PLATELETS ARE NECESSARY FOR SURVIVAL AND TISSUE INTEGRITY IN A MURINE MODEL OF *ASPERGILLUS FUMIGATUS* INFECTION

by

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A Dissertation

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Tobias M. Hohl, MD, PhD Date

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DEDICATION

This dissertation is dedicated to my grandparents, Dr. Bluma Tischler (of blessed memory), Dr. Isaak Tischler (of blessed memory), Immanuel Braverman (of blessed memory), and Sorkie Braverman. It is to them that I owe my love of learning, my thirst for knowledge, and passion for discovery.

Dr. Bluma Tischler (1924-2015) co-authored 36 peer-reviewed articles on intellectual and developmental disabilities, focusing on phenylketonuria, and was instrumental in the introduction of PKU screening for newborns in British Columbia in 1964. In 1977, she received the Queen's Jubilee Medal, and in 1978 received the annual research award from the American Association on Intellectual and Developmental Disabilities. In recognition of her service, the University of British Columbia established the Bluma Tischler Postdoctoral Fellowship, which continues to be awarded to MD or PhD graduates conducting research on intellectual developmental or other neurological disorders. While my publication record pales in comparison to hers, I am proud to have continued her legacy in biomedical research.

Though I am leaving the bench, I hope to channel all of my grandparents as I continue to work to ensure the betterment of human health.

ABSTRACT

Aspergillus fumigatus is a ubiquitous environmental mold and an opportunistic human pathogen. As part of its reproductive cycle, *A. fumigatus* releases spores, called conidia, into the environment. Humans inhale hundreds to thousands of these conidia daily. Immune competent individuals clear conidia asymptomatically through alveolar macrophages and neutrophils. However, in the context of innate immune injury, such as neutropenic chemotherapy patients and those receiving organ or bone marrow transplants, the conidia can escape inactivation, germinate into tissue and angioinvasive hyphae, and cause the disease invasive aspergillosis.

Invasive aspergillosis affects over 250,000 patients annually with unacceptably high mortality rates and inefficient therapeutic approaches. As such, it is critical to understand the immune response to *A. fumigatus*. Beyond their established roles in hemostasis and thrombosis, platelets are increasingly recognized to play roles in the immune and inflammatory response, both by directly targeting invading pathogens, and indirectly by recruiting and activating immune cells such as neutrophils. Thrombocytopenia often co-occurs with neutropenia, itself a primary risk factor for developing invasive aspergillosis, in at risk patient groups, making clinical studies of the role of platelets in anti-*Aspergillus* host defense challenging.

In this dissertation, I utilized two complementary approaches to induce thrombocytopenia in mouse models in the absence of neutropenia. Thrombocytopenic mice were highly susceptible to *A. fumigatus* challenge and rapidly succumbed to challenge in a strain independent manner. Though platelets seemed to transiently regulate early neutrophil phagocytosis (but not that of other cell types) via a spleen

tyrosine kinase (Syk)-dependent mechanism, the immunomodulatory function of platelets appeared to be dispensable for survival.

Instead, the data presented herein suggest that platelets primarily function to maintain hemostasis and tissue integrity during pulmonary inflammation. When challenged with live conidia, mice exhibited severe hemorrhage into the airways and lung parenchyma in the absence of overt germination and angioinvasion. In agreement, challenge with non-viable, but immunogenic, conidia induced similar levels of mortality, a finding that could be rescued by treatment with purified platelets. The increase in hemorrhage was not coincident with epithelial or endothelial cell apoptosis, although thrombocytopenic mice did exhibit higher levels of certain inflammatory cytokines.

In aggregate, these data establish a crucial role of platelets in anti-*Aspergillus* host defense and expand the role of platelets in to a new class of pathogens, the filamentous molds.

BIOGRAPHICAL SKETCH

Benjamin (Ben) Tischler was born and raised in Vancouver, British Columbia, Canada. From his earliest years, he had a great interest in the natural world. On family camping trips as a young child, he could often be found conducting “experiments” in his “lab.” These early forays into scientific research included the analysis of spores on sword ferns or assessing the biodiversity of beaches and tide pools. After attending Vancouver Talmud Torah for elementary school, where he had the opportunity to raise salmon in the classroom and release them into the wild, Ben engaged deeply with all the scientific opportunities available at St. George’s School as a high school student. Ben’s path to becoming a scientist began in earnest as a Grade 9 student, when his teachers Shawn Lawrence and Dr. Anthony Mercer organized a trip to the Galapagos in Ecuador to see the islands and animals that led Darwin to formulate his theory of evolution. After that trip, Ben registered for as many STEM courses as he could, including Biology, Physics, Geography, AP Calculus, and AP Chemistry (taught by Dr. Mercer), eventually graduating in June 2008 with a Headmaster’s Leadership Award and Educational Excellence Medal.

After a gap-year attending classes at the Hebrew University of Jerusalem and volunteering with *Magen David Adom* in Israel under the auspices of The Nativ College Leadership Program, Ben matriculated into the Joint Program between the Albert A. List College of Jewish Studies and the Columbia University School of General Studies in the Fall of 2009. While an undergraduate student, Ben majored in Jewish History at List, and Biochemistry at Columbia. After 2 summers as a founding staff member at Ramah Outdoor Adventure (now Ramah in the Rockies, a Jewish outdoor education camp in Sedalia, Colorado) in 2010 and 2011, Ben began his laboratory science journey under

the supervision of Dr. Terry Snutch (University of British Columbia) in the summers of 2012 and 2013 where he learned the basics of molecular biology in a neuroscience environment. In the spring of 2013, Ben was first exposed to microbiological research through the Project Laboratory in Microbiology course taught by Dr. Lars Dietrich, where he conducted basic research on *Pseudomonas aeruginosa*. In his final semester as an undergraduate, Ben volunteered in the laboratory of Dr. Riccardo Dalla-Favera at the Columbia University Medical Center studying B-cell lymphomas. Ben completed his undergraduate studies in December 2013 and proceeded to work in Dr. Dalla-Favera's lab as a technician until July 2014. These experiences combined to lead Ben to apply to graduate school intending to work on solving today's biomedical research problems.

Ben joined the Louis V. Gerstner, Jr. Graduate School of Biomedical Sciences in July 2014. After a 2-year period in the laboratory of Dr. Mary Goll studying the primary immune deficiency disease ICF syndrome, Ben joined the laboratory of Dr. Tobias Hohl. Since joining the Hohl lab, Ben has presented his research on the role of platelets in the anti-*Aspergillus* immune response at national and international meetings, written a literature review, published original research in PLOS Pathogens, and served as a teaching fellow for the GSK core course. After graduation, Ben will apply his scientific knowledge to help life science companies bring their treatments to the clinic in his new role as a Life Sciences Specialist at L.E.K. Consulting in New York.

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There are far too many people to thank for bringing me to this moment. Firstly, I would like to thank my thesis mentor, Tobias Hohl. When I needed to change labs in 2017, Tobias was one of the few PIs I reached out to that was willing to set up time to meet. Two hours into a meeting originally scheduled for one, I knew that I had to figure out a way to join his lab. To Tobias, thank you for taking a risk on me: not only was I coming in late in my PhD, but I was also your first ever graduate student. Your mentorship has been critical these past few years in keeping me on track, continually improving my writing, aligning my figures, and ensuring that I became a better scientist than I thought possible only 3 years ago. Beyond the work required to be a PI and mentor, Tobias worked tirelessly to keep the patients of MSK safe during the COVID-19 pandemic, and I will always appreciate his commitment to patient care. To the rest of the Hohl Lab, past and present, you have all been instrumental in helping me out with experiments when needed, being an open ear for ideas and conversations, and welcoming me in to one of the warmest environments I've ever had in science. Shinji Kasahara, Bing Zhai, Nick Tosini, Thierry Rolling, Neta Shlezinger, Yahui Guo, Mariano Aufiero, Kathleen Mills, Mergim Gjonbalaj, Simone Becattini, and Lena Heung: each of you has contributed in ways I cannot begin to describe.

To my current and past thesis committee members, Eric Pamer, Joe Sun, Ross Levine, Richard White, and Jeffrey Berger, thank you for the countless hours you have spent with me, both in committee meetings, and in informal discussions. Without your guidance, this project would not be where it is today.

I also would like to thank Mary and the rest of the Goll Lab. Mary, without your mentorship and guidance those first years, I can safely say that I would not be the scientist that I am today. You helped me grow from a nervous but excited second year PhD student, into a skilled researcher that was able to hit the ground running in a new environment. Varsha, Kathrin, Cheng, Paige, Lianna, and Jun, you were all critical to the second and third years of my PhD.

To the GSK entering class of 2014, thank you for being there for me these past six years. From the nights at Allie Way, to vegetarian dim sum, to super bowl parties, to my wedding, we have made some incredible memories. You were there when we were struggling through problem sets, when we were stressed by our TPEs, when I changed labs, and when I just needed friends. To Chris, Nick, Nayan, Michelle, Jake, Xiaoyi, Yuchen, and Steve, thank you for your friendship and support. You have all meant a lot to me, and I look forward to keeping in touch and seeing all of your accomplishments.

To the graduate school staff, current and historical, thank you for all your work to make GSK what it is. Ken, Linda, Mike, and the rest of the GSK staff, thank you for the hard work you have done over the years to further graduate education at MSKCC. To Tom Magaldi and Thalyana Stathis, you have been amazing resources to me as I prepared to make the jump into the non-academic world; thank you for your help in making the transition as easy as possible.

To my friends outside of science, thank you for bearing with me as I've completed this PhD. There are too many of you to list individually, but know that I cherish each of our friendships, and whether we have seen each other recently or not, you are the reason I was able to complete this dissertation.

Finally, I'd like to thank my family. To my parents, Neri and Aron Tischler, my brother Raphy, and sister Yael: you have been the longest and most constant part of my journey. You have loved me and supported me through the highs and lows of my education. You have done so much for me, and I can only hope that I have sufficiently returned the love you have showed me. To my cousins Daniel, Jesse, Jonah, Toby, Ezra, and Adin, Uncle Ronnie, Auntie Sharon, Uncle Fred, and Auntie Aimee, and the rest of my extended family, thank you for always being there and for the endless good times we have had together over the years. To my in-laws, Karen and Herb Berkowitz, Asher, Jared, Ilana, Yehuda, Aviva, and Avigail, thank you for welcoming me into your family with open arms. Bala Cynwyd has been a source of renewal, both spiritual and culinary, for me. To Yoda, my first dog, thank you for teaching me love and empathy, and for ensuring that most days of the week I get out of the lab at lunch.

Lastly, and most importantly, to my wife Alyssa, thank you for everything you have done for me. We moved in together on my first day of graduate school, and since that moment, you have been critical to my physical (marathons) and mental (you're always willing to listen) well-being. You've kept me nourished and rested, you've picked me up when I've been down, you've dealt with the late nights and early mornings, and you've let me drag you up and down mountains. I don't think I could have done this without you, and words cannot describe how grateful I am that you came into my life over a decade ago, and that you took a leap of faith to stay with me through this PhD. You carried and brought Noah Isaak into our lives, and I love both of you more than words can express.

To everyone who has touched my life, mentioned here or not, you will always have my utmost gratitude.

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LIST OF ABBREVIATIONS

ABPA	Allergic bronchopulmonary aspergillosis
ADP	Adenosine diphosphate
AF633	Alexa Fluor 633
AIDS	Acquired immunodeficiency syndrome
AIM2	Absent in melanoma 2
AML	Acute myeloid leukemia
ASC	Apoptosis-associated speck like protein containing a caspase recruitment domain
BALF	Bronchoalveolar lavage fluid
BM	Bone marrow
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CARD	Caspase Recruitment Domain Family Member
CCL	C-C Motif Chemokine Ligand
CCR	C-C chemokine receptor type
CD	Cluster of differentiation
CFU	Colony forming unit
CGD	Chronic granulomatous disease
CLEC-2	C-type lectin-like receptor 2
CLR	C-typle lectin receptor
COPD	Chronic obstructive pulmonary disease
CR3	Complement receptor 3
CXCL	chemokine (C-X-C motif) ligand
DC	Dendritic cell

DHN	1,8-dihydroxynaphthalene
DMSO	Dimethyl sulfoxide
DT	Diphtheria toxin
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FLARE	Fluorescent aspergillus reporter
GAG	Galactosaminogalactan
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMS	Grocott's methenamine silver
GPVI	Glycoprotein VI
H&E	Hematoxylin and eosin
HCT	Hematopoietic cell transplant
HIF	Hypoxia inducible factor
IA	Invasive aspergillosis
ICU	Intensive care unit
iDTR	Inducible diptheria toxin receptor
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
IV	Intravenous
KO	Knock out

LC3	Microtubule-associated protein 1A/1B-light chain 3
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
MBL	Mannose binding lectin
MFI	Mean fluorescence intensity
MO	Monocyte
Mo-DC	Monocyte-derived dendritic cell
MSKCC	Memorial Sloan Kettering Cancer Center
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NK	Natural killer
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline - Tween20
pDC	Plasmacytoid dendritic cell
PF4	Platelet factor 4
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
PTX	Pentraxin
RAG	Recombination-activating gene
RBC	Red blood cell
RCD	Regulated Cell Death

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SYK	Spleen tyrosine kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
XTT	2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxide

CHAPTER 1: INTRODUCTION

I. *Aspergillus* Pathogenesis

Fungal Infections

Eukaryotic fungi were first recognized to cause disease in the 19th century by Robert Remak, who identified *Trichophyton schoenleinii* as the cause of the skin disease, favus (Grzybowski & Pietrzak, 2013). Since then, fungi have been found to cause a wide range of diseases of varying severity, including skin, mucosal, and invasive infections. These infections are typically manageable (such as athlete's foot, vulvovaginal candidiasis, and ringworm), but both genetic and pharmacological immune deficiency can lead to negative outcomes (G. D. Brown et al., 2012). Multiple medical advances over the 20th century have led to an increase in fungal infection cases, including myeloablative chemotherapies, immune modulatory drugs, and solid organ and hematopoietic cell transplants (Lionakis, Iliev, & Hohl, 2017b). As a result of these advances, as well as the AIDS epidemic, more than 150 million people globally have severe fungal disease, with more than 1.5 million people dying every year (Bongomin, Gago, Oladele, & Denning, 2017). As there are only 3 major drug classes targeting 2 biosynthetic pathways (Tischler & Hohl, 2019), this unacceptably high mortality rate has stimulated immense interest in understanding the immune response to fungi.

Aspergillus fumigatus as a Human Pathogen

Pathogenic fungal species present in 3 general forms: yeasts, filamentous molds, and dimorphic fungi (species that exhibit one form or the other depending on the environmental context). The genus *Aspergillus* is a filamentous mold that is ubiquitous in the environment. As part of the *Aspergillus* lifecycle, asexual spores, called conidia, are released into the air. Humans inhale hundreds, if not thousands, of conidia on a daily basis. Because of their small size (2-3 microns), conidia can bypass mucociliary clearance in the airways. In immunocompetent individuals, the cells of the innate immune response (see below) efficiently clear the conidia before they can germinate into tissue-invasive hyphae. Despite the existence of over 200 *Aspergillus* species on Earth, only a few are associated with human disease. *Aspergillus* species can cause allergic diseases such as allergic bronchopulmonary aspergillosis (ABPA, occurs primarily in cystic fibrosis patients), keratitis (ocular growth of hyphae, primarily impacting agricultural workers), chronic pulmonary aspergillosis (hyphal growth in a pre-existing cavity, typically in patients with underlying lung disease including COPD or treated tuberculosis), or the most severe form, and the focus of this chapter and thesis, invasive aspergillosis (IA) (Tischler & Hohl, 2019).

IA is characterized by tissue-invasive hyphae with or without angioinvasion, which primarily leads to pneumonia (since the lung is the primary site of entry and infection) and potential systemic dissemination (Tischler & Hohl, 2019). IA impacts over 250,000 patients annually (Bongomin et al., 2017). Despite the relative rarity of IA, the fungus-attributable mortality can exceed 20% in at-risk populations (Garcia-Vidal et al., 2015), and is a highly expensive disease to manage (Latgé & Chamilos, 2019). *A. fumigatus* is the primary etiologic agent accounting for the majority of IA cases, although

other non-fumigatus *Aspergillus* species have been reported to cause invasive disease, including *A. flavus*, *A. terreus*, *A. niger*, and *A. nidulans*, as well as other, rarer, *Aspergillus* species (Seyedmousavi, Lionakis, Parta, Peterson, & Kwon-Chung, 2018).

Although immune competent mice and humans are inherently resistant to *A. fumigatus* and to IA, injury to the innate immune system allows inhaled conidia to germinate. Specifically, profound and prolonged neutropenia is the canonical risk factor for the development of IA and is a commonly associated side effect of chemotherapy and hematopoietic cell transplantation (HCT). Qualitative, or functional, defects in the immune response, rather than numerical deficiencies in cell types, can also predispose human patients to disease. Chronic granulatomous disease (CGD), caused by deficiencies in NADPH oxidase function, and corticosteroid treatment, render myeloid cells unable to perform their requisite antifungal functions, and can lead to IA (Dagenais & Keller, 2009). CGD patients in particular have an incidence rate of 2.6 cases per 100 patient years, and in a cohort of 268 patients diagnosed with CGD between 1969 and 2012, 44% of patients had at least one confirmed case of *Aspergillus* infection (Marciano et al., 2015). In part as a result of the associated immune suppression, solid organ transplant recipients are increasingly recognized as being at risk for IA (Morgan et al., 2005)

Beyond the canonical risk factors, patients that receive ibrutinib (a Bruton's tyrosine kinase, or Btk, inhibitor), were recently shown to be at risk for developing IA. In one study, 7 of 18 patients developed IA, with 2 of those patients receiving only ibrutinib and dexamethasone, a corticosteroid (Lionakis et al., 2017a), with a second study showing 8/378 patients undergoing ibrutinib therapy being diagnosed with IA (Varughese et al., 2018). As well, COPD patients and ICU patients (particularly those with influenza)

have become increasingly recognized as being at risk for IA development (Baddley, 2011; van de Veerdonk et al., 2017).

Fungal Growth and Adaptation in the Lung

Like other fungi, *A. fumigatus* has a polysaccharide-rich cell wall (Erwig & Gow, 2016). The outermost layer consists of hydrophobic rodlets called hydrophobins, in particular RodA. This external-most layer is not recognized by the immune system, and allows the conidium to escape immune detection, phagocytosis, and inactivation (see below) (Aimanianda et al., 2009; Dagenais et al., 2010). Beneath the RodA layer is a melanin layer, consisting of dihydroxynapthalene (DHN) melanin in *A. fumigatus* (Pal, Gajjar, & Vasavada, 2014). Though dispensable for fungal growth, deletion of the *pksP* gene results in amelanotic conidia that also disrupts the rodlet layer (Langfelder et al., 1998). Beneath these outer layers is a carbohydrate rich layer consisting of β-1,3-linked glucans, galactomannan, and mannoproteins. These polysaccharides cover a layer consisting of chitin (Fontaine et al., 2000), a polymer of *N*-acetylglucosamine and is adjacent to the plasma membrane (Gow, Latgé, & Munro, 2017). Upon deposition in a suitable environment for growth such as soil, compost, or other decaying organic matter, the conidia begin to germinate. In the process, the outermost hydrophobin and melanin layers are shed, thereby exposing the immunoreactive polypeptides of the cell wall core. As the germlings become hyphae, they synthesize an outer galactosaminogalactan layer (Fontaine et al., 2011; Latgé, Beauvais, & Chamilos, 2017) (Figure 1).

The lung itself is a relatively hostile environment for the germinating fungus. As necessary micronutrients and macronutrients are present in the lung at lower levels than ideal, *A. fumigatus* has evolved multiple mechanisms to thrive in these conditions. In

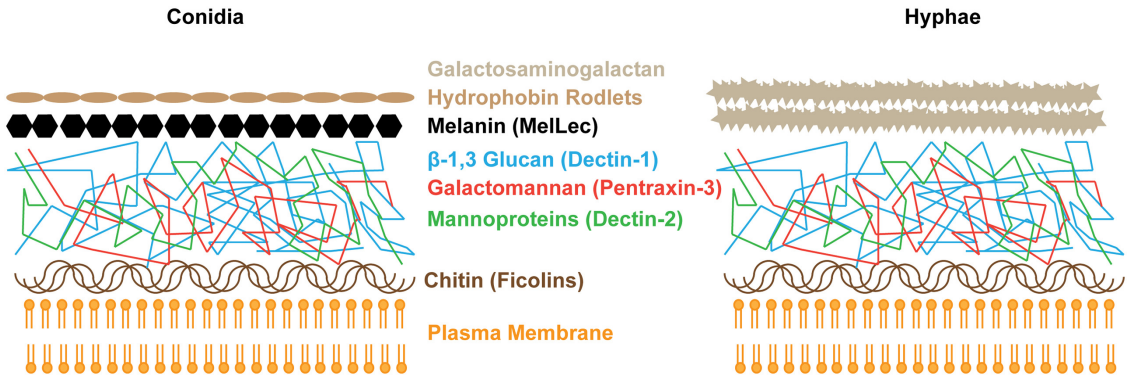

Figure 1: Schematic of the *A. fumigatus* cell wall

The composition of the *A. fumigatus* cell wall depends on the fungal morphotype. In conidia, a hydrophobin and a melanin layer shields the immunogenic core of the cell well, consisting of carbohydrates and glycoproteins. Germlings and hyphae lose the outer conidial layer, leading to exposure of immunoreactive polysaccharides and the eventual synthesis of an outer galactosaminogalactan layer. The indicated host cell receptors trigger innate immune activation in response to exposure of defined polysaccharide and melanin ligands. Reprinted from Tischler, B. Y. & Hohl, T. M. Menacing Mold: Recent Advances in Aspergillus Pathogenesis and Host Defense. J. Mol. Biol. (2019). doi:10.1016/j.jmb.2019.03.027

order to obtain macronutrients from the mammalian lung, *A. fumigatus* is able to utilize extracellular proteases to extract glycoproteins from the mucus layer (Ries, Beattie, Cramer, & Goldman, 2018). Iron is critical for growth of *Aspergillus* and defective uptake renders fungi hypovirulent in immune deficient hosts (Schrettl et al., 2010). In agreement, lung transplant recipients are vulnerable to IA at anastomotic sites, which is consistent with experimental models of infection wherein iron application to lung grafts increase susceptibility to infection (Hsu et al., 2018). These observations raise the possibility that angioinvasion or induction of hemorrhage, micro or otherwise, may be beneficial to invading *A. fumigatus* (Figure 2).

Beyond the availability of nutrients, the host response induces multiple stressors to the lung environment, including hypoxia and oxidative stress. The ability of *Aspergillus* to adapt to hypoxic environments impacts the virulence of particular strains (Kowalski et al., 2016). Multiple pathways are important for this adaptation. As part of the adaptation to the hypoxic environment, *A. fumigatus* can switch engage the fermentation pathway for carbon assimilation, and proper growth depends on the expression of AlcC (Grahl et al., 2011). Correct sterol synthesis is also important, as both SrbA and RbdA are required for optimal fungal adaptation under hypoxic and non-hypoxic conditions (Dhingra et al., 2016; Vaknin et al., 2016). With respect to oxidative stress, the transcription factor Yap1 controls multiple stress response genes, including superoxide dismutases, catalases, and thioredoxins (Hillmann et al., 2016; Leal et al., 2012), and is itself regulated by cofilin, an actin polymerizing factor (Jia et al., 2018) (Figure 2).

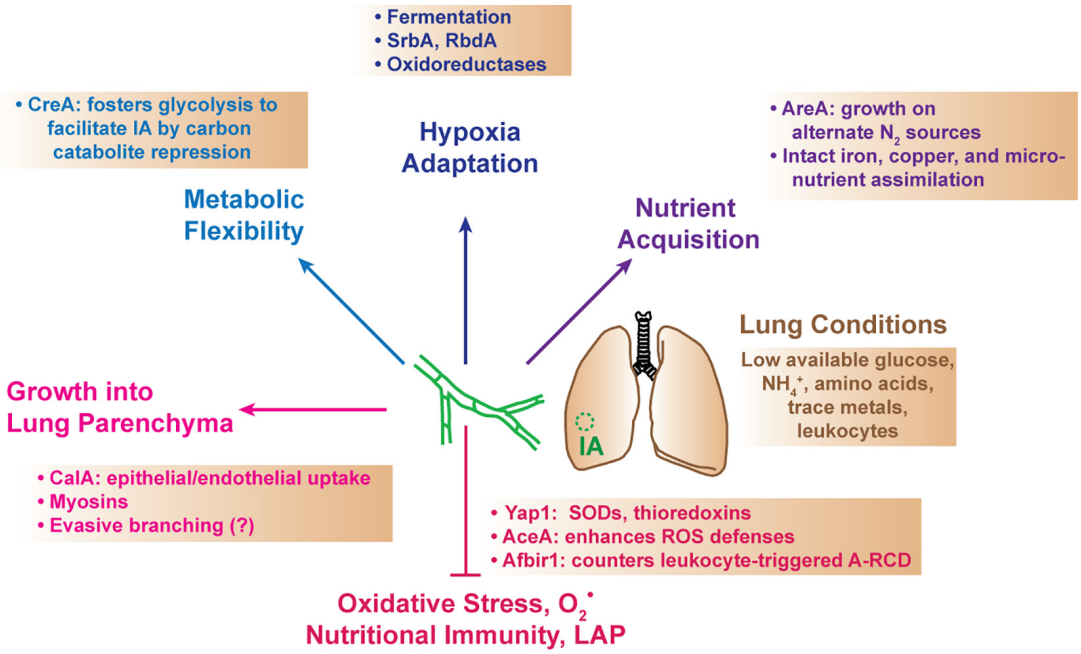

Figure 2: *Aspergillus* adaptations and gene programs that promote invasive pulmonary aspergillosis

Aspergillus can establish IA under specific host conditions by overcoming scarce nutrient availability, exhibiting metabolic flexibility and adaptation of tissue hypoxia, invading the lung parenchyma, and neutralizing oxidative and non-oxidative host defense mechanisms. The panels indicate a selected set of fungal genes and fungal properties that are essential to promote invasive disease and to counter host defense mechanisms. SODs, superoxide dismutases; ROS, reactive oxygen species; A-RCD, apoptosis-like regulated cell death; LAP, LC3-associated phagocytosis. Reprinted from Tischler, B. Y. & Hohl, T. M. Menacing Mold: Recent Advances in Aspergillus Pathogenesis and Host Defense. J. Mol. Biol. (2019). doi:10.1016/j.jmb.2019.03.027

Strain Selection, Host Context, Tissue Damage, and Virulence

All of these factors combine to give *A. fumigatus* the ability to survive in patient lungs. However, how *A. fumigatus* grows and damages its host is dependent on the specific characteristics of the strain used and the form of immune injury. There are multiple strains used for *in vivo* and *in vitro* research on IA. Two of the most common isolates utilized in laboratory research were originally isolated from patients: the original Af293 reference genome strain (Nierman et al., 2005) isolated from a rheumatoid arthritis patient, and the CEA10 strain, isolated from an acute leukemia patient (which is more typical of the IA patient). Of the two, CEA10 is more virulent within the context of a murine infection (Table 1) (Keller, 2017). In agreement with this, CEA10 conidia germinate and grow hyphae more rapidly *in vitro*, as well as in mice (Caffrey-Carr, Kowalski, et al., 2017c) and zebrafish (Rosowski et al., 2018). Perhaps as a result of the increase in germination and growth rates, CEA10 is cleared more quickly than Af293 (Rosowski et al., 2018). Consistent with this, evidence has emerged that CEA10 and Af293 induce differing cellular and immune responses. Specifically, lung damage mediators such as hypoxia inducible factor 1α (HIF-1α), and neutrophil chemotaxis markers including chemokine (C-X-C motif) ligand (CXCL) 1 and 2, and leukotriene B4 (LTB_4), are more highly upregulated in models of CEA10 challenge compared with Af293, and the regulatory cytokine IL-10 is reduced. (Caffrey-Carr, Hilmer, Kowalski, Shepardson, Temple, Cramer, & Obar, 2017a; Rizzetto et al., 2013). The differential induction of these molecules is directly correlated with host survival, as loss of HIF-1α directed LTB_4 synthesis renders mice more susceptible to CEA10 compared with Af293, suggesting that host hypoxia signaling is critical for murine survival in some infectious

Strain	CEA10	Af293
Virulence	High	Low
Germination	Fast	Slow
Elimination in the lung	More rapid	Less rapid
Lung Inflammation	Higher levels	Lower levels
Hypoxia fitness	High	Low, can be increased by serial passaging in hypoxia

Table 1: Comparison of *A. fumigatus* CEA10 and Af293 strains

Reprinted from Tischler, B. Y. & Hohl, T. M. Menacing Mold: Recent Advances in Aspergillus Pathogenesis and Host Defense. J. Mol. Biol. (2019). doi:10.1016/j.jmb.2019.03.027

contexts but not others (Caffrey-Carr, Hilmer, Kowalski, Shepardson, Temple, Cramer, & Obar, 2017b). Adapting Af293 to a hypoxic environment renders it similarly virulent to CEA10 in mice, consistent with the idea that there are specific virulence factors that are unique to each strain (Kowalski et al., 2016). For these reasons, it has become common in the fungal pathogenesis field to test phenotypes in multiple strains.

It is not enough to simply consider the impact of strain selection on virulence; the host context must be considered as well. As pharmacologic immune suppression is inherently pleiotropic, many studies have sought to address *in vivo* pathogenesis and host responses by studying immune competent mice, though approximately 75% of published studies use some type of immune suppression (Desoubeaux & Cray, 2017). It is challenging to do so, however, as immune competent mice are highly resistant to disease (Hohl, 2014), necessitating inocula as high as 10^8 conidia when combined with genetic alterations to observe differences in experimental outcomes such as mortality. Immune suppression requires lower inocula, as low as 10^4 – 10^7 conidia depending on the type of injury, with CGD mice requiring fewer conidia than neutropenic or steroid treated mice (Sugui et al., 2017).

The exact cause of morbidity and mortality of the host depends on the nature of immune injury, as does disease progression. Upon infection of neutropenic mice with *A. fumigatus*, conidia germinate into extensive and angioinvasive hyphae, that in turn cause tissue necrosis (Sugui et al., 2017). Transfusion of myeloid cells to neutropenic mice rescues this phenotype and improves survival after *A. fumigatus* challenge. On the other hand, treatment of mice with corticosteroids have appropriate, if not excessive, neutrophil recruitment, and limited hyphal growth, leading to tissue hypoxia. As such, additional myeloid cells have no bearing on infection outcome (Kalleda et al., 2016).

CGD mice exhibit similar phenotypes to corticosteroid-treated mice, with limited hyphal growth and angioinvasion (Sugui et al., 2017). Together, these data suggest that phagocytes can play both positive and detrimental roles in IA pathogenesis. Consistent with the damage response framework of microbial pathogenesis (Casadevall & Pirofski, 2003), the absence of neutrophils represents an quantitatively inadequate immune response that results in the overgrowth of the pathogen. In contrast, impaired function of neutrophils and other phagocytes allows for the control of hyphal growth, but at the expense of increased inflammation. This in turn can lead to harmful immune pathology, and the death of the host.

Summary

Though often underappreciated, fungal pathogens represent a global public health threat. In particular, *A. fumigatus*, an environmental mold, is an opportunistic pathogen that primarily impacts immune compromised individuals. Over the last decades, the number of cases has increased substantially, with mortality remaining exceptionally high for an infectious disease. *A. fumigatus* is inhaled in its conidial form, where it is protected by immunologically inert melanin and hydrophobin layers. Upon germination, these external layers are lost, exposing immunogenic polypeptides, and then replaced by galactosaminogalactans. Throughout their life cycle in the lung, *A. fumigatus* must adapt to the inherently nutrient poor environment of the lung, as well as the stressors instigated by the host immune system, in a strain dependent manner. Depending on the underlying host condition, *A. fumigatus* can induce death either by extensive angioinvasive hyphal growth, or by causing pathogenic inflammation.

II. Host Immune Responses to *Aspergillus*

Innate and Adaptive Immune Cells

As suggested in Part I, neutrophils and monocytes are critical components of the innate immune response to *A. fumigatus*. Inducing neutropenia prior to or immediately following *A. fumigatus* challenge results in severe susceptibility to Af293 infection, and is characterized by the extensive hyphal growth described above (Mircescu, Lipuma, van Rooijen, Pamer, & Hohl, 2009). Similarly, depletion of C-C chemokine receptor type 2 (CCR2)-expressing monocytes through a CCR2 promoter-driven diphtheria toxin receptor (DTR) strategy, but not $CCR2^{+}$ natural killer (NK) cells or innate lymphoid cells (ILC) results in a survival defect and invasive hyphal growth indistinguishable from that observed in neutropenic mice. Monocytes, once recruited to the lung, can differentiate into monocyte-derived dendritic cells (Mo-DCs). Both monocytes and their Mo-DC derivatives are able to kill phagocytosed conidia (Espinosa et al., 2014). A specific subset of DCs, plasmacytoid dendritic cells (pDCs), are also critical for host defense, as antibody-mediated depletion of pDCs causes increased susceptibility to *A. fumigatus* challenge (Ramirez-Ortiz et al., 2011). Though alveolar macrophages have fungicidal activity (Bhatia et al., 2011; Jhingran et al., 2012), other cells of the innate immune response, such as neutrophils, appear to be sufficient to compensate for their loss (Mircescu et al., 2009).

The clear importance of the innate immune system for anti-*Aspergillus* immunity is in stark contrast to the relative insignificance of the adaptive immune system. As RAG2- and IL-2 receptor common γ chain-deficient mice are equally sensitive to fungal challenge as their wildtype counterparts, it can be concluded that T cells, B cells, and

other cells of the lymphoid lineage do not play a critical role in countering *Aspergillus* challenge (Espinosa et al., 2014). This is not to say that T cells are completely irrelevant, as *Aspergillus*-specific T cells can augment the response of neutrophils to *Aspergillus in vitro* (Beck et al., 2006). Though seemingly dispensable for invasive disease, T cells can drive immunopathology seen in non-invasive forms of aspergillosis (Latgé & Chamilos, 2019).

Innate Immune Recognition of Aspergillus

A key hallmark of the innate immune system is the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Multiple PRRs have been associated with anti-*Aspergillus* immune recognition, but the C-type lectin receptor (CLR) family has become increasingly recognized as a critical component of the innate immune response (Tischler & Hohl, 2019). The CLR Dectin-1, encoded by *CLEC7A*, recognizes β-1,3-glucans, which are expressed by germinating conidia, is the major receptor for *A. fumigatus* recognition (Steele et al., 2005). Dectin-1 generally exists as a monomer on the cell membrane, but can form homo-oligomers after recognition of its ligand (J. Brown et al., 2007). Polymorphisms in human *CLEC7A* are correlated with IA susceptibility in HCT patients, with those with wildtype loci having fewer infections by 24 months post-transplant (Fisher et al., 2017).

The FcRγ-coupled receptor Dectin-2 (*CLEC6A*) recognizes cell wall mannans (McGreal et al., 2006), and is activated by *A. fumigatus* (Loures et al., 2015). Dectin-3 (*CLEC4D*) has been reported to form heterodimers with Dectin-2 to aid in *C. albicans* recognition (Zhu et al., 2013), though it is dispensable for *C. neoformans* immunity (Campuzano, Castro-Lopez, Wozniak, Leopold Wager, & Wormley, 2017), and thus the

implications for *A. fumigatus* recognition remain unclear. Dectin-3 is also reported to form a complex with Mincle (*CLEC4E*) (Miyake, Masatsugu, & Yamasaki, 2015) and the two are coregulated (Kerscher et al., 2016), though its role to date in anti-*Aspergillus* immunity is mostly putative, with some data suggesting a protective role in *Aspergillus* keratitis (Yu et al., 2018). CD23 (*CLEC4J*), also a CLR coupled to FcRγ, recognizes fungal β-glucans and α-mannans and is required for survival after intravenous murine *Aspergillus* infection (Guo et al., 2018). Fungal galactomannans are also recognized by the CLR DC-SIGN (*CLEC4L*), the absence of which leads to impaired *in vitro* phagocytosis (Serrano-Gómez et al., 2004). Though DHN melanin was previously thought to largely be inert from the standpoint of innate immune recognition, the recently described CLR MelLec (*CLEC1A*) recognizes conidial melanin, and when absent, immunocompetent mice are susceptible to systemic models of *Aspergillus* challenge, and polymorphisms in the *CLEC1A* locus double the risk of IA in human patients (Stappers et al., 2018). Loss of surfactant protein D (SP-D), a soluble CLR that may recognize melanin, leads to impaired cytokine responses (Wong et al., 2018) (Figure 1).

Beyond the CLRs, the toll-like receptor (TLR) family has also been implicated in protection against IA. However, TLR polymorphisms are only correlated with increased risk in otherwise susceptible hosts (Park & Mehrad, 2009). The TLR4 S4 haplotype, for example, has a 2-20-fold increase in IA risk in allogeneic HCT patients (Bochud et al., 2008). In contrast, neither loss of TLR4 nor TLR2 expression in an immune competent murine model results in susceptibility to *Aspergillus* challenge (Dubourdeau et al., 2006). TLR4 is required for murine survival in the context of cyclophosphamide immune suppression. In the absence of suppression both TLR4 and TLR2 knockouts exhibit impaired fungal clearance (Bellocchio et al., 2004). TLR3 deficiency in both immune deficient mice (cyclophosphamide) or humans (certain SNPs in HCT transplant donors)

also increases susceptibility to *Aspergillus* infection with protection dependent on adaptive immune memory, specifically the priming of CD8 T-cells by myeloid DCs (Carvalho et al., 2012). Unlike TLR2, TLR3, and TLR4, TLR9 deficiency appears to be beneficial in some invasive murine models of aspergillosis, with knockouts surviving longer than wildtypes when neutropenic mice are infected with resting conidia. These benefits are reduced when mice are challenged with pre-swollen conidia (Ramaprakash, Ito, Standiford, Kunkel, & Hogaboam, 2009). As TLR deficiency phenotypes are generally present only in the context of neutropenia, they likely reflect impacts on general immune modulation rather than intrinsic anti-*Aspergillus* pathways.

Integrin signaling is important for anti-*Aspergillus* immunity as well. Complement receptor 3 (CR3), also known as Mac-1, consists of the CD11b and CD18 subunits and binds β-glucan in addition to the complement proteins (O'Brien et al., 2012). In the absence of Mac-1, neutrophils fail to recruit to *A. fumgatus*-infected corneas resulting in more severe disease (Leal et al., 2012). This phenotype is not observed in pulmonary models of infection, as neutrophils are recruited normally, albeit with impaired fungal phagocytosis (Jhingran et al., 2012). The lack of a severe phenotype in the lung model of infection was confirmed by a separate group in 2019, where a loss of CD11b did not cause impaired survival, but an increase in neutrophil recruitment was accompanied by increased colony forming units (CFU) of fungi, a less inflammatory environment, and impaired phagocytosis (Teschner et al., 2019), a finding similar to *Escherichia coli* and *Streptococcus pneumoniae* pneumonia (Mizgerd et al., 1997). The disparate phenotypes observed in different sites of infection suggests that there are tissue specific requirements for immunity to *A. fumigatus* and other pathogens. This underscores the necessity to assess the immune response to *Aspergillus* in clinically relevant models utilizing physiological routes of infection.

Soluble receptors are also important in innate immune recognition of *Aspergillus* species. Pentraxin-3 (PTX-3), secreted by macrophages in response to inflammatory stimulus, recognizes galactomannan on *A. fumigatus* conidia, but, likely due to the de novo synthesis of galactosaminogalactans (GAGs) on the hyphal surface, PTX does not bind hyphae. PTX-3 directly binds conidia *in vitro*, and in its absence, mice are susceptible to pulmonary fungal challenge (Garlanda et al., 2002). Other types of soluble factors, such as the ficolins and mannose binding lectin (MBL), have proven to be important for anti-*Aspergillus* host defense. Both of these receptors activate the lectin complement pathway. M-ficolin is reported to interact with the inner chitin layer (Jensen et al., 2017), and ficolin deficient mice have delayed fungal clearance, normal neutrophil recruitment, and impaired inflammatory cytokine production (Genster et al., 2016). MBL directly binds *A. fumigatus in vitro* (Neth et al., 2000), and enhances phagocytosis. *In vivo*, MBL aids in host defense in corticosteroid-treated animals, as treatment with recombinant human MBL increases murine survival from 0% to 80% in conjunction with an increase in the inflammatory microenvironment (Kaur, Gupta, Thiel, Sarma, & Madan, 2007). Whether through CLRs, TLRs, soluble receptors or integrins, PRRs play important, if often overlapping, roles in *Aspergillus* recognition and host immunity.

Anti-Fungal Innate Immune Signaling

Both Dectin-1 and FcRγ-coupled receptors (CD23, Dectin-2, Dectin-3, Mincle) signal through intracellular immunoreceptor tyrosine-based activation motif (ITAM) domains, either attached directly to the receptor (like Dectin-1) or through the associated FcRγ adapter (Tischler & Hohl, 2019). ITAMs contain repeated sequences of YXXI/L that are phosphorylated by Src kinases upon stimulation and recruit other kinases such as

spleen tyrosine kinase (Syk) (Drummond & Brown, 2011) in a SHP-2 dependent manner (Deng et al., 2015). Upon Syk recruitment to the phosphorylated ITAM, Syk itself is phosphorylated and activated. Upon activation, Syk initiates a downstream signaling cascade that begins with the phosphorylation of protein kinase C delta type (PKCδ). Further downstream, PKCδ then phosphorylates CARD9 (Strasser et al., 2012). CARD9, itself an adaptor protein, complexes with BCL10 and MALT1, which then induces translocation of the NF-κB transcription factor to the nucleus and its subsequent activation (Gross et al., 2006). CARD9-dependent NF-κB signaling then leads to the activation of myeloid cells, independently of lymphocyte activation, indicating the importance of this pathway to anti-fungal immune defense (Hara et al., 2007). Dectin-1/Syk/CARD9-dependent NF-κB signaling then leads to the transcription and eventual secretion of various mediators of anti-fungal immunity, including TNF, IL-12, IL-6, CXCL1, CXCL2, and pro-IL-1β (Tischler & Hohl, 2019).

In vivo data support the importance of the Dectin-1/Syk/CARD9 pathway in anti-*Aspergillus* immunity. Recognition of the germinating conidium and initiation of the Syk signaling cascade begins with the ITAM receptors, particularly Dectin-1. The requirement for Dectin-1 is strain and host dependent. Infection of immunocompetent Dectin-1 knockout 129/SvEv mice with the isolate 13073 results in increased mortality with an impaired immune response compared to wildtype controls (Werner et al., 2009). In contrast, Dectin-1 deficient C57BL/6 mice exhibit no increased mortality in response to infection with the Af293 strain (Jhingran et al., 2012). In agreement with the Jhingran findings, resistance to IV *C. albicans* infections is dependent on cooperation between the various CLRs, with single knockout mice of Dectin-1, Dectin-2, or Mincle having reduced susceptibility compared to multiple knockouts (Thompson et al., 2019). Beyond *Aspergillus* and *Candida*, in response to the dimporphic fungus *Histoplasma capsulatum*,

CR3 and Dectin-1 collaborate in a Syk-dependent manner, suggesting synergy between these two important anti-fungal receptors (Huang et al., 2015). Consistent with the idea of redundancy amongst CLRs, otherwise immunocompetent Syk-deficient mice exhibit increased susceptibility to pulmonary *A. fumigatus* challenge (Jhingran et al., 2012). Further downstream, deletion of CARD9, in conjunction with loss of MyD88 signaling, also results in diminished survival after *A. fumigatus* inoculation. Underscoring the importance of non-TLR pathways in anti-*Aspergillus* immunity, MyD88's critical role is downstream of IL-1R rather than TLRs (Jhingran et al., 2015). Once dissemination occurs, the Dectin-1/Syk/CARD9 pathways remains critical, as extrapulmonary infections are reported in human patients with CARD9 deficiency (Rieber et al., 2016). Together, these reports confirm the critical nature of CLR-induced Syk signaling during IA.

In parallel to the CARD9-dependent and NF-κB-coupled transcription of inflammatory mediators, activated Syk also induces the activation of PLCγ2 and a subsequent increase in intracellular Ca^{2+} levels after zymosan treatment (Xu, Huo, Lee, Kurosaki, & Lam, 2009). This signaling step leads to calcineurin-dependent activation of the NFAT pathway (Goodridge, Simmons, & Underhill, 2007). Activation of NFAT-dependent transcription leads to an increase in CD23 transcription and surface expression (Le Roy et al., 2012) as well as PTX-3 transcription and secretion (Zelante et al., 2017) (Figure 3).

Adding to the complexity of the anti-*Aspergillus* response, NFAT signaling also appears to be dependent in part on the action of BTK. Inhibition of BTK through ibrutinib treatment, or otherwise impairing the function of BTK, blocks *in vitro* activation of NFAT and NF-κB through endosomal activation of TLR9 (Bercusson, Colley, Shah, Warris, & Armstrong-James, 2018). Intriguingly, BTK drives calcineurin/NFAT signaling, apparently

independent of Dectin-1, in the context of *in vitro Aspergillus* challenge (Herbst et al., 2015), highlighting the importance of model selection discussed above.

Both the PKCδ and PLCγ2 arms of Syk signaling lead to IL-1β activation separately from their roles in NFAT and NF-κB signaling (Tischler & Hohl, 2019). In the PKCδ pathway, the cysteine protease caspase-8 is recruited to the CARD9-BCL10-MALT1 complex along with the ASC upon stimulation with curdlan independently of phagocytosis. *In vitro*, swollen *A. fumigatus* conidia drive the recruitment and activation of caspase-8, which subsequently leads to cleavage of pro-IL-1β to its active form (Gringhuis et al., 2012). In parallel, germination of *A. fumigatus* leads to Syk-dependent generation of reactive oxygen species (ROS) and K^+ release (Saïd-Sadier, Padilla, Langsley, & Ojcius, 2010), and ROS generation is downstream of PLCγ2 in non-fungal models of inflammation and infection (Graham et al., 2007). The generation of ROS leads to the formation of the NLRP3 and AIM2 inflammasomes (multiprotein complexes that drive generation of proinflammatory cytokines), and subsequently leads to the formation of a single inflammasome consisting of ASC, caspase-1, and caspase-8. Activated caspase-1 (possibly activated by caspase-8) is then able to cleave IL-1β to its active form. The NLRP3 and AIM2 inflammasomes, while redundant with each other, are together required for survival in immunosuppressed mice (Karki et al., 2015) (Figure 3).

Innate Immune Cell Recruitment

The first major step in recruitment and activation of the innate immune response to *A. fumigatus* is the release of IL-1α (Caffrey et al., 2015) and IL-1β (Jhingran et al., 2012). Though the exact cellular sources of IL-1 signaling are not yet clear, work from

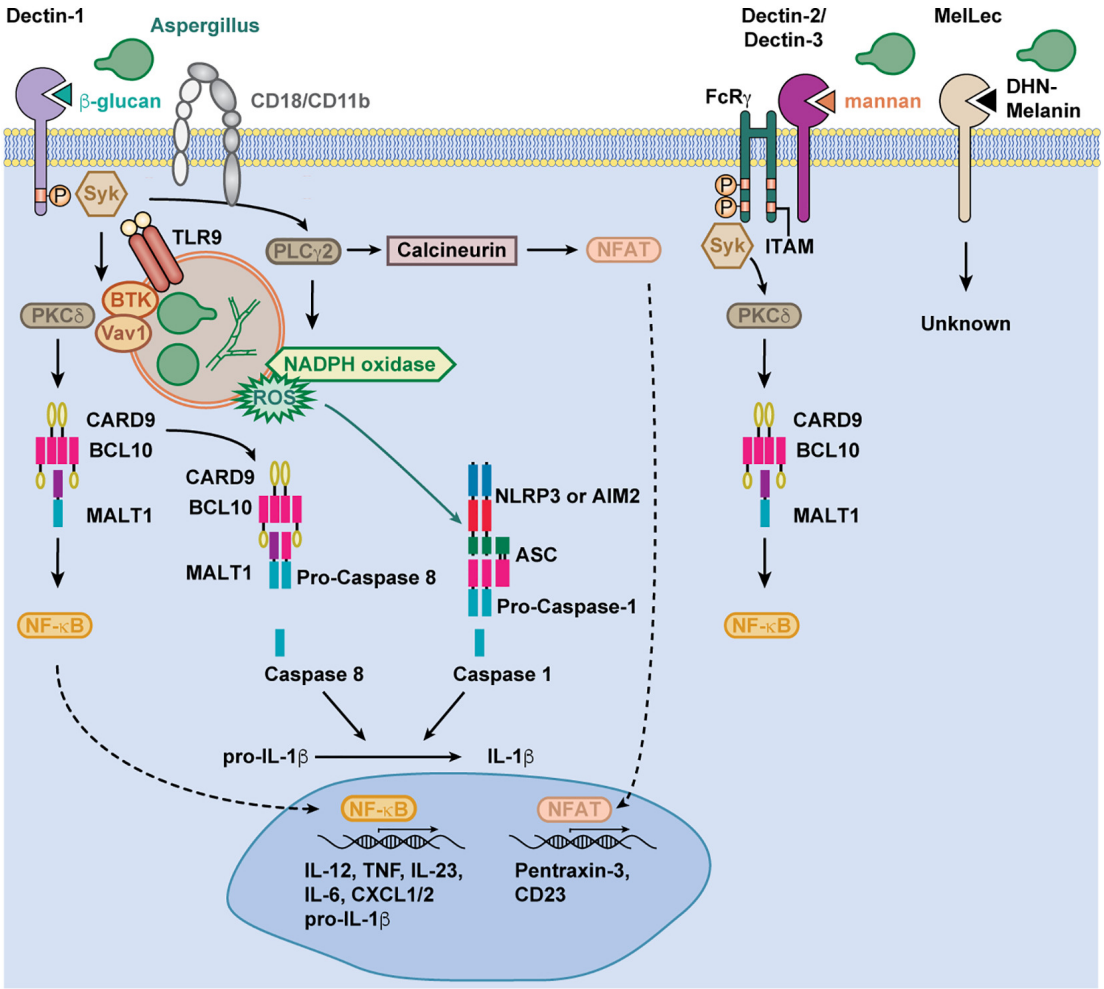

Figure 3: *Aspergillus* ligands activate C-type lectin receptor signaling in myeloid cells

A. fumigatus β-glucans activate Dectin-1 and CR3 signaling, resulting in Syk phosphorylation. *A. fumigatus* can activate the FcRy-coupled receptors Dectin-2 and Dectin-3 via mannan moieties, and MelLec via DHN-melanin exposure. Dectin-1/Syk and Dectin-2/Syk activation is transduced via PKCδ and CARD9 for assembly of the ternary CARD9/BCL10/MALT1 complex, NF-κB activation, and the transcription of NFκB-dependent genes, including pro-IL-1β and other cytokines. The assembly of NLRP3 and AIM2 inflammasomes results in proteolytic activation of caspase-1 and the production of bioactive IL-1β. Alternatively, ASC and caspase-8 recruitment to the CAD9/BCL10/MALT1 complex gives rise to active caspase-8 and bioactive IL-1β. CR3/Syk-dependent PLCγ2 activation is linked to NADPH oxidase assembly, the production of reactive oxygen species, and calcineurin-dependent NFAT activation. NFAT-dependent genes include pentraxin-3 and CD23. Dectin-1 can interact with VAV1 and BTK to facilitate macrophage fungal phagocytosis. BTK may also promote calcineurin activation and the production of NFAT-regulated cytokines. The model depicts fungal killing in the phagosome. Reprinted from Tischler, B. Y. & Hohl, T. M. Menacing Mold: Recent Advances in Aspergillus Pathogenesis and Host Defense. J. Mol. Biol. (2019). doi:10.1016/j.jmb.2019.03.027

Caffrey-Carr and colleagues suggested that a hematopoietic cellular population is responsible for the production of both IL-1α and IL-1β. Intriguingly, only IL-1α is required for non-immunosuppressed murine survival, and only when challenged with a virulent *A. fumigatus* isolate, highlighting the importance of strain and host model selection (Caffrey-Carr, Kowalski, et al., 2017c). MyD88, an adaptor protein downstream of both IL-1R and Toll-like receptors, is required for optimal immune responses to *A. fumigatus* challenge (Bretz et al., 2008).

Though IL-1α and IL-1R signaling are important for survival and neutrophil recruitment in otherwise immune competent mice, the same signal could be detrimental in immune suppressed states with impaired fungicidal immune activity, such as corticosteroid or CGD patients. Highlighting the requirement for IL-1 signaling in infection, there has been a report of *Aspergillus* pneumonia in a Still's disease-patient treated with anakinra, a modified recombinant human IL-1R antagonist, even though this is not widely recognized as a risk factor (Bilgin et al., 2018). On the other hand, in corticosteroid-treated mice, anakinra is able to inhibit the development of hypoxia and marginally extend murine survival (Gresnigt et al., 2016). Similar findings are seen in CGD mice, with high doses of anakinra extending survival, while increasing autophagy, and reducing neutrophil recruitment, inflammasome activation, as well as lung IL-1β and IL-17A levels (De Luca et al., 2014). These observations are consistent with a model where IL-1R signaling is important for the initiation of inflammation, but that continued activation of this pathway in specific host contexts such as CGD or steroid treatment results in neutrophil-driven immunopathology.

In high inoculum models of infection, IL-1R drives MyD88 signaling in radioresistant lung cells, leading to the induction of CXCL1 and CXCL5. The induction of

these cytokines leads to the first wave of neutrophil recruitment to the lung, the absence of which impairs murine survival in immunocompetent mice (Jhingran et al., 2015). In line with this finding, the receptor for CXCL1 and CXCL5, CXCR2, is critical for neutrophil recruitment and murine survival (Mehrad et al., 1999). CARD9 is dispensable for the initial phase of recruitment, suggesting an as yet unidentified mechanism for initial fungal detection or molecular redundancy. Continued recruitment of neutrophils to the lung is reliant on leukocyte secretion of CXCL1, CXCL2 (also a CXCR2 ligand), and CXCL5 in a CARD9-dependent manner. In the absence of these pathways, *Aspergillus* challenge is lethal (Jhingran et al., 2015). Accordingly, while CARD9 deficiency is not associated with pulmonary IA, likely due to normal early neutrophil recruitment, there have been reports of patients with CARD9 mutations developing intra-abdominal aspergillosis, as noted above (Rieber et al., 2016).

In parallel to the IL-1R/MyD88/CXCR2 axis, the transcription factor hypoxia inducible factor 1α (HIF1α) drives neutrophil recruitment pathways. In the absence of HIF1α, otherwise immune competent mice are highly vulnerable to *Aspergillus* challenge and exhibit a higher fungal burden. The decrease in survival is associated with reduced neutrophil recruitment early in infection without impacts on phagocytosis or killing of conidia, and the neutrophils that are present are more apoptotic. Consistent with the neutrophil recruitment defect, there is less CXCL1 present in the airways (Shepardson et al., 2014). This suggests that early neutrophil recruitment is, in part, mediated by HIF1α-dependent regulation of CXCR2 cytokines. Beyond the canonical CXCR2 cytokine-driven neutrophil recruitment, the virulent CEA10 strain induces LTB_4 synthesis in a HIF1α-dependent manner. In the absence of LTB_4 receptor, neutrophil recruitment is impaired, coincident with an increase in fungal burden, with analogous results in the

absence of all leukotrienes (Caffrey-Carr, Hilmer, Kowalski, Shepardson, Temple, Cramer, & Obar, 2017a).

Beyond neutrophils, inflammatory monocytes must also be recruited for optimal host defense. In the absence of CCR2, monocyte recruitment to the lung is impaired, as is further differentiation to Mo-DCs. Mo-DCs, rather than being recruited, differentiate from CCR2-positive monocyte progenitors (Hohl et al., 2009). The inability of inflammatory monocytes to migrate to the lung is likely tied to the fact that in the absence of CCR2 signaling, monocytes accumulate in the bone marrow and are at lower levels in the blood at steady state and in response to bacterial *Listeria monocytogenes* infection. However, adoptive transfer experiments suggest that CCR2 is not required for recruitment to sites of infection after egress from the bone marrow, indicating a different chemotactic pathway is necessary for the final steps of inflammatory monocyte recruitment (Serbina & Pamer, 2006).

Phagocytosis and Killing of Conidia and Hyphae by Neutrophils

Multiple innate immune cell types engage in fungal phagocytosis and conidial killing after infection. Professional phagocytes such as alveolar macrophages, monocytes, and neutrophils, each play important roles in the anti-*Aspergillus* response. Although dispensable for high inoculum models of IA, as chlodronate liposome mediated depletion of alveolar macrophages exhibit no severe phenotypes (Mircescu et al., 2009), alveolar macrophages are able to phagocytose conidia independent of the Dectin-1/Syk/CARD9 axis (Jhingran et al., 2012). Not only is phagocytosis of conidia by alveolar macrophages not dependent on the canonical recognition pathway, it is also independent of β-glucan exposure, as conidial phagocytosis occurs within 2 hours of *in*

vitro challenge (Philippe et al., 2003). Upon engulfment, alveolar macrophages form an acidified phagolysosome containing the conidia (Ibrahim-Granet et al., 2003). Subsequent killing of conidia requires germination (Philippe et al., 2003), and recognition by Dectin-1 occurs intracellularly within the phagolysosome after several hours (Faro-Trindade et al., 2012). This process leads to the development of reactive oxygen intermediates (the “respiratory burst”) which can kill conidia (Philippe et al., 2003), highlighting the importance of oxidative killing.

After MyD88 and CXCR2-dependent, but monocyte-independent, recruitment (Espinosa et al., 2014; Jhingran et al., 2015), neutrophils engage conidia in a process dependent on the Dectin-1/Syk/CARD9 axis. In the absence of Dectin-1, neutrophils exhibit a delay in conidial uptake. Although conidial uptake is 60% of normal levels at 12 hours post infection, by 36 hours, phagocytosis is normal. CARD9 and Syk, downstream of Dectin-1 and other ITAM-coupled receptors, is required for proper phagocytosis and conidial killing throughout infection. In their absence, otherwise immunocompetent mice have evidence of tissue-invasive hyphae and an increase in CFU. Further, though NADPH oxidase is important for neutrophil conidial killing, neutrophils retain reduced fungicidal capabilities (Jhingran et al., 2012), underscoring the fact that non-oxidative mechanisms of conidial killing are important for host defense, and perhaps offering an explanation of why CGD patients have lifetime incidence rates of up to 50% rather than 100% (Henriet, Verweij, Holland, & Warris, 2013).

The induction of ROS then leads to a variety of possible downstream events. Recent studies by Shlezinger and colleagues suggest that host ROS production mediates the induction of an apoptosis-like regulated cell death (RCD) pathway. Upon conidial killing by neutrophils, there is an increase in caspase activity and TUNEL

staining. This can in part be overcome by overexpression of the *AfBIR1* anti-apoptotic protein. Increased resistance to RCD increases fungal virulence independent of host NADPH oxidase function (Shlezinger et al., 2017). Unlike conidia, hyphae are too large to be efficiently phagocytosed. Though the exact mechanism is unclear, NADPH oxidase, which generates hydrogen peroxide, and myeloperoxidase (MPO), which converts H_2O_2 into HOCl, are both required for *in vitro* fungal hyphal killing (Gazendam et al., 2016b).

It is clear that ROS-independent mechanisms of conidial killing exist, as human neutrophils are reported to kill conidia in a CR3-dependent non-oxidative manner (Gazendam et al., 2016b). Lactoferrin, a mediator of iron depletion, has been reported have anti-fungal activity (Leal et al., 2013), and appears to be partially responsible for conidial killing rather than ROS (Gazendam et al., 2016b). While there are important differences between the experimental systems of Jhingran and Gazendam which could explain the differences, these data do suggest the potential role of nutritional immunity in anti-*Aspergillus* host defense. In agreement with this idea, calprotectin (a zinc and manganese chelator) is necessary for killing corneal hyphae *Aspergillus* keratitis models of infection, though it is dispensable for phagocytosis and killing of conidia in the lungs (Clark et al., 2016). The exact contributions of these and other non-oxidative mechanisms of fungal killing remain an important topic of current research.

Monocytes are critical for the innate immune response to *A. fumigatus*. In the absence of $CCR2^+$ monocytes, otherwise immunocompetent mice are susceptible to *A. fumigatus* infection. Though the depletion of monocytes does not impact neutrophil recruitment to the infected lung, normally recruited neutrophils exhibit impaired fungicidal activity (Espinosa et al., 2014). The exact mediator of this phenotype was later revealed

to be type I and type III interferons. Upon detection of *A. fumigatus* monocytes release interferons α and β. These type I interferons then signal to both hematopoietic and non-hematopoietic pulmonary cells to release IFN-λ. Neutrophils themselves are responsive to IFN-λ signaling, and in the absence of their cognate receptor, IFNLR1, mice are highly susceptible to *A. fumigatus* challenge. This is concurrent with a strong decrease in neutrophil ROS production and a corresponding increase in pulmonary fungal burden (Espinosa et al., 2017). Other extracellular signaling pathways are important to enhancing the respiratory burst. Though the source of GM-CSF remains to be uncovered, GM-CSF receptor is required for optimal conidial phagocytosis, ROS production and conidial killing by neutrophils (Kasahara et al., 2016). These data emphasize that intercellular crosstalk has profound implications for the killing of fungi and control of infection.

Summary

Upon engulfment and germination, conidia trigger IL-1 release by a hematopoietic phagocyte, likely the alveolar macrophage. Non-hematopoietic IL-1R signaling triggers CXCL1 and CXCL5 release, allowing the recruitment of neutrophils to the lung in a HIF-1α-dependent manner. Other critical cells, such as monocytes, are recruited independently. While there are many receptors that recognize germinating *A. fumigatus* conidia, conidia trigger the canonical Dectin-1/Syk/CARD9 pathway, resulting in phagocytosis, additional secretion of CXCL1, CXCL2, and CXCL5, and further recruitment of neutrophils. Neutrophils then kill conidia largely, but not exclusively, via ROS production that is regulated by GM-CSF signaling and monocyte-initiated interferon signaling. The phagocyte oxidative burst then induces a regulated cell death pathway in conidia (Figure 4).

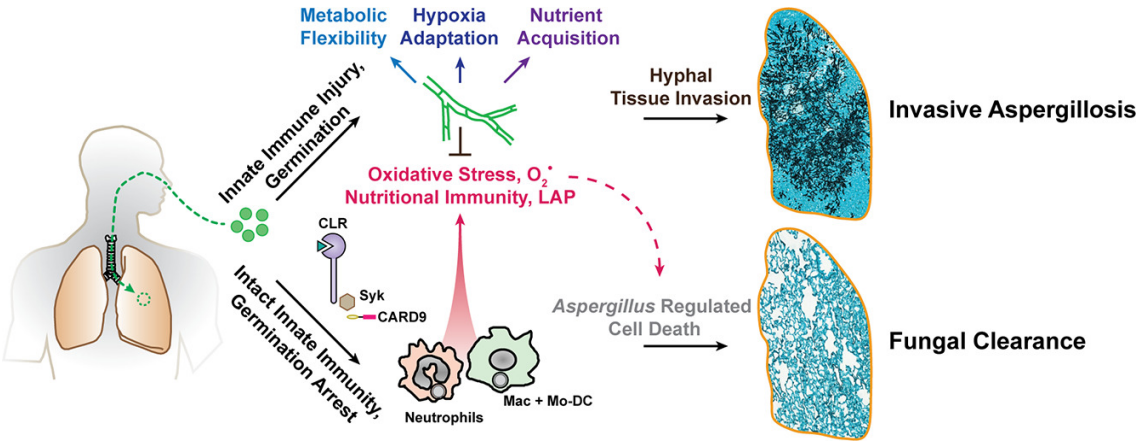

Figure 4: Summary of *A. fumigatus* pathogenesis

Upon inhalation, an immune competent host can arrest germination through the Dectin 1-Syk-CARD9 axis and the action of neutrophils, macrophages, monocytes, and monocyte-derived dendritic cells. Through a combination of nutritional immunity, LC3-associated phagocytosis, and oxidative stress, the immune system can induce a form of regulated cell death in the invading *Aspergillus* pathogen and clear the fungus. In the context of innate immune injury, the germinating conidium adapts to the hypoxic and nutrient deficient environment to form invasive hyphae and to cause IA. Reprinted from Tischler, B. Y. & Hohl, T. M. Menacing Mold: Recent Advances in Aspergillus Pathogenesis and Host Defense. J. Mol. Biol. (2019). doi:10.1016/j.jmb.2019.03.027

III. The Role of Platelets in Antimicrobial Host Defense

Platelets

Platelets are small (~2 μm in humans), non-nucleated, discoid-shaped, cells that are derived from polyploid megakaryocyte progenitors in the bone marrow and lung of mammals (Lefrançais et al., 2017). As part of platelet biogenesis, megakaryocytes generate extensions called proplatelets that extend from their site of residence into the blood stream. Once in the circulation, proplatelets continue to fragment into mature platelets. Platelets are the second most common cell type in the blood, with counts in humans reaching 400 x 10^9/L and up to 1600 x 10^9/L in mice. These cells, while numerous, are temporally limited, with a lifespan of approximately 1 week, necessitating constant production. Although these cells are small and have no nucleus, and thus no genomic DNA, platelets retain many characteristics of their nucleated counterparts. The cytoplasm contains both mature and pre-mRNA, and retains all cellular infrastructure necessary for de novo protein synthesis, allowing platelets to serve their role as an acute phase reactant (Semple, Italiano, & Freedman, 2011).

The primary role of platelets has long been thought to be in hemostasis. Upon disruption of the vasculature, platelets bind to exposed collagen and extracellular matrix proteins. Platelets then aggregate and release activation mediators, eventually resulting in the production of thrombin and subsequent activation of the coagulation cascade. At the end of the process, the platelets form a plug that also includes fibrin, leukocytes and red blood cells (Semple et al., 2011).

Platelets as Immune Cells

Platelets are increasingly recognized for roles beyond their canonical functions in hemostasis. In particular, they appear to function as immune or inflammatory cells, despite their lack of nucleus. Multiple immune receptors such as TLRs (Cognasse et al., 2005), FcγRII (Rosenfeld et al., 1985), CR3 (Cosgrove, d'Apice, Haddad, Pedersen, & McKenzie, 1987), and various chemokine receptors (Clemetson et al., 2000), are expressed on the surface of platelets, allowing platelets to respond directly to pathogens and indirectly via extracellular signaling pathways. Though their contribution to the immune response is unknown, platelet surface receptors CLEC-2 (a c-type lectin) and GPVI (an integrin) signal through ITAMs, suggesting that Syk signaling is relevant for proper platelet function (Ichinohe et al., 1997; Suzuki-Inoue et al., 2006).

Platelets also appear to have the ability to be directly recruited to sites of inflammation, as pulmonary installation of LPS leads to increased recruitment to the lungs and airways (Cleary et al., 2019). Like neutrophils, platelets contain several classes of granules that are released in response to external stimulus. Dense granules contain vascular mediators such as ADP, lysosomal granules that contain proteases and glycosidases, and, most notably for the purposes of this discussion, alpha granules contain many proteins that are important for immune functionality, including adhesion mediators, platelet microbicidal peptides, and kinocidins (chemokines that also exert antimicrobial functions, with CXCL4/PF4 being the primary example) (Yeaman, 2014). Moreover, platelets generate ROS through oxidases (Begonja et al., 2005), suggesting that platelets could function through direct killing and by recruiting other immune cell subtypes.

Importantly for this discussion of antifungal immunity, part of the contribution of platelets to inflammation is the recruitment and activation of neutrophils. Platelets are reported to be involved in this process both through direct and indirect mechanisms. Upon platelet activation, P-selectin (CD62P) is transferred from platelet alpha granules to the cell surface and can bind to PSGL-1 on neutrophils. Of the many chemokines released by activated platelets, platelet derived CXCL4 and CCL5 have been reported to be necessary for neutrophil recruitment to sites of inflammation. Moreover, once recruited, platelets assist in neutrophil phagocytosis, ROS generation and neutrophil extracellular trap (NET) formation (Rossaint, Margraf, & Zarbock, 2018). While the ability of platelets to potentiate NET formation is intriguing, the evidence for anti-*Aspergillus* functionality of these structures is controversial. Although a case report suggests that restoration of NET formation appeared to be beneficial to a CGD patient with IA (Bianchi et al., 2009), a number of studies suggest that NETs possess limited (or no) fungicidal activity (Bruns et al., 2010; Clark et al., 2018; Gazendam et al., 2016b; Gazendam, van de Geer, Roos, van den Berg, & Kuijpers, 2016a).

Platelets in Host Defense

Various *in vivo* models of bacterial infection have underscored the importance of platelets for host defense. Using a murine PF4 promoter-regulated inducible diphtheria toxin receptor, Wuescher and colleagues showed that continued depletion of megakaryocytes results in profound thrombocytopenia (Wuescher, Takashima, & Worth, 2015). In the absence of platelets, mice are highly vulnerable to IV *Staphylococcus aureus* infection coincident with an increase in bacterial burden in various organs, including the heart, kidney, and liver. Perhaps due to the increased bacterial burden, IFN-γ, IL-10, and TNF-α are elevated after infection (Wuescher et al., 2015). Similar to

the IV model, a pulmonary model of *Pseudomonas aeruginosa* infection suggests that platelets are important for immunity in a physiological route of infection. Pulmonary infection with *P. aeruginosa* leads to increases in airway PF4 levels, as well as increases in platelet recruitment. In an antibody model of thrombocytopenia, fewer neutrophils are recruited after infection, which aligns with a higher bacterial burden and a slight but significant decrease in murine survival (Amison et al., 2018).

Host defense by platelets is not strictly limited to their antimicrobial roles. During immune thrombocytopenia, *Klebsiella pneumoniae* is highly virulent in murine pulmonary infection, with almost all mice rapidly succumbing to disease. Though this is concurrent with an increase in bacterial burden, mice with very low platelet counts exhibit severe lung hemorrhage in response to infection (de Stoppelaar et al., 2014), suggesting that maintaining tissue integrity is a critical role for platelets in certain models of infection. Similarly, a genetic model of thrombocytopenia induced by deletion of the thrombopoietin receptor (which reduces circulating platelet counts to 10% of normal levels) predisposes mice to *P. aeruginosa*. Unlike the Amison study, Bain and colleagues found that the cause of death in thrombocytopenia is due to severe pulmonary hemorrhage caused by apoptosis of the epithelial barrier induced by a soluble factor secreted by the bacterium (Bain et al., 2019). In line with this study, LPS is reported to induce pulmonary hemorrhage in thrombocytopenic mice (Goerge et al., 2008; Hillgruber et al., 2015), as does *Streptococcus pneumoniae* (van den Boogaard et al., 2015). Together, these data suggest that platelets play bimodal roles in protecting against pathogen induced inflammation: 1) platelets can directly and indirectly kill invading pathogens, and 2) platelets maintain tissue and vascular integrity during the immune response.

Platelets in Invasive Aspergillosis

The potential role of platelets in host defense in IA is increasingly recognized. A retrospective clinical study revealed a low platelet count prior to induction of chemotherapy and before diagnosis of IA to be predictive of a negative outcome in patients with IA. Post chemotherapy platelet counts were not assessed (Nouér et al., 2012). Furthermore, thrombocytopenia has been identified as a univariate risk factor for developing IA in liver transplant patients (Rosenhagen, Feldhues, Schmidt, Hoppe-Tichy, & Geiss, 2009). Prolonged thrombocytopenia is also a risk factor for developing invasive fungal infections when AML patients undergo myeloablative induction chemotherapy (Lien et al., 2018). However, evaluating the role of platelets in preventing IA is somewhat challenging in human patients, as thrombocytopenia is often associated with other cytopenias, including neutropenia, the major clinical risk factor for IA (Lien et al., 2018; Rosenhagen et al., 2009).

Over the last 20 years, multiple groups have attempted to clarify the precise activity of platelets in response to *Aspergillus* challenge. These studies have largely fallen into two categories of *in vitro* experiments: (1) assessing the direct antifungal capacity of platelets and (2) determining whether there is any crosstalk with other cell types. The first reports of direct antifungal activity of platelets began with the observation by Christin and colleagues in 1998 that human platelets directly interact with *A. fumigatus* hyphae. Through this interaction, platelets release their granules and induce hyphal damage, as measured by cell wall protein release and a decrease in XTT reduction activity, a marker of fungal metabolic activity (Christin et al., 1998).

Various morphotypes of *A. fumigatus* activate platelets *in vitro*, as measured by surface CD62P (Rødland et al., 2010). Platelets assessed after intravenous inoculation of Balb/c mice with *A. fumigatus* conidia show analogous results (Speth et al., 2013). Activation occurs upon direct interaction with fungal galactosaminogalactan on hyphae or melanin on conidia (inhibited by hydrophobins) (Rambach et al., 2015), but does not strictly require direct fungus-platelet contact, since an *A. fumigatus* supernatant activates platelets *in vitro* via gliotoxin or an unknown secreted protease(s) (Speth et al., 2013), or secreted galactosaminogalactan (Deshmukh et al., 2020). Interaction between *Aspergillus* and platelets then leads to impaired hyphal germination and elongation, in conjunction with reduced galactomannan release (Perkhofer et al., 2008), and increased transcription of *A. fumigatus* genes involved in the stress response (Perkhofer et al., 2015). The mechanism of platelet-mediated fungal impairment occurs independent of known actions and targets of antifungal drugs, since platelets in conjunction with amphotericin B, as well as certain echinocandin and azole drugs, additively reduce the germination rate of *Aspergillus* (Perkhofer et al., 2013; Perkhofer, Trappl, Nussbaumer, Dierich, & Lass-Flörl, 2010; Perkhofer, Trappl, Striessnig, Nussbaumer, & Lass-Flörl, 2011).

In parallel to direct antifungal activity, Christin and colleagues hypothesized that platelets could supplement fungal killing by neutrophils and observed increased aggregation of assay components (likely consisting of neutrophils, platelets and *A. fumigatus* but not shown or described in detail). However, they were unable to see additive effects of platelets on neutrophil anti-fungal activity due to aggregate-related assay variability (Christin et al., 1998). Since then, a series of *in vitro* studies that point to a potential role in priming the immune system have been published. THP-1 cells and monocytes increase IL-8 secretion after stimulation with platelet-rich plasma and *A.*

fumigatus hyphae, compared with control conditions (Rødland et al., 2010). Moreover, co-culture of DCs with platelet-rich plasma and *A. fumigatus* increases the expression of the CD83 and CD86 activation markers on DCs, relative to control conditions. Platelet-rich plasma also improves macrophage phagocytosis of conidia, and enhances the antifungal activity of macrophages and DCs (Czakai et al., 2017). Recently, *in vitro* coagulation was found to cause neutrophils to aggregate around platelets and conidia, pointing to a potential role for platelets in augmenting neutrophil activity (Frealle et al., 2018).

Summary

Platelets are anucleate cells that are produced from megakaryocytes and are the most numerous component of the blood other than red blood cells. Platelets are typically thought of in their roles in maintaining hemostasis after vascular injury. Increasingly, however, platelets are recognized for their roles in immunity and inflammation. Not only do platelets maintain a full repertoire of cellular material to respond to inflammatory and infectious stimuli, in vivo and in vitro evidence supports both antimicrobial and tissue protective roles for platelets after challenge. In line with clinical data identifying platelet counts as a risk factor for IA, in vitro studies suggest a role for platelets in directly impairing *A. fumigatus* and assisting leukocytes in their antifungal activity (Figure 5).

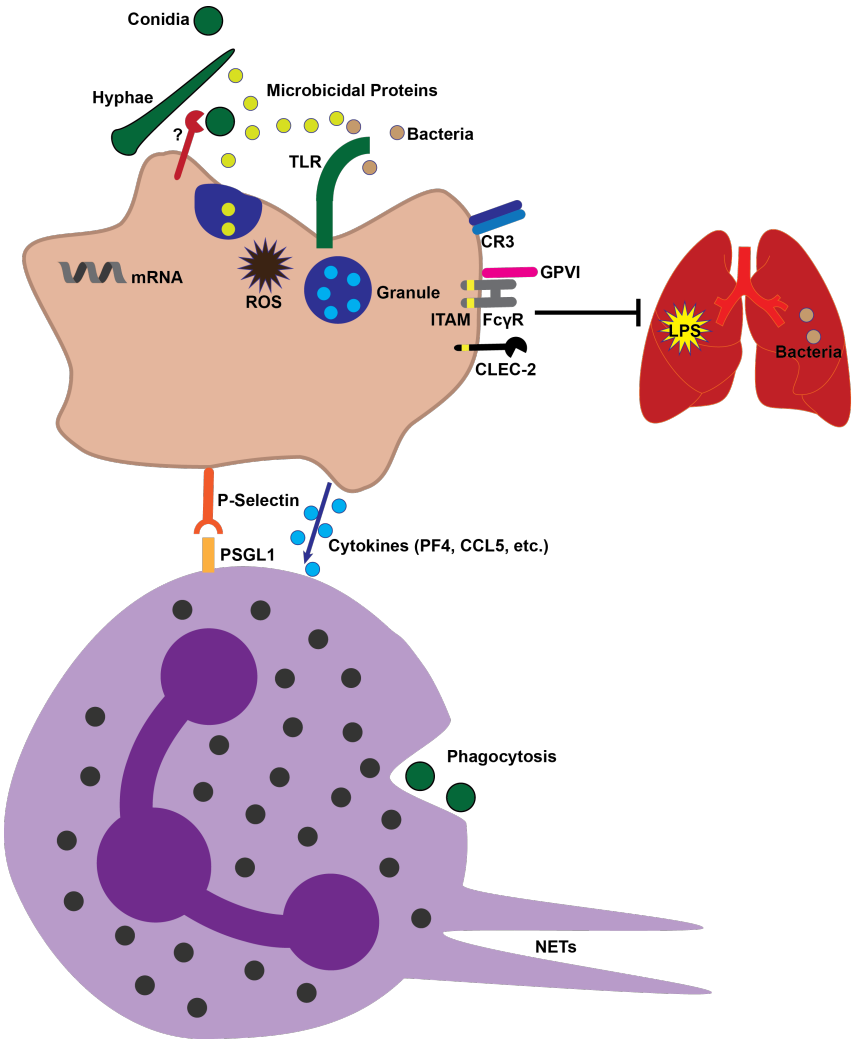

Figure 5: Summary of platelet immune function

Platelets (light brown) are anucleate cell fragments that react acutely to host damage beyond their canonical roles in hemostasis and thrombosis. Platelets contain mRNA, granules, can synthesize ROS, and express multiple surface receptors (including TLRs, integrins (CR3, GPVI, FcγR), and C-type lectins (CLEC-2)). Many of these receptors signal through ITAMs. Upon activation with microbial ligands, platelets release their granules containing microbicidal proteins and cytokines, and upregulate surface P-Selectin expression. This enables the recruitment and activation of leukocytes such as neutrophils and can induce the formation of NETs and phagocytosis. In line with their canonical roles, platelets maintain hemostasis in response to inflammatory stimulus such as LPS or live bacteria at various sites of inflammation including the lung.

IV. Summary and Thesis Goals

Invasive aspergillosis is an infectious disease with a high mortality rate; the most common etiologic agent is the filamentous mold *A. fumigatus*. Thrombocytopenia, or a low platelet count, has been recognized in multiple clinical studies as a risk factor for IA acquisition and IA progression. Complicating efforts to understand the role of platelets within anti-fungal host defense is that neutropenia, itself the primary risk factor for developing invasive fungal infections, often co-occurs with thrombocytopenia. Although there is increasing evidence that suggests a role for platelets in the immune system, evidence for platelet anti-*Aspergillus* activity remains limited to *in vitro* studies. The objective of this dissertation is to integrate the role of platelets into a comprehensive *in vivo* model of pulmonary *A. fumigatus* infection. In Chapter 2, I describe the materials and methods utilized in these studies. In the first part of Chapter 3, I assess the necessity of platelets to anti-*A. fumgatus* host defense. In the second part of Chapter 3, I determine whether platelets exhibit direct or indirect effects on the immune response to *A. fumigatus* infection. In the third part of Chapter 3, I quantify the role of platelets in maintaining lung tissue integrity and hemostasis during the inflammatory response. In Chapter 4, I review my results and place them into the scientific context, discuss the implications of my findings, and suggest future directions.

CHAPTER 2: MATERIALS AND METHODS

Chemicals and Reagents

Unless indicated otherwise, chemicals were purchased from Fisher Scientific or Sigma Aldrich, cell culture reagents from Thermo Fisher Scientific, and microbiological culture media from BD Biosciences. Antibodies for flow cytometry were acquired from one of BD Biosciences, Thermo Fisher Scientific, or Tonbo (Table 2).

Fungal Strains, Growth and Preparation

In the studies conducted in this dissertation, the *A. fumigatus* strains CEA10 and Af293 were used. For experiments involving leukocyte function (FLARE), a DsRed strain of Af293 described in (Jhingran et al., 2012) was utilized. The melanin-deficient Δ*pksP* (AF293.1 background) strain was a generous gift from Dr. Robert Cramer of Dartmouth College, and was generated by replacing the endogenous locus of the *pksP* gene with *pyrG*.

To grow the conidia, glycerol stocks of the relevant conidia were streaked onto agar slants and allowed to grow for approximately one week at 37 degrees Celsius. For the majority of experiments, conidia were grown on glucose minimal media prepared by Hohl Laboratory technicians. For experiments involving FLARE conidia, DsRed conidia were grown on BD Difco™ Sabouraud Dextrose Agar slants (Catalog Number BD 211661).

Antibody	Manufacturer	Clone	Catalog Number
CD41-FITC	Thermo Fisher	MWReg30	MA5-16874
CD41-APC	BioLegend	MWReg30	133914
CD41-Alexa700	BioLegend	MWReg30	133926
Ly6G-FITC	BD	1A8	551460
Ly6G-Alexa700	BD	1A8	561236
Ly6C-PE-Cy7	BD	AL-21	560593
CD11b-vF450	Tonbo	M1/70	75-0112
CD45-BV605	BioLegend	30-F11	103139
CD45-APC-Cy7	BD	30-F11	557659
CD45.2-PerCP-Cy5.5	Tonbo	104	65-0454
CD11c-PE-Cy7	BD	HL3	558079
CD11c-BV605	BioLegend	N418	117334
MHCII-Alexa700	Thermo Fisher	M5/114.15.2	56-5321-82
MHCII-APC-780	Thermo Fisher	M5/114.15.2	47-5321-82
Live/Dead (APC780)	Tonbo	N/A	13-0865
Live/Dead (506)	Thermo Fisher	N/A	65-0866-14
Live/Dead (510)	Tonbo	N/A	13-0870
EpCAM-EF450	Thermo Fisher	G8.8	48-5791-82
CD31-APC	BioLegend	390	102409

Table 2: List of antibodies

Conidia were harvested by flooding the slants with 5 mL of PBS supplemented with 0.025% Tween-20 (PBS-T) (Fisher Scientific Catalog Number BP337-500) and vortexing vigorously. The suspension was filtered through a 40 μm filter and centrifuged at 2000 RPM for 5 minutes and resuspended in 5 mL PBS-T and counted using a hemacytometer. The conidial suspension was centrifuged again and resuspended in PBS-T according to experimental needs, discussed in more detail in the relevant sections. For *in vivo* experiments, the conidial concentration was verified by plating serial dilutions on Sabouraud dextrose agar plates and counting the colonies.

For experiments involving resting heat-killed conidia, the conidia were prepared as above, and then boiled at 100 degrees Celsius for 30 minutes. In order to generate swollen, heat-killed conidia, conidia were suspended in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 mmol/L HEPES [pH 7.4], 1.1 mmol/L L-glutamine, 0.5 U/ml penicillin, 0.5 μg/ml gentamicin, 50 mg/ml streptomycin, and 50 mol/l 2- mercaptoethanol, and 1mM sodium pyruvate (RPMI+ medium), as well as 0.5 μg/mL voriconazole (to prevent hyphal formation) at a concentration of ~$5x10^6$ conidia per mL in 50 mL conical tubes. The conidial suspension was incubated overnight on rotation. The next morning, the suspension was centrifuged at 2000 RPM for 5 minutes, suspended in 1-2 mL PBS-T, and boiled for at least 30 minutes at 100 degrees Celsius. To confirm killing, the suspension was plated on Sabouraud dextrose agar plates to ensure no colonies were present.

To generate FLARE conidia, DsRed-expressing Af293 conidia were suspended in 0.05M $NaHCO_3$ (pH 8.3) at a concentration of 7.5 x 10^8/mL. After adding 10 μL of a 10 mg/200μL DMSO stock of Biotin-XX, SSE (Molecular Probes B-6352), the conidial

suspension was incubated for at least 2 hours at 4 degrees Celsius, up to overnight. After incubation, the suspension was centrifuged and the pellet resuspended in 1mL of 100 mM Tris-HCl (pH 8.0) to inactivate free biotin, and centrifuged again. After centrifugation, the conidial pellet was resuspended in 1 mL of normal PBS, and incubated with 10 µL of a 2mg/mL stock of streptavidin-conjugated Alexa Fluor 633 (AF633, Molecular Probes S-21375) for 1 hour at room temperature, shielded from light. After incubation, the suspension was centrifuged and resuspended in PBS-T, counted, adjusted, and verified by plating serial dilutions on Sabouraud dextrose agar plates and counting the colonies for *in vivo* experiments. Proper labelling was verified by flow cytometry.

Mice

Ethics Statement

All animal studies were approved by the MSKCC Institutional Animal Care and Use Committee under protocol number 13-07-008. All procedures were compliant with state and federal regulations including all applicable provisions established by the Animal Welfare Act and the Public Health Services (PHS) Policy on the Humane Care and Use of Laboratory Animals. Throughout all experimental procedures, mice were monitored for stress, pain, or discomfort, and euthanized upon severe morbidity.

General Information

To conduct the studies described in this dissertation, C57BL/6J (stock #00664), PF4-iCre (stock #008535), $Syk^{Fl/Fl}$ (stock # 017309), mTmG (stock #007676) and

ROSA26iDTR (stock #007900) mice were purchased from the Jackson Laboratory, and C57BL/6-Ly5.1 (CD45.1, stock #564) was purchased from Charles River Laboratories. After arrival, the mice adjusted to the MSKCC vivarium for one week prior to any experimentation. Age, sex, and/or littermate controls were used for experiments as appropriate and available. If advised by MSKCC veterinary staff at any point during infection, mice were euthanized by CO_2 asphyxiation. For certain experiments, mice were euthanized at specific time points by either CO_2 asphyxiation or pentobarbital injection.

Bone Marrow Chimera Generation

To generate bone marrow (BM) chimeras, $CD45.1^+$ recipient mice were lethally irradiated with 9 Gy. After irradiation, 1-5 x 10^6 $CD45.2^+$ BM cells of the relevant genotype were infused into the tail vein of each mouse in a volume of 200 µL PBS. Subsequently, chimeric mice were treated with water supplemented with Baytril (enrofloxacin) for 3 weeks post irradiation to prevent infection. Mice were allowed to recover for at least 6 weeks to ensure full reconstitution of the hematopoietic compartment. See the below section entitled “Bone Marrow Harvest” for details on isolation.

Platelet Depletion and Counting

In these experiments, two parallel methods of inducing thrombocytopenia were utilized. To induce thrombocytopenia via antibody-mediated depletion, C57BL/6J mice were injected intraperitoneally with 100 µL of rabbit anti-mouse platelet serum (Accurate Chemical and Scientific Corporation, AIA31440) diluted 1:4, or an equivalent volume of

diluted normal rabbit serum (Accurate Chemical and Scientific Corporation, JNZ000120). As a second model, PF4-iCre mice were crossed to ROSA26-iDTR (iDTR) mice ($iDTR^{Pf4}$) to enable targeted depletion of platelet-progenitor megakaryocytes. On the first day of depletion, 400 ng of diphtheria toxin (DT) (List Biological Laboratories, 150) was injected intraperitoneally into Cre-positive mice and Cre-negative littermates, followed by additional doses of 200 ng DT on day 2 and day 4.

To monitor platelet counts, mice were lightly anesthetized by isoflurane, nicking the lateral tail vein, and collecting 2 µL of peripheral blood. The collected blood was placed into FACS buffer (0.5% BSA, 0.1 mM EDTA, 1X PBS) containing 2 µL of fluorescent beads (Spherotech, Inc., ACFP-70-10) and FITC-conjugated anti-CD41 antibody (see Table 2) and kept on ice until analysis by flow cytometry (LSR II). The absolute platelet count was determined according to manufacturer's instructions.

Infection

6-12-week-old mice, or mice that were at least 6 weeks post-transplant, were lightly anesthetized by isoflurane. Once the breathing rate slowed to approximately 1 breath per second, individual mice were held vertically and 50 µL of the conidial suspension was placed on the nares. After inhalation of the entire suspension, mice were returned to their cages and monitored for discomfort.

Survival Experiments

To monitor survival, mice were depleted of platelets as discussed above. For antiserum-mediated thrombocytopenia, mice were injected 2 hours prior to infection, with

additional injections every 24 hours. For the DT-mediated thrombocytopenia, mice were infected 6 days after the initial DT dose. Throughout the experiment, mice were monitored twice daily. Moribund mice were humanely euthanized upon consultation with MSKCC veterinary services and recorded as deaths. For platelet rescue experiments as indicated in the text, 1 x 10^8 platelets were infused by intravenous injection through the tail vein (see below for details on platelet isolation). In some experiments, the mice were weighed and normalized to the starting average to monitor morbidity. In the event of the death of a mouse, its weight at death was used to determine a cohort's average weight on subsequent days.

Tissue Harvest and Analysis

Bone Marrow Harvest

To obtain bone marrow (BM) cells, 6-12-week-old mice were euthanized by CO_2 asphyxiation. Post euthanasia, tibias and femurs were removed from the animals. The ends were removed from each animal and flushed with either sterile PBS or sterile RPMI+. The BM was then centrifuged for 5 minutes at 1500 RPM and resuspended in 1 mL of 1X sterile RBC Lysis Buffer (Tonbo Biosciences catalog number TNB-4300-L100) for 5 minutes. After 5 minutes, 9 mL of sterile PBS was added to the cell suspension, filtered through a 100 μm filter, and centrifuged as before. The cell pellet was then suspended in either sterile PBS (for *in vivo* generation of BM chimeras) or RPMI+ medium (for *in vitro* assays).

Platelet Isolation

C57BL/6J mice were euthanized by pentobarbital injection. After ensuring non-responsiveness, blood was drawn from the vena cava into syringes containing 3.8% sodium citrate as an anticoagulant. Citrated blood from multiple mice was pooled and centrifuged for 10 minutes at room temperature at 100 x g, and with no brake. After centrifugation, the top layer of platelet-rich plasma was removed and mixed with an equivalent volume of HEP buffer (140mM NaCl, 2.7mM, 3.8 mM HEPES, 5 mM EGTA, pH 7.4) and supplemented with 1 μM prostaglandin E1 for *in vitro* use. The plasma suspension was then centrifuged at 100 x g for 15 minutes, with no brake, to pellet any contaminating large cells such as RBCs or leukocytes. The resulting platelet suspension was then centrifuged at 800 x g for 15 minutes and no brake to pellet the platelets. The platelet pellet was washed twice in platelet wash buffer (10mM sodium citrate, 150 mM NaCl, 1mM EDTA, 1% (w/v) dextrose, pH 7.4) without resuspension. After the washes, the platelets were resuspended in Tyrode's buffer (130 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) supplemented with 3 mg/mL BSA, 5 mM glucose, as well as 1 μM prostaglandin E1 for *in vitro* use. Platelets were enumerated by flow cytometry as above and adjusted to the proper concentration in Tyrode's buffer with supplements.

Western Blot Analysis

Cell lysate for Western blotting was prepared by incubating the platelet suspension with 2x lysis buffer (2% NP40, 30 mM HEPES, 150 mM NaCl, 2 mM EDTA, pH 7.4) supplemented with HALT protease and phosphatase inhibitor (Thermo Fisher Scientific Catalog Number 78440) on ice for 20 minutes. The lysate was then centrifuged

for 20 minutes at full speed on a microcentrifuge. The supernatant was combined with Bolt LDS Sample Buffer (Thermo Fisher Scientific Catalog Number B0007) and sample reducing agent (Thermo Fisher Scientific Catalog Number B0004), heated according to manufacturer's instructions, and separated by SDS-PAGE and transferred to nitrocellulose membranes using Bolt MES reagents from Thermo Fisher Scientific. Membranes were probed using the primary and secondary antibodies listed in Table 2. After blotting, the membranes were imaged on a LiCor Odyssey Infrared Imager.

Fungal Burden

Mice were infected as above and euthanized by CO_2 asphyxiation at predetermined time points. After verification of death, lungs, brain, spleen, and kidney were removed and placed in 2 mL of cold PBS. The harvested organs were homogenized using a Power Gen 125 homogenizer (Fisher). Colony forming units (CFU) were determined by plating serial dilutions on Sabouraud dextrose agar plates and manual counting.

Preparation of Mice for Histopathology (and Enumeration of Germination)

Infected mice were euthanized at 2 days post infection by pentobarbital, the vena cava was cut, and the lungs were perfused with copious amounts of PBS (8-10 mL) through the right ventricle. The lungs were then inflated using 4% paraformaldehyde (PFA) while additional organs were harvested and placed in PFA. The inflated lungs were then placed in PFA. After 72h fixation at 4 degrees Celsius, the fixed tissues were washed in deionized water and stored in 70% ethanol prior to processing by the MSKCC Molecular Cytology Core Facility. The Core embedded the tissues in paraffin, generated

sections, and stained with with hematoxylin & eosin (H&E) or Grocott's methenamine silver stain (GMS). The Core also acquired whole slide images using a Zeiss Mirax Midi slide scanner and assisted with image processing using Panoramic Viewer. Using the GMS-stained slide sections, the germination rate was determined by counting the number of germlings and hyphae and dividing by the total number of fungal particles counted using ImageJ software.

Analysis of Leukocyte Function

Thrombocytopenic or control mice were infected with FLARE conidia described above. After euthanasia with pentobarbital, lungs were perfused with PBS through the right ventricle and removed. Lungs were then dissociated using a gentleMACS tissue dissociator and digested in PBS supplemented with 5% PBS, 2.2 mg/mL collagenase type IV, and 100 µg DNase I for 30-60 minutes at 37 degrees Celsius under gentle rotation. After incubation, lungs were homogenized again, and centrifuged for 5 minutes at 1500 RPM and 4 degrees Celsius. The cell pellet was then suspended in 1 mL RBC lysis buffer. After 5 minutes of room temperature incubation, 9 mL PBS supplemented with 5% FBS was added to each cell suspension and filtered through a 100 µm filter. The filtrate was then centrifuged as above, and the cell pellet resuspended in FACS buffer.

The single cell suspension was stained with fluorescent antibodies to Ly6G, Ly6C, CD11b, CD45, CD11c, MHCII, as well as a live-dead stain (see Table 2 for specifics). Neutrophils were identified as live $CD45^+$ $CD11b^+$ $Ly6G^+$ cells, monocytes as live $CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$ $CD11c^-$ cells (some experiments did not distinguish CD11c-positive and negative cells due to available antibodies and are indicated as

"monocytes plus derivatives" in the text), and monocyte-derived dendritic cells as live $CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$ $CD11c^+$ $MHCII^+$ cells (some experiments did not include Ly6G or Ly6C markers). Data were acquired on a BD LSRII flow cytometer and analyzed on FlowJo v9 (Treestar). Phagocytosis and conidial viability were assessed as in (Jhingran et al., 2012) and is described in the text in more detail. The total lung cell count was determined by Z2 Coulter counter, and cellular recruitment was determined by multiplying the percentage of cells by the total cell count. Infection was verified by plating the single cell suspension on Sabouraud dextrose agar plates.

Assessment of Tissue Damage and Vascular Permeability

Thrombocytopenic mice were challenged with *A. fumigatus* conidia as indicated in the text. 60 minutes prior to euthanasia with pentobarbital, mice were lightly anesthetized with isoflurane and inoculated intranasally with 50 µL of 4 mg/mL FITC-Dextran MW 400 (Sigma Aldrich Catalog Number 46944). After euthanasia, blood was obtained from the vena cava or heart and drawn into syringes containing 3.8% sodium citrate. Subsequently, bronchoalveolar lavage fluid (BALF) was obtained by inserting a tracheal catheter and inflating and deflating the lungs with 3 mL PBS in 0.5 mL steps. Subsequently, the lungs were perfused with PBS through the right ventricle, removed, and imaged. Airway hemorrhage was assessed by measuring BALF absorbance at 410 nm, a peak characteristic of hemoglobin, in serial dilutions. The BALF protein content was determined by a commercially available BCG Albumin Assay according to the manufactuer's instructions (Sigma, MAK124). Lactate dehydrogenase (LDH) levels in the BALF were determined by a commercially available kit, utilized according to the manufacturer's instructions (Promega, G1780). To assess vascular permeability, plasma was obtained by centrifuging peripheral blood at 7000 RPM for 10 minutes, and the MFI

at 485 nm excitation and 528 nm emission was determined by using a fluorescent plate reader based on the studies in (Chen et al., 2014). Similar to the above studies, thrombocytopenic and mock infected mice were normalized to controls to account for variability between experiments.

Isolation and Assessment of Pulmonary Epithelial and Endothelial Cells

Thrombocytopenic or control mice were challenged with *A. fumigatus* as indicated in the text. After incubation, mice were euthanized with pentobarbital individually. After euthanasia, the vena cava was transsected and the lungs perfused with 2 mL PBS. The lungs were then perfused with 1 mL of dispase. 0.7 mL of dispase was inoculated into the lungs via a BAL catheter, and subsequently clamped for 5 minutes. After incubation, leaving the catheter in place, 0.5 mL 1% low melting point agarose in PBS was added to the lungs. The trachea was clamped shut and ice placed on top of the lungs to allow solidification. After the solidification of the agarose, the lungs were removed and placed in PBS. After harvest, the lungs were diced in a 5-cm dish with a razor blade allowed to digest without agitation in a buffer consisting of PBS, 0.5% BSA, 5 mg/mL collagenase type I, 20 µL/mL dispase, and 100 µg/mL DNase for 45 minutes. Each lung was then dissociated by passing the tissue through a 10-mL syringe equipped with a 16-gauge needle. After RBC lysis, 9 mL PBS supplemented with 5% FBS was added and filtered through a 100 µm filter. After 2 rounds of centrifugation and washing (1500 RPM, 5 minutes) the cells were suspended in 2 mL PBS/FBS.

For experiments involving cell death assessment, 40 µL of the cells were aliquoted to wells of a 96-well plate. FITC-DEVD-FMK (Thermo Fisher Catalog Number 88-7004-42) was diluted 1:260 in RPMI and added to a total volume of 300 µL and

incubated for 30 minutes at 37 degrees Celsius (5% CO_2). After incubation, the cells were spun and washed twice with the supplied wash buffer, and the cells were resuspended in the normal FACS buffer used above (see table for antibody list). Following staining, the cells were washed with cold PBS, and stained with 100 µL of 1:20 Annexin V PE in binding buffer (Fisher Scientific Catalog Number BDB559763) in the dark for 15 minutes on ice. 400 µL of binding buffer was added and the samples assessed by flow cytometry.

Analysis of Lung Protein Levels

Thrombocytopenic or control mice were infected as indicated in the text. At the indicated time points, the mice were euthanized, and the lungs were removed and placed in PBS supplemented with 1x cOmplete protease inhibitor (Millipore Sigma Catalog Number 11697498001). The lungs were mechanically homogenized as above. After plating serial dilutions on Sabouraud dextrose agar plates to confirm infection, the homogenate was centrifuged for 5' at 1500 RPM. The supernatant was removed and centrifuged at full speed for 5 minutes. The supernatant was then frozen at -80 degrees Celsius until use. To assess lung protein content, the ELISAs or cytokine arrays were utilized according to the manufacturer's instructions. Inverted pixel densities were determined for all cytokine spots. To generate a list of proteins upregulated, noise was reduced by excluding proteins with an inverted pixel density in thrombocytopenic mice of less than 5, and the $\log_2$ fold change was determined in Microsoft Excel.

Statistics

All results are expressed as mean (± standard error of the mean, SEM) using pooled results from 1-3 experiments as noted. For all comparisons between two groups, a Mann-Whitney U test was utilized. Comparison between survival groups was accomplished utilizing a Mantel-Cox test. All statistical analyses were performed with GraphPad Prism, version 7.0.

CHAPTER 3: RESULTS

I. Platelets and Aspergillosis

Platelets are essential for survival following A. fumigatus challenge (antiserum model)[1]

To induce thrombocytopenia in mice, I utilized two experimental strategies in parallel. In the first model, I induced thrombocytopenia by injecting mice with a platelet-targeting antiserum (AS model) on three consecutive days. In the AS model, platelet counts fell to < 3% of starting levels within 2 hours of the first injection (Figure 6A). In the AS model, the onset of platelet recovery began on day +3 (Figure 6A). To assess the importance of platelets for survival in the AS model, I infected thrombocytopenic or control mice with the virulent *A. fumigatus* clinical isolate CEA10 2 hours after the initial antiserum administration. Thirteen of fifteen thrombocytopenic mice succumbed to infection with a median survival time of 2.5 days (Figure 6B). Only two of fifteen platelet-sufficient mice died from infection (Figure 6B). Importantly, no uninfected thrombocytopenic mice died during the experiment, indicating that the platelet-depleting strategy did not account for murine mortality (Figure 6B). This phenotype was strain-independent since infection with the less virulent clinical isolate Af293 yielded a similar result (Figure 7). Eight of ten thrombocytopenic mice died in comparison to no mortality in the control group (Figure 7).

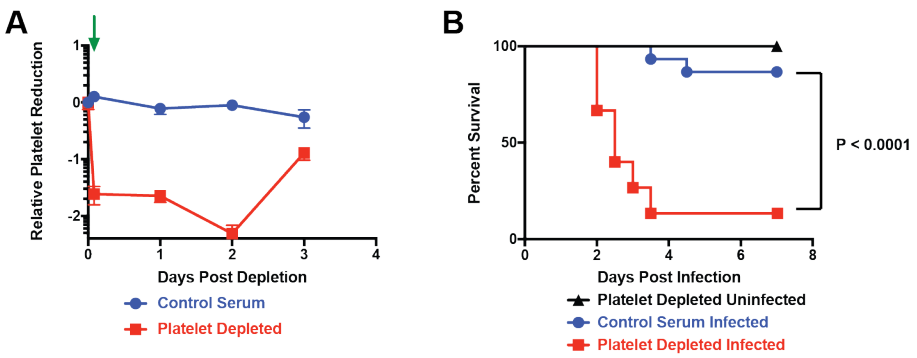

Figure 6: Thrombocytopenia (AS model) predisposes mice to *Aspergillus*[1]

(A) Relative platelet levels (log_{10} scale) in C57BL/6J mice injected with platelet targeting serum (red) or normal rabbit serum (blue) on day 0, day +1, and day +2. (n = 4-5 mice per group). The green arrow indicates the timing of infection in B. (B) Kaplan-Meier survival curve of mice injected with normal rabbit serum (blue) or platelet targeting serum (red, black), that were infected with ~6-10^7 *A. fumigatus* CEA10 conidia (blue, red) or left uninfected (black). Data are pooled from 2 experiments (n = 10-15 per group). For survival experiment comparison, a Mantel-Cox test was used.

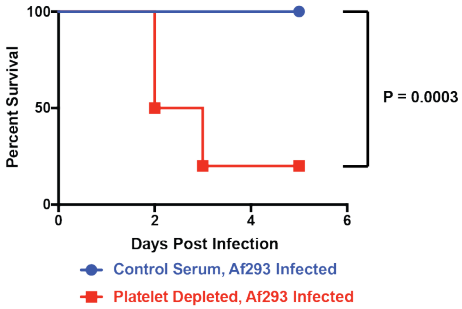

Figure 7: *A. fumigatus* susceptibility is strain independent in thrombocytopenic mice[1]

Kaplan-Meier survival curve of C57BL/6J mice treated with normal rabbit serum (blue) or platelet targeting serum (red) and infected with ~6x10^7 A. fumigatus Af293 conidia. Data are from one experiment (n = 10 mice per group). For survival experiment comparisons, a Mantel-Cox test was used.

Thrombocytopenic mice lose weight at similar rates to wildtype mice

Since the loss of platelets in the AS model led to a severe, rapid, survival defect, I assessed the morbidity of the mice using weight loss as a proxy. I found that over the course of the first 2 days of infection, thrombocytopenic mice lost weight at the roughly same rate as their platelet sufficient counterparts (Figure 8). However, almost all surviving mice began to recover on day 4, though 1 out of 2 surviving thrombocytopenic mice did not begin to recover until the termination of the experiment at day 7 (data not shown). These data suggest that the thrombocytopenic mice die too rapidly for differences in weight loss to become apparent.

Platelets are essential for survival following A. fumigatus challenge (iDTR model)[1]

In the second model of thrombocytopenia, I injected iDTR^{Pf4} mice or non-Cre littermate controls with 3 doses of diphtheria toxin, each 2 days apart (DT model). Six days after the initial DT treatment, platelet levels were reduced to < 1% of normal levels in Cre-positive mice (Figure 9A). Mice remained thrombocytopenic for an additional 24 h, before recovering to normal platelet levels on day +9 (Figure 9A). To confirm that the infectious susceptibility in the AS model (Figure 6) was not due to off target effects, I challenged thrombocytopenic mice with CEA10 conidia in the DT model on day +6 after the first DT dose to target Pf4^{+} megakaryocytes. All thrombocytopenic mice infected with conidia died with a median survival time of one day, compared with only one of eight infected controls (Figure 9B). No uninfected controls died, indicating that mortality was not a result of the depletion strategy (Figure 9B). Regardless of the mechanism of platelet depletion, thrombocytopenic mice were highly susceptible to *A. fumigatus* challenge with 2 different clinical isolates.

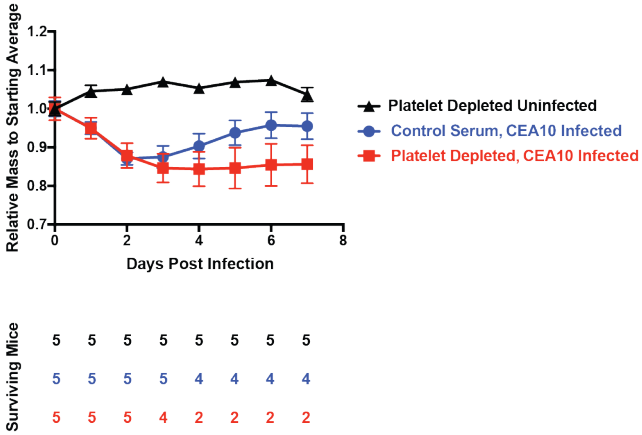

Figure 8: Thrombocytopenic mice lose weight a similar rate to controls

Weight of mice injected with normal rabbit serum (blue) or platelet targeting serum (red, black), that were infected with ~6-10^7 A. fumigatus CEA10 conidia (blue, red) or left uninfected (black). A relative weight was determined by comparing to the average starting weight of the cohort. The weight of mice upon death was carried forward until the final day of the experiment. The bottom chart indicates surviving mice at the beginning of the day.

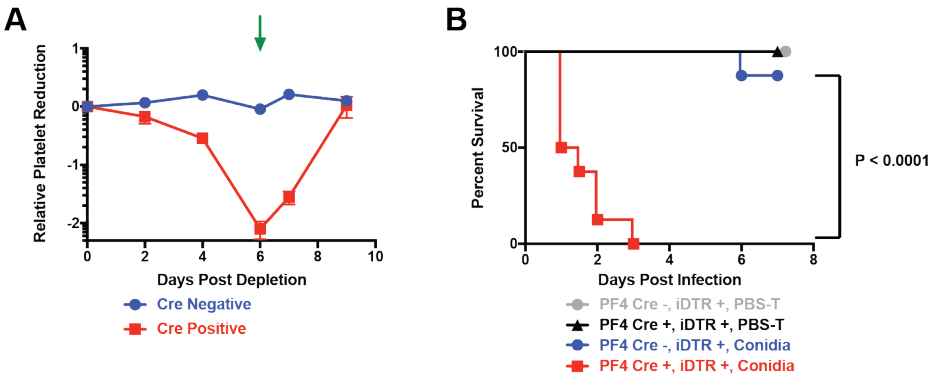

Figure 9: Thrombocytopenia (DT model) predisposes mice to *A. fumigatus*[1]

(A) Relative platelet levels (log_{10} scale) of PF4-iCre-positive, iDTR positive, mice (red) or Cre-negative, iDTR positive, littermates (blue) injected with 400 ng diphtheria toxin (DT), and subsequent doses of DT on day 2 (200 ng), and day 4 (200 ng). (*n* = 4-6 mice per group). The green arrow indicates the timing of infection in D. (B) Kaplan-Meier survival curve of PF4-iCre positive or negative, iDTR positive mice, injected with DT and either infected with ~6x10^7 *A. fumigatus* CEA10 conidia (blue, red) or left uninfected (grey, black). Uninfected mice were from 1 experiment (*n* = 4-6 mice per group), and infected mice were pooled from 2 experiments (*n* = 8 mice per group). For survival experiment comparison, a Mantel-Cox test was used.

Platelets reduce fungal burden after A. fumigatus challenge[1]

To examine the impact of thrombocytopenia on fungal growth and dissemination in the AS model, I challenged thrombocytopenic or control mice with CEA10 conidia and quantified organ fungal CFU at 2 days post infection. I observed a higher lung fungal burden in thrombocytopenic mice (1,260,000 ± 270,000 CFU) compared with controls (370,000 ± 100,000 CFU) (Figure 10A). However, I did not observe notable dissemination to the kidney (Figure 10B), spleen (Figure 10C), or brain (Figure 10D). These data suggest that platelets contribute to controlling fungal growth in the lung only.

II. Platelets and the Immune System During Infection

Platelets regulate early neutrophil function during A. fumigatus lung infection[1]

Prior studies reported that platelets regulate neutrophil recruitment to sites of inflammation (Pitchford, Pan, & Welch, 2017). Thus, I examined whether thrombocytopenia is associated with defective innate immune cell recruitment in the context of respiratory *A. fumigatus* infection. In both models of thrombocytopenia, platelets were dispensable for neutrophil migration to the lung when infected with Af293 conidia, with the thrombocytopenic mice having 1.23 (± 0.18) and 1.97 (± 0.65)-fold higher neutrophil counts in the AS and DT models respectively. This difference did not reach statistical significance (Figure 11D). These data suggest that neutrophils are recruited to the lung in a platelet-independent manner during *A. fumigatus* infection.

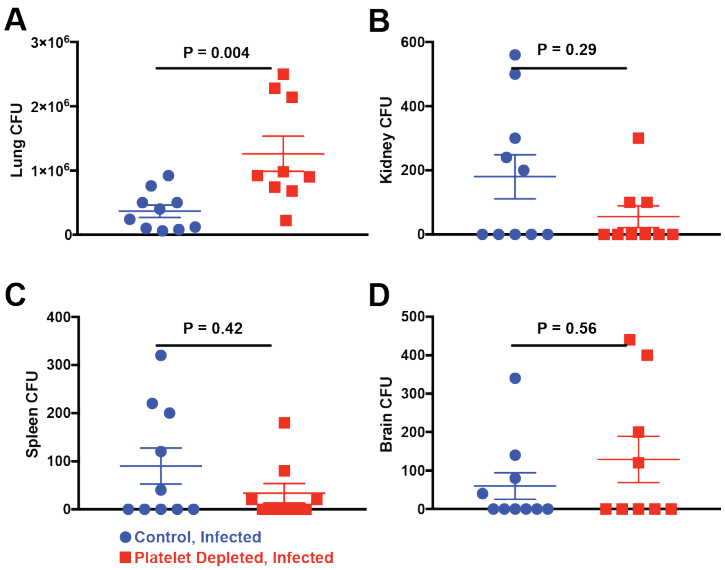

Figure 10: Platelet depletion and organ fungal burden[1]

Colony forming units (CFU) in C57BL/6J mice treated with normal serum (blue) or platelet targeting antiserum (red) and infected with ~3-6x10^7 *A. fumigatus* CEA10 conidia. CFU levels in the (A) lung, (B) kidney, (C) spleen, and (D) brain, 2 days after infection. Error bars are expressed with mean ± SEM. Data are pooled from 2 experiments (*n* = 9-10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

Since platelets may enhance microbial phagocytosis and the antimicrobial activity of leukocytes (Czakai et al., 2017), I next assessed the ability of lung neutrophils to phagocytose and kill conidia in the absence and presence of platelets. I inoculated thrombocytopenic or control mice with FLARE conidia, a DsRed (a conidial viability marker) transgenic Af293 strain labeled with Alexa Fluor 633 (a tracer fluorophore that does not degrade upon conidial killing) (Figure 11A) (Jhingran et al., 2012). These conidia allow discrimination of bystander leukocytes, fungus-engaged leukocytes with live conidia, and fungus-engaged leukocytes with killed conidia, at single cell resolution by flow cytometry (Figure 11B). Neutrophil conidial uptake was reduced to 80% (± 5%) of controls in the AS model, and 75% of controls (± 6%) in the DT model (Figure 11D). These data indicate that lung neutrophils display modest defects in conidial uptake in thrombocytopenic mice compared to platelet-sufficient littermates. In contrast, neutrophil conidial killing was similar in both thrombocytopenic and controls (1.08 ± 0.06-fold higher in the AS model, and 1.03 ± 0.08 higher in the DT model), suggesting platelets are dispensable for neutrophil conidiacidal activity (Figure 11F).

Platelets do not modulate monocyte or Mo-DC function early in infection[1]

As monocytes are required for optimal lung neutrophil function during *A. fumigatus* challenge (Espinosa et al., 2014), I sought to quantify their recruitment, uptake, and conidial killing capacity. Similar to the neutrophil data, thrombocytopenia had no bearing on the recruitment of monocytes in either the AS model (1.30 ± 0.22 higher) or the DT model (1.30 ± 0.35 higher) at 12 hours post infection. However, unlike neutrophils, thrombocytopenic mice exhibited normal phagocytosis of *A. fumigatus* conidia in both the AS model (1.20 ± 0.13 higher) and the DT model (1.22 ± 0.49 higher). The data were inconsistent with respect to the conidicidal activity, with the AS model

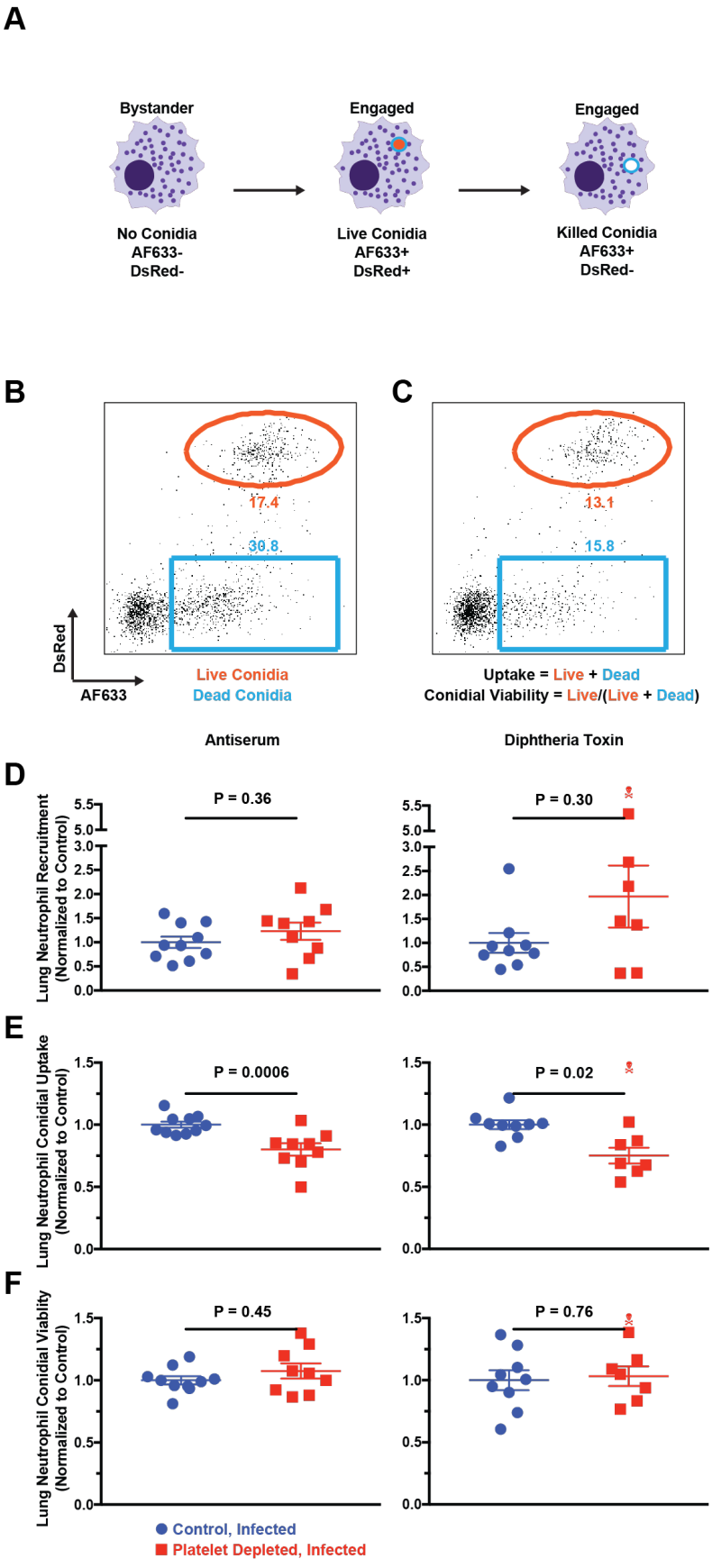
A
Bystander
Engaged
Engaged
No Conidia
AF633-
DsRed-
Live Conidia
AF633+
DsRed+
Killed Conidia
AF633+
DsRed-
B
17.4
30.8
DsRed
AF633
Live Conidia
Dead Conidia
C
13.1
15.8
Uptake = Live + Dead
Conidial Viability = Live/(Live + Dead)
Antiserum
Diphtheria Toxin
D
Lung Neutrophil Recruitment
(Normalized to Control)
P = 0.36
P = 0.30
E
Lung Neutrophil Conidial Uptake
(Normalized to Control)
P = 0.0006
P = 0.02
F
Lung Neutrophil Conidial Viablity
(Normalized to Control)
P = 0.45
P = 0.76
Control, Infected
Platelet Depleted, Infected

Figure 11: Platelets regulate neutrophil function after *A. fumigatus* infection[1]

(A) Scheme to monitor leukocyte conidial uptake and killing with FLARE conidia that express the viability fluorophore DsRed and are labeled with the tracer fluorophore Alexa Fluor 633 (AF633). Bystander lung leukocytes are $DsRed^-$ and $AF633^-$. Upon engulfing live FLARE conidia, leukocytes become $DsRed^+$ and $AF633^+$. After killing phagocytosed conidia, leukocytes lose DsRed fluorescence and are $DsRed^-$ and $AF633^+$.
(B-C) Representative flow cytometry plots of $Ly6G^+$ neutrophils analyzed for DsRed and AF633 fluorescence. The red gates indicate the frequency of neutrophils that contain live conidia, and the blue gates indicate the frequency of neutrophils that contain killed conidia in (B) control and (C) thrombocytopenic mice. (D-F) Neutrophil recruitment (D), lung neutrophil conidial uptake (E) and lung neutrophil conidiacidal activity (F) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 12 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 8-10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

showing no difference between thrombocytopenic mice (0.95 ± 0.05 of controls) and controls, while monocytes in the DT model had more viable conidia (1.70 ± 0.16, P = 0.01) (Figure 12). While this difference may be due to the experimental approach (DT vs. AS models), an alternate explanation in the AS model is that I did not exclude $CD11c^+$ events in the monocyte gate, and thus analyzed a heterogeneous population that included both monocytes and Mo-DCs.

Lung monocytes differentiate into dendritic cells upon *A. fumigatus* challenge (Hohl et al., 2009; Rivera et al., 2011). I therefore quantified the activity of Mo-DCs early in infection. Although both there was an increase in Mo-DC recruitment in the DT model (2.02 ± 0.68 higher, P = 0.0164), the relative increase in Mo-DC numbers in the AS model did not reach statistical significance (1.37 ± 0.27 higher, P = 0.4002). In agreement with the data for monocytes, Mo-DCs showed no difference in phagocytic efficiency in both the AS model (1.02 ± 0.12 higher) and DT model (0.85 ± 0.06 of controls). The Mo-DCs isolated from thrombocytopenic mice were equally efficient as controls in killing internalized conidia in both the AS model (1.01 ± 0.06 of controls) and DT model (1.29 ± 0.17 of controls) (Figure 13). Together, these data suggest that platelets are dispensable for the recruitment, phagocytosis, and killing ability of monocytes and their Mo-DC derivatives.

Platelet modulation of the innate immune response is transient[1]

Since the mortality phenotype was consistent with a severe impairment in host defense, I next tested whether the neutrophil phagocytic defect continued throughout infection. In line with data at 12 hours post infection, thrombocytopenic mice exhibited no difference in neutrophil recruitment to the lungs in either the AS model (1.67 ± 0.29 of

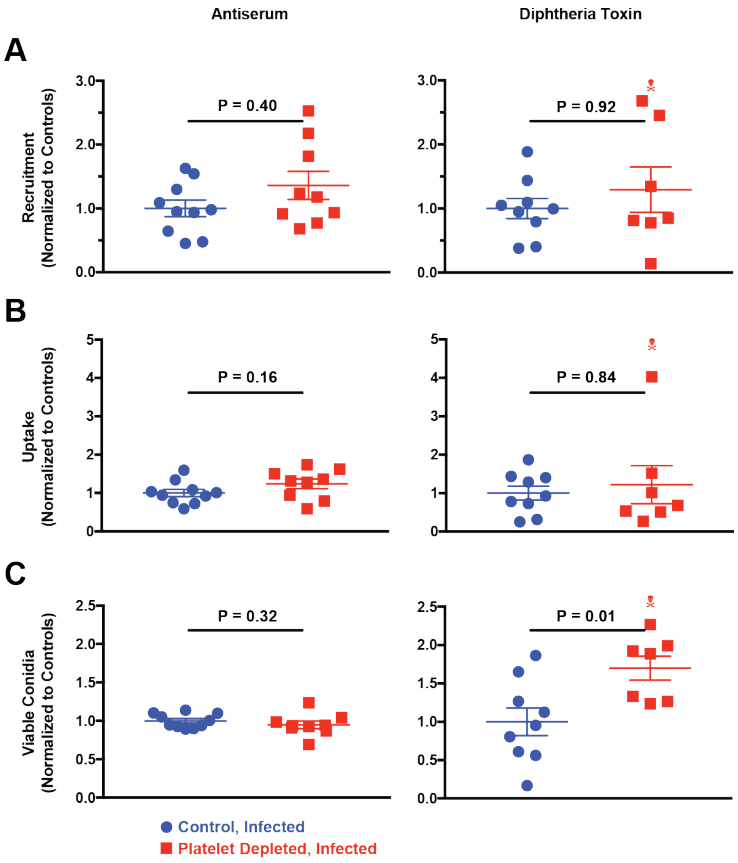

Figure 12: Platelets do not regulate monocyte function after *A. fumigatus* infection[1]

Monocyte recruitment (A), lung monocyte conidial uptake (B) and lung monocyte conidiacidal activity (C) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 12 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 8-10 mice per group). AS mice do not have Mo-DCs gated out. For comparisons between two groups, a Mann-Whitney U test was used.

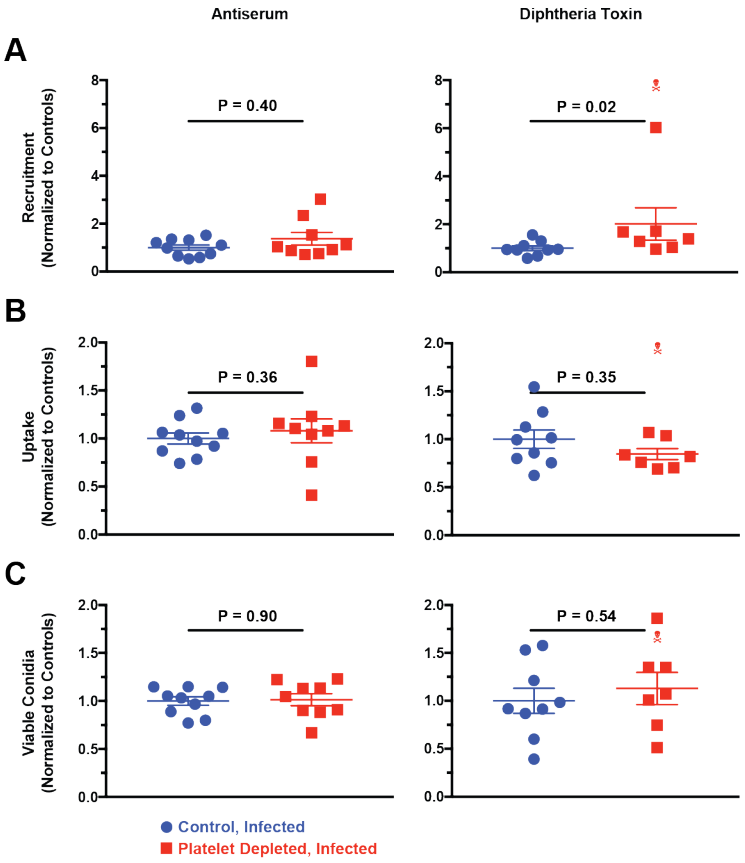

Figure 13: Platelets do not regulate Mo-DC function after *A. fumigatus* infection[1]

Monocyte-derived dendritic cell recruitment (A), lung Mo-DC conidial uptake (B) and lung Mo-DC conidiacidal activity (C) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 12 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 8-10 mice per group). AS mice do not have Ly6C as a marker. For comparisons between two groups, a Mann-Whitney U test was used.

controls) or the DT model (1.17 ± 0.25 of controls) at 40 hours post infection (Figure 14A). Additionally, neither model of thrombocytopenia impacted neutrophil conidial killing at 40 hours (AS model: 0.92 ± 0.11 of controls, DT model: 0.96 ± 0.09 of controls) (Figure 14C). However, in contrast to the data obtained at 12 hours post infection, neutrophil phagocytosis of conidia returned to control levels by later time points of infection in both the AS model (0.98 ± 0.09 of controls) and DT model (0.93 ± 0.09 of controls) (Figure 14B). These results suggest that the impact of platelets on neutrophil function in response to *A. fumigatus* challenge is transient.

To ensure that the other leukocyte subsets tested were not impacted later in infection, I also analyzed the recruitment, uptake, and conidial killing of monocytes and Mo-DCs at 40 hours post infection. In line with the data obtained at 12 hours post infection, monocytes and Mo-DCs had no deficiencies in the AS model (Figure 15-16). Although thrombocytopenic mice showed defects in monocyte uptake (0.56 ± 0.11 of controls, P = 0.0465) and Mo-DC recruitment (0.65 ± 0.09, P = 0.0381) in the DT model (Figure 15B, 16A), the inconsistencies between the AS and DT models suggest that these observations were more likely experimental artifacts than biologically relevant phenotypes. Otherwise, the phenotypes of leukocytes isolated from thrombocytopenic mice were normal in the DT model (Figure 15-16), consistent with the idea the platelets do not impact non-neutrophil leukocyte populations during *A. fumigatus* infection.

Thrombocytopenia Induces Inflammatory Cytokines in the Lungs

Because neutrophil recruitment was normal, I assessed whether the source of the lethal immunopathology was due to a dysregulated cytokine response. I isolated lung

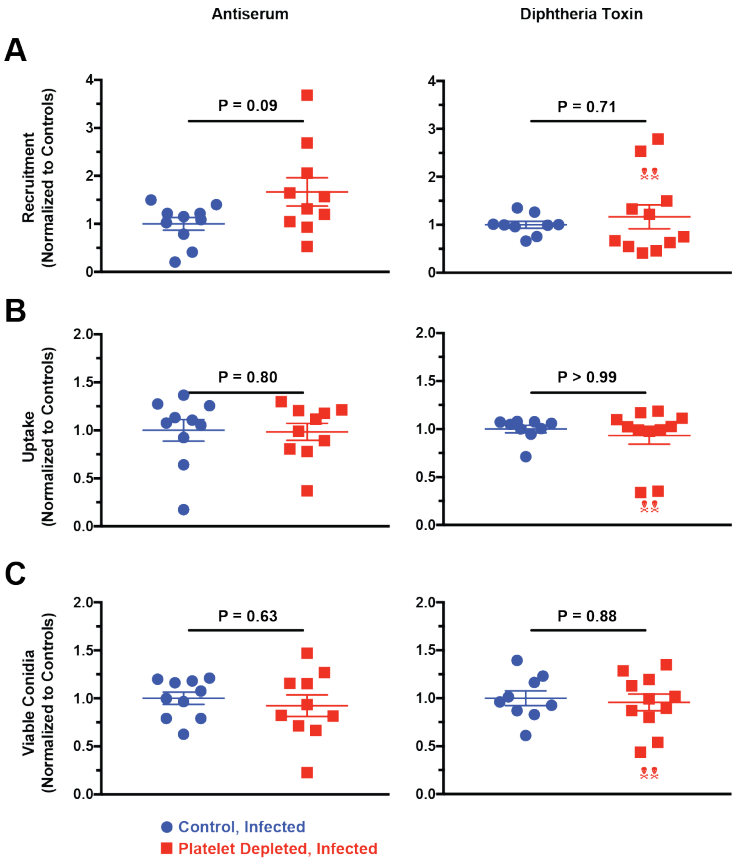

Figure 14: Platelet regulation of neutrophil function is transient[1]

Neutrophil recruitment (A), lung neutrophil conidial uptake (B) and lung neutrophil conidiacidal activity (C) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 9-13 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

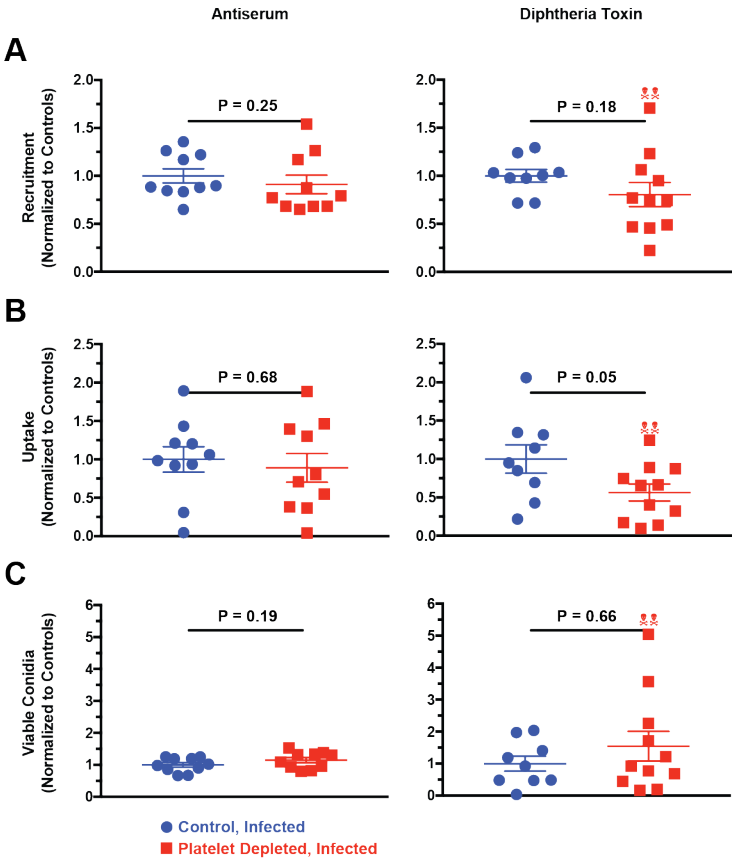

Figure 15: Platelets do not regulate monocyte function during late infection

Monocyte recruitment (A), lung monocyte conidial uptake (B) and lung monocyte conidiacidal activity (C) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 9-13 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

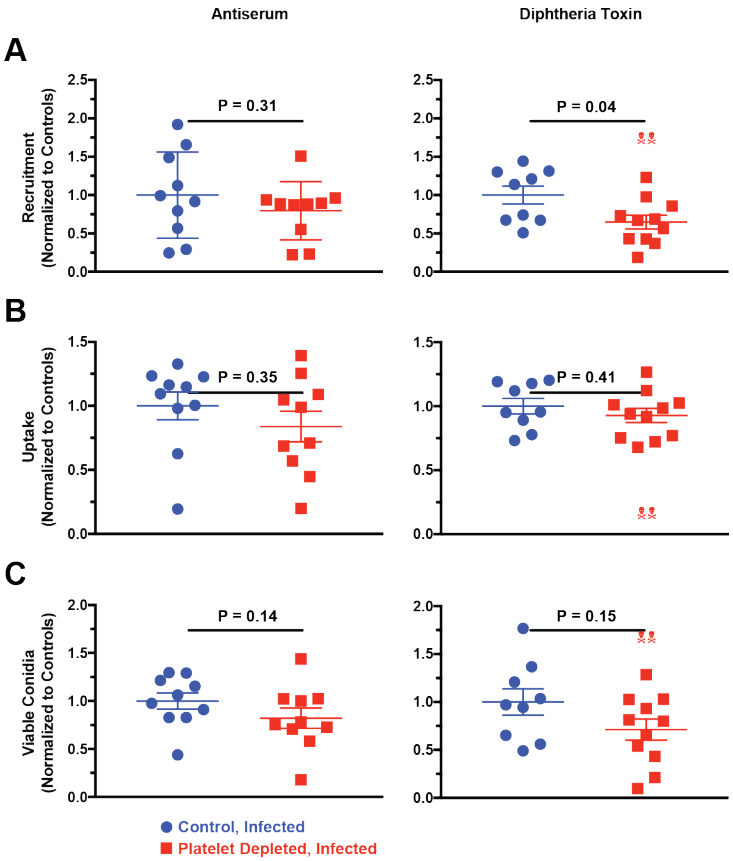

Figure 16: Platelets do not regulate Mo-DC function late in infection

Monocyte-derived dendritic cell recruitment (A), lung Mo-DC conidial uptake (B) and lung Mo-DC conidiacidal activity (C) in the AS model of thrombocytopenia (left) and DT model of thrombocytopenia (right). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 9-13 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

homogenate at approximately 48 hours post infection and performed a cytokine array (Figure 17A-C). I did not observe decreases in any protein tested of greater than 50%. However, CXCL2 (2.99), CCL2 (2.41), CXCL1 (1.82), CCL20 (1.29), and TNF-α (1.15), all had a $\log_2$ fold change of greater than 1 in thrombocytopenic mice compared with controls (Figure 17D, Appendix Table 3). Despite this, neutrophil recruitment is not significantly increased. Given the importance of CXCL1 and CXCL2 in the innate anti-*Aspergillus* neutrophil response (Jhingran et al., 2015), these results are consistent the hypothesis that there may be compensation from non-platelet sources to ensure proper neutrophil recruitment.

Syk signaling in the megakaryocytic lineage promotes neutrophil conidial uptake[1]

Conidial germination results in surface exposure of β-glucan polysaccharides that trigger innate immune signaling through Dectin-1, Syk, and CARD9 (Drummond & Brown, 2011). Previously, the Hohl lab reported that genetic defects in the Dectin-1/Syk/CARD9 signaling pathway leads to impaired conidial phagocytosis by neutrophils (Jhingran et al., 2015). Because Syk integrates signaling from C-type lectin and integrin receptors expressed both on leukocytes (Jhingran et al., 2015) and platelets (Spalton et al., 2009), I deleted Syk in Pf4-expressing cells, resulting in a loss of platelet Syk expression (Figure 18). I then assessed the role of platelet/megakaryocyte Syk signaling in neutrophil conidial phagocytosis by infecting $Syk^{\Delta Pf4}$ chimeras, or Cre-negative $Syk^{fl/fl}$ control chimeras with FLARE conidia. Conditional Syk deletion in $Pf4^{+}$ cells reduced neutrophil conidial uptake to 80% (± 6%) of Syk-sufficient controls (Figure 19B). This result suggests that Syk expression in platelets regulates neutrophil conidial phagocytosis in a manner similar to complete platelet loss.

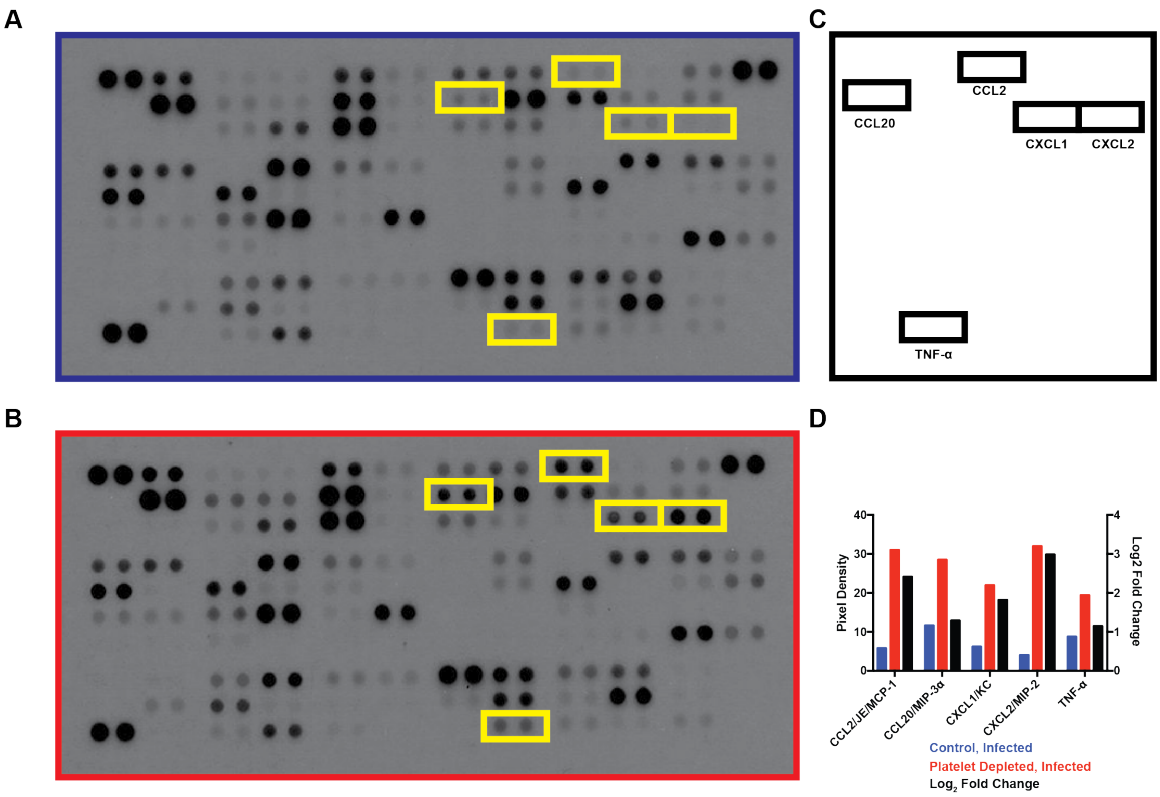

Figure 17: Differential cytokine levels in control and thrombocytopenic mice

(A-B) Cytokine array of lung homogenate from AS model control (A, blue) or thrombocytopenic (B, red) mice pooled from 3 animals at 48 hours post infection with ~3-6 x 10^7 CEA10 conidia. Yellow rectangles represent differentially expressed cytokines. (C) A map of the cytokines indicated in panels (A) and (B). (D) The pixel density of the selected cytokines in (A, blue), or (B, red), and the $\log_2$ fold change of cytokine levels in thrombocytopenic mice (black).

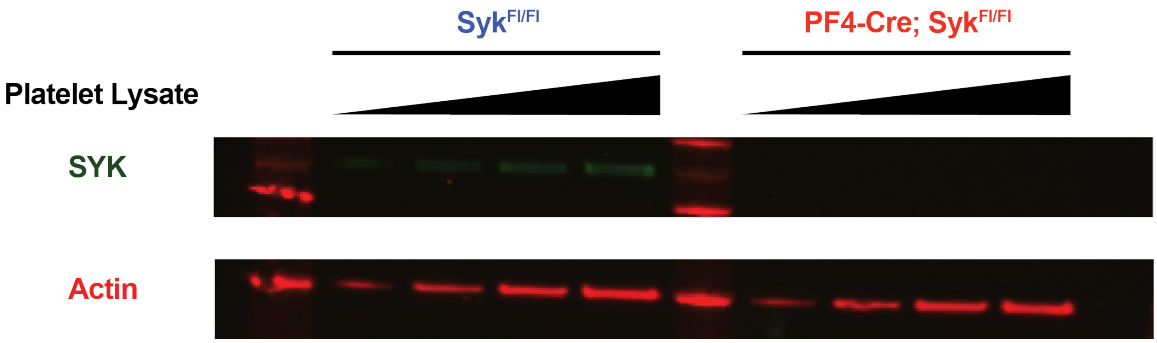

Figure 18: Pf4 driven Cre efficiently deletes Syk from platelets[1]

Western blot of increasing amounts of platelet lysate obtained from $Syk^{Fl/Fl}$ control mice (left) or platelet lysate from $Syk^{\Delta Pf4}$ mice (right). The upper band (green) shows Syk expression, and the lower band (red) shows β-actin as a loading control. Platelets were pooled from 2 mice.

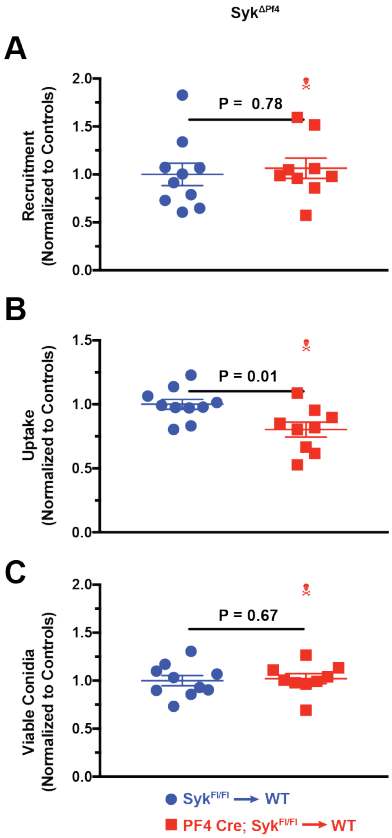

Figure 19: Platelet and/or megakaryocyte Syk is necessary for early neutrophil phagocytosis[1]

Neutrophil recruitment (A), lung neutrophil conidial uptake (B) and lung neutrophil conidiacidal activity (C) in bone marrow chimeric mice ($Syk^{\Delta Pf4}$ → CD45.1 or $Syk^{Fl/Fl}$ → CD45.1) All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used. Panel B is reprinted from (Tischler et al., 2020).

Megakaryocytic Syk signaling has a transient impact on neutrophil conidial phagocytosis

In the ensuing series of experiments, I assessed the impact of megakaryocyte lineage Syk signaling on lung neutrophil recruitment and lung neutrophil conidial killing capacity at 12 hours post infection. I found that neither neutrophil recruitment (1.06 ± 0.10 of controls) nor conidial killing ability (1.021 ± 0.05 of controls) were affected by loss of Syk in Pf4-expressing cells (Figure 19 A, C). Although the number of monocytes present was slightly elevated (1.81 ± 0.26 of controls) in $Syk^{\Delta Pf4}$ chimeras, phagocytosis (0.59 ± 0.12 of controls) and killing (0.85 ± 0.16 of controls) were normal (Figure 20). $Syk^{\Delta Pf4}$ chimeras exhibited normal Mo-DC function, with lung recruitment (1.26 ± 0.17 of controls), conidial uptake (0.98 ± 0.05 of controls), and conidial killing (1.01 ± 0.09 of controls) identical to Syk sufficient counterparts (Figure 21).

As the effect of platelet loss was largely transient, I examined whether this was true for $Syk^{\Delta Pf4}$ chimeras as well. I found that neutrophil recruitment (0.93 ± 0.13 of controls), uptake (0.95 ± 0.08 of controls), and killing (0.99 ± 0.10 of controls) were norma (Figure 22A-C). Assessment of monocyte recruitment (0.98 ± 0.10 of controls), uptake (1.20 ± 0.21 of controls), and killing (0.75 ± 0.13 of controls), revealed no difference compared with Syk-sufficient mice (Figure 22D-F). Similarly, Mo-DC recruitment (0.94 ± 0.13 of controls), uptake (0.97 ± 0.08 of controls), and killing (0.97 ± 0.12 of controls), showed identical results to control mice (Figure 22G-I). Together, these results suggest that Pf4-driven Syk expression regulates neutrophils in a transient manner, with a minor impact on other leukocyte subsets.

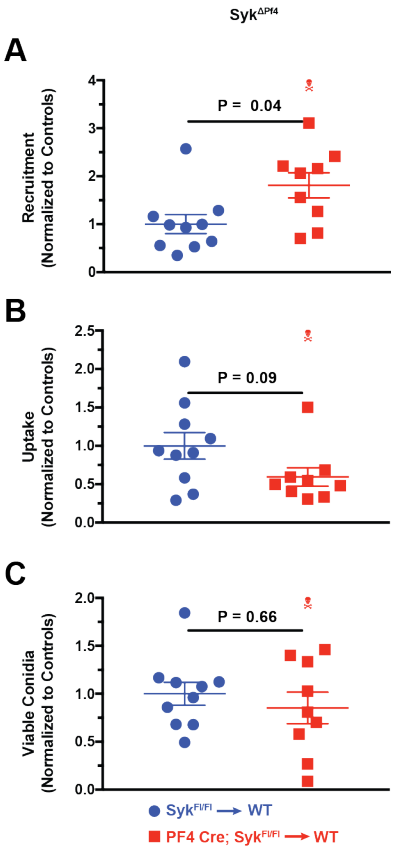

Figure 20: Platelet and/or megakaryocyte Syk is dispensable for early monocyte function

Monocyte recruitment (A), lung monocyte conidial uptake (B) and lung monocyte conidiacidal activity (C) in bone marrow chimeric mice ($Syk^{\Delta Pf4}$ → CD45.1 or $Syk^{Fl/Fl}$ → CD45.1) All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

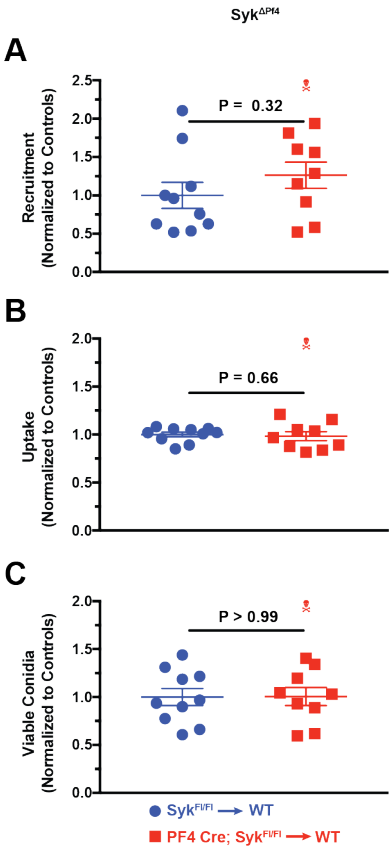

Figure 21: Platelet and/or megakaryocyte Syk is dispensable for early Mo-DC function

Monocyte-derived dendritic cell recruitment (A), lung Mo-DC conidial uptake (B) and lung Mo-DC conidiacidal activity (C) in bone marrow chimeric mice ($Syk^{\Delta Pf4}$ → CD45.1 or $Syk^{Fl/Fl}$ → CD45.1) All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 40 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data are pooled from 2 experiments (*n* = 10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

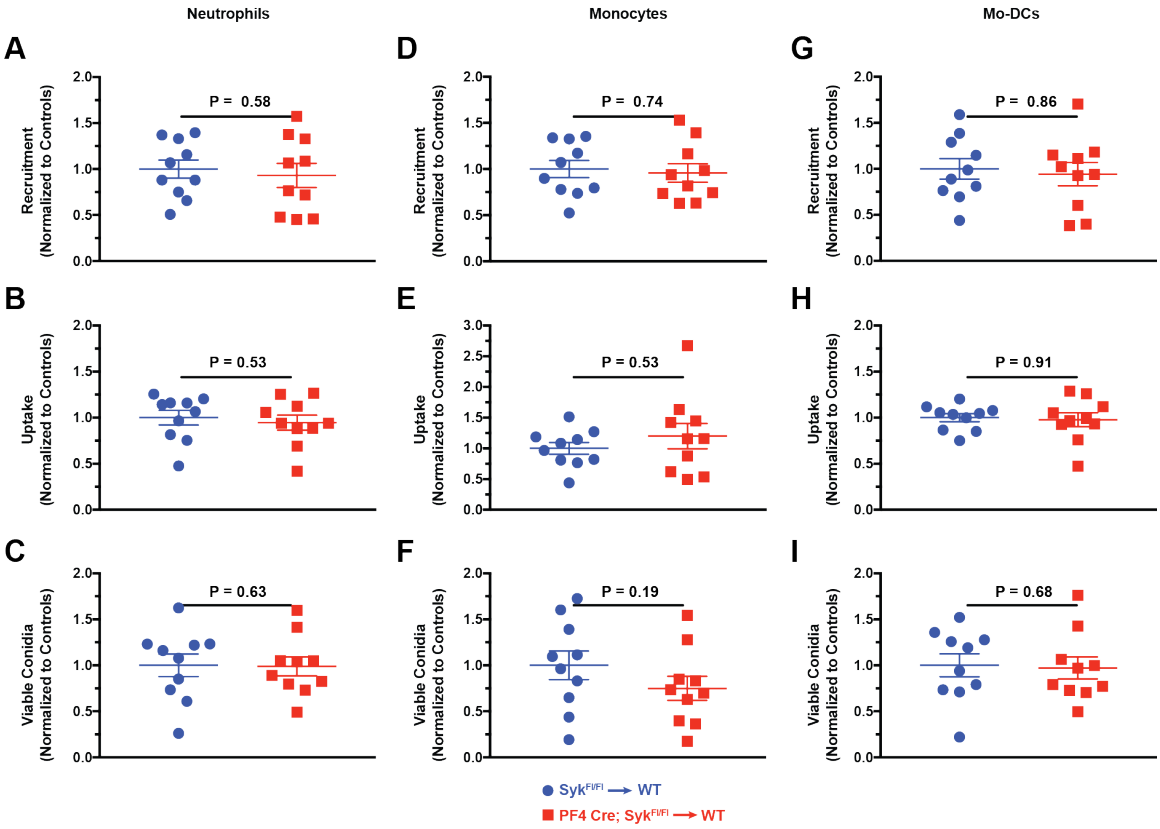

Figure 22: Platelet and/or megakaryocyte Syk expression is dispensable for leukocyte function at late time points post-infection

Immune cell recruitment (A, D, G), conidial uptake (B, E, H) and conidiacidal activity (C, F, I) in bone marrow chimeric mice ($Syk^{\Delta Pf4}$ → CD45.1 or $Syk^{Fl/Fl}$ → CD45.1) for lung neutrophils (A-C), lung monocytes (D-F) and lung Mo-DCs (G-I). All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at 36 hrs post infection. Error bars are expressed with mean ± SEM. Data are pooled from 2 experiments (*n* = 10 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

Syk-dependent neutrophil phagocytosis does not contribute to murine survival[1]

To assess the contribution of megakaryocytic lineage Syk signaling to fungal susceptibility, I challenged $Syk^{\Delta Pf4}$ chimeras, or Cre-negative $Syk^{fl/fl}$ control chimeras with CEA10 conidia. There is no significant difference in survival among both groups (Figure 23). These data indicated that Syk signaling in megakaryocytes and platelets and its role in mediating neutrophil conidial phagocytosis were not responsible for the mortality phenotype seen in both the AS and DT models of thrombocytopenia.

CXCL4 levels increase in response to infection

Given the transient and relatively minor nature of the neutrophil phagocytic defect, I hypothesized that direct action of platelets might impact fungal burden, as has been reported in the literature *in vitro* (Christin et al., 1998). As platelet anti-*Aspergillus* activity appears to be modulated by granule release (Perkhofer et al., 2008), I sought to quantify the release of CXCL4 (PF4) during over the course of infection. CXCL4 is one of the most abundant components of α-granules, and is released upon platelet activation (Kowalska, Rauova, & Poncz, 2010). Additionally, CXCL4 functions in part as a kinocidin and has antimicrobial activity (Love et al., 2012; Yue et al., 2018). I determined that over the first two days of infection, the concentration of CXCL4 increased by more than 2-fold, before peaking almost 10-fold higher than steady state amounts after 3 days (Figure 24). While my in vivo approach did not allow for testing whether CXCL4 has direct anti-*Aspergillus* activity, my results are consistent with the notion that platelets may kill conidia independently of leukocytes, though this idea will need to be tested *in vivo* in *Cxcl4* knockout mice.

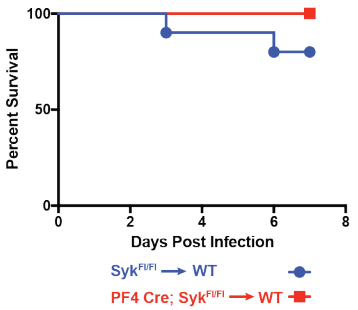

Figure 23: Platelet and/or megakaryocyte Syk signaling is dispensable for survival[1]

Kaplan-Meier survival curve of bone marrow chimeric mice ($Syk^{\Delta Pf4}$ → CD45.1 or $Syk^{Fl/Fl}$ → CD45.1) infected with ~3-6x10^7 *A. fumigatus* CEA10 conidia. Data are pooled from 2 experiments (*n* = 9-10 mice per group). For survival experiments, a Mantel-Cox test was used.

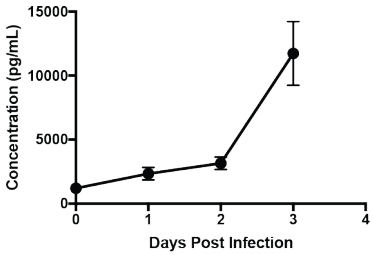

Figure 24: CXCL4 levels increase throughout infection

ELISA showing the concentration of protein in homogenized mouse lungs at the indicated time points post infection (*n* = 4-5 mice per time point). Day 0 represents uninfected mice.

III. Platelets and Hemostasis During Fungal Challenge

Platelets Maintain Hemostasis During A. Fumigatus Challenge[1]

Since functional defects in innate immunity did not explain the mortality phenotype in thrombocytopenic mice, I next explored the contribution of platelets to lung tissue integrity. Since thrombocytopenia can promote pulmonary hemorrhage after instillation of inflammatory stimulus (e.g., LPS (Goerge et al., 2008)), I harvested bronchoalveolar lavage fluid (BALF) and lung tissue after CEA10 infection to qualitatively assess pulmonary hemorrhage and tissue damage. I found that in both the AS (Figure 25A-B) and DT models (Figure 26A-B) of thrombocytopenia, a loss of platelets led to severe hemorrhage in the airways and lung parenchyma. Hemorrhage was readily evident in thrombocytopenic AS model mice compared with the control mice in H&E stained lung sections (Figure 25C). I found a significant increase in hemorrhage in thrombocytopenic infected mice compared to platelet-sufficient controls as assessed by BALF OD_{410} absorbance measurements. Thrombocytopenic mice exhibited a 49.44 (± 8.48)-fold higher OD_{410} reading at 48 hr post infection in the AS model, and a 34.46 (± 12.12)-fold higher OD_{410} reading at 24 hr post infection in the DT model (Figure 25D and 26C). These data demonstrate that platelets maintain hemostasis in the lung during respiratory fungal infection.

Platelets Reduce Vascular Permeability During Infection[1]

In agreement with these findings, a BCG assay found an increase in albumin levels in the BALF of infected thrombocytopenic animals compared to infected platelet-sufficient control mice, with a 14.58 (± 2.15)-fold increase in the AS model, and a 12.74

(± 3.81)-fold increase in the DT model (Figure 25E and 26D). Similarly, I found an increase in the BALF LDH level in infected thrombocytopenic mice compared with infected platelet-sufficient controls, with a 4.81 (± 0.21)-fold increase in the AS model, and 6.45 (± 1.50)-fold increase in the DT model. (Figure 25F and 26E). Thus, the presence of platelets reduced lung tissue damage during respiratory *Aspergillus* infection.

Given the presence of blood in the airways, I assessed the permeability of the lung airway barrier by assessing diffusion of FITC-dextran particles from the airways to the vasculature. I found a significant increase in FITC fluorescence in peripheral plasma (Figure 25G and 26F), suggesting a breakdown in the barrier between the airways and the blood in the absence of platelets (3.30 (± 0.31)-fold higher in the AS model, and 2.36 (± 0.40)-fold higher in the DT model). As expected from the survival data (Figures 6-9), uninfected thrombocytopenic mice had no evidence of hemorrhage or tissue damage (Figure 25).

Germling and Hyphae Formation is Unaffected in Thrombocytopenic Mice[1]

To assess the contribution of fungal germination to lung tissue damage, I quantified the conidial germination rate in the AS model on GMS-stained lung sections. I did not observe a statistically significant difference in fungal germination in the lungs of thrombocytopenic mice compared to the lungs of control mice (Figure 27). I did not observe overt evidence of fungal angioinvasion in either group, consistent with the model that inflammatory lung hemorrhage was the primary cause of mortality in thrombocytopenic mice.

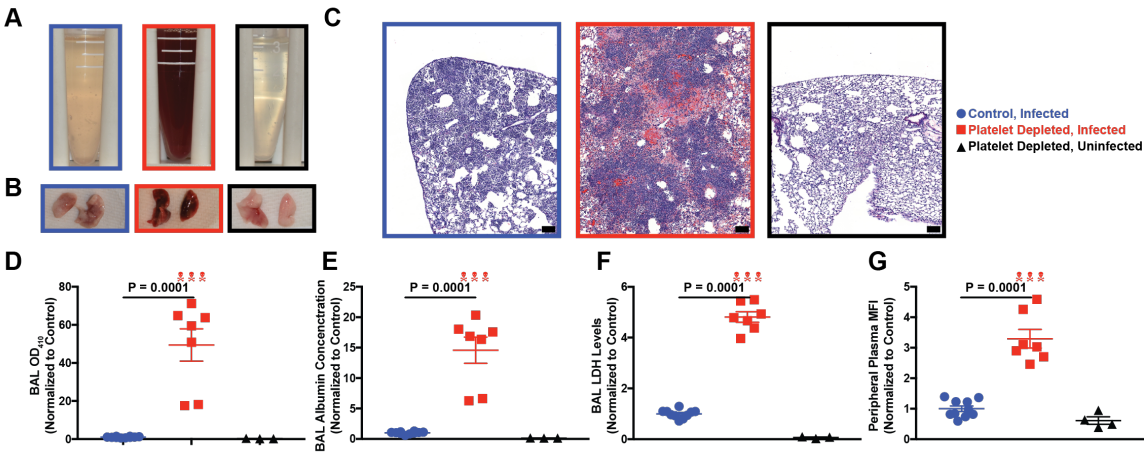

Figure 25: Thrombocytopenia impairs lung tissue integrity after *Aspergillus* challenge[1]

(A) Representative images of bronchoalveolar lavage fluid (BALF), (B) whole perfused lungs, (C) H&E stained lung sections (scale bar: 100 μm), (D) BALF OD_{410} absorption measurements, (E) BALF albumin levels, and (F) BALF LDH levels 2 days after infection of platelet sufficient mice infected with ~3-6 x 10^7 *A. fumigatus* CEA10 conidia (blue), thrombocytopenic (AS model) mice infected (red) or uninfected thrombocytopenic mice (black).

(G) Vascular permeability assessed by monitoring peripheral plasma fluorescence after intranasal installation of FITC-Dextran prior to euthanasia. All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) and uninfected thrombocytopenic mice (black) as a percentage of infected controls at approximately 48 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data from (D-G) are pooled from 2 experiments for infected mice (*n* = 10 mice per group), and 1 experiment for uninfected mice (*n* = 3-4 mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

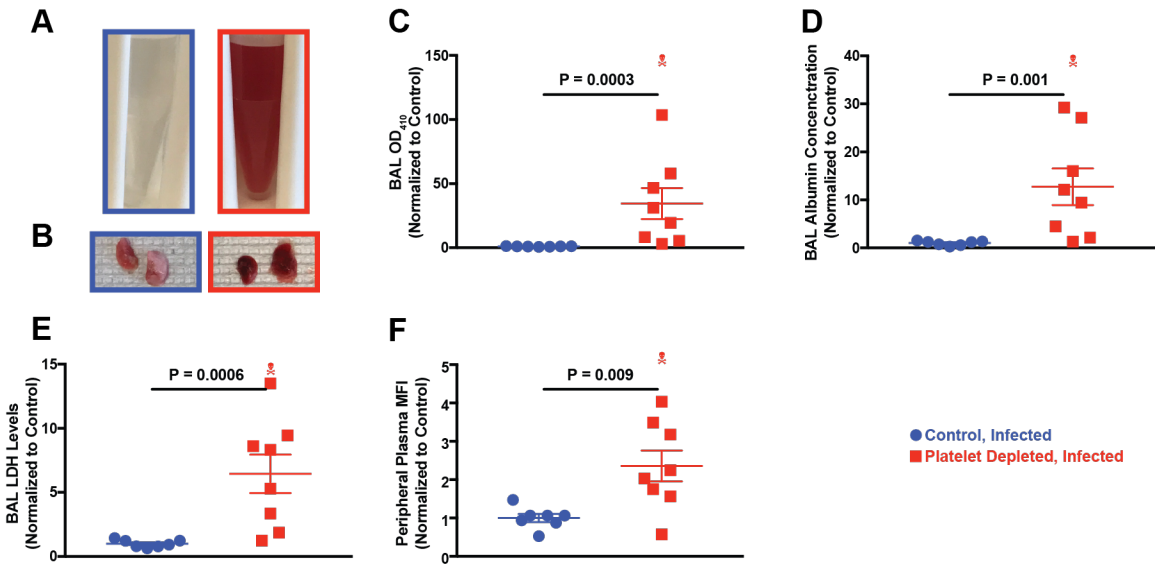

Figure 26: A second model of thrombocytopenia impairs lung tissue function integrity *Aspergillus* challenge early in infection[1]

(A) Representative images of bronchoalveolar lavage fluid (BALF) and (B) perfused lungs 1 day after infection of Cre negative (blue) or iDTR^{Pf4} (red) mice injected with DT and infected with ~3-6 x 10^7 *A. fumigatus* CEA10 conidia. (C) Lung airway bleeding (from A) quantified by measuring BALF OD_{410} absorption, (D) airway vascular leakage determined by BALF albumin levels, (E) airway LDH levels, and (F) vascular permeability assessed by monitoring peripheral plasma fluorescence after intranasal installation of FITC-Dextran prior to euthanasia in thrombocytopenic and control mice. All data are expressed as relative values, with control mice (blue) having a value of 1.00, and thrombocytopenic mice (red) as a percentage of controls at approximately 24 hrs post infection. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest (excluded from analysis). Data from (C-F) are pooled from 2 experiments (*n* = 7-9 total mice per group). For comparisons between two groups, a Mann-Whitney U test was used.

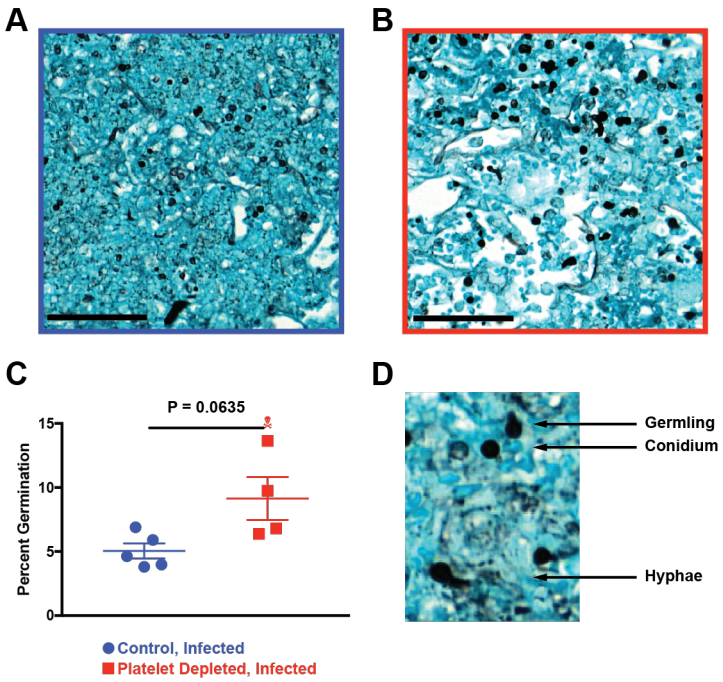

Figure 27: The fungal burden in thrombocytopenic mice is not coincident with an increase in fungal germination[1]

(A-B) Representative GMS staining of control infected (blue, A), or thrombocytopenic infected (red, B), C56BL/6J mouse lung 2 days after infection with ~3-6 x 10^7 *A. fumigatus* CEA10 conidia in the AS model. The scale bars represent 50 μm. (C) Quantification of the germination rate in control infected (blue) and thrombocytopenic infected (red) C57BL/6J mice. Error bars are expressed with mean ± SEM. The skull symbol indicates a mouse died prior to harvest and was excluded from analysis. Data are from one experiment (*n* = 5 mice per group). (D) Germination rate in C was determined by counting all fungal particles and dividing the number of germlings and hyphae by the total number. For comparisons between two groups, a Mann-Whitney U test was used.

Epithelial and Endothelial Cell Death is not Affected by Thrombocytopenia

Because previous reports have suggested that apoptosis in airway barrier cells causes hemorrhage in pulmonary *P. aeruginosa* challenge (Bain et al., 2019), I assessed apoptosis in lung epithelial and endothelial cells. After challenge with live *A. fumigatus* neither platelet-sufficient nor thrombocytopenic mice (AS model) had differences in the proportion of Cleaved-Caspase 3^+ Annexin V^+ epithelial cells or endothelial cells (Figure 28). These results suggest that it is unlikely that *A. fumigatus* conidia induce programmed host cell death in the pulmonary epithelial or endothelial compartments.

Fungal Germination is Required for Mortality in Thrombocytopenic Mice[1]

To determine whether fungal germination and viability were necessary to induce mortality in thrombocytopenic mice, I compared murine survival in thrombocytopenic mice challenged with heat-killed resting conidia and heat-killed swollen conidia, the first step in germination and hyphal growth. Inoculation with resting heat-killed conidia failed to induce any mortality in either thrombocytopenic (0/8) or control (0/12) mice in the DT model (Figure 29A). This suggests that fungal germination or metabolic activity is necessary to induce inflammation-dependent mortality in thrombocytopenic mice.

In resting conidia, the rodlet and melanin layers mask the internal immunogenic polysaccharides, primarily β-glucans (Aimanianda et al., 2009; Bayry et al., 2014). A mutant deficient in melanin synthesis (Δ*pksP*) has exposed β-glucans in the resting state (Langfelder et al., 1998). Infection of thrombocytopenic mice with heat-killed resting

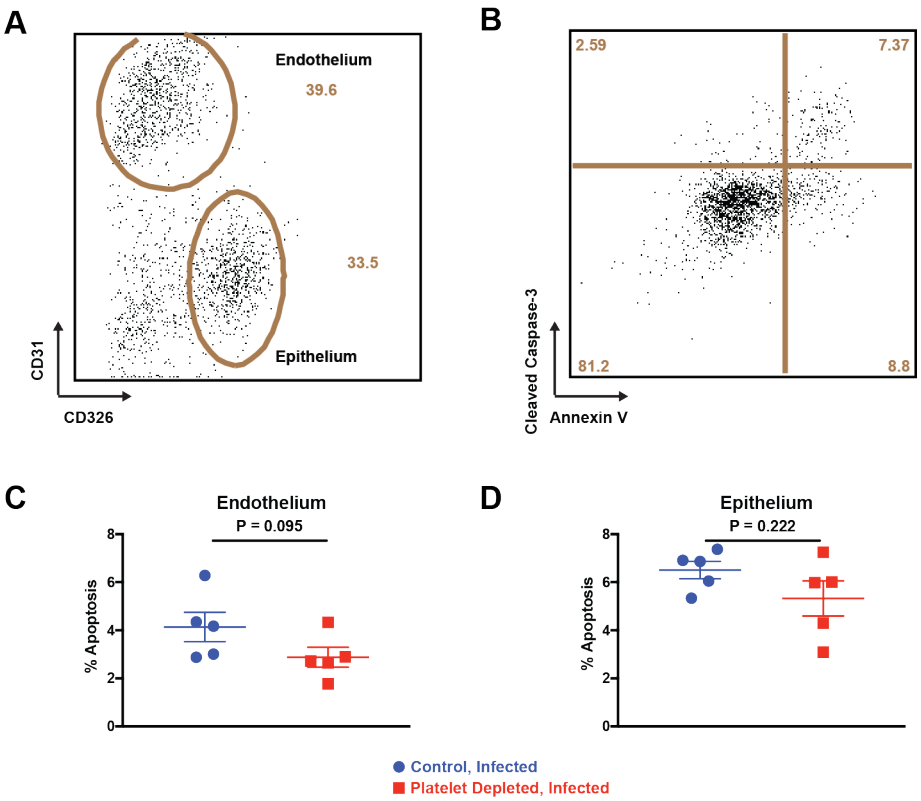

Figure 28: *A. fumigatus* does not induce apoptosis in thrombocytopenic mice

(A) Representative flow cytometry plot of lung CD45$^-$ cells stained for the epithelial marker CD326 and the endothelial marker CD31. (B) Representative flow cytometry plot showing the staining strategy for apoptosis. Cleaved caspase-3 and Annexin V double positive cells (top right quadrant) were identified as apoptotic. (C) The percentage of double positive lung endothelial cells in control (blue) or thrombocytopenic (red) AS-treated mice. (D) The percentage of double positive lung epithelial cells in control (blue) or thrombocytopenic (red) AS-treated mice. Error bars are expressed as mean ± SEM

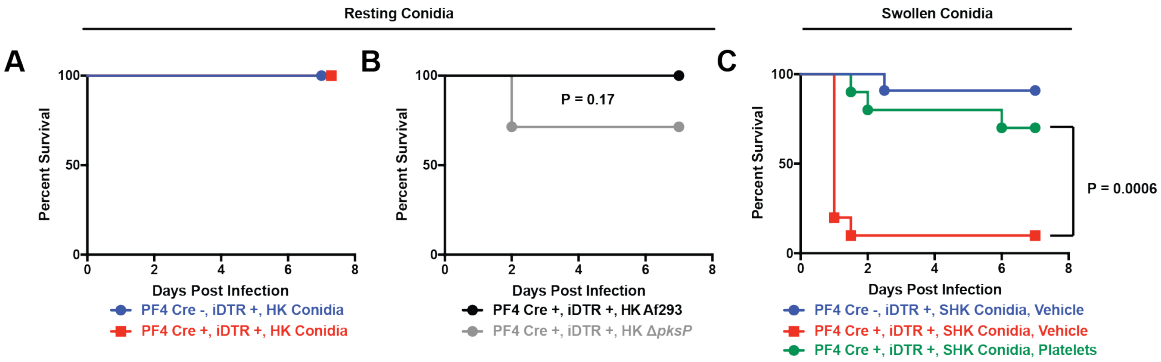

Figure 29: Fungus-induced inflammation is necessary and sufficient to drive mortality in the diphtheria toxin model of thrombocytopenia[1]

(A) Kaplan-Meier survival curve of Cre negative (blue) or iDTR^{Pf4} (red) mice infected with ~6x10^{7} resting, heat-killed CEA10 conidia. Data are pooled from 2 experiments (*n* = 8-12 mice per group). (B) Kaplan-Meier survival curve of iDTR^{Pf4} mice infected with ~6 x 10^{7} resting, heat-killed, WT Af293 (black) or a Δ*pksP* mutant strain of Af293 (grey). Data are pooled from 2 experiments (*n* = 6-7 mice per group). (C) Kaplan-Meier survival curve of Cre negative (blue) or iDTR^{Pf4} (red, green) mice infected with ~6x10^{7} swollen, heat-killed CEA10 conidia. 1 hour after infection, mice were injected with 1 x 10^{8} platelets (green) or Tyrode's buffer supplemented with BSA and glucose (blue, red). Data are pooled from 3 experiments (*n* = 10-11 mice per group). For survival experiment comparisons, a Mantel-Cox test was used.

Δ*pksP* conidia killed a portion of thrombocytopenic mice in the DT model (2/7 compared to 0/6, P = 0.17), but did not completely recapitulate the survival defect seen in live infections with resting wild-type conidia (Figure 29B). This result suggested that surface exposure of cell wall carbohydrates in amelanotic resting conidia is not fully sufficient to induce lung hemorrhage and death observed in thrombocytopenic hosts infected with live wild-type conidia that have the capacity to germinate.

During conidial swelling, fungal cells lose their melanin and hydrophobin layers and increase in diameter from ~2 to ~4 μm, and thereby increasing the amount (through de novo synthesis) and exposure of immunoactive β-glucan carbohydrates (Hohl et al., 2005) (Figure 1). Thrombocytopenic mice (DT model) were highly susceptible (90%) to challenge with heat-killed swollen conidia compared with platelet-sufficient controls (9%; Figure 29C). This finding indicated that fungal germination-induced inflammation, but not fungal growth per se, resulted in lethal lung hemorrhage in thrombocytopenic mice.

This model predicts that platelet transfusion should reverse the susceptibility of platelet depletion followed by challenge with swollen, heat-killed conidia. To test this conjecture, I transfused 1 x 10^8 platelets 1 hour after inoculation of heat-killed swollen conidia in thrombocytopenic mice (DT model). Following platelet transfusion, murine mortality was severely mitigated and only 3 of 10 transfused mice succumbed to this challenge (Fig. 29C). This finding suggested that a low level of platelets is sufficient to overcome pathologic lung inflammation and confirmed the tissue-protective role of platelets in this model.

Transfusion Partially Reconstitutes Platelet Counts

As previous reports suggested that transfusion of 2 x 10^8 platelets only raised the peripheral platelet count to 18% (Boulaftali et al., 2013), I assessed circulated platelet counts. I transfused mice with 1 x 10^8 fluorescent platelets from mTmG mice. The analyzed mice had a non-fluorescent platelet count of 721 ± 112 x 10^9 platelets/L and a fluorescent platelet count of 36 ± 8.8 x 10^9 platelets/L (Figure 30A). When the fluorescent platelet count in an individual mouse was normalized to its corresponding WT platelet count, infusion of 1 x 10^8 platelets reconstituted platelets to 5.04% ± 0.85% of normal (Figure 30B). These data are consistent with the idea that a low platelet level is sufficient to prevent inflammatory hemorrhage (Goerge et al., 2008).

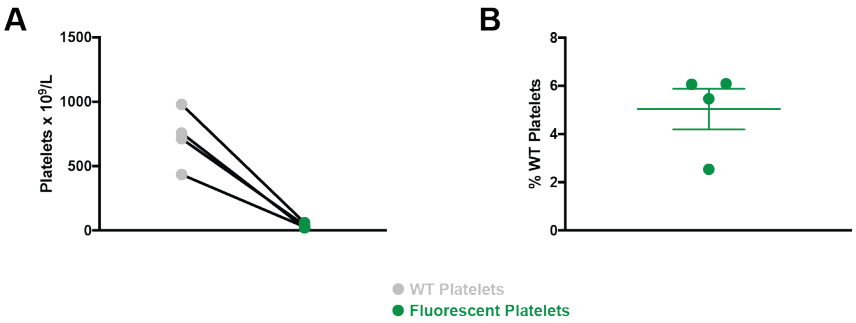

Figure 30: Platelet counts post-transfusion

WT mice were transfused with 1 x 10^8 fluorescent platelets. (A) Platelet counts of endogenous WT platelets (grey) and transfused fluorescent platelets (green). The paired bars indicate the counts came from the same mouse. (B) The count of platelets in A normalized to the WT count in A. Error bars represent mean ± SEM.

CHAPTER 4: DISCUSSION

Summary of Thesis Data

In this dissertation, I assessed the hypothesis that platelets contribute to host defense during murine pulmonary *Aspergillus* challenge. In using both a DT and antiserum-based approach, I determined that platelets are necessary for survival independent of the fungal strain used to infect mice. In conjunction with a survival defect, thrombocytopenic mice have a higher fungal burden and impaired early neutrophil phagocytosis of conidia *in vivo*. However, leukocyte recruitment and conidial killing is not different in thrombocytopenic mice compared to controls. Moreover, this phenotype is transient and disappears by 36 hours post infection. The recruitment, phagocytic capacity, and conidiacidal activity of the other major immune cell types is either unaffected or inconsistent between models.

The ability of platelets to regulate immune cell function is dependent on Syk signaling, as Pf4 Cre-mediated deletion phenocopies early neutrophil phagocytosis defects but survival is normal. In contrast, the mortality in thrombocytopenic mice instead appears to be mediated by inflammation induced hemorrhage, as swollen, heat-killed conidia are able to kill thrombocytopenic mice, and live conidia induce a stronger inflammatory phenotype and hemorrhage in the absence of overt angioinvasion.

Contextualizing the Platelet Requirement

In my studies, I determined that platelets are necessary for anti-*Aspergillus* host defense through the maintenance of hemostasis and tissue integrity, with a minor role for immune regulation and possible direct action. Through studies similar to the DT

model used in this dissertation, Wuescher and colleagues induced thrombocytopenia through a Pf4-cre system, mice were infected via IV inoculation with *S. aureus.* In line with my pulmonary cytokine array data, TNF-α is increased (in the plasma), there is a higher bacterial kidney burden, and there is evidence of increased tissue damage (including blood urea nitrogen, phosphate, and creatinine) (Wuescher et al., 2015). However, the authors did not report hemorrhage into any organ systems, including sites of colonization. This likely reflects the differences between pulmonary infections and bacteremia, and the authors concluded that a combination of septic shock and kidney failure result in the mortality of the infected mice. The inflammation induced hemorrhage seen in my model, however, may be the cause of lung failure and the ultimate death of the thrombocytopenic mice rather than septic shock and organ failure per se.

For a better comparison between platelet-based responses to bacteria and fungi, it is important to look at pulmonary infection models rather than systemic infection. Using the same methods and materials, van den Boogaard and colleagues induced thrombocytopenia and challenged mice with *S. pneumoniae* intranasally. My data are partially in line with their results: thrombocytopenic mice rapidly succumbed to infection with a median survival time of around 3 days, with evidence of intrapulmonary hemorrhage and 24 and 48 hours post infection. In contrast to my data, van den Boogard observed that TNF-α is not increased in lung homogenates, but is in the plasma (which I did not assess), and while there is increased systemic bacterial load, the bacterial burden at the site of infection is unaffected and neither is neutrophil recruitment (van den Boogaard et al., 2015). The differences with my study likely reflect the differences between prokaryotic and eukaryotic pathogens such as surface proteins and recognition by PRRs, metabolism, toxin production and/or growth. Given the similarities,

it is reasonable to hypothesize that the primary role of platelets during pulmonary infection is to prevent inflammatory hemorrhage.

Gram-negative pathogens such as *P. aeruginosa* exhibit similar host requirements for platelets during infection. In line with my study, CXCL4 increases in the lung following infection, and a loss of platelets through antibody depletion leads to an increased pulmonary bacterial burden, and increased mortality. However, in contrast, the authors report decreased neutrophil recruitment and did not comment on hemorrhage beyond suggesting that barrier breakdown may lead to increased systemic bacterial dissemination (Amison et al., 2018). In a subsequent study, *Mpl* knockout mice, which constitutively have platelet counts 20% of WT levels, were susceptible to *P. aeruginosa* infection due to a soluble factor initiating barrier apoptosis, a finding that I was unable to replicate in a fungal model. As well, the hemorrhage seen in the *Mpl* model of gram negative pneumonia appears to occur at a much higher baseline platelet count than observed in this dissertation, highlighting the differences between inflammatory models. Despite this, like the data presented here, a platelet transfusion ameliorated the phenotype, emphasizing that the tissue protective role of platelets can be harnessed therapeutically (Bain et al., 2019). That Bain and colleagues did not observe hemorrhage for *K. pneumoniae* emphasizes that even within the gram-negative family of bacteria there are differences in pathogenesis. However, my results clearly expand the tissue protective and antimicrobial role of platelets to medically relevant fungi.

Other groups, however, have reported important roles for platelets during anti-*K. pneumoniae* host defense. In an antibody-depleted model of thrombocytopenia, mice were susceptible to *Klebsiella* challenge, in conjunction with increased bacterial burdens at both the site of infection (in the lung) and at secondary sites. As well, similarly to my

study, a very low platelet count induces severe pulmonary hemorrhage after infection to the exclusion of other sites in conjunction with an increase in pulmonary TNF-α (de Stoppelaar et al., 2014). Though the exact details differ, as might be expected from the differences between fungi and bacteria, it is clear that I have extended a previously appreciated role of platelets to a pathogenic mold.

Anti-fungal Effector Functions

My data suggest that the direct and/or indirect antifungal role of platelets, although identified *in vitro* (Speth, Rambach, & Lass-Flörl, 2014), is secondary to the primary role of tissue protection,. However, it is important to contextualize my results in the broader sphere of platelet mediated immunity in general, and to fungi in particular. The indirect antifungal effector functions of platelets can be broadly broken down into three categories: recruiting immune effector cells, stimulating phagocytic uptake, and enhancing microbicidal activity.

Multiple reports have indicated that platelets play a critical role for neutrophil recruitment to sites of inflammation (Rossaint et al., 2018). In my study, I found that neutrophil recruitment was independent of platelet levels, both early and late in infection, and regardless of model. The finding implies that cellular redundancies exist in the sources of neutrophil recruitment factors that include CXCL1, CXCL2, CXCL5 (Jhingran et al., 2015), IL-1α (Caffrey et al., 2015) and LTB_4 (Caffrey-Carr, Hilmer, Kowalski, Shepardson, Temple, Cramer, & Obar, 2017a). In agreement, my data suggest that CXCL1 and CXCL2 are increased in response to antiserum-mediated thrombocytopenia, pointing to a possible platelet independent source of neutrophilic cytokines. Therefore, neutrophil recruitment to sites of inflammation can occur via platelet-dependent (Amison

et al., 2018; Asaduzzaman et al., 2009; Kornerup, Salmon, Pitchford, Liu, & Page, 2010; Zarbock, Singbartl, & Ley, 2006) and -independent pathways (de Stoppelaar et al., 2014; van den Boogaard et al., 2015). Importantly, none of these studies used live fungi or fungal ligands, highlighting the novelty of my findings. Uncovering the pathway underlying platelet-independent recruitment of neutrophils could have implications beyond IA, as these as yet recruitment factors could improve outcomes in thrombocytopenic patients with other infections. Also noteworthy is that my data do not suggest that monocytes or their derivatives have impairments in recruitment.

My data indicate that platelets are necessary for early neutrophil phagocytosis in a Syk-dependent manner, as complete loss of platelets exhibits a similar immune phenotype to platelet-specific Syk loss. This phenotype that appears to be transient and exclusive of monocytes and monocyte-derived DCs. The available anti-*Aspergillus* literature suggests that when platelet-rich plasma is added to in vitro macrophage cultures, a statistically significant increase in phagocytosis was noted, as measured by the percentage of GFP-conidia-positive cells. DCs showed a similar trends but this finding did not reach statistical significance (Czakai et al., 2017). Similar bacterial *in vitro* studies showed that thrombin-stimulated platelets increased the phagocytosis of *S. aureus* by macrophages by up to 70% (Ali, Wuescher, Dona, & Worth, 2017). Though I did not assay lung or alveolar macrophages, these *in vitro* gain of function studies correlate nicely with the *in vivo* loss of function results described herein.

The potential role of Syk partially answers the question as to the lineage in which Syk is important during anti-*Aspergillus* host defense. The Hohl laboratory previously showed that hematopoietic Syk is necessary for survival and to prevent hyphal tissue invasion during *A. fumigatus* infection, with severe defects in neutrophil fungal uptake

observed in this setting (Jhingran et al., 2012). Subsequent unpublished experiments revealed that this is a cell-extrinsic capability, with no differences observed between wild-type and knockout cells in a mixed chimeric setting, implying a soluble factor is necessary. Placing Syk under the control of various myeloid cell-specific Cre promoters (LysM-Cre, CD11c-Cre, Mrp8-Cre, and CCR2-Cre) did not impact murine survival or leukocyte conidial uptake (Unpublished Hohl Lab data, not shown). The data presented herein suggest that platelet or megakaryocyte Syk signaling enabled optimal early neutrophil phagocytosis but was dispensable for survival. In combination with unpublished data, it is clear that a defect in Syk signaling in one hematopoietic lineage can be largely compensated for by expression in other hematopoietic cells. The exact nature of these soluble Syk-dependent factors, particularly those that come from Pf4-expressing cells, remains unknown and future studies assessing differences in cytokine or other signaling molecule expression may reveal their identity.

In these experiments, I did not find evidence that platelets influence neutrophil or other immune cell fungicidal activity, either early or late in infection. This finding contrasts to previous reports in which platelet-rich plasma impaired fungal metabolism when combined with either macrophages or dendritic cells (Czakai et al., 2017). My findings are also different from an *in vitro* bacterial study where addition of activated platelets reduces intracellular growth of *S. aureus* (Ali et al., 2017). While the differences between bacteria and fungi may explain the differences between the Ali study and mine, the divergence of the Czakai paper to mine underlines the importance of studying host-microbe interactions in a live animal. In particular, the differences between *in vitro* and *in vivo* approaches include numeric differences in the effector to fungal cell ratios found in the test tube compared with the lung, as well as functional differences between recruited neutrophils and cultured phagocytes. Moreover, it important to note that fungistatic

activity as assessed by metabolism is a different output from killing as measured by FLARE. XTT and similar assays cannot be conducted *in vivo* and so the role of platelets in *in vivo* fungistasis cannot be excluded in my model.

Given the relative lack of impact of platelet loss on the cells of the innate immune response, the origin of the difference between thrombocytopenic and control mice in CEA10 fungal burden in the lung may rest with the platelets themselves. Various *in vitro* studies have suggested a direct fungicidal and/or fungistatic role. Christin and colleagues reported a decrease in XTT reduction in *A. fumigatus* hyphae when treated with platelets (Christin et al., 1998), which later studies by the Lass-Flörl group suggested was due to platelet granule release (Perkhofer et al., 2008). No study to date has suggested what the platelet-derived fungicidal or fungistatic factor may be. It is tempting to speculate that CXCL4 may be at least partially responsible due to its increase over the course of infection, abundance in granules, and reported anti-bacterial activity (Yeaman et al., 2007). Further functional studies are needed to determine the identity of this factor and will likely require an RNA-Seq or proteomics approach.

Though the molecular mechanism of Syk-dependent neutrophil phagocytosis and/or direct killing is unknown, it is clear from my data that the role of platelets in modulating the immune response is minor in comparison to the role in tissue integrity, discussed in detail below.

Inflammatory Hemorrhage

In the absence of an overt immunomodulatory phenotype, my data suggest a major role for platelets in maintaining hemostasis and tissue integrity during

inflammatory challenge, as discussed above. The lack of overt germination in thrombocytopenic mice seen on GMS-stained sections suggests that it is the inflammation per se, rather than angioinvasion, that causes hemorrhage and ultimately the death of the host. This is supported by my finding that swollen, heat-killed conidia, which are immunogenic but nonviable, are able to induce kill thrombocytopenic mice. This idea is in line with an in vitro study that suggested that thimerosal-killed germ tubes can induce endothelial injury (Lopes-Bezerra & Filler, 2004), implying that *A. fumigatus* germination is harmful under specific host conditions, which in my case is thrombocytopenia. My data do suggest, however, that *A. fumigatus* is not triggering apoptosis in the host.

The exact cause of bleeding is unclear. In certain models of inflammation, such as pulmonary LPS administration, hemorrhage can be abrogated by antibody mediated depletion of neutrophils (Hillgruber et al., 2015). In others, such as *P. aeruginosa* or *K. pneumoniae* infection, hemorrhage was independent of neutrophil recruitment (Bain et al., 2019; Claushuis et al., 2018). Understanding the role of platelets in preventing inflammatory hemorrhage is of critical importance however, as hemoptysis is a known symptom of IA, despite modern therapeutics (Kousha, Tadi, & Soubani, 2011). During chemotherapy-induced neutropenia, hemoptysis a more common symptom and often temporally corresponds with neutrophil recovery (Albelda, Talbot, Gerson, Miller, & Cassileth, 1985). Given that neutropenia and thrombocytopenia are often coincident in this patient group (Lien et al., 2018), and baseline platelet counts determine risk levels (Nouér et al., 2012), it is important to understand whether neutrophils play a role in this process. As neutropenic mice are susceptible to *A. fumigatus* challenge, swollen, heat-killed conidia can be inoculated into thrombocytopenic mice in the presence or absence

of neutrophils to answer this question[1]. Should neutropenia not impact hemorrhage, it may be the result of the endothelial damage discussed above in an apoptosis-independent manner.

Some studies suggest that the (hem)ITAM receptors CLEC-2 and GPVI are responsible for maintaining hemostasis during inflammatory challenge (Boulaftali et al., 2013) . However, transfusion of 8×10^8 CLEC-2 deficient platelets, JAQ-1 treated (GPVI-deficient), or SLP76-deficient (downstream of Syk) platelets did not rescue LPS induced hemorrhage. As a control, WT platelets did rescue bleeding, analogous to the transfusion survival data in this dissertation (Boulaftali et al., 2013). Similar results were seen in an immune complex model, suggesting that platelets can seal in the vasculature caused by invading neutrophils via GPVI (Gros et al., 2015). This reported finding would appear to contrast with my data showing that platelet-specific Syk deletion does not impact survival, which likely means that there is no hemorrhage (experiment not done).

It is possible that, in my model, endogenous levels of Syk-deficient platelets exhibit normal hemostasis, given the severity of thrombocytopenia that is necessary for hemorrhage. Impaired vascular integrity may only be reliant on the (hem)ITAM receptors during pulmonary inflammation in thrombocytopenic hosts. In a pulmonary *K. pneuomniae* model of infection, depletion of CLEC-2 with an INU-1 antibody did not impact hemorrhage, and JAQ-1-mediated suppression of GPVI had notably less hemorrhage than fully thrombocytopenic mice (Claushuis et al., 2018). My data are also in line with the suggestion by Claushuis and colleagues that the nature of sterile

[1] Please note that this experiment was terminated due to the COVID-19-related laboratory shutdown.

inflammation compared with live infection may explain the disparate data with the LPS experiments. This may be an additional explanation for the differences between the studies. That some of my experiments were conducted with swollen, heat-killed conidia does not affect this possibility, as swollen, heat-killed, conidia induce the same amount of inflammation as resting live conidia that swell *in vivo* (Hohl et al., 2005). Even though I did not observe a difference in pulmonary hemorrhage in mice with Syk-sufficient or deficient platelets, it is possible that CLEC-2 or GPVI impact vascular integrity in a Syk-independent manner. Further studies on this question are warranted.

Origin of Vascular Permeability and Reversibility

The initial source of vascular permeability is important to consider, as in the absence of platelet, it may lead to a loss of vascular integrity and hemorrhage during inflammation. The early neutrophilic cytokines may be responsible for increasing vascular permeability to aid in neutrophil recruitment. The Hohl lab previously showed that IL-1 release after germination leads to IL-1R-depending MyD88 signaling and release of CXCL1 and CXCL5 early in infection. In a subsequent CARD9-dependant manner, CXCL1, CXCL2, and CXCL5 are released (Jhingran et al., 2015). This leads to the hypothesis that one of these factors could stimulate the vascular permeability. IL-1 is a notable possibility as both IL-1α and IL-1β have been reported to increase vascular permeability (Martin, Maruta, Burkart, Gillis, & Kolb, 1988).

Beyond IL-1R signaling, loss of CXCR2 in nonhematopoietic cells (that is to say, endothelial or epithelial cells) not only reduces neutrophil recruitment, but also Evans blue extravasion after pulmonary LPS installation, indicating an increase in vascular permeability (Reutershan et al., 2006). It is possible to speculate that small amount of

permeability LPS causes in a platelet sufficient host may become hemorrhagic in the context of thrombocytopenia. As CXCL1, CXCL2, and CXCL5 are all ligands for CXCR2, the early release of these chemokines enables a hypothesis as to the origin of the inflammatory hemorrhage and possible reversibility.

Testing this axis will require generation of both genetic models and purchase of pharmaceutical reagents. I am currently in the process of crossing *Clec7a*$^{-/-}$ iDTR^{Pf4} mice as well as *Card9*$^{-/-}$ iDTR^{Pf4} mice. The use of the iDTR model in conjunction with swollen heat-killed conidia is necessary as live fungi cause mortality in *Card9*$^{-/-}$ mice. Should the reduction of inflammation be insufficient to rescue mortality and/or hemorrhage, it is possible to generate hematopoietic chimeras with IL-1R recipients and either Dectin-1 or *Card9* iDTR^{Pf4} donors[2]. This would completely shut down the early inflammatory response. I hypothesize that barring epithelial or endothelial damage induced by germinating *A. fumigatus* conidia, inhibition of the innate response may be sufficient to rescue survival of the host. If this hypothesis proves to be correct, it opens up possibilities to reverse the hemorrhage pharmaceutically. IL-1R blocking antibodies such as JAMA147 (Castro-Dopico et al., 2019) or CXCR2 small molecule inhibitors with *in vivo* activity such as SB225002 are possible options either as a combination treatment or individually[2]. To be clear, there are many safety issues which would have to be assessed and mitigated, as interfering with neutrophil recruitment is likely to be detrimental in a human patient with active IA and thrombocytopenia.

[2] Please note that this experiment was terminated due to COVID-19-related shutdowns.

Conclusions and Future Directions

In many clinical settings, thrombocytopenia is coincident with neutropenia, and together both conditions represent risk factors for IA. In this study, my work finds a major role for platelets in maintaining pulmonary tissue integrity during *A. fumigatus* infection. Although platelets were found to be involved in the regulation of the early innate immune response against *Aspergillus,* this role was dispensable for murine survival. Though these studies have established a clear, non-redundant, requirement for platelets *in vivo*, there are multiple open questions which remain to be addressed. First, it remains unclear whether there are direct interactions between fungi and platelets *in vivo*. Three-dimensional histology may assist with assessing these interactions during infection and at steady state. Second, platelets possess many receptors not canonically involved in the immune response, such as the thrombin receptors and the ATP-sensing P2 receptors. Given the primary role of platelets in maintaining hemostasis during inflammation, assessing these functions during *Aspergillus* challenge may reveal the molecular mechanisms that underlie the maintenance of lung tissue integrity. Finally, as platelet transfusion improves outcomes for mice challenged with IA, further work may reveal platelet-based therapeutics to have beneficial outcomes in patients at risk for, or diagnosed with, IA.

APPENDIX

Cytokine Array Summary

Table 3: Cytokine array summary

	CTRL	PD	Fold Change	Log2 Fold Change
Adiponectin/Acrp30	33.2065	35.108	1.057262885	0.080334143
Amphiregulin	6.698	5.8005	0.866004778	-0.207553111
Angiopoietin-1	6.4955	4.798	0.73866523	-0.437007426
Angiopoietin-2	32.444	31.463	0.969763284	-0.044295461
Angiopoietin-like 3	9.155	9.378	1.024358274	0.034720393
BAFF/BLyS/TNFSF13B	20.295	16.906	0.833013057	-0.263588985
Blank	3.3775	2.3529	0.696639526	-0.521515763
C-Reactive Protein/CRP	19.234	11.0975	0.576973069	-0.793424115
C1q R1/CD93	21.703	21.324	0.982536976	-0.025416393
CCL11/Eotaxin	11.3415	17.1675	1.513688666	0.598068503
CCL12/MCP-5	9.28	16.029	1.727262931	0.788487713
CCL17/TARC	51.1245	61.478	1.202515428	0.266055404
CCL19/MIP-3β	7.365	7.933	1.077121521	0.107181024
CCL2/JE/MCP-1	5.8215	31.023	5.329038907	2.413875366
CCL20/MIP-3α	11.654	28.5115	2.446499056	1.290718726
CCL21/6Ckine	53.8485	40.2565	0.747588141	-0.419684411
CCL22/MDC	30.547	28.3355	0.927603365	-0.108420041
CCL3/CCL4/MIP-1α/β	1.8015	4.4105	2.44823758	1.291743566
CCL5/RANTES	14.401	11.695	0.812096382	-0.300277134
CCL6/C10	60.2045	60.1855	0.999684409	-0.000455373
CD14	8.1885	11.246	1.37338951	0.457740849
CD160	5.737	6.2925	1.09682761	0.133336793
CD40/TNFRSF5	12.252	10.083	0.822967679	-0.281092324
Chemerin	8.941	7.7525	0.867073034	-0.205774577
Chitinase 3-like 1	24.0995	22.8145	0.946679392	-0.079052179
Coagulation Factor III/Tissue Factor	54.845	54.72	0.99772085	-0.003291872
Complement Component C5/C5a	6.1515	5.5335	0.899536698	-0.152745955
Complement Factor D	13.1355	18.664	1.420882342	0.506787095
CX3CL1/Fractalkine	3.1815	5.3815	1.691497721	0.758301234
CXCL1/KC	6.22	21.9885	3.535128617	1.821762705
CXCL10/IP-10	23.3555	21.486	0.919954615	-0.120365407
CXCL11/I-TAC	5.7695	4.927	0.853973481	-0.227736825

CXCL13/BLC/BCA-1	49.5115	37.8715	0.764903103	-0.386651094
CXCL16	17.8065	15.35	0.862044759	-0.214165316
CXCL2/MIP-2	4.0415	31.998	7.917357417	2.985018981
CXCL9/MIG	27.818	20.671	0.74308002	-0.428410516
Cystatin C	8.3285	7.1945	0.863841028	-0.211162256
DKK-1	4.571	4.6905	1.026143076	0.037231901
DPPIV/CD26	15.5115	15.317	0.987460916	-0.018204448
E-Selectin/CD62E	3.669	5.987	1.631779777	0.706446366
EGF	4.8575	4.797	0.987545033	-0.018081556
Endoglin/CD105	31.752	25.194	0.793461829	-0.333767273
Endostatin	23.359	20.442	0.875123079	-0.19244216
Fetuin A/AHSG	10.421	10.668	1.02370214	0.033796005
FGF acidic	38.7385	34.0925	0.880067633	-0.184313696
FGF-21	4.555	4.3715	0.959714599	-0.059322655
Flt-3 Ligand	34.263	29.528	0.861804279	-0.214567834
G-CSF	5.614	6.772	1.206270039	0.270552909
Gas 6	11.814	10.6805	0.904054512	-0.14551833
GDF-15	4.6525	4.7715	1.025577646	0.036436722
GFBP-1	3.2005	6.228	1.945945946	0.960471636
GM-CSF	4.1455	4.088	0.986129538	-0.020150923
HGF	13.4205	11.009	0.820312209	-0.285754994
ICAM-1/CD54	35.3065	34.7825	0.98515854	-0.021572181
IFN-γ	4.121	3.653	0.886435331	-0.17391271
IGFBP-2	11.3115	14.754	1.304336295	0.383315885
IGFBP-3	6.52	10.278	1.576380368	0.656615688
IGFBP-5	7.217	8.69	1.204101427	0.267956922
IGFBP-6	20.3655	19.691	0.966880263	-0.048590855
IL-10	3.5295	4.398	1.246068848	0.317383783
IL-11	2.5045	3.94	1.573168297	0.653673018
IL-12 p40	6.656	6.8405	1.027719351	0.039446348
IL-13	6.3335	5.7005	0.900055262	-0.151914512
IL-15	4.097	4.4485	1.085794484	0.11875106
IL-17A	3.9055	4.835	1.237997696	0.308008629
IL-1ra/IL-1F3	37.7765	37.5605	0.99428216	-0.008272774
IL-1α/IL-1F1	55.197	50.095	0.90756744	-0.139923243
IL-1β/IL-1F2	7.353	5.9605	0.810621515	-0.302899628
IL-2	3.9225	3.4155	0.870745698	-0.199676655
IL-22	4.1865	4.094	0.977905171	-0.032233523
IL-23	4.933	4.226	0.856679505	-0.22317252

IL-27 p28	4.691	4.332	0.923470475	-0.114862258
IL-28A/B	5.25	4.51	0.859047619	-0.219189989
IL-3	4.474	3.8735	0.865780063	-0.207927517
IL-33	35.9155	35.041	0.975651181	-0.035562653
IL-4	6.023	5.7635	0.956915159	-0.063537076
IL-5	3.185	2.8495	0.89466248	-0.16058458
IL-6	1.5135	2.352	1.554013875	0.635999385
IL-7	2.266	1.795	0.792144748	-0.336164017
LDL R	11.2735	12.453	1.104625893	0.14355785
Leptin	3.056	4.6985	1.537467277	0.620555705
LIF	3.242	5.456	1.682911783	0.750959554
Lipocalin-2/NGAL	16.2315	15.0935	0.929889413	-0.104868941
LIX	22.1585	31.5035	1.421734323	0.507651896
M-CSF	6.719	10.326	1.536835839	0.619963068
MMP-2	6.764	8.0225	1.186058545	0.246175225
MMP-3	53.116	54.598	1.027901197	0.039701598
MMP-9	36.8955	38.6375	1.04721443	0.066556882
Myeloperoxidase	30.0015	17.9115	0.597020149	-0.744148473
Negative Control	-0.048	0.776	-16.16666667	#NUM!
Osteopontin (OPN)	27.602	22.9765	0.832421564	-0.264613757
Osteoprotegerin/TNFRSF11B	2.71	2.514	0.927675277	-0.108308202
P-Selectin/CD62P	7.281	9.4765	1.30153825	0.380217711
PD-ECGF/Thymidine phosphorylase	0.626	1.7365	2.773961661	1.471947848
PDGF-BB	3.565	4.971	1.394389902	0.479634027
Pentraxin 2/SAP	9.2365	10.3645	1.122124181	0.166232342
Pentraxin 3/TSG-14	20.1025	22.7645	1.132421341	0.179410842
Periostin/OSF-2	5.8215	7.9525	1.366056858	0.450017533
Pref-1/DLK-1/FA1	4.1475	4.6805	1.128511151	0.174420674
Proliferin	4.6545	4.3345	0.931249329	-0.102760615
Proprotein Convertase 9/PCSK9	8.6595	11.0275	1.273456897	0.348750129
RAGE	35.685	34.2405	0.959520807	-0.059614004
RBP4	10.655	8.965	0.841389019	-0.249155105
Reference Spots	54.483	49.32633333	0.90535274	-0.143448096
Reg3G	46.631	40.7455	0.873785679	-0.194648634
Resistin	5.322	2.2345	0.419860955	-1.252016466
Serpin E1/PAI-1	23.747	25.3495	1.067482208	0.094212025
Serpin F1/PEDF	4.2565	5.6135	1.318806531	0.399232937

Thrombopoietin	3.8405	3.2665	0.850540294	-0.23354851
TIM-1/KIM-1/HAVCR	4.396	4.781	1.087579618	0.121121019
TNF-α	8.772	19.4245	2.214375285	1.146899746
VCAM-1/CD106	12.2305	8.697	0.711091125	-0.491893645
VEGF	7.355	4.938	0.671380014	-0.574798505
WISP-1/CCN4	5.4215	5.742	1.059116481	0.082861264

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