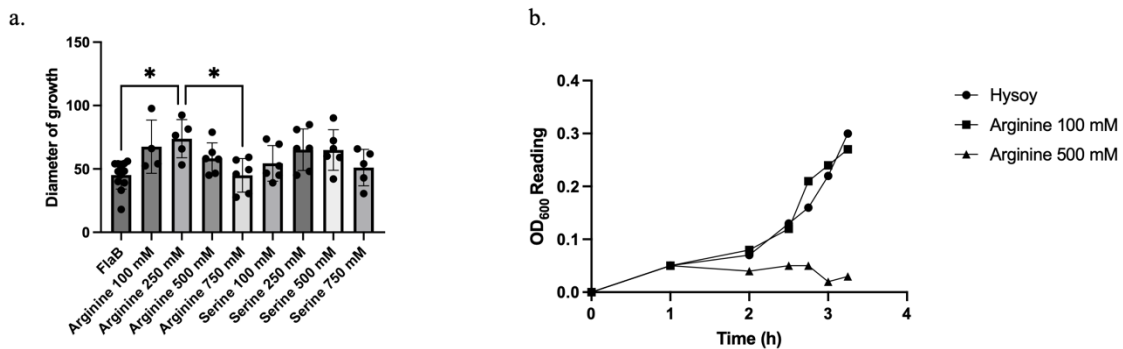
Arginine 0/7) (Figure 3-7b). Arginine was also delivered systemically via i.p. injection immediately post-B/I, but, here, too, there was no impact on mortality (No Arginine 0/7 vs. Arginine 0/14) (Figure 3-7c). Based on previous data showing the presence of PA in the blood at 12 h post-B/I, arginine was administered i.p. at 12 h post-B/I and observed prolonged survival at day 1 in the arginine-treated mice (No Arginine 3/7 vs. Arginine 10/10) and that one of the mice even survived to day 7,  $p=0.0085$  (Figure 3-7d). Administering a similar dose of serine s.c. at the burn site at the time of infection did not impact survival (Figure 3-7a).

**Figure 3-7 Arginine restored survival in B/I mice and decreased dissemination.**

Mice were administered 200  $\mu\text{L}$  of 0.125 g/mL of arginine or serine immediately or 12 h post-B/I; the number of mice in each treatment is indicated in the legend. Figures represent combined data from 2-3 individual experiments. (a) Arginine, serine, or PBS was mixed with the PA inoculum and immediately delivered s.c. at the burn site. (b) Arginine was administered s.c. in the scruff of the neck, distal to the burn and infection site, immediately post-B/I. (c) To measure systemic effects, arginine was administered i.p. immediately post-B/I. (d) Arginine was administered i.p. 12 h post-B/I. Arginine restored survival to mice when administered s.c. immediately post-B/I and i.p. 12 h post-B/I (Day 1: No Arginine 3/7 vs. Arginine 10/10). One mouse survived for seven days. (e) Mice were burned and either infected s.c. with PA in PBS or PA in arginine. At 24 h post-B/I, skin, liver, spleen, and blood samples were homogenized in PBS, serially diluted, and plated on TSA agar plates. After overnight incubation at 30° C, individual colonies were counted, and log transformed. Significance was determined using an REML mixed model [16]. Although there was active infection in the skin of all mice, there was a significant decrease in dissemination to distal tissues in mice that received arginine. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



To determine if the delayed deaths were due to decreased PA dissemination, arginine was administered s.c. at the burn site immediately post-B/I and measured bacterial burden in tissue samples (0.025 g or 100 uL) at 24 h post-treatment. While there was still active infection at the burn site in all mice at 24 h, arginine induced a significant decrease in dissemination of the organism to organs (Fig 3-7e). In some mice, there were no CFUs found in distal tissues. Administering arginine s.c. at the burn site at the time of infection decreased dissemination of PA and increased survival in 50% of mice, suggesting that supplemental arginine in the skin B/I environment decreased dissemination of PA to distal tissues. We speculate that the arginine effect in our studies was due to its local effect on PA, impacting motility (Figure 3-8a) and inhibiting growth (Figure 3-8b), rather than acting on a host defense response.



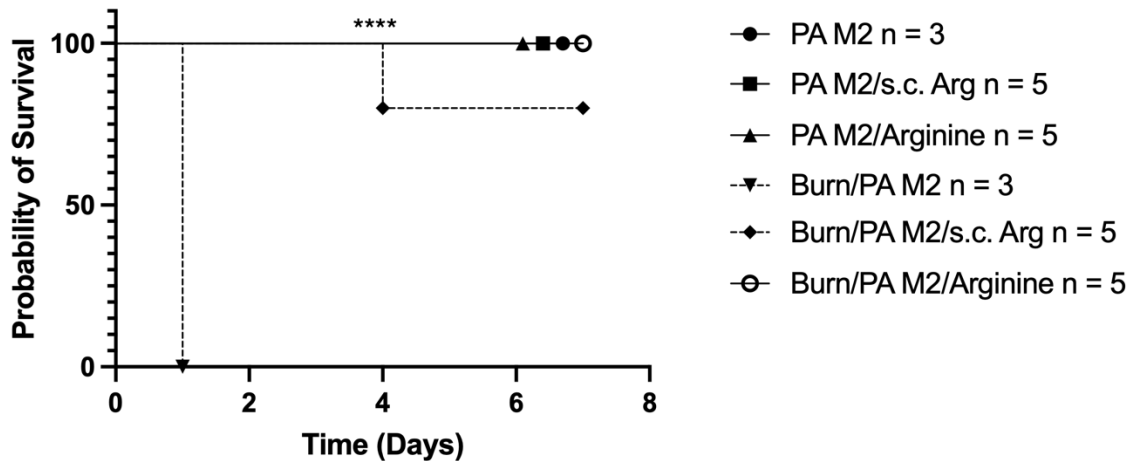
**Figure 3-8 Arginine inhibits motility and growth of *Pseudomonas aeruginosa*.**

(a) PA was grown in Tryptic Soy Agar (TSA) wells supplemented with rabbit sera with FlaB antibodies (FlaB) or increasing doses (100-750 mM) of arginine or serine diluted in normal rabbit serum. PA had high motility at 250 mM which decreased in a dose-dependent manner at 750 mM. Serine did not have this effect. (b) PA grown in the bacterial culture medium, Hy-Soy Broth, supplemented with 100mM or 500mM of arginine. Spectrophotometer readings were taken at OD<sub>600</sub> to measure PA growth. The lower dose of arginine allowed growth at a rate comparable to that in Hy-Soy Broth, however the higher dose inhibited growth. \* p < 0.05, One-way ANOVA with multiple comparisons.

**Mice infected with *Pseudomonas aeruginosa* incubated with arginine are fully protected**

To test the hypothesis that the observed delay in death in mice that were administered arginine was due to its impact on PA motility, the same experiment as described above was repeated with the added variable of infection with PA that were incubated with the same dose of arginine for 1 h prior to the burn. The mice were then either infected with PA and given a s.c. dose of arginine (PA M2/s.c. Arg) or infected with PA from the incubation (PA M2/Arginine). This was conducted in Sham mice and mice that were burned. There were also control groups of mice that were administered PA without arginine (PA M2).

As previously observed, the untreated B/I mice (inverted triangle) succumbed at 24 h post-treatment, but the B/I mice that received s.c. arginine (diamond) had a high survival rate and delayed time to death (Figure 3-9). As expected, the mice that were infected but not burned had 100% survival as well (closed circle). Interestingly, the mice that were burned and infected with the arginine-incubated PA also exhibited 100% survival (open circle). These results indicate that survival in these mice was most likely due to direct interaction between PA and arginine.



**Figure 3-9 Burned mice infected with *Pseudomonas aeruginosa* incubated with arginine had 100% survival.**

Mice (+/- Burn) were infected with  $10^6$  CFU of PA M2, infected and administered 0.125 g/mL subcutaneously (PA M2/s.c. Arg) or infected with PA which had been incubated with 0.125 g/mL of arginine for 1 h (PA M2/Arginine). The mice were monitored for survival for 7 days. All mice that had not been burned survived (closed circle, square, and triangle). Mice that were burned and infected without arginine died by 24 hours (inverted triangle). Mice that received arginine s.c. at the time of B/I had prolonged survival (diamond). The burned mice infected with PA incubated with arginine (PA M2/Arginine) had 100% survival (open circle). \*\*\*\*  $p < 0.0001$

## Discussion

The goal of the present study was to determine if our previously observed host/pathogen interactions were due to changes in gene expression, in either the host or PA, that may explain the increased mortality in mice that were burned and infected. Gene expression in the host was impacted by both burn alone and infection alone. When done concurrently, however, a burn and infection caused changes in gene expression in each host tissue interrogated that were not seen in the other conditions. Further, in burned and infected animals, there were genes that were significantly up-regulated in PA that were not up-regulated during an infection alone. This shows that the burned state of the skin impacted gene expression not only in the host, but also in PA. The combination of burn and PA infection significantly and uniquely impacted both the host and the pathogen at the level of gene expression.

We previously hypothesized that the skin served as a reservoir for PA growth and dissemination. Our NanoString™ data support this hypothesis. Over time, there was a global down-regulation of host genes in the skin. This could not be attributed to dead cells: while the epidermis and dermis were destroyed in our burn model, the underlying support structures remained intact [174]. When B/I skin was H&E stained, there was an increase in the number of recruited lymphocytes in comparison to Burn only skin [174]. This suggests that the changes in gene expression in the host skin were due to cells that were recruited to the burn site, such as neutrophils and monocytes. Further, in the Burn alone and Infection alone conditions, there was significant up-regulation of host genes at 12 h, but when the B/I condition was compared to the Burn or Infection alone conditions, expression of these same host genes were significantly decreased in expression. PA had one gene significantly expressed in reference to Sham, *oprL*, which is an outer membrane bound lipoprotein. Significant expression of *oprL* at 12 hours post-B/I support the hypothesis that the increase in transcripts is likely due to an increase in total number of PA rather than an upregulation of gene expression in individual bacteria. Because of its role in peptidoglycan synthesis, the *opr* operon has been utilized as a vaccine target [187-189].

When investigating the changes in gene expression in the skin over time, gene expression of the pathogen can be better observed. Using B/I 6 h as the baseline reference point for PA gene expression, at 18 hours there was a significantly increased expression of *lpxC* and *pagL* genes involved in altering the structure of LPS. This could result in immune evasion [18, 190]. As the B/I condition progressed over time, there was a significant upregulation of pili genes *pilC* and *pilQ*, protease *ftsH*, and toxin *exoT*, a virulence factor

shown to aid in dissemination of PA [32]. The *Pseudomonas aeruginosa* M2 strain lacked the *exoU* gene.

Previous studies using dual-RNASeq have shown that PA gene expression and kinetics vary based on the wound and disease type [16, 23, 191-196]. In models of pneumonia and cystic fibrosis, PA has been found to increase iron acquisition gene expression [16, 192]. In the comparison of gene expression in an acute scald burn wound and chronic surgical wound on the same animal, it was found that PA had greater gene expression in genes related to cell motility and cell envelope biogenesis in the burn wound than in the chronic wound [23]. Similar to our work, a study compared the transcriptome of PA grown in burn wound exudate (BWE) or in broth [193]. They found that there were genes uniquely altered in the BWE condition, similar to our B/I findings [193]. These works all show that PA and the host alter gene expression differently based on the various underlying conditions. We suggest that the relationships could be better studied and compared using a standardized NanoString™ panel. Understanding these specific host and pathogen gene changes, could result in more precise treatment for specific disease/infection states.

RNA was isolated from whole blood. The assumption was that any gene changes observed in the blood were due to circulating leukocytes since red blood cells and platelets do not have nuclei, although the possibility that these transcripts can be due to preformed mRNA or parenchymal cells in circulation because of the burn cannot be ignored. Changes in gene expression in the blood were minimal; there was a significant decrease in *Il1b* (IL-1 $\beta$ ) expression when comparing the B/I to the Infection condition at 12 h. This finding would support dissemination of PA. High circulating IL-1 $\beta$  levels have been positively

correlated with infectious disease severity [197-199]. Also, IL-1 $\beta$  is important in protection against infection [200]. The fact that *I1b* expression decreased in the B/I condition, and that circulating concentrations of IL-1 $\beta$  protein did not significantly increase post-B/I [172], indicates an inadequate immune response which may predispose the mice to bacterial dissemination and lethality. This can also account for, in part, the decreased LD<sub>50</sub> of PA infection post-burn.

When investigating the transiently increased infection susceptibility post-burn, we looked for significant gene expression that would contribute to an anti-inflammatory response. In previous work, IL-10 was elevated in the blood at 18 h post-burn and infection and remained elevated until the mice succumbed [172]. IL-10 is an autoregulatory cytokine released primarily by monocytes [86]. It inhibits the synthesis of cytokines in the Th1 response through activating STAT3, which is the expression factor for anti-inflammatory cytokines [86, 201]. It has been found in the circulation of trauma patients, in correlation with injury severity [202]. It has also been positively associated with post-traumatic infection susceptibility and mortality [38, 142, 202, 203]. While expression in the liver and spleen was not as impacted by the B/I condition as the skin, *I10* (IL-10) was expressed significantly in both distal tissues. Using anti-IL-10 neutralizing antibodies, neutralizing IL-10 post-B/I increased survival when administered 12 h post-B/I, which was the observed clinical onset of symptoms. Giving an additional dose at 24 h, or doubling the initial IL-10 dose, did not further increase survival, or extend mean time to death, possibly due to the continued expression of *I10* by the spleen. Higher and earlier doses of anti-IL-10 may be able to further improve survival in our burn model.

Another relationship investigated was the effect of arginine on infection susceptibility post-burn. In the host, arginase is an enzyme involved in the urea cycle in the liver and in arginine metabolism in immune cells [204]. Recruited macrophages alter the metabolism of arginine based on the surrounding cytokines [184, 186, 204]. Pro-inflammatory mediators cause an up-regulation of iNOS, resulting in nitric oxide production, which is bactericidal. In anti-inflammatory environments, arginase is up-regulated, which uses arginine to form precursors to collagen, thereby promoting wound healing [204]. The pathogen, PA is able to utilize arginine as a source of ATP in anaerobic conditions through the arginine deaminase pathway [205]. Arginine inhibited swarming motility in a dose-dependent manner and did so more significantly than other amino acids at the same dose [29]. It has also been associated with increased biofilm formation [28, 29, 206]. PA with an induced loss of swarming motility were found to have decreased dissemination to distal tissues [6, 24, 30].

Previous work in a non-lethal scald burn and infection model implicated arginine as a factor in PA pathogenesis post-burn [177]. It was shown to limit the motility of PA *in vitro* when grown on arginine-enriched agar [177]. Myeloid-derived suppressor cells (MDSCs) were hypothesized as the source of arginase activity, which would decrease the arginine availability and aid in PA dissemination.

In our studies, administering arginine i.p. immediately at the burn site or at 12 h post-B/I significantly prolonged and increased survival. This effect was location- and time-dependent, as administration of arginine in the scruff (superior to the burn site) or immediately after burn and infection i.p. did not improve survival. The arginine dose used also decreased dissemination of PA, likely due to an inhibition of growth and motility.

Further, administration of PA incubated for one h with arginine resulted in 100% survival of the mice. These results suggest that the PA and arginine must be co-located physically and temporally for the effect on motility and dissemination to be observed. The increased survival in our study is more likely due to the arginine/PA interaction rather than being attributed to the effect of arginine on host defenses. And while MDSCs express arginase [96, 207, 208], and have been shown to be recruited to a burn site by day 3 post-burn [209], the interaction between arginine and PA appeared to be more acute.

Our study had several limitations. We wanted to investigate specific pathways known to be affected by burn and infection alone, such as those involved with innate immunity, cell death, inflammatory mediators, and bacterial virulence factors [210-212]. While the use of NanoString™ allowed us to evaluate a custom panel of genes quickly and with high sensitivity, it introduced selection bias. RNA-Seq would remove selection bias through interrogating all transcripts over the threshold of sensitivity, however NanoString™ allowed us to analyze data without the need for steps such as alignment and assembly which RNASeq requires. Further, multi-species RNASeq requires additional adjustments in sample processing [213].

Since previous work has shown dissemination of PA in the blood, liver, and spleen post-burn [172], these sites were chosen for sample collection. In patients with burn wounds, positive PA cultures were found in skin, urine, sputum, and blood [34]. The most common cause of death post-burn is sepsis with multi-organ failure [33, 117, 131, 182]; patients who died from sepsis post-burn had symptoms of kidney and liver failure [144]. Consequently, there may not have been a complete picture of host/pathogen interactions by not sampling additional sites (*e.g.* kidneys).

While fold change can be calculated using housekeeping genes as the baseline, the housekeeping genes that were chosen, both for the host and the pathogen, were not consistently expressed, especially in the skin. Further, external variables such as carbon source and antibiotics can alter the expression of common housekeeping genes in PA [214, 215]. The variations in gene expression for the selected “housekeeping” genes were insufficient to serve as a basis of comparison. For these reasons, the individual genes at the indicated baseline conditions (Sham, Burn, etc.) were used as the baseline for ratio calculations.

Gene expression of this set of genes was impacted in a significantly different manner when mice were concurrently burned and infected with PA than if they were burned alone or infected alone. This was observed in both the host and the pathogen. If mice were dosed with anti-IL-10 or arginine at specific sites and timepoints, increased survival was observed. Future directions should include further optimization of the anti-IL-10 doses, determining if other anti-inflammatory mediators impact survival, and investigating if additional gene pathways are impacted by burn and infection.

Importantly, our work suggests that underlying disease states in combination with infection can alter gene expression both in the pathogen and host, which may affect outcomes. It may be that different disease states such as trauma or diabetes mellitus, while altering host gene expression in a disease-specific manner, like burn injury, also may impact the expression of genes in a pathogen-specific manner. The host environment may significantly alter components of the bacterial cell wall as may have occurred in our studies with an increase in *oprL* and *lpxC* which affect cell integrity and lipid A synthesis respectively. Further, pathogen gene expression in one disease state may differ from sets

of genes expressed in other disease states. If true, this introduces an entirely new complexity in studying host-pathogen interactions. While many papers similarly examine host and bacterial gene expression under various conditions, few have systematically compared specific gene expression under different disease states. Such a study could identify novel disease-associated host immune pathways that may be associated with specific pathogens. NanoString™ provides the opportunity to make these standardized observations. Such insights could lead to unique therapeutic interventions.

## **Chapter 4. Circulating Naïve Neutrophil Function Is Impaired in Response to Non-lethal Flame Burn**

### **Abstract**

In response to trauma and burns, patients experience a transient decrease in host defenses. This is mediated by a decrease in neutrophil counts and neutrophil function. In a non-lethal, 10% total burn surface area (TBSA) flame mouse model, increased mortality post-burn and infection with *Pseudomonas aeruginosa* (PA) has been observed. This is significant, as this TBSA in patients is not considered to be a severe burn and suggests an impaired host immune response. The presence of a seroma that forms beneath the burn wound, which is comprised of CD45<sup>+</sup> cells was previously reported. In the case of burn and infection, PA was found to be in close proximity to these cells, but was not phagocytosed, suggesting neutrophil dysfunction. In this study, phagocytosis in neutrophils isolated from both the circulation and seroma of burned mice had a decreased ability to kill PA compared to Sham mouse circulating neutrophils. Further, both Sham and burned mouse neutrophils completely lost the ability to kill when incubated with seroma fluid. In a separate assay, neutrophils in the seroma had a decreased ability to produce reactive oxygen species (ROS) when compared to neutrophils in circulation from the same mice. Sham neutrophils incubated with sera from burned mice and burned and infected mice, but not sera from Sham mice, significantly produce ROS at rest which may be correlated with the pro-inflammatory DAMP HMGB1, found in the sera of burned mice. These data suggest that a non-lethal burn is able to prematurely activate neutrophils while in circulation, reducing their functionality at the infected burn site. To conclude, burns that are not considered severe can prematurely activate circulating neutrophils, resulting in an

impaired immune response, increased infection propensity, and development of lethal sepsis.

## **Introduction**

Neutrophils are cells in the innate immune system that are critical to the resolution of bacterial infection. They utilize a variety of functions to accomplish this: production of reactive oxygen species (ROS), phagocytosis, neutrophil extracellular traps (NETs), and the formation of nitric oxide [99, 107, 109, 216]. Dysfunction of these cells has been implicated in sepsis, a dysregulated, systemic immune response [217-219].

A leading cause of mortality worldwide is burns, causing over 100,000 deaths annually [132]. Patients experiencing burn wounds are evaluated based on the depth and total body surface area (TBSA) involved in the injury. There is a positive correlation between the extent of TBSA and risk for mortality [33, 131, 134, 220]. Burn wounds also release danger-associated molecular patterns (DAMPs), such as mitochondrial DNA (mtDNA), High Mobility Group Box 1 (HMGB1), and S100A into circulation [67, 72, 74]. Increased levels of circulating DAMPs post-burn serve as a predictor of mortality [74, 153]. Specifically, HMGB1 is released into circulation post-burn at levels that bind to MD-2 and signal through TLR4 [172].

In burn patients, one of the most common causes of death is sepsis caused by the opportunistic pathogen *Pseudomonas aeruginosa* (PA) [33, 35, 182]. PA is a ubiquitous organism commonly found in the skin flora. While PA infections are less common in hosts with an intact immune system, it is able to establish infection in hosts with an impaired neutrophil response [10, 221-223]. Patients with chemotherapy-induced neutropenia or following burn injury are particularly prone to PA infection [14, 33-36, 173, 195].

To study the immune response to burns and the propensity for infection with PA, a 10% TBSA non-lethal, full-thickness murine burn model was used. In this model, mice immediately infected with PA post-burn experience 100% mortality within 36 hours [172]. These mice had a transiently decreased LD<sub>50</sub> (the dose of bacterium that causes 50% mortality); mice infected with the same PA challenge at 72 hours post-burn have restored survival [172]. There was also a collection of gelatinous fluid between the skin and dorsal fascia at the burn site [174]. This seroma appears at approximately 3 hours post-burn and begins to recede at approximately 18 hours [174]. There was an influx of CD45<sup>+</sup> cells, visualized with immunohistochemistry [174]. Collected seroma fluid was able to support the growth of multiple strains of PA comparable to HySoy broth [174]. With Gram stain, these cells were found near PA within the seroma [174]. Also within this model, while HMGB1 is released into circulation post-burn (approximately 50 ng/mL immediately post-burn, returning to baseline after 3 hours), a 10-fold increase from baseline following a burn and infection was observed, which steadily increased and remained elevated until death [172]. When burned and infected mice were administered P5779, which competes with HMGB1 for its binding site on MD-2, survival was restored [172].

These observations lead to the hypothesis that neutrophils may be experiencing early activation in circulation and arrive at the burn site “exhausted” or having utilized their prepared functions. Combined with the ability of the seroma fluid to support the continued growth of PA, these factors may contribute to the observed increased mortality in our model. To assess this, the function of neutrophils from the circulation and from the seroma of burned mice was compared to neutrophils isolated from Sham mice. While the ability to kill was similar between neutrophils in circulation and seroma neutrophils, they were

significantly decreased compared to naïve mice. Additionally, we observed that naïve neutrophils resuspended in seroma fluid have a decreased ability to kill, either due to inhibition by the seroma fluid or the continued growth of PA. There was a decrease in ROS production in neutrophils in the seroma when compared to neutrophils in the circulation of the same mice. Naïve mice incubated with sera from burned and infected mice with quantified HMGB1 produced ROS without stimulation. Together, a non-lethal burn is sufficient to impact neutrophil function not only at the burn site but also in circulation.

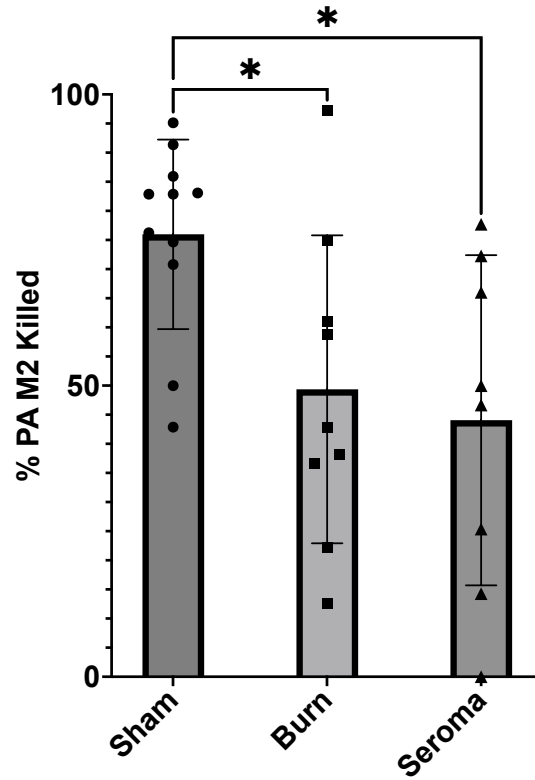
## **Results**

### **Burn alone is sufficient to impact neutrophil killing**

To determine if our burn model impacts the ability of neutrophils to kill, isolated neutrophils from the circulation and seroma of the same burned mice were incubated with PA. These findings were compared to the circulating neutrophils of Sham mice. These neutrophils were incubated with PA at an MOI of 1 for two hours at 37° C. Surprisingly, the neutrophils in circulation were impacted by the burn alone, having a significant decrease in bacterial killing when compared to neutrophils in circulation of Sham mice (Figure 4-1). The initial hypothesis was that the seroma environment negatively impacted neutrophil function, but neutrophils in circulation were affected before they reached the burn site. These data suggest that substances in the circulation from a non-lethal burn are sufficient to impact neutrophil function comparably to the seroma environment

### **In seroma fluid, neutrophils lose the ability to kill PA**

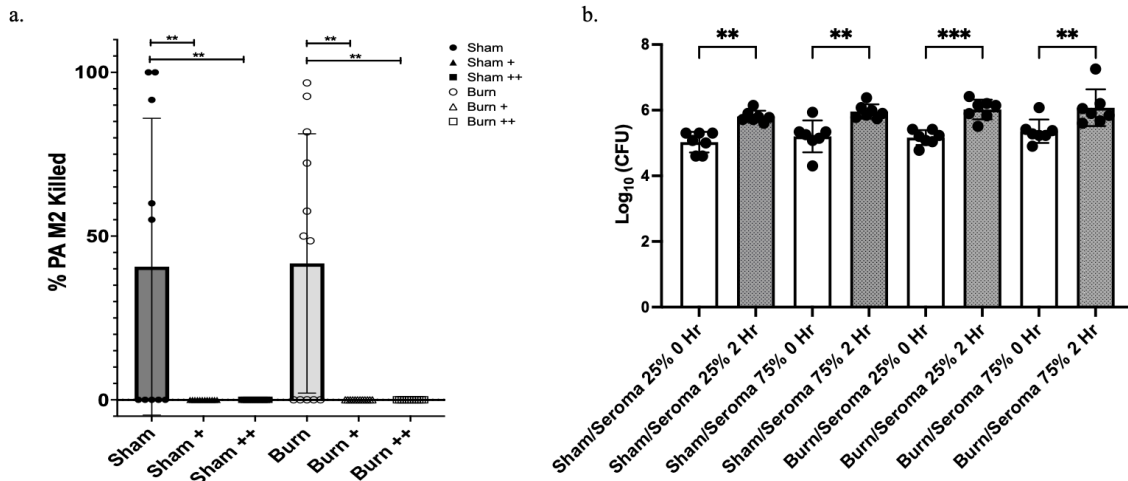
To assess the role of the seroma environment in the killing function of neutrophils, seroma fluid was collected from burned mice and used to resuspend isolated neutrophils from the circulation of Sham and burned mice. The cells were resuspended in HBSS<sup>+</sup>



**Figure 4-1 Phagocytic activity of neutrophils from the circulation of Sham mice, and from the circulation and seroma of burned mice resuspended in HBSS+.**

Neutrophils pooled from five mice (the circulation of Sham mice, the circulation of burned mice [Burn] and from the seroma from the same burned mice [Seroma]) were incubated with PA M2 in HBSS<sup>+</sup> at 37° C for two hours. Samples were taken at time 0 and at the end of the 2 hours. Serial dilutions of the 10 µL samples were plated on tryptic soy agar and incubated overnight. The CFUs were counted and calculated to adjust for dilution. The data charted is 1-(Time 2 h/Time 0 h). Bar height is the mean and error bars indicate standard deviation. \* p < 0.05.

enriched with either 25% or 75% seroma fluid. After two hours of incubation, PMNs from the circulation of Sham and burned mice could kill PA, however, in the HBSS/seroma-enriched treatments, neutrophils lost the ability to kill (Figure 4-2a). In fact, quantification showed that there were more CFUs present after two hours in all samples that were incubated in seroma fluid (Figure 4-2b), confirming previous work showing that the seroma environment supports PA growth [174]. It is unclear if the seroma fluid directly inhibited killing by neutrophils or if the PA was simply able to continue to grow in the seroma environment.

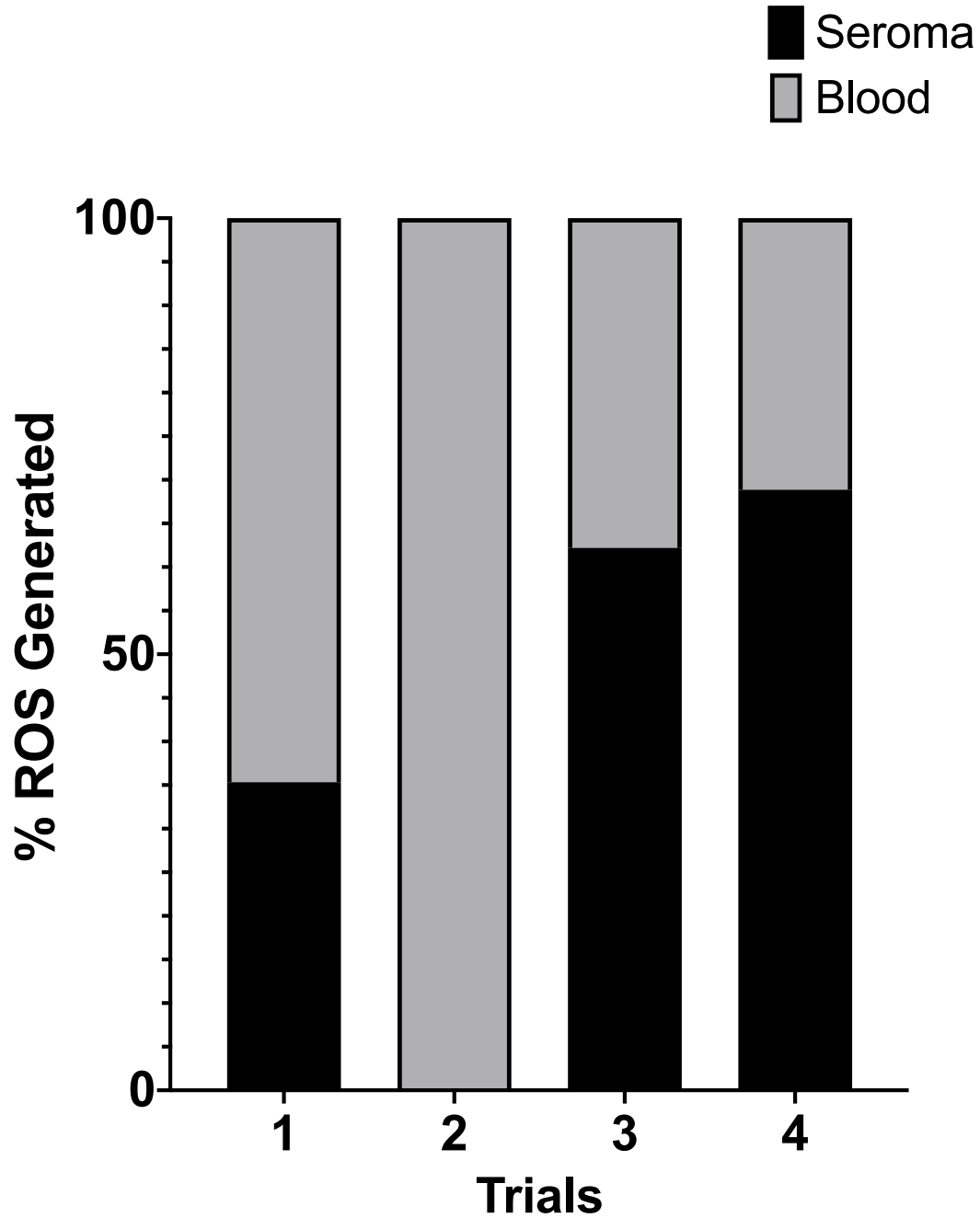


**Figure 4-2 Phagocytic activity of neutrophils from peripheral blood of Sham and from burned mice resuspended in seroma fluid.**

Neutrophils were incubated with PA M2 in HBSS<sup>+</sup> and seroma (25%, 75%) at 37°C for two hours. Samples were taken at time 0 and at the end of the 2 hours. Serial dilutions of the 10  $\mu$ L samples were plated on tryptic soy agar and incubated overnight. The CFUs were counted and calculated to adjust for dilution. The data charted is 1-(Time 2h/Time 0h). (a) Percent of PA killed after two hours. (b) Actual CFU counts from all samples after 2 hours. \*\* p < 0.01, \*\*\* p < 0.001.

### Neutrophils in the seroma have a decreased ability to generate ROS

To address this possibility, we utilized a cytochrome C assay to measure reactive oxygen species (ROS) production. Neutrophils release ROS into the phagosome, which are toxic to pathogens by causing oxidative stress which damages membranes, proteins, and DNA. We compared pooled neutrophils (resuspended in HBSS<sup>+</sup>) from the circulation of



**Figure 4-3 ROS production in seroma-isolated neutrophils in relation to those isolated from circulation.**

Each trial consists of the pooled neutrophils of 5 mice, comparing the activity of neutrophils collected from seroma and blood. Both blood and seroma were collected from five mice at 18 hours post-burn. The production of neutrophils isolated from blood was normalized to 100% and the activity of the seroma neutrophils is shown as a fraction. Reduction was measured through colorimetry at 550 nm.

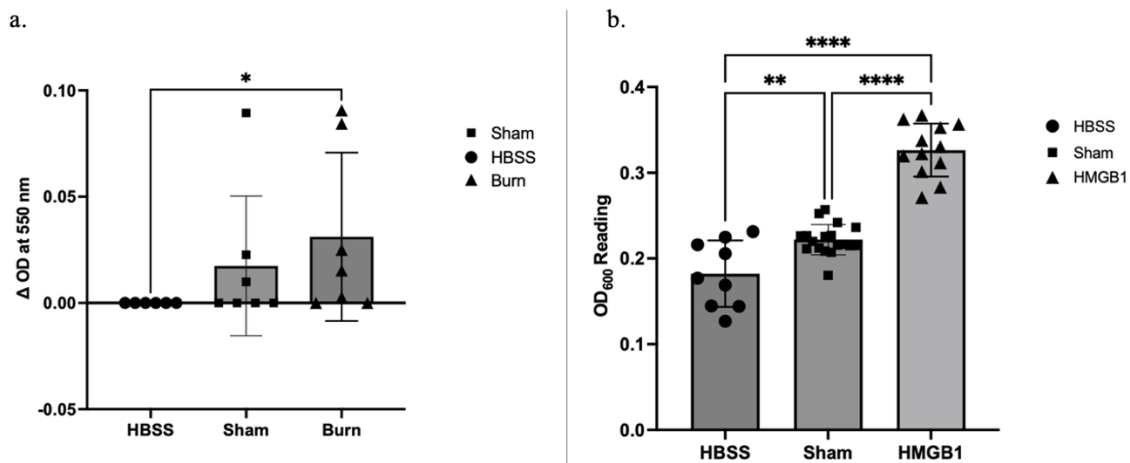
five burned mice to neutrophils isolated from the seroma of the burn site of the same mice. The neutrophils were stimulated with PMA and incubated for 20 minutes at 37° C. We set the ROS production of the circulating neutrophils as 100% and expressed the production of the neutrophils in the seroma as a percentage (Figure 4-3). In each instance, the seroma neutrophils had a decreased ability to produce ROS. In one trial, the seroma neutrophils were unable to reduce cytochrome C. This could be due to insufficient stimulation or lack of response by the isolated cells.

#### **Naïve neutrophils incubated in sera with quantified HMGB1 reduce cytochrome C at rest**

Because the bacterial killing of neutrophils isolated from burned mice was decreased, we wanted to determine if the burn impacted ROS production. Using neutrophils isolated from Sham mice, we incubated the cells with cytochrome C in either HBSS<sup>+</sup> or sera from Sham or burned mice. We found that the sera from burned mice, without the addition of PMA, was able to cause the production of ROS and the reduction of cytochrome C at rest (Figure 4-4a).

Burn injury has been previously reported to release HMGB1, a pro-inflammatory DAMP, into the circulation. In the absence of infection, HMGB1 returned to baseline levels after 6 hours [172]. During burn and infection, HMGB1 is released at higher levels, which continued to increase until death [172]. Administration of a competitive peptide, P5779, prolonged survival of PA-infected, burned mice [172]. The hypothesis from these findings was that the HMGB1 released into the circulation of burned mice may have stimulated the PMNs in the circulation such that they may have reduced their bactericidal capacity before they entered the PA-infected seroma. After collecting sera at 18 hours post-burn and

infection, HMGB1 was quantified. Resting neutrophils from Sham mice were resuspended in either HBSS<sup>+</sup>, sera from Sham mice (Sham), or sera from burned and infected mice (HMGB1), then incubated with cytochrome C without stimulation by PMA (Figure 4-4b). These resting PMNs were able to significantly produce ROS when incubated with sera from burned and infected mice, suggesting that neutrophils could be activated while still in circulation, prior to arriving at the burn site.



**Figure 4-4 Resting neutrophils from Sham mice are able to be activated with sera from burned mice.** Pooled neutrophils from Sham mice were incubated with HBSS<sup>+</sup>, sera from Sham mice, or sera from burned mice (a) for 20 minutes at 37° C. The neutrophils incubated with sera from burned mice were able to produce ROS and reduce cytochrome C. Further, Sham neutrophils incubated in sera from burned and infected mice with quantified HMGB1 were able to reduce cytochrome C.

## Discussion

After an initial hemostasis period following burn injury, where platelets and clotting factors are activated to prevent blood loss, there is an inflammatory response at the burn site. Resident macrophages, endothelial cells, and dendritic cells respond to DAMPs by recruiting monocytes and neutrophils to the burn wound to remove cellular debris and begin the repair process [68, 138, 224, 225]. This inflammatory stage can last for days or months, depending on the severity of the wound [138, 152, 226, 227].

Severe trauma, including burns, has been shown to cause a systemic activation of neutrophils, resulting in the sequestration of neutrophils in healthy tissues and causing

tissue damage [69, 72, 73, 228, 229]. This immune response can be triggered and sustained by various factors, but DAMPs are a major contributor. Released by necrotic cells and activated neutrophils, DAMPs activate immune cells which express pattern recognition receptors. Ideally, neutrophils would be activated at the site where their action is needed. However, with prolonged inflammatory mediator and vasodilator release into circulation, activated neutrophils can localize to healthy tissue, where they can cause tissue damage leading to organ injury. These off-target effects on a large scale can cause dangerous sequelae in burn patients, such as multisystem organ failure [74, 230]. The neutrophils have also been shown to have decreased bactericidal function post-trauma [230].

In our non-lethal flame burn mouse model, there is evidence of this as well. There was an observed relative neutropenia in our model, which is more severe when the mice are infected [174]. Further, these neutrophils appear to be sequestered in the seroma [174]. Because of this, a superimposed PA infection in this model may become 100% fatal. The lethal PA dose is approximately 1000-fold less than in mice that are not burned [172]. This effect is temporally related, as mice at infected 72 hours post-burn have fully restored survival. This transient increase in infection propensity mimics the temporary decrease in bactericidal activity seen in severe trauma and burns; however, our burn model is only 10% TBSA. These findings support the conclusion that even a minor burn alters the immune response to superimposed infection by sequestering neutrophils to the seroma and prematurely activating neutrophils while still in circulation, thereby decreasing their bactericidal capacity.

First, bacterial killing by neutrophils was investigated. the initial hypothesis was that the seroma fluid somehow inhibited neutrophil-mediated bacterial killing. However,

when neutrophils isolated from the circulation of burned mice and neutrophils from the seroma of burned mice were incubated with PA, we found that their ability to kill was not significantly different. They were both significantly decreased when compared to mice that were not burned. This suggests that neutrophils were being activated while still in circulation, decreasing their killing ability.

To determine the effect of the seroma on bacterial killing, we resuspended neutrophils from the circulation of Sham and of burned mice in varying concentrations of seroma fluid. Neutrophils were unable to kill PA when seroma fluid was present. In fact, in each instance, there was an increase in CFUs after the two-hour incubation. It is unclear if the seroma fluid inhibits neutrophil-mediated bacterial killing, if the PA was able to grow at a rate that the neutrophils could not control, or a combination of these effects.

ROS production is a critical mechanism of neutrophil-mediated bactericidal activity. Using a colorimetric assay to measure cytochrome C reduction, ROS production neutrophils isolated from the seroma was compared to neutrophils isolated from the circulation of the same burned mice. Initially, experiments were conducted to determine the role of circulating sera on Sham neutrophils, where these neutrophils were resuspended in sera from either Sham or burned mice. However, the “resting” neutrophils, neutrophils without PMA stimulation in the well, were observed to be able to generate ROS. This was measured by loading neutrophils from Sham mice resuspended in either HBSS<sup>+</sup>, sera from Sham mice, or sera from burned mice and measuring the OD at time 0 and after 20 minutes of incubation. The sera from burned mice was able to cause a production of ROS. This was not entirely surprising, as it has been previously shown that sera from mice burned in this model was able to activate HEK-Blue<sup>TM</sup>-mTLR4 reporter cells [172].

It was previously reported that, within this model, there is the release of HMGB1 systemically post-burn. In the burn alone group, approximately 50 ng/mL of HMGB1 was measured immediately post-burn which returned to levels comparable to baseline (20 ng/mL). When mice were burned and infected, however, HMGB1 was persistently released into the bloodstream, increasing to over 200 ng/mL until death. To determine the role of HMGB1 in neutrophil function, we plan to use P5779, a peptide that competes for the binding site of HMGB1 on MD-2. HMGB1 has been successfully quantified from sera of burned and infected mice and observed its ability to activate resting Sham neutrophils. In the future, this assay will be repeated to include a dose-response curve for P5779, to determine if this activation can be reduced or eliminated by this specific HMGB1 inhibitor.

In severe burns, patient outcomes are well studied. The correlation between TBSA and mortality has been frequently enumerated. What is shown is that a non-lethal burn can significantly alter neutrophil function, increasing the propensity for bacterial infection. Understanding the balance between the burn wound and the immune system, including in smaller burns, will better inform the care of these patients.

## Chapter 5. Discussion

We utilized a non-lethal flame burn model, which gave us the ability to examine the effect of burn alone and burn with superimposed infection on innate immune responses. In this model, one can elucidate the state of the burned patient and the relationship of the burned patient with *Pseudomonas aeruginosa* infection. Previous work studying the interaction of burned mice and PA have utilized various burn models, including scald and contact. The flame burn model is significant, as it is the most common burn modality adult patients face. In this model, the effect of a full-thickness flame burn on subsequent infection susceptibility can be studied.

Many animal models have characterized immune suppression in response to burns [13, 143, 224, 225, 231, 232]. Work in models and with patients shows decreased immune function, decreased numbers of circulating immune cells, and increased cytokine production post-burn. The prevailing hypothesis is that because of this suppressed immune state, bacteria are more likely to be able to infect and cause more severe disease. It has been thought that the immunocompromised state of the burned patient allows for infection by an opportunistic pathogen. However, our findings suggest that it is an active interaction between both the host and the pathogen which creates a permissive environment for increased PA virulence. The B/I condition uniquely impacts both the host and pathogen, and resulted in increased mortality.

In this model, cytokine production, circulating DAMP levels, and LD<sub>50</sub> differed in burned mice compared to Sham, burned mice, and B/I mice [172]. B/I mice produced IL-6 sooner, in higher amounts, and for longer than mice that were burned alone [172]. There were also significant levels of TNF- $\alpha$  and IL-10 in circulation late in the burn and infection

process [172]. Significantly, burned mice experienced a transient increase in susceptibility to PA infection: in Sham mice, the LD<sub>50</sub> was 10<sup>4</sup> times higher than in burned mice when infected subcutaneously at the burn site. This trend was also observed to a lesser degree when PA was delivered intravenously, intraperitoneally, and subcutaneously distal to the burn site [172]. When mice were infected at 48-72 hours post-burn, survival was restored. These results were due to infection with live PA, as PA LPS and heat-killed PA had no impact on the survival of burned mice [172].

The presence of a seroma was also characterized. It forms directly inferior to the burn site. This seroma appears at approximately 3 hours post-burn and is comprised of cellular debris, fluid from circulation, and CD45<sup>+</sup> cells [174]. When collected, the seroma fluid can support the growth of PA at a rate comparable to HySoy broth [174]. When mice were burned and infected, PA was shown to be present within the seroma in close proximity to granulocytes via Gram stain [174]. The seroma was also found to have a significant and unique cytokine panel when mice are burned and infected. There were significant levels of CXCL10, CCL2, CCL3, CCL4, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  when compared to Sham, burn alone, and infection alone mice [174]. These data lead us to hypothesize that the burn and infection together creates a unique environment that impacts both the host and the pathogen, resulting in increased virulence and mortality.

This is a multifactorial process causing the observed increased mortality. When P5779, a novel peptide that inhibits the TLR4 signaling by binding to the same site on MD-2 as HMGB1, was administered in our B/I model, survival was prolonged in 50% of mice and was restored in 12% of mice [172]. The mice that survived were bacteremic, however, suggesting that inhibiting TLR4 signaling did not inhibit PA dissemination or aid in

bacterial clearance, and that some other mechanism aside from dissemination may affect mortality.

In this dissertation work, the host-pathogen interactions that result in increased mortality in our burn model were further characterized. Because the increased mortality was a result of infection with live bacteria, potential expression changes in both the host and pathogen were measured to determine if the burn and infection condition uniquely impacted either the host, pathogen, or both. There have been previous publications that investigated gene expression in burns using RNA-seq, which discovered physiological and metabolic changes in PA in burn wounds [193, 195]. Other studies found differential gene expression in *Pseudomonas aeruginosa* based on the modality or duration of the wound [23, 194].

Not only is gene expression in the B/I condition significantly different from Sham, Burn, and Infection, but it also significantly impacted each tissue we sampled. In the skin, there is a period of relative quiescence in gene expression in both the host and pathogen, with significant and sustained divergence after 12 hours, when clinical symptoms begin to appear in our model: there was qualitative downregulation of gene expression in the host and upregulation in PA. Over time, gene expression differed significantly in each tissue.

But how did these findings relate to the observed mortality in the model? While previous work showed high amounts of IL-10 in circulation, it was surprising to find that the increased in *Il10* expression occurred in the spleen, not the skin. Systemically, IL-10 supports an anti-inflammatory response [201]. When neutralizing anti-IL-10 antibodies were delivered in our burn and infection model, there was a one-day delay in mortality. The increased expression of *Il10* in the spleen may lead increased IL-10 protein in

circulation that overwhelms the single dose of neutralizing antibodies. When P5779 was administered previously, the mice received multiple doses. Future work in this project would include a treatment of multiple doses of anti-IL-10 to observe mortality in this model.

Published work in a scald model showed that arginine inhibited dissemination of PA post-burn by inhibiting bacterial motility [177]. It was also shown to restore survival in mice [177]. This work also suggested that the utilization of arginine by myeloid-derived suppressor cells (MDSCs) increased the innate immune response to PA [177]. The NanoString™ data did not support this; there was no significant increase in *Arg1* or *iNos* expression in the B/I condition. There was, however, restored survival in our model when mice were administered arginine. This was possibly due to arginine acting on PA, inhibiting motility and growth, rather than enhancing the innate immune response, as the arginine restored survival when colocalized with PA.

There was suspicion that the dysfunction of neutrophils had a role in the increased mortality observed in our model. The observed seroma formation was comprised of CD45<sup>+</sup> cells which originated from the circulation [174]. PA was in close proximity to these cells, indicating a dysfunction (*i.e.* the PA was not being taken up by nearby PMNs). Neutrophils are required for clearance of PA infection. In cystic fibrosis, PA has been shown to induce neutrophil apoptosis in order to evade the immune response [222]. Burn wounds have been shown to cause an initial hyperactive immune response, followed by late immunosuppression, which leads to increased infection susceptibility [35, 166]. In a scald burn model, mice were found to have decreased neutrophil function, including chemotaxis and phagocytosis [228]. Neutrophils isolated from burned patients expressed increased

markers of degranulation compared to healthy patients, with a decreased ability to produce ROS and NETs [233]. There is evidence to support the conclusion that innate signaling contributes to increased mortality post-burn. In *Nlrp3*<sup>-/-</sup> mice, there was an increase in bacterial clearance and survival [164]. In our own work, inhibiting HMGB1:TLR4 signaling also resulted in restored survival [172]. This could be partially ascribed to circulating DAMPs. DAMPs have been shown to be associated with inflammation and immune cell activation post-burn [67, 72, 74, 153, 234]. The relationship between pro-and anti-inflammatory mediators and immune cells post-burn, and the effects of imbalance on infection propensity, is unclear.

In this model, a non-lethal burn was shown to impact the function of neutrophils. In bacterial killing, neutrophils in the circulation of burned mice had a decreased ability to kill PA. It was anticipated that neutrophils from the seroma would have decreased function based on previously observed dissemination. Surprisingly, there was significantly decreased killing by neutrophils still in circulation. Additionally, when ROS production was measured, there was decreased ROS production in the seroma neutrophils compared to neutrophils in the circulation of burned mice. Sham neutrophils incubated with sera from burned mice were able to produce ROS without stimulation. These data suggest that the burn wound released activating factors into circulation, which can cause ROS production. The hypothesis was that this premature activation of neutrophils causes them to arrive at the burn site unable to effectively kill PA, with the seroma environment further inhibiting bactericidal function.

The role of *Pseudomonas aeruginosa* cannot be discounted in these observations. The burn and infection condition had significant gene expression changes when compared to

burn alone and infection alone. This indicates that the relationship between the burned host and PA results in a transcriptome that differs from burn alone and infection alone and relates to our previous work showing differential cytokine profiles when comparing burned mice to B/I mice. The live PA, with active expression changes, interacting with the burned host results in increased mortality.

In totality, a non-lethal flame burn is able to decrease host defenses and provide a favorable environment for bacterial growth. The association shown in the literature consistently is that the extent of TBSA is positively correlated with mortality [33, 135, 148, 235]. With a non-lethal, 10% TBSA flame burn, sera from burned mice, and B/I mice, are able to activate neutrophils prematurely while in circulation and thereby decrease bactericidal functions. Also, gene expression in both the host and the pathogen differ when they occur concurrently. Finally, the survival of mice can be restored when arginine is colocalized with *Pseudomonas aeruginosa*, inhibiting growth and motility.

## Appendix A – NanoString™ Panel and Experiment Design of Aim 1

Official Symbol	Official Full Name	Official Symbol	Official Full Name
<i>Abl1</i>	c-abl oncogene 1, non-receptor tyrosine kinase	<i>Il1b</i>	interleukin 1 beta
<i>Bcl2</i>	B cell leukemia/lymphoma 2	<i>Il4</i>	interleukin 4
<i>C1qa</i>	complement component 1, q subcomponent, alpha polypeptide	<i>Il6</i>	interleukin 6
<i>C3</i>	complement component 3	<i>Irak1</i>	interleukin-1 receptor-associated kinase 1
<i>C8a</i>	complement component 8, alpha polypeptide	<i>Irak2</i>	interleukin-1 receptor-associated kinase 2
<i>C9</i>	complement component 9	<i>Irak3</i>	interleukin-1 receptor-associated kinase 3
<i>Casp1</i>	caspase 1	<i>Irak4</i>	interleukin-1 receptor-associated kinase 4
<i>Casp3</i>	caspase 3	<i>Myd88</i>	myeloid differentiation primary response gene 88
<i>Casp8</i>	caspase 8	<i>Nod2</i>	nucleotide-binding oligomerization domain containing 2
<i>Ccl2</i>	chemokine (C-C motif) ligand 2	<i>Pecam1</i>	platelet/endothelial cell adhesion molecule 1
<i>Ccl20</i>	chemokine (C-C motif) ligand 20	<i>Rag1</i>	recombination activating gene 1
<i>Cd40</i>	CD40 antigen	<i>Rag2</i>	recombination activating gene 2
<i>Cd44</i>	CD44 antigen	<i>Tgfb1</i>	transforming growth factor, beta 1
<i>Cxcr1</i>	chemokine (C-X-C motif) receptor 1	<i>Tlr2</i>	toll-like receptor 2
<i>Cxcr2</i>	chemokine (C-X-C motif) receptor 2	<i>Tlr4</i>	toll-like receptor 4
<i>Cxcr5</i>	chemokine (C-X-C motif) receptor 5	<i>Tlr5</i>	toll-like receptor 5
<i>Ddx58</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	<i>Tlr9</i>	toll-like receptor 9
<i>Fasl</i>	Fas ligand (TNF superfamily, member 6)	<i>Tnf</i>	tumor necrosis factor
<i>Fcer1a</i>	Fc receptor, IgE, high affinity I, alpha polypeptide	<i>Traf1</i>	TNF receptor-associated factor 1
<i>Fcer1g</i>	Fc receptor, IgE, high affinity I, gamma polypeptide	<i>Traf2</i>	TNF receptor-associated factor 2
<i>Fcgr1</i>	Fc receptor, IgG, high affinity I	<i>Traf3</i>	TNF receptor-associated factor 3
<i>Fcgr2b</i>	Fc receptor, IgG, low affinity IIb	<i>Traf4</i>	TNF receptor associated factor 4
<i>Fcgr3</i>	Fc receptor, IgG, low affinity III	<i>Traf5</i>	TNF receptor-associated factor 5
<i>Fcgr4</i>	Fc receptor, IgG, low affinity IV	<i>Traf6</i>	TNF receptor-associated factor 6
<i>Fcgrt</i>	Fc receptor, IgG, alpha chain transporter	<i>Ptgs2</i>	prostaglandin-endoperoxide synthase 2
<i>Ifng</i>	interferon gamma	<i>Cxcl1</i>	chemokine (C-X-C motif) ligand 1
<i>Il10</i>	interleukin 10	<i>Nos2</i>	nitric oxide synthase 2, inducible
<i>Il18</i>	interleukin 18	<i>Cxcl10</i>	chemokine (C-X-C motif) ligand 10
<i>Il1a</i>	interleukin 1 alpha	<i>Pparg</i>	peroxisome proliferator activated receptor gamma
<i>Arg1</i>	arginase, liver	<i>Igf2r</i>	insulin-like growth factor 2 receptor
<i>Retnla</i>	resistin like alpha	<i>Il13</i>	interleukin 13
<i>Chil3</i>	chitinase-like 3		

Table 6-1 *M. musculus* genes included in NanoString™ panel.

Official Symbol	Official Full Name
<i>exoT</i>	putative exoenzyme T
<i>lasI</i>	N-(3-oxododecanoyl)-L-homoserine lactone synthase @ N-acyl-L-homoserine lactone synthase, LuxI family (EC 2.3.1.184)
<i>pcrV</i>	Type III secretion cytoplasmic LcrG inhibitor (LcrV, secretion and targeting control protein, V antigen)
<i>phoP</i>	Opacity protein and related surface antigens
<i>fliH</i>	Flagellar basal body-associated protein FliH
<i>pilQ</i>	Type IV pilus biogenesis protein PilQ
<i>tse3</i>	Bacterial lysozyme Tse3, effector of type VI secretion system
<i>plcH</i>	Phospholipase C (EC 3.1.4.3) => hemolytic PlcH
<i>plcN</i>	Phospholipase C (EC 3.1.4.3) => non-hemolytic PlcN
<i>eptA</i>	Lipid A phosphoethanolamine transferase, putative
<i>pchC</i>	Pyochelin biosynthetic protein PchC, predicted thioesterase @ Thioesterase in siderophore biosynthesis gene cluster
<i>feoB</i>	Ferrous iron transporter FeoB
<i>cupB5</i>	Large exoproteins involved in heme utilization or adhesion
<i>pvdL</i>	Pyoverdine chromophore precursor synthetase PvdL @ Siderophore biosynthesis non-ribosomal peptide synthetase modules
<i>phzS</i>	FAD-dependent monooxygenase PhzS
<i>ftsX</i>	Cell-division-associated, ABC-transporter-like signaling protein FtsX
<i>tolC</i>	Type I secretion outer membrane protein, TolC family
<i>rhaS</i>	T6SS PAAR-repeat protein / RhaS protein
<i>pvdE</i>	PvdE, pyoverdine ABC export system, fused ATPase and permease components
<i>pilQ</i>	Type IV pilus biogenesis protein PilQ
<i>BNR</i>	FIG002465: BNR repeat protein
<i>pilC</i>	Type IV fimbrial assembly protein PilC
<i>t1SS</i>	T1SS secreted agglutinin RTX
<i>pvdL</i>	Pyoverdine sidechain non-ribosomal peptide synthetase PvdI @ Siderophore biosynthesis non-ribosomal peptide synthetase modules
<i>pcaH</i>	Protocatechuate 3,4-dioxygenase beta chain (EC 1.13.11.3)
<i>cobH</i>	Precorrin-8X methylmutase (EC 5.4.99.61) biosynthetic pathway to cobalamin (vitamin B12) in aerobic bacteria
<i>gntR</i>	Transcriptional regulator, GntR family
<i>lysR</i>	Transcriptional regulator, LysR family
<i>pmrA</i>	Two-component system response regulator QseB
<i>pagL</i>	Lipid A 3-O-deacetylase
<i>lpxC</i>	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase (EC 3.5.1.108)
<i>fabZ</i>	3-hydroxyacyl-[acyl-carrier-protein] dehydratase, FabZ form (EC 4.2.1.59)
<i>waaA</i>	3-deoxy-D-manno-octulosonic acid transferase (EC 2.4.99.12)(EC 2.4.99.13)
<i>ftsH</i>	Cell division-associated, ATP-dependent zinc metalloprotease FtsH
<i>pagP</i>	hypothetical protein (PagP)

**Table 6-2 *P. aeruginosa* genes included in NanoString™ panel**

Internal Reference Genes	Official Full Name
<i>Gapdh</i>	glyceraldehyde-3-phosphate dehydrogenase
<i>Polr1b</i>	polymerase (RNA) I polypeptide B
<i>Rpl19</i>	ribosomal protein L19
<i>Tbp</i>	TATA box binding protein
<i>oprL</i>	Tol-Pal system peptidoglycan-associated lipoprotein PAL
<i>algD</i>	GDP-mannose 6-dehydrogenase (EC 1.1.1.132)

**Table 6-3 *M. musculus* and *P. aeruginosa* housekeeping genes included in NanoString™ panel.**  
*P. aeruginosa* genes are underlined.

	Blood	Liver	Spleen	Skin
<b>Sham</b>	5	5	5	5
<b>Burn</b>	5	5	5	5
<b>Infection</b>	5	5	6	5

Table 6-4 Number of mice sampled in each reference condition and tissue.

	0 Hr	2 Hr	6 Hr	7 Hr	12 Hr	18 Hr	20 Hr	22 Hr	24 Hr
<b>Blood</b>	2	2	3	2	5	4	2	2	3
<b>Liver</b>	0	0	3	0	5	4	2	2	4
<b>Spleen</b>	0	0	3	0	5	4	2	1	4
<b>Skin</b>	0	0	1	3	5	5	3	3	4

Table 6-5 Number of mice sampled in the B/I condition in each tissue per timepoint.

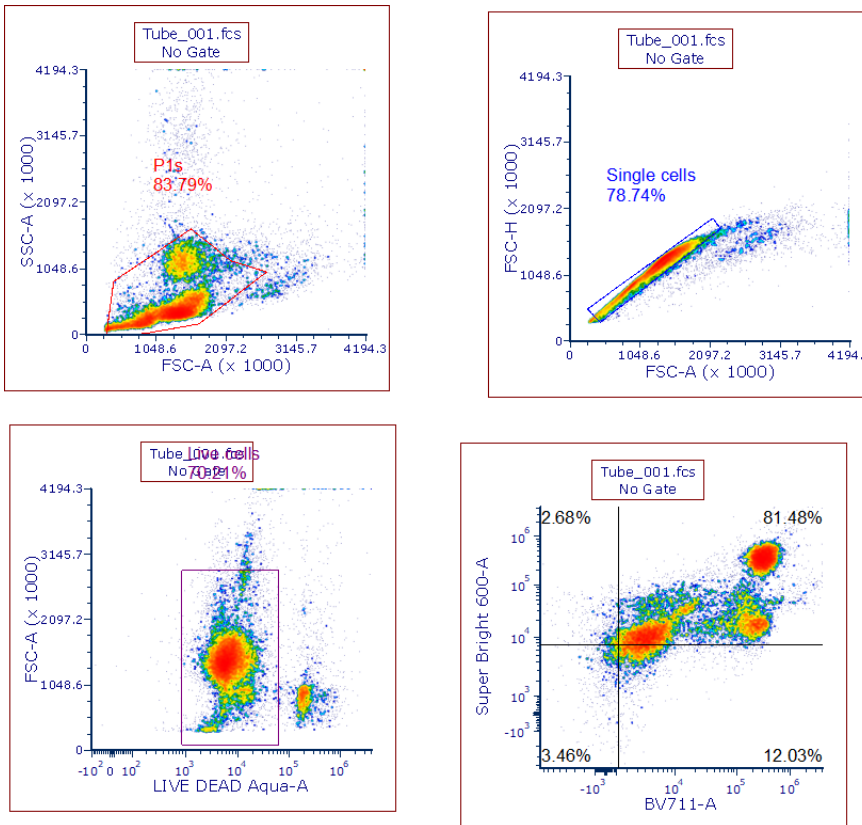


Figure 6-1 Flow cytometry of cells isolated with Histopaque density gradient

Cells collected between density phases were resuspended in HBSS and analyzed through flow cytometry. The cells were gated by size (FSC-A vs SSC-A), singularity (FSC-A vs FSC-H), and viability (Aqua-A). Finally, CD11b<sup>+</sup> and Lyg6G<sup>hi</sup> cells were counted.

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