EVASION OF ADAPTIVE IMMUNE DEFENSES BY THE LETHAL CHYTRID FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS*

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

May, 2014

Nashville, TN

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DEDICATION

To my family,

My loving wife Meg,

My newborn son Peter,

My parents Jeff and Robin,

And my sister Kateri.

ACKNOWLEGMENTS

I have many people to acknowledge for the achievements I have made in graduate school both personal and academic. I could not have accomplished a paper in *Science* or completed my Ph. D. thesis work without their assistance and support.

I practiced karate for eight years from elementary school through high school. At every promotion to a higher rank, my karate instructor would remind all the students that they did not make achievements alone and that they must "put all their ducks in a row" to thank all the people responsible helping us along our way. He would say that we should start by thanking the oldest living members of our families, our parents, grandparents, and great-grandparents because, "if it wasn't for our great-grandparents, our grandparents wouldn't be here; and if it wasn't for our grandparents, our parents wouldn't be here; and if it wasn't for our parents, we wouldn't be here."

In such fashion, I will begin by thanking my oldest living relatives, my grandparents, Jack and Pauline Fites. When I get a chance to call up my grandparents I often discuss my research or scientific research in general with my granddad. My grandparents are by no means scientists, but my granddad would always discuss research and graduates school with great interest. When I told them that I had published a paper in *Science*, they did not really understand what that meant, but they were both excited and proud when I told them.

My parents, Jeff and Robin, have been a great support system for my sister and me throughout all of our years of schooling, now about 42 years for the both of us combined. They have given my sister and me great education opportunities that most kids never have. My dad's favorite past-time has always been travel, and he would spend months researching interesting

locations around the world to find affordable rates to show my sister and me the world from a young age. Travel was the best educational experience I had growing up. An appreciation for nature came from the pristine waters of the Antarctic, the thick canopy of the Amazon rainforests, and the majestic Himalaya. An understanding of history flourished in the temples of Luxor, the streets of Pompeii, and the plazas of Teotihuacán. An openness to the excitement of the world came floating over Victoria Falls in a microlight, meeting Shaolin monks, and experiencing the Christkindlmarkt.

I must not forget to thank my wife, Meg. For all of the times when lab got in the way and for all the weekends I spent writing or working on data, I thank you for your patience and understanding. It has been rough these last few months with the pregnancy, and I hope this thesis did not keep me from supporting you and the baby. I also must thank you for loving me and for moving to Tennessee. I know that science will be a part of my life for the rest of my life, and I appreciate that you understand that. Finally, I must thank you the most for bringing little Peter into the world and for pushing on through one of the most difficult pregnancies ever.

To little Peter, although you came early and greatly complicated my dissertation defense, you are a wonderful addition to my life. Your early appearance has greatly diverted my anxiety about my defense; I have probably stressed out less about my defense than any other graduate student in the history of Ph. D dissertations. Peter, your presence has made me so happy these last few weeks; I look forward to watching you grow.

Kateri, thank you for being a great sister. It was fun growing up with you. You have become a strong, intelligent, and beautiful young woman. You are going to be a great biomedical engineer. I wish you and Ben the best in Seattle.

I would not have even made it to graduate school if it had not been for all of the countless teachers and mentors I had along the way. To all the teachers of St. Susanna, Cardinal Ritter, and the University of Evansville, I thank you. I would especially like to recognize Linda Bloomer, Kathy Chapin, Jean Reed, Vicky Branchini, Jill Wynalda, Mary Pat O'Connor, Linda Mann, Michael Cullen, Pat Acrobawi, Joyce Stamm, Mark Davis, Cris Hoechwender, and Phil Morgan. Thank you especially to the research mentors I had as an undergraduate, Jae-Ho Lee, Wen-Chen Xiong, Dale Edwards, and Brian Ernsting. The research experiences I had as an undergraduate sparked my development as a scientist and prepared me well for graduate school.

In completion of my Ph. D. dissertation thesis, I owe the most gratitude to my research advisor Louise Rollins-Smith. Near the end of my first year at Vanderbilt, I came to Louise and told her that I wanted to pursue this project to investigate the inhibition of lymphocytes by *Batrachochytrium dendrobatidis*. Despite all of the funding issues, Louise found a way to keep me and my project funded. Also, it was a long, difficult struggle to publish our paper in *Science*. I was very anxious to publish a first author publication; especially because I had no first author publication at the beginning of my fifth year in graduate school. Thank you Louise for continuing to push to publish in a high impact journal; all of the stress and hard work paid off in the end. I also would like to thank Louise for being available and working to prepare me into the scientist I am today. I hope that I can continue to represent you well in my future research endeavors.

Along with Louise, I have received much support and assistance from all of the members of the Rollins-Smith lab. Laura Reinert, our lab manager, deserves recognition for all that she does in keeping the Rollins-Smith lab running. Laura, it was a pleasure working with you. Jeremy Ramsey, a previous graduate student in the Rollins-Smith lab, initiated the research

which eventually evolved into my Ph. D thesis. Jeremy, you gave a great start to this amazing story about chytridiomycosis and were a great mentor to me during my early years in the lab. Whitney (Gammill) Holden, you have been a great colleague to have in the lab, and it has been wonderful collaborating with you. I would also like to acknowledge Jim Pask, another former graduate student in the Rollins-Smith lab. Rotation students and undergraduates who have contributed to tangential projects were Diana Contreras, Tim Chappell, Heather Wells, Julia Coleman, and Jack Lee.

I would have not had the great success without all of collaborators I had through my thesis work. Kyra Oswald-Richter completed the experiments determining the effects of *B. dendrobatidis* supernatants on human $CD4^+$ T cells. Sarah Parker Collier and the Aune lab provided me with mouse splenocytes and aided in designing the experiments I completed with mouse lymphocytes. Danica Sutherland in the lab of Terrence Dermody worked with me to test my factors on two cell lines with which she had experience: CHO and HeLa cells. Sophia Gayek completed several experiments present in the *Science* manuscript during her rotation in the Rollins-Smith lab. Michelle Reyzer completed MALDI/MS to analyze the components of supernatants. Thomas Umile, a post-doc in the lab of Kevin Minbiole at Villanova, has been working to understand the small molecule metabolites produced by *B. dendrobatidis*. Thomas has identified some of the small molecule components of *B. dendrobatidis* supernatants and is continuing to identify others. Alex Roberts, a post-doc in the lab of Lee Berger at James Cook University in Townsville, Australia, has been working to isolate the large molecular factors. Pavlina Ivanova in the lab of Alex Brown at Vanderbilt completed the analysis to determine if lipids might be responsible for inhibition of lymphocytes. John Galgiani, the director of the Valley Fever Center for Excellence at the University of Arizona also provided the nikkomycin Z

drug which was essential for key components of my research. Thank you all for your assistance and open collaboration.

Thank you also to my committee who has helped me in my success. Kathy Friedman, my chair, has aided me in many things including obtaining a Dissertation Enhancement Grant from the graduate school. Julián Hillyer provided me with great advice and the facilities of his lab so that I could complete necessary experiments for the *Science* manuscript. Borden Lacy has given great advice about how to proceed along my project. Clint Carter stayed on my committee even after retiring and was an important component of my committee having served on Jeremy Ramsey's committee.

I have received much assistance in completing my work at Vanderbilt by other labs and core facilities. I have had to rely on the generosity of these labs and cores to allow me to use the equipment necessary to obtain the necessary data. I used a scintillation counter in the lab of Luc Van Kaer to complete many of the proliferation assays throughout my time at Vanderbilt. I would like to also thank the members of the Van Kaer lab, Vrajesh Parekh and Danyvid Olivares-Villagomez. The Skaar lab has been a great neighbor to have. The members of the Skaar lab have been very friendly and generous to allow me to use some of their equipment from time to time. Julián Hillyer also provided me access to his awesome microscope so that I could follow phagocytosis in amphibian leukocytes. I would also like to thank Tania Estévez-Lao and Garrett League from the Hillyer lab for their assistance. The Vanderbilt Flow Cytometry Core has been very helpful to me in both working through issues with my project and assisting in teaching; thank you Kevin Weller, David Flaherty, Brittany Matlock, and Christian Warren. I would like to thank the lab of Daniel Liebler and the Ayers Institute for allowing me heavy use of a lyophilizer. I also benefited from the use of equipment in the Cell and Developmental

Biology Core, the Mass Spectroscopy Core, and the High-Throughput Screen Core at Vanderbilt. I was lucky to be working at such an amazing institution where not only are there all of the great tools to do outstanding research but also a cooperative spirit to allow students to learn and use new techniques outside of their own laboratory.

I have had some amazing experiences teaching at Vanderbilt. I was lucky enough to teach some amazing undergraduates. I had the chance to teach a group of talented undergraduates in a research laboratory course where we investigated the ability of amphibian skin bacteria to inhibit the growth of *B. dendrobatidis*. I would like to thank the staff of the BSCI 111 lab and Steve Baskauf for such a wonderful experience. I would also like to thank Chris Janetopoulos who taught the cell biology lab with whom I assisted teaching. Along with teaching undergraduates, I participated as a teaching assistant in the Vanderbilt Summer Academy. The VSA was a wonderful experience and I would like to thank Holly Algood and David Dunn for the opportunity to teach such brilliant high school students in an amazing summer program.

I would lastly like to thank my department of Biological Sciences. When funding was questionable, the Biological Sciences Department accepted me as a teaching assistant to cover much of my costs. I had an amazing experience as a teaching assistant. The department was always supportive of my work and genuinely interested in the Rollins-Smith lab's research. I would like to acknowledge a few people from the department who have assisted me along the way. Charles "Bubba" Singleton worked very hard to give me the opportunity to be a part of this great department. Kathy Friedman, outside of being a great committee chair, was always accessible and helpful as the Director of Graduate Studies. I owe Leslie Maxwell a huge debt of gratitude as well. Any time I had an issue, big or small, Leslie was there to help and always did it with a smile. I would not have been able to make the most of the travel funds I was able to obtain

without the help of Leslie. In the Biological Sciences Department, I must also recognize Seth Bordenstein, Patrick Abbot, and all of the students serving on the GSA board. I would finally like to acknowledge the Gisela Mosig Fund that provided me with excellent chances to travel and speak at conferences and meetings. I was able to speak at meetings in Japan, California, New York, and Arizona due to this amazing resource in the Biological Sciences Department.

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7-AAD	7-Aminoactinomycin D
ACN	acetonitrile
A. h.	*Aeromonas hydrophila*
AMP	antimicrobial peptide
ANOVA	analysis of variance
APBS	amphibian phosphate buffer saline (amphibian cell tonicity)
APC (cell)	antigen presenting cell
APC (fluorophore)	allophycocyanin
ATP	adenosine triphosphate
BCA	bicinchonic acid
BCR	B-cell receptor
Bd	*Batrachochytrium dendrobatidis*
BID	BH3-interacting domain death agonist
BSA	bovine serum albumin
C18	octadecyl (18) carbon chain (type of reverse-phase column)
casp.	caspase
CBA	cytokine bead array
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of (cellular) differentiation
CE	collision energy (in tandem mass spectrometry)
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming units
CH	Swiss (*Bd* lineage)

CHCA	α-cyano-4-hydroxycinnamic acid
CHO	Chinese hamster ovary
CLR	C-type lectin receptor
CN	cyano column
CPM	counts per minute (scintillation counter readout)
CTL	cytotoxic (T) lymphocyte
Cyclo(His-Pro)	cyclo-L-histidyl-L-proline (diketopiperazine)
Cyclo(Phe-PrO	cyclo-L-phenylanalanyl-L-proline (diketopiperazine)
Da	Dalton (molecular mass)
DAMP	damage (danger)-associated molecular patterns
DC	dendritic cell
DHB	2,5-dihydroxybenzoic acid
DIC	differential interference contrast (microscopy)
DMSO	dimethyl sulfoxide
DNSA	dinitrosalicylic acid
EDTA	ethylenediaminetetraacitic acid
FACS	fluorescence-activated cell sorter
Fc	fragment crystallizable (antibody constant region)
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FT	Fourier transformation ion cyclotron resonance (mass spectrometry)
GalXM	galactoxylomannan
GM-CSF	granulocyte macrophage-colony stimulating factor
GPL	global panzootic lineage (*Bd* strain)

GXM	glucuronoxylomannnan
Hp	*Homolaphlyctis polyrhiza* (designated as strain JEL142)
HPLC	high performance liquid chromatography
h.p.t.	hours pre-treatment
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
iNKT	invariant natural killer T (cell)
iono	ionomycin
i.p.	intraperitoneal
JEL	Joyce E. Loncore (who isolated the chytrid)
L-15 (medium)	Leibovitz-15 medium
LC	Langerhans cell
LC/MS	liquid chromatography mass spectrometry
LPS	lipopolysaccharide
LTQ	linear trap quadrupole (mass spectrometry)
m/z	mass-to-charge ratio
Mø	macrophage
MALDI/MS	matrix-assisted laser desorption/ionization mass spectrometry
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction (response)
MR	mannose receptor
MS-222 (TMS)	ethyl-m-aminobenzoate methanesulfonate salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide

MYD88	myeloid differentiation primary response 88
Nec-1	necrostatin-1
NF-κB	nuclear factor κ B (transcription factor)
NK	natural killer (cell)
Nrf2	nuclear factor erhytroid 2—related factor 2
NZ	nikkomycin Z (chitin synthase inhibitor)
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline (mammalian cell tonicity)
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohemagglutinin
PI	propidium iodide
PKC	protein kinase C
PL	peritoneal leukocyte
PMA	phorbol-12-myristate 13-acetate
PMN	polymorphonuclear cell (neutrophil)
ppb	parts per billion
PRR	pathogen recognition receptor
PS	phosphatidylserine
qPCR	quantitative polymerase chain reaction
RIP	receptor-interacting serine/threonine-protein (kinase)
RFU	relative fluorescence units
RLU	relative light units (for luminescence)

RNase A	ribonuclease A
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI (medium)	Roswell Park Memorial Institute (where medium was developed)
SALT	skin-associated lymphatic tissue
SAX	strong anionic exchanger
SEM	standard error of the mean
SPE	solid phase extraction
Spl	splenocytes
Sup	supernatant
T broth	tryptone (1%) broth
TCR	T-cell receptor
TFA	trifluoroacetic acid
TGF	transforming growth factor
Th	T-helper (in reference to the cell or response mediated by helper T cells)
TLR	Toll-like receptor
TNF	tumor necrosis factor
TOF	time-of-flight (mass spectrometry)
UV	ultraviolet
Zsp	zoospore

CHAPTER I

INTRODUCTION AND RESEARCH AIMS

The Threat of Fungal Disease

Fungal diseases are an important contributor to declines in wildlife populations and have the potential to cause extinction of many species (Daszak & Cunningham, 2000). Fungi are common pathogens of plants, but only recently has the threat of fungal diseases in animals been appreciated (Fisher et al., 2012). Corals (Rosenberg & Ben-Haim, 2002), crayfish (Daszak & Cunningham, 2000), snails (Cunningham & Daszak, 1998), sea turtles (Sarmiento-Ramírez et al., 2010), platypuses (Obendor et al., 1993), bats (Foley et al., 2011), and amphibians (Daszak & Cunningham, 2000) are all threatened by fungal diseases. Fungal pathogens may have even contributed to the extinction of the dinosaurs (Casadevall, 2012). The most widespread and ecologically destructive fungal diseases have been *Pseudogymnoascus* (previously *Geomyces*) *destructans* (Minnis & Lindner, 2013) and *Batrachochytrium dendrobatidis* (Daszac & Cunningham, 2000) causing white-nose syndrome in bats (Foley et al., 2011) and chytridiomycosis in amphibians, respectively.

The chytrid fungus *Batrachochytrium dendrobatidis* is an emergent fungal disease pathogen of amphibians. One-third of all amphibian species are currently threatened with extinction (Stuart et al, 2004), and the loss of amphibians represents a large proportion of the current sixth historical mass extinction event (Wake & Vredenburg, 2008). Infectious diseases have greatly contributed to amphibian declines, and chytridiomycosis is the primary pathogenic

contributor (Rachowicz et al., 2006; Voyles et al., 2012). *B. dendrobatidis* infects amphibians on all six continents where amphibians are present (Speare & Berger, 2000) and is believed to be responsible for at least 90 extinction events (Pennisi, 2009).

This review highlights the immune defenses, both characterized and theoretical, which could protect amphibians from chytridiomycosis. Jawed vertebrates, especially tetrapods, share an overwhelmingly similar immune system allowing for comparative studies (Robert & Ohta, 2009; Robert & Cohen, 2011). Of fungal diseases in animals, those that infect humans and mouse models of fungal diseases have been well characterized in both host and pathogen. Some themes of pathogen recognition, immune response, and immune evasion are recurrent in fungal diseases and may be applicable to chytridiomycosis. Despite the overwhelming greater understanding of human mycoses, relatively little is understood about fungal diseases in animals compared to bacterial and viral diseases (Casadevall, 2013), and only about 2% of the funding for research on infectious diseases in the United States and United Kingdom are dedicated to studying fungal pathogenesis (Brown et al., 2012). A much greater focus on the interactions between vertebrate hosts and fungal pathogens will be necessary to find to find cures for current and future mycoses.

Chytridiomycosis, a Major Contributor to Amphibian Declines

Nearly a third of all amphibian species are considered threatened, endangered, or critically endangered (Houlahan et al., 2000; Stuart et al., 2004; Wake & Vredenburg, 2008). Amphibian declines have been linked to various factors (reviewed in Collins, 2010) including habitat loss (Collins & Storfer, 2003), climate change (Pounds et al., 2006), chemicals and pesticides present in the environment (Davidson et al., 2002; Blaustein et al., 2006), introduction

of exotic species (Vredenburg, 2004), and infectious diseases (Carey et al., 1999; Daszac et al., 2003; Rachowicz et al., 2006). The emerging fungal disease chytridiomycosis, caused by *B. dendrobatidis*, is the most serious infectious disease of amphibians and is implicated in mass mortality events in more than 200 species (Wake & Vredenburg, 2008).

Batrachochytrium dendrobatidis was characterized as a new species in 1999 and identified as the suspect causative agent of several amphibian population declines (Berger et al., 1998; Longcore et al., 1999; Pessier et al., 1999). *Batrachochytrium dendrobatidis* is a member of the Chytridiomycota, a paraphyletic, basal group of fungi defined by a motile zoospore life stage (Longcore et al., 1999, James et al., 2006). Chytrid fungi are largely saphrophytic but can be parasitic on phytoplankton, zooplankton, fungi, plants and invertebrate animals. Only *B. dendrobatidis* and *Batrachochytrium salamandrivorans*, another amphibian pathogen (Martel et al., 2013), infect vertebrates (Gleason et al., 2008). The life cycle of *B. dendrobatidis* (Fig. 1-1) begins as a free-living zoospore containing a single flagellum (Longcore et al., 1999; Berger et al., 2005a). At the surface of the skin, a zoospore encysts and forces a germination tube into the skin so that it can infect an immature keratinocyte inside the *stratum granulosum* (Van Rooij et al., 2012; Greenspan et al., 2012). As the host cells keratinize and move toward the *stratum corneum*, *B. dendrobatidis* cells mature into zoosporangia filled with many zoospores (Longcore et al., 1999; Berger et al., 2005a). Once fully matured, zoosporangia will form a discharge tube at the top of the epithelium to release zoospores into the surrounding environment (Berger et al., 2005a). Zoospores released from the discharge tube can re-infect the same host or be transmitted to a nearby host.

Chytridiomycosis has caused amphibian declines globally. Large population declines in Australia, Central America, the western United States, Europe, and Africa have been linked to

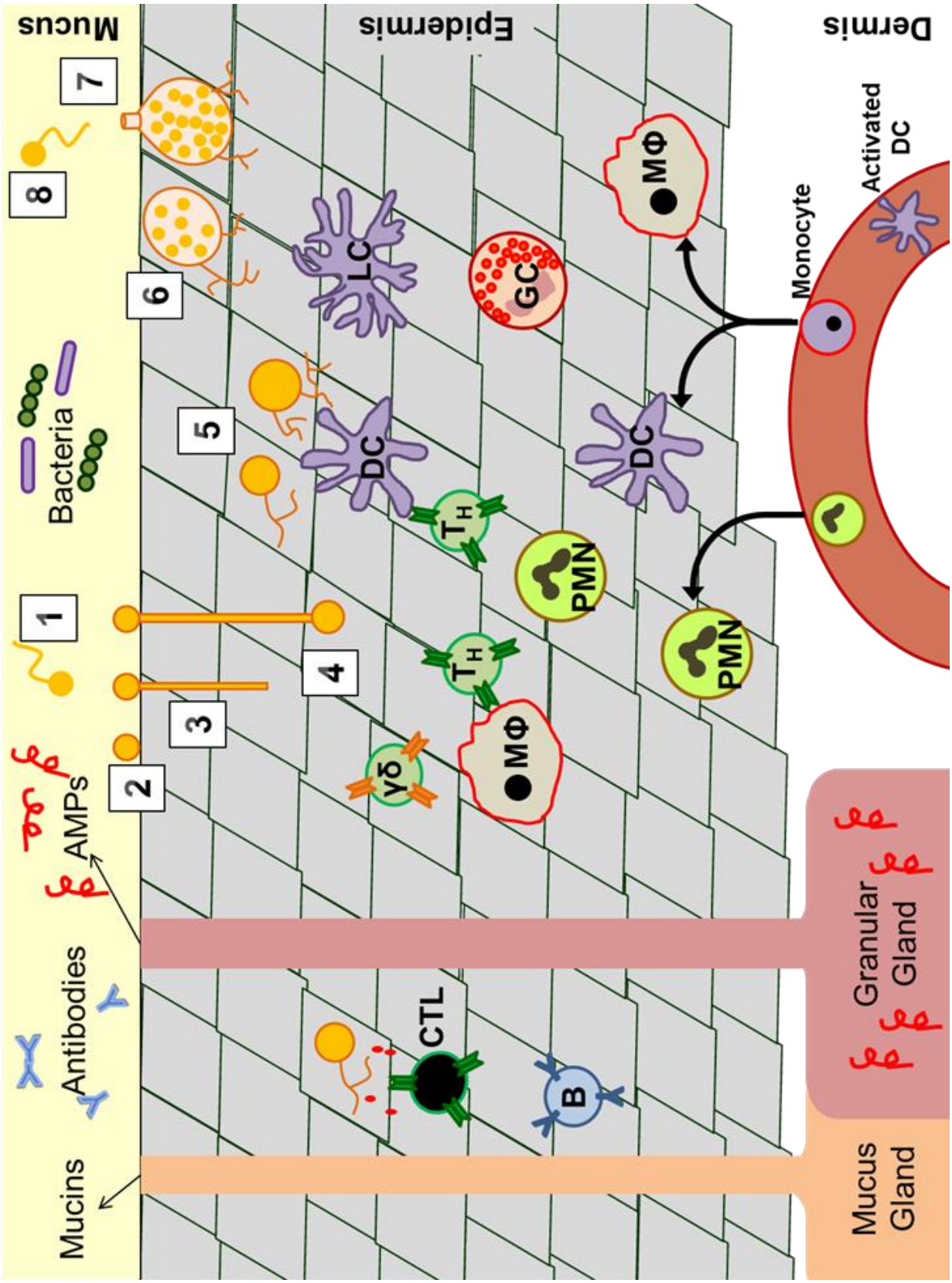

Fig. 1-1. Immune defenses to chytridiomycosis; see following page for details.

Fig 1-1. The immune defenses present in amphibian skin against *B. dendrobatidis* (*Bd*) infection.

The life-cycle of *Bd*: (**1**) *Bd* zoospores from the environment enter the mucus layer covering the skin. (**2**) *Bd* zoospores encyst on the surface of the skin and form a cell wall. (**3**) The encysted cell forms a germinal tube which pushes into the epithelium. (**4**) *Bd* injects itself into a living epithelial cell via the germ-tube. (**5**) *Bd* cells grow and develop inside host cells as the host cell is pushed superficially. Root-like rhizoids form on these growing *Bd* germlings and thalli. (**6**) The matured *Bd* cells, now zoosporangia, become filled with zoospores. (**7**) The host cell is pushed to the surface of the skin and zoosporangia form discharge papillae which allow for the release of zoospores. (**8**) Zoospores pass again through the mucus to re-infect the same individual or to infect another individual.

The surface of the skin is coated in mucin-rich mucus which is secreted from dermal mucus glands. The skin mucus contains symbiotic bacteria; some amphibians have skin bacteria that secrete antifungal metabolites. Antibodies are also present in the mucus; mucosal antibodies from *X. laevis* have been found to be specific against *Bd*. Dermal granular glands release antimicrobial peptides (AMPs) into the mucus; mucosal AMPs from many amphibian species have been shown to inhibit *Bd* growth and survival.

The epidermis likely contains a variety of immune cells that promote and coordinate immune response. Dendritic cells (DC), especially those resembling Langerhans cells (LC), have been identified in the skin of amphibians; dendritic cells are important for recognizing foreign antigens and presenting these to lymphocytes. Macrophages (MΦ) and neutrophils (polymorphonuclear cells, PMN) infiltrate tissue during infection and kill pathogens. B cells secrete antibodies that opsonize and neutralize pathogens; mucosal antibodies are probably produced by B cells in the skin. T cells are important at mediating immune responses by producing cytokines and interacting with other immune cells. T cells expressing the γδ T-cell receptor are typically associated with mucosal epithelia and have been identified in amphibian skin. $CD8^+$ T cells do infiltrate amphibian skin during rejection of a skin transplant, so it is likely that more than just γδ T cells are important effectors in the skin. Helper T cells (T_H) produce cytokines that promote phagocyte and other innate immune responses to clear infections. Cytotoxic lymphocytes (CTL) such as $CD8^+$ T cells and natural killer cells kill host cells infected with pathogens by activating cell death receptors and releasing perforin and granzyme; these cells could limit *Bd* infection by killing infected epithelial cells. Granulocytes (GC) likely contribute to skin immunity, playing roles in alarming the immune system and pathogen destruction.

B. dendrobatidis (Berger et al., 1998; Speare & Berger, 2000; Skerratt et al., 2007). Chytridiomycosis is defined by excessive skin sloughing, thickening and discoloration of the skin, and hyperkeratosis (Berger et al., 1998; Nichols et al., 2001). Amphibian skin is an important surface for the exchange of gases, water, and ions (Evans, 2009), and chytridiomycosis disrupts this normal function of amphibian skin (Voyles et al., 2009). The disruption of the skin is the most likely cause of death from chytridiomycosis due to electrolyte depletion and dehydration resulting in cardiac arrest (Voyles et al., 2009; Marcum et al., 2010; Voyles et al., 2012).

Amphibian Immune Defenses

The role of immune defenses and symbiotic skin bacteria in protection against chytridiomycosis is not well understood, but the current state of knowledge has been previously reviewed (Rollins-Smith et al., 2009; Richmond et al., 2009; Voyles et al., 2011; Rollins-Smith et al., 2011). In adult amphibians, *B. dendrobatidis* invades host epithelial cells in the skin epithelium and develops inside host keratinocytes as these move toward the more superficial layer of the skin (Berger et al., 2005a). Before infecting host cells, *B. dendrobatidis* must first pass through a layer of mucus on top of the skin, and then project a germ-tube into the tissue so as to insert itself into a host cell (Van Rooij et al., 2012; Greenspan et al., 2012). Thus, *B. dendrobatidis* must survive in the two different environments of the mucus and the skin epithelium. The defenses present in these two locations are very different, and pathogenesis studies should recognize that both *B. dendrobatidis* virulence features and host adaptive responses are likely to be site-specific (Fig. 1-1).

Recognition of *B. dendrobatidis* by the immune system

Recognition of *B. dendrobatidis* is likely to be mediated by pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). PRRs are expressed typically by phagocytes and epithelial cells to recognize pathogens and promote an immune response. The most important PRR families for recognizing fungal PAMPs are Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (reviewed by Brown, 2011).

Xenopus expresses 20 TLRs sharing homology with mammalian and other vertebrate TLRs (Ishii et al., 2007). TLRs 1, 2, 3, 4, 6, and 9 have been shown to be important in mammalian recognition of fungal pathogens (Garcia-Vidal et al., 2013), and orthologues of all of these TLRs are present in amphibians (Ishii et al., 2007). Although the general structure of amphibian TLRs are conserved (Roach et al., 2005), PAMP recognition may not necessarily be shared between mammals and amphibians. For example amphibians have minimal responses to the TLR4-ligand lipopolysaccharide (LPS) (Bleicher et al., 1983), but still have strong phagocyte and B-cell responses to Gram-negative bacteria (Morales et al., 2003; Nedelkovska et al., 2010; Chapter II and III).

CLRs are essential for immune activation against fungal pathogens in mammals (Hardison & Brown, 2012). The *Xenopus tropicalis* genome, the only completed amphibian genome, has at least 200 genes with C-type lectin homology (Joint Genome Institute, KOG classification, http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Xentr4), and a specific CLEC orthologue has been identified as well (Robert & Ohta, 2009). The C-type lectin receptor Dectin-1 (CLEC7a) recognizes β-1,3-glucan in fungal cell walls and aids in signaling immune responses against fungi (Hardison & Brown, 2012). *Batrachochytrium dendrobatidis* lacks the fungal genes to synthesize β-1,3-glucan and has been proposed to lack this cell wall component (Ruiz-Herrera

& Ortiz-Castellanos, 2010). The *B. dendrobatidis* cell wall has chitin (Ruiz-Herrera & Ortiz-Castellanos, 2010; Holden et al., 2013), but it is still uncertain which PRRs, if any, recognize chitin (Brown, 2011). Mannose and mannans are important components of fungal cell walls, as well, and are recognized by several PRRs including the mannose receptor, Decitn-2, and Mincle (Hardison & Brown, 2011). Mannan structures are thought to be present in the *B. dendrobatidis* cell wall, and likely are important in the interaction between *B. dendrobatidis* and the amphibian immune system.

Recognition of intracellular fungi can activate the inflammasome to induce release of active pro-inflammatory cytokines such as interleukin (IL)-1 and IL-18 (Romani, 2011; Garcia-Vidal et al., 2013). Nod-like receptors (NLRs) are important activators of the inflammasome, and some evidence suggests that NLRs recognize fungal PAMPs because fungal pathogens can activate the NLRP3 inflammasome (Saïd-Sadier et al., 2010; Mao et al., 2013; Pietrella et al., 2013; Tavares et al., 2013). Because of its intracellular location, *B. dendrobatidis* would be expected to activate the inflammasome of host epithelial cells. Increased expression of IL-1β in *B. dendrobatidis*-infected skin (Rosenblum et al., 2012a) suggests that *B. dendrobatidis* might activate the inflammasome or other intracellular pathogen recognition signaling pathways.

Damage-associated (or danger-associated) molecular patterns (DAMPs) are also important in pathogen recognition and activation of immune responses (Bianchi, 2007; Zitvogel et al., 2010). DAMPs are released due to damage to cells or cellular structures that is often correlated with virulence, especially during fungal infection (Romani, 2011). *B. dendrobatidis* enters skin epithelial cells via a germination tube that grows into the epidermis (Van Rooij et al., 2012; Greenspan et al., 2012); the ensuing damage from germination tube protrusion may produce DAMPs that would activate immune signaling pathways. Protease-activated receptors

(PARs) are activated by protease released from host or pathogen cells during pathogenesis (Romani, 2011). *Batrachochytrium dendrobatidis* has genomic expansions in proteases that likely contribute to virulence (Joneson et al., 2011; Sun et al., 2011); these proteases likely activate PARs in amphibian skin and may activate immune responses.

Defenses within the mucus

The mucus covering amphibian skin provides the first barrier to invading zoospores. This mucus provides both a chemical and physical barrier. Mucus is mainly composed of highly-glycosylated mucins (Schumacher et al., 1994) released by dermal mucus glands (Fig. 1-1). The network of mucins provides a matrix for chemical defenses (Antoni et al., 2013) and has been shown to promote tolerance in gut epithelia (Shan et al., 2013). The mucus layer also contains antimicrobial peptides (Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009), symbiotic bacteria (Bletz et al., 2013), and antibodies (Ramsey et al., 2010) (Fig. 1-1). Antimicrobial peptides (AMPs) are amphipathic peptides produced by granular glands in amphibian skin (reviewed by Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009). Many AMPs secreted by amphibian species are very effective against *B. dendrobatidis in vitro* (Rollins-Smith & Conlon, 2005). The *in vitro* inhibitory properties of AMPs against *B. dendrobatidis* have also been correlated with host resistance (Woodhams et al., 2006; Woodhams et al., 2007a).

In 1965, Farquhar and Palade identified bacteria in close association with amphibian skin in electron micrographs. Only recently have studies begun to identify and characterize these symbiotic bacteria (Harris et al., 2006, Lauer et al., 2007). Some of the bacteria living in the mucus layer of the skin have been shown to produce metabolites that inhibit *B. dendrobatidis* growth (Harris et al., 2006; Woodhams et al, 2007b; Lauer et al., 2008; Becker & Harris, 2010;

Lam et al., 2010). Probiotic skin bacteria and their potential use in bioaugmentation have recently been reviewed by Bletz et al. (2013). Bacteria in the mucus may create a hostile environment to invading *B. dendrobatidis* zoospores, and conservation efforts may one day incorporate cutaneous symbionts to limit chytridiomycosis in maintenance and repopulation strategies.

Antibodies of the three main amphibian classes have been identified in the mucus of *Xenopus laevis*. When exposed to *B. dendrobatidis*, *X. laevis* have *B. dendrobatidis*-specific mucosal antibodies (Ramsey et al., 2010). How these antibodies are transported into the skin mucus is not known. In the mammalian gut, epithelial cells transcytose antibodies into the gut mucosa (Lamm, 1997). B cells producing IgX, analogous to mammalian secretory IgA, and IgM have been identified along intestinal mucosal surfaces in *X. laevis* (Mussman et al., 1996). Mucosal antibodies probably are produced by B cells present in the skin. Cutaneous antibodies may protect against *B. dendrobatidis* by neutralization of virulence factors, by potentially inhibiting zoospore adherence, or by blocking germination tube formation. Mucosal antibodies may also opsonize *B. dendrobatidis* cells and activate killing by complement (Ogundele et al., 2001). The protective role of *B. dendrobatidis*-specific mucosal antibodies remains to be determined.

B. dendrobatidis probably only spends a small portion of its lifecycle in the mucus. Greenspan et al. noted that infection of epidermal cells can occur as soon as 12 hours after inoculation with zoospores (2011). Van Rooij et al. (2011) shown even more rapid initiation of the germ tube formation at 2 hours after exposure to amphibian skin *in vitro.* In this time, zoospores must survive any onslaught by AMPs, bacterial metabolites, complement, antibodies, and any other molecules that may inhibit or kill the zoospores (Fig. 1-1). Some evidence

suggests that zoospores can move away from high concentrations of these inhibitory molecules (Lam et al., 2011), but it is likely that the presence of these defenses is important for ceasing or delaying colonization of the epithelium by *B. dendrobatidis*.

Defenses within the skin

Very little is known about how the amphibian host responds to *B. dendrobatidis* infection of epithelial cells. The first stage of infection is when the encysted zoospore invades an immature keratinocyte in the *stratum granulosum* via a germination tube that penetrates the *stratum corneum* (Van Rooij et al., 2012; Greenspan et al., 2012). *Batrachochytrium dendrobatidis* is an intracellular pathogen (Berger et al., 2005a) but does not appear to be quite like typical intracellular bacteria or viruses. Much damage occurs to the infected host cell and the *B. dendrobatidis* cell or cells can occupy a large amount of the cytoplasm (Berger et al., 1998; Berger et al., 2005). Between the germ tube puncture and any rhizoids extending out of the host cell (Pessier et al., 1999), it is possible that the host cell is necrotic having lost membrane integrity and simply provides space and nutrients for the pathogen.

Keratinocytes are capable of mounting innate responses to pathogens. Human keratinocytes are known to express PRRs and can respond to cutaneous pathogens by producing AMPs and inflammatory cytokines (Hau et al., 2011; Gallo & Nakatsuji, 2011). Some evidence suggests that a single family of AMPs and inflammatory cytokines are up-regulated in *B. dendrobatidis*-infected skin (Rosenblum et al., 2012a). Skin sloughing may be another mechanism to protect against cutaneous pathogens. In cane toads, skin sloughing decreases the amount of cutaneous bacteria (Meyer et al., 2012). Increased skin sloughing, a symptom of chytridiomycosis (Berger et al., 1998), either may be a defense mechanism to remove infected

cells from the epithelium before *B. dendrobatidis* can complete its lifecycle (Berger et al., 2005b) or could simply be a result of the damage caused by *B. dendrobatidis* infection.

All exterior surfaces of vertebrates (the gastrointestinal tract, respiratory tract, skin, ect.) have associated immune cells to maintain these barriers and prevent microbial invasion. All the major leukocyte populations present in mammals have been identified in the amphibian immunological model *X. laevis* (Robert & Ohta, 2009) and many types of leukocytes are present in amphibian skin. Skin-associated lymphatic tissue (SALT) has been described in fish and mammals and has been shown to be important in interactions with skin symbionts (Xu et al., 2013; Egawa & Kabashima, 2011). Components of SALT have been identified in amphibians as well (Carrillo-Farga et al., 1990; Castel-Rodriguez et al. 1999; Mescher et al., 2007). Thus, immune cells are normally present in amphibian skin and will infiltrate the skin when foreign antigens are detected (Fig. 1-1).

Phagocytes are an important component of immune surveillance along mucosal surfaces. Phagocytic leukocytes—dendritic cells, macrophages, and neutrophils—are important in killing fungal pathogens via phagocytosis, reactive oxygen and nitrogen production, release of antimicrobial peptides and enzymes, and nutritional immunity (Brown, 2011). Phagocytes are likely the first leukocyte responders in the skin responding to a foreign allograft. In *X. laevis*, skin allografts are infiltrated by MHC class II-expressing cells, many of which are probably phagocytes, likely macrophages, Langerhans cells, or dendritic cells (Ramanayake et al., 2007). During chytridiomycosis, phagocytes likely play a role in recognition, immune activation, and antigen presentation after recognizing PAMPs from *B. dendrobatidis* or after engulfing dead cells infected with *B. dendrobatidis*. Macrophages are likely important in immunity to *B. dendrobatidis*. Macrophages, with T cell assistance, can activate protective killing mechanisms

shown to be important during fungal pathogenesis (Romani, 2011). Macrophages are an important component of the amphibian immune system important for pathogen clearance (Chen & Robert, 2011), and are also likely important in immunity to chytridiomycosis. Amphibian macrophages engulf *B. dendrobatidis* cells *in vitro* (Chapter III) and may play a similar role *in vivo* to kill *B. dendrobatidis* cells and activate immune responses.

Dendritic cells (DCs) are sentinel cells that both detect pathogens and activate adaptive immunity (Banchereau & Steinman, 1998). Langerhans cells (LCs) are a type of DC present in the skin and play a major role in skin immunity (Igyáró & Kaplan, 2013). DCs and LCs are important for recognition and presentation of fungal antigens to T cells to promote antifungal immunity in mammalian systems (Roy & Klein, 2012; Wüthrich et al., 2012a). Both DCs and LCs have been identified in histological studies of *Xenopus* and *Rana* skin (Carrillo-Farga et al., 1990; Castel-Rodriguez et al. 1999; Mescher et al., 2007). DCs are likely to be important for recognition of *B. dendrobatidis* infections, but their role has not yet been studied.

Neutrophils are important mediators of pathogen killing, especially during fungal infections. Along with typical phagocytic killing mechanisms, neutrophils can also kill fungi through extracellular chromatin traps (neutrophil extracellular traps) (Brinkmann et al., 2004; Brinkman and Zychlinsky, 2007). Neutrophils promote epithelial barrier maintenance by producing IL-22 which promotes AMP production by epithelial cells in mammals (Zindl, et al., 2013). T helper 17 (Th17) responses typically are protective against fungal pathogens, and neutrophil recruitment is an important component of the Th17 response (Wüthrich et al., 2012a). Much less is known, but neutrophils are also important in the immune responses of amphibians (Robert & Ohta, 2009). Although little is known about what the role neutrophils play in chytridiomycosis, amphibian neutrophils can engulf *B. dendrobatidis* cells *in vitro* (Chapter III).

T cells are important in regulating and coordinating immunity in the skin. Humans have about 20 billion T cells in the skin, substantially more than the number in blood circulation (Egawa & Kabashima, 2011). T cells are also present in amphibian skin. T cells expressing the γδ T cell receptor, important for mucosal immunity (Ismail et al., 2011), have been identified in *X. laevis* skin (Mescher et al., 2007). Cytotoxic ($CD8^+$) T cells infiltrate *X. laevis* skin allografts to reject the foreign tissue (Ramanayake et al., 2007). CD4, the marker for helper T cells, is well conserved in structure and function between amphibians and mammals (Chida et al., 2011); however, because no amphibian CD4-specific antibodies are available (Robert & Cohen, 2011), the presence of helper T cells in amphibian skin is still hypothetical. Invariant T cells have recently been described in *X. laevis*; these cells have been proposed to recognize MHC class Ib and be important for larval immunity against viruses (Edholm et al., 2013). Clearance of *B. dendrobatidis* from amphibian skin would be expected to require robust T cell responses. Th1 and Th17 responses, although not fully characterized in amphibians, are typically protective against fungal pathogens (Romani, 2011; Wüthrich et al., 2012a). The important cytokines for these responses appear to be conserved among vertebrates (Kaiser et al., 2004; Qi & Nie, 2008; Savan et al., 2009). Cytotoxic T cells may also contribute to protection against chytridiomycosis, especially if these cells are capable of killing *B. dendrobatidis*-infected epithelial cells. Successful strategies attempting to induce immunity and protection from *B. dendrobatidis* infection will probably require activation of T cell-mediated responses.

Amphibian B cells produce antibodies of three main immunoglobulin classes: IgM, IgY (IgG homologue) and IgX (IgA analogue) and two other recently identified classes of IgD and IgF (Robert & Ohta, 2009). Amphibians have many Fc receptors to recognize these antibodies that have targeted pathogens for destruction (Guselnikov et al., 2003). The presence of B cells in

the skin of amphibians has not been determined, but populations of B cells have been identified in the skin of fish (Xu et al., 2013) and mammals (Geherin et al., 2012). IgX-producing plasma cells are present in the gut of *X. laevis* (Mussman et al., 1996), and similar cutaneous B cells probably are secreting IgM, IgY, and IgX due to the presence of these antibodies in the skin mucus (Ramsey et al., 2010).

Natural killer (NK) cells have been described in *X. laevis* (Horton et al., 2000). Although NK cells have not been identified in the skin, they have been identified in amphibian intestinal epithelia (Horton et al., 2000). NK cells are functionally conserved between amphibians and mammals releasing interferon γ (IFN-γ) and cytotoxic mediators to promote immune response and kill tumorigenic or infected cells (Robert & Ohta, 2009; Chen & Robert, 2011). NK cells have not been identified in the skin of amphibians, but putatively may act to kill *B. dendrobatidis*-infected cells or augment a Th1-type response by producing IFN-γ.

Granulocytes play an important role in initiating and promoting immune responses in all vertebrates. Granulocytes are innate immune cells with granules that can release immune activators, like histamine, or pathogen-killing molecules (Rothenberg & Hogan, 2006; Perrigoue et al., 2009; Urb & Sheppard, 2012). Mast cells are granulocytes that occupy barrier tissues like skin and gut mucosal epithelia and typically act as alarms for immune activation. Although poorly understood, mast cells can recognize fungi via PRRs and potentially coordinate a response against fungal pathogens (Urb & Sheppard, 2012). In amphibians, mast cells have been found in many tissues including along the gastrointestinal tract (Baccari et al., 1998). Basophils and eosionophils are other types of granulocytes important for anti-parasitic defense and allergic responses (Rothenberg & Hogan, 2006; Perrgoue et al., 2009). Basophils may counteract an immune response to fungi due to their involvement in Th2 responses (Perrgoue et al., 2009;

Yoshimoto et al., 2009) which typically are not protective against fungal infections (Szymczak & Deepe, 2009; Wüthrich et al., 2012a). Basophils and eosinophils have been identified in the blood and peritoneum of *X. laevis* (Hadji-Azimi et al., 1990; Robert & Ohta, 2009). The presence of granulocytes in amphibian skin is unknown, but it is possible that such cells may help initiate or mediate a response against *B. dendrobatidis*.

Immune responses against *B. dendrobatidis* in the skin

Several studies have investigated the cutaneous immune responses in the skin of *B. dendrobatidis*-infected frogs (Table 1-1). Early histology studies of chytridiomycosis noted a mild infiltration of leukocytes into infected skin (Pessier et al., 1999; Berger et al., 2005b). Later microarray studies of *B. dendrobatidis*-infected skin noted very little up-regulation of cytokines and immune related genes especially those related to a robust lymphocyte-mediate response (Rosenblum et al., 2009; Rosenblum et al., 2012a). These studies provide insights into some of the skin responses, but changes in immune cell expression would probably be indistinguishable over the noise from transcripts of other epidermal and dermal cells. Some attempts have been made to immunize frogs to protect them from subsequent infections with *B. dendrobatidis*. An immunization protocol that induced strong antibody responses in *X. laevis* was unable to protect boreal toads from lethal infections (Rollins-Smith et al., 2009). Immunization of mountain yellow legged frogs with formalin-fixed *B. dendrobatidis* also failed to protect this species from a subsequent *B. dendrobatidis* infection (Stice & Briggs, 2010). The failure to immunize and induce a protective response in amphibians against *B. dendrobatidis* results from an incomplete understanding of the pathogenesis of *B. dendrobatidis* and amphibian immune responses. Immunization protocols using killed fungi have been mostly unsuccessful in mammals (Cassone

Table 1-1. Evidence for and against the capability of the amphibian immune system to clear *B. dendrobatidis* infection. Likely, the response is multifactorial where low infection loads that are maintained by innate immune defenses will lead to clearance or at least survival. When *B. dendrobatidis* loads are high, virulence factors may inhibit lymphocyte-mediated responses preventing robust immune responses against *B. dendrobatidis*.

No Clearance	Clearance
Very little leukocyte infiltration in the skin of infected individuals (Pessier et al., 1999; Berger et al., 2005b).	Temporarily knocking out adaptive immunity with X-irradiation prior to infection increases *B. dendrobatidis* load (Ramsey et al., 2010).
Prior exposure and clearance in some instances is not protective (Cashins et al., 2013) and immunization attempts have not been protective (Rollins-Smith et al., 2009; Stice & Briggs, 2010)	Instances where previous exposure to *B. dendrobatidis* with clearance aided in later resistance (Richmond et al., 2009; Bishop et al., 2009; McMahon et al., submitted).
Little transcriptional evidence of an immune response (Rosenblum et al., 2009; Rosenblum et al., 2012a)	MHC class II alleles correlated with survival of one species (Savage & Zamudio, 2011).
Lymphocytes from *B. dendrobatidis*-immunized frogs do not respond to the fungus *in vitro* (Rollins-Smith et al., 2009).	Mucosal antibodies are produced by individuals exposed to *B. dendrobatidis* (Ramsey et al., 2010).
Difficult to clear infection without treatment, even in resistant species (Mazzoni et al., 2003; Ramsey et al., 2010)	*B. dendrobatidis* does not appear to inhibit phagocyte functions (Chapter III of this thesis).
B. dendrobatidis inhibits lymphocytes responses (Chapter II of this thesis).	

& Casadevall, 2012; Wüthrich et al., 2013). Similar immunizations using killed *B. dendrobatidis* will likely fail. Development of a systemic antibody response to *B. dendrobatidis* may not be protective unless defenses are activated in the skin.

Despite the fairly consistent observation of very limited immune responses during chytridiomycosis, several studies do suggest that adaptive immune responses are present and can be important in survival and decreasing pathogen burden. Temporarily impairing lymphocyte populations with sub-lethal X-irradiation in *X. laevis* greatly increased *B. dendrobatidis* infection loads (Ramsey et al., 2010). Frogs that have survived *B. dendrobatidis* infection can mount better immune responses likely mediated by adaptive immunity (Richmond et al., 2009; McMahon et al., submitted). *X. laevis* previously exposed to *B. dendrobatidis* produce more *B. dendrobatidis*-specific mucosal antibodies than unexposed controls suggesting that lymphocyte responses may mediate protection (Ramsey et al., 2010). Also MHC class II alleles, important for antigen presentation to T cells, have been correlated with surviving chytridiomycosis (Savage and Zamudio, 2011).

Although amphibians appear to be capable of mounting adaptive immune responses against *B. dendrobatidis*, infection continues to persist even in highly resistant species such as *X. laevis* and *Rana catesbeiana* (Ramsey et al., 2010; Mazzoni et al., 2003). Studies investigating whether prior *B. dendrobatidis* exposure and clearance provides protection from chytridiomycosis show mixed results (Table 1-1) (Bishop et al., 2009; Cashins, et al., 2013). The best hypothesis to explain why amphibians can only mount a minimal or inconsistent response is that *B. dendrobatidis* is able to inhibit development of an effective immune response. *Batrachochytrium dendrobatidis* appears to inhibit adaptive immune responses by inducing apoptosis in lymphocytes (Chapter II). Interestingly, *B. dendrobatidis* does not appear to impair

phagocyte activities suggesting that by targeting lymphocyte responses, *B. dendrobatidis* is able to persist in the skin of its hosts. *B. dendrobatidis* likely evades detection by phagocytes in the skin by being an intracellular pathogen (Berger et al., 2005a) subtly injecting itself into epidermal cells via a germ tube (Van Rooij et al., 2012; Greenspan et al., 2012). Responses against an intracellular fungus typically require T cell responses in order to clear the infection (Romani, 2011; Wüthrich et al., 2012a). Occupying an epithelial cell may be sufficient to evade some detection and destruction by phagocytes but would not be protective once the adaptive arm was activated; therefore, inhibiting lymphocytes is quite necessary for *B. dendrobatidis* to continue colonization of the skin epithelium.

IV. Vertebrate Immune Responses against Fungi

The best studies of immune responses against fungal diseases have been those directed at human mycoses and using murine models of fungal diseases. The conservation of pathogen recognition systems and immune responses among vertebrates allows for speculation about how the amphibian immune system might respond to chytridiomycosis based on what is known about how mice and humans respond to fungal pathogens. Each fungal disease seems to vary in the way it is manifested and the way the immune system responds and clears the fungal burden. Of the well-studied fungal diseases, the majority are caused by higher fungi in the Ascomycota and Basidiomycota phyla (James et al., 2006). *Batrachochytrium dendrobatidis*, as a chytrid fungus, is very distantly related to the fungi known to cause disease in humans. *Batrachochytrium* species are the only known members of the Chytridiomycota to infect vertebrates, but other chytrids are known to be pathogens of plants and arthropods (Gleason et al., 2008; Martel et al., 2013). The way a vertebrate immune system recognizes and responds to a chytrid fungus is

likely to be very different from a response to higher fungi. Another major difference between the response of mammalian species and those of amphibians is that amphibians are "cold-blooded", and body temperature fluctuate with environmental temperature, but generally are well below 37 °C. Thus, differences between immunity to chytridiomycosis and immunity to human mycoses are likely due to differences in the fungal pathogen, and differences in the temperature-limited host response. Despite these differences, the similarities in fungal pathogenesis and common features of all vertebrate immune defenses may provide some new insight into host-pathogen interactions during chytridiomycosis.

Fungal diseases in humans

The occurrence of fungal disease in humans is on the rise due to an increased number of immunocompromised persons and invasive therapies (Cassone & Casadevall, 2012). Also the threat of endemic fungal disease may be increasing due to climatic shifts causing ambient temperatures to approach the human body temperature (Garcia-Solache & Casadevall, 2010). The most common fungal pathogen of humans is *Candida*, most typically *C. albicans*, typically causing mucocutaneous or vulvovaginal candidiasis (Cassone & Casadevall, 2012). Candidemia is a common hospital-acquired infection and has become the second most common infection in premature infant deaths and the fourth most common bloodstream infection in hospital patients (Spellberg, 2011; Roy & Klein, 2012). *Candida* typically exists as a human commensal in the yeast-like form, but becomes pathogenic in a transition to hyphae or pseudohyphae as it invades host tissue (Romani, 2004; Filler & Sheppard, 2006). *Aspergillus* species are the next most important fungal pathogen of humans followed by *Cryptococcus* species and endemic mycoses such as *Histoplasma capsulatum*, *Coccidioides immitus*, *Blastomyces dermatitidis*, and

Paracoccidioides brasiliensis (Spellberg, 2011; Romani, 2004; Romani, 2011). Other frequent fungal pathogens, *Malasezia* species, are human skin commensals that can cause skin infections (Romani, 2011). Typically individuals who are immunocompromised develop severe mycoses; the exception being candidiasis where only a small percentage of *Candida* infections can be directly linked with immune deficiencies (Spellberg, 2011; Iannitti et al., 2012).

Fungal recognition and immune activation

Studies of human mycoses using murine models suggest the use of certain common PRRs and activation signals in responses to several fungal diseases (Brown, 2011; Romani, 2011; Wüthrich et al., 2012a). Variations in host responses may result from evasion strategies employed by the pathogen. Common themes are also noted in immune evasion strategies among fungal pathogens (Table 1-2). In general, inflammatory immune responses are typically protective against fungal pathogens; however, these inflammatory responses can lead to immunopathology (Romani, 2011; Wüthrich et al., 2012a). In fungal disease, an in all infectious disease, a delicate balance exists such that the immune response must be strong enough to remove the pathogen without causing too much damage to the host.

Immune recognition of fungi typically occurs via PRRs on or inside of host cells, usually epithelial or phagocytic cells. Most fungal PAMPs are located in the fungal cell wall. The fungal cell walls are structurally supported by a fibrillar structure typically composed of chitin at the base, an intermediate layer of β-1,3-glucans with differing β-1,6-branching, and a superficial layer of mannosylated proteins (Yin et al., 2005; Latgé et al., 2010; Hardison & Brown, 2012). Many fungi also have a layer superficial to this fibrillar cell wall. *Cryptococcus* fungi form polysaccharide capsules mainly composed of glucuronoxylomannan (GXM) and

Table 1-2: Immune evasion strategies employed by fungi to suppress vertebrate immune defenses (1/4).

Evasion Strategy	Pathogen	Mechanism of Evasion	Reference
Preventing detection of PAMPs	*B. dendrobatidis*	Appears to lack β-1,3- or β-1,6-glucan in its cell wall (although dectin-1 homologue yet to be identified in amphibians or fish).	Ruiz-Herra & Ortiz-Castellanos, 2010; Aoki et al., 2008
	Candida albicans	Hyphal β-glucan in cell wall is masked preventing detection by dectin-1.	Gantner et al., 2005
	Aspergillus fumigatus	Galactosaminogalactan on hyphae mask β-glucan exposure.	Gravelat et al., 2013
	A. fumigatus	Hydrophobin on conidia mask cell wall PAMPs especially β-glucan.	Aimanianda et al., 2009
	Cryptococcus neoformans	Produces a capsule which covers cell-wall PAMPs (capsule components are also PAMPs but tend to not be as immunogenic)	Zaragoza et al., 2009
	Histoplasma capsulatum, *Paracoccidioides* species	α-1,3-glucan in the cell wall masks β-1,3-glucan in cell wall.	Rappleye et al., 2007; Puccia et al., 2011
	Fonsecaea pedrosoi	Infection does not activate TLR signaling likely due to the lack of TLR-agonist PAMPs.	Sousa et al., 2011.
	Pneumocystis carinii	Glycoproteins on surface block mannose receptor recognition.	Pop et al., 2006
Inhibition of phagocyte killing	*Candida* species	Catalase, superoxide dismutase, thioredoxin proteins, glutaredoxin proteins.	Brown et al., 2009
	C. neoformans	Have superoxide dismutase, thioredoxin proteins and up-regulate anti-oxidants, like melanin, to protect against ROS and RNS.	Brown et al., 2009; Coelho et al., 2013
	C. neoformans	Capsule protects against ROS and RNS.	Zaragoza et al., 2009
	H. capsulatum	Catalases and superoxide dismutase target ROS. Flavoproteins resist RNS.	Holbrook et al., 2013; Subramanian Vignesh et al., 2013
	Blastomyces dermatitidis	Yeasts inhibit inducible nitric oxide synthase activity in alveolar macrophages.	Rocco et al., 2011

Table 1-2 Continued: Fungal immune evasion strategies (2/4).

Evasion Strategy	Pathogen	Mechanism of Evasion	Reference
Evasion of phagocytosis or escape from phagocytes	*B. dendrobatidis*	Infects keratinocytes from the surface via germ-tubes potentially evading phagocyte recognition.	Van Rooij et al., 2012; Greenspan et al., 2012
	C. albicans, *A. fumigatus*, *C. neoformans*, *H. capsulatum*, *Paracoccidioides brasiliensis*, *Sporothriz schenckii*, *Rhizopus oryzae*	Can invade epithelial or endothelial cells where phagocytic recognition would not occur.	Filler & Sheppard, 2006
	C. albicans	Induces the cycling of phagosomal maturation markers out of the phagosome.	Fernandez-Arenas, 2009
	C. albicans, *A. fumigatus*	Growing hyphae can break out of phagosomes (hyphal growth is inhibited by neutrophils).	Brown, 2011
	H. capsulatum	Inhibits phagosome-lysosome fushion.	Woods, 2003
	C. neoformans	Large cell phenotype (up to 100 μm in diameter) prevents phagocyte engulfment.	Zaragoza & Nielsen, 2013
	Cryptococcus species	Produce a polysaccharide capsule to prevent phagocytosis.	Zaragoza et al., 2009
	C. neoformans	Phagosomes in infected macrophages tend to be 'leaky.'	Tucker & Casadevall, 2002
	C. neoformans	Nonlytic exocytosis from phagocytes.	Coelho et al., 2013
Destruction/ evasion of chemical defenses	*B. dendrobatidis*	Genome expansion in proteases. Some of these proteases have been shown to degrade amphibian antimicrobial peptides.	Joneson et al., 2011; Thekkiniath et al., 2013
	B. dendrobatidis	Zoospores evade antifungal metabolites in mucus.	Lam et al., 2011.
	Candida species	Secrete a variety of proteases and other enzymes linked with virulence including degradation of complement and antimicrobial peptides	Garcia-Vidal, et al., 2013; Speth et al., 2008; Meiller et al., 2009
	Candida species	Produce regulatory proteins that inhibit complement activation	Speth et al., 2008; Luo et al., 2009
	Aspergillus species	Secretes proteases that degrade complement.	Rambach et al., 2010

Table 1-2 Continued: Fungal immune evasion strategies (3/4).

Evasion Strategy	Pathogen	Mechanism of Evasion	Reference
Decreasing inflammatory responses	*A. fumigatus*	Gliotoxin inhibits activation of NF-κB.	Ben-Ami et al., 2010
	C. albicans, *A. fumigatus*	Hyphae promote a Th2 (less inflammatory) response. However, this may be an appropriate response to filamentous fungi.	d'Ostiani et al., 2000; Netea et al., 2003; Chai et al., 2011
	C. albicans	Modification of tryptophan metabolism by virulence factors inhibits IL-17 production.	Cheng et al., 2010
	C. albicans	Natural tolerance to commensal yeast could reduce responses to invasive hyphae or pseudohyphae.	Romani, 2011
	C. neoformans	Capsular polysaccharide GXM induces production of IL-10 and TGF-β while decreasing TNF-α, IL-1 and IL-12 and generally inhibits leukocyte infiltration.	Dong & Murphy, 1995; Vecchiarelli & Monari, 2012
	B. dermatitidis	BAD-1 on surface activates signal in macrophages to decrease TNF-α production.	Finkel-Jimenez et al., 2002.
	B. dermatitidis	Chemokines are impaired preventing recruitment of inflammatory monocytes.	Wüthrich et al., 2012b.
	P. brasiliensis	Soluble factors drive IL-4 production to dampen inflammatory response.	Cavassani et al., 2011
Induction of phagocyte apoptosis	*A. fumigatus*	Galactosaminogalactan on hyphae induces apoptosis of neutrophils and dendritic cells	Fontaine et al., 2011; Lee et al., 2013
	A. fumigatus	Gliotoxin has toxic effects on phagocytes.	Ben-Ami et al., 2010.
	C. neoformans	Capsular polysaccharide GXM induces phagocyte apoptosis.	Villena et al., 2008
Evasion of nutritional immunity	*A. fumigatus*, *H. capsulatum* *B. dermatitidis*	Secrete siderophores to scavenge metal nutrients (mainly iron).	Kornitzer, 2009; Gauthier et al., 2010
	C. albicans, *C. neoformans*	Can obtain iron from host iron binding proteins (hemoglobin, ferritin, transferrin)	Kornitzer, 2009

Table 1-2 Continued: Fungal immune evasion strategies (4/4).

Evasion Strategy	Pathogen	Mechanism of Evasion	Reference
Impairment of lymphocytes	*B. dendrobatidis*	Soluble molecules induce apoptosis in lymphocytes.	Chapter II this thesis.
	C. neoformans	Capsule polysaccharide GXM induces FasL expression on macrophages and dendritic cells.	Monari et al., 2005; Piccioni et al., 2011
	C. neoformans	Capsule polysaccharide GalXM directly induces apoptosis in lymphocytes.	De Jesus et al., 2009 Pericolini et al., 2009
	B. dermatitidis	BAD-1 (adherence factor) inhibits T-cell activation through interactions with CD47	Brandhorst et al., 2013.
	A. fumigatus	Produces gliotoxin which activates apoptosis in lymphocytes.	Fox & Howlett, 2008; Ben-Ami et al., 2010
	P. brasiliensis	Produces prostaglandins suspected to suppress lymphocyte immunity in paracoccidiomycosis.	Campanelli et al., 2003; Bordon et al., 2007

galactoxylomannan (GalXM) (De Jesus et al., 2010). *Aspergillus* species produce exopolysaccharides on the cell wall surface such as galactomannan, α-1,3-glucan, and galactosaminogalactan (Latgé, 2010). The glucans and mannans of fungal cell walls are typically recognized by PRRs to activate downstream immune responses (reviewed by Brown, 2011). C-type lectin receptors (CLRs) are very important for recognizing carbohydrate structures of fungal cell walls. CLRs involved in fungal recognition include Dectin-1 which recognizes β-1,3-glucan, Dectin-2 which recognizes α-mannan, the mannose receptor (MR) which recognize mannans, Mincle which recognizes mannose, and DC-SIGN which recognizes mannans (Hardison & Brown, 2012).

Toll-like receptors (TLRs) are also important for recognizing fungal PAMPs. Several TLRs recognize mannan structures present on fungal surfaces. TLR2 binds to phospholipomannan and GXM ligands, and TLR4 recognizes O-linked mannans and GXM as well (Brown, 2011). TLRs 1 and 6 are also important in fungal recognition and often form heterodimers with TLR2 (Garcia-Vidal et al., 2013). PAMPs outside of the fungal cell wall can also be detected by PRRs. For example, TLR9 has been shown to recognize DNA from *Aspergillus fumigatus* and *C. albicans* but may be dispensable for protection (Ramirez-Ortiz et al., 2008; van de Veerdonk, 2008). TLR3 and TLR7, which typically recognize foreign RNA, also appear to be important for detection of fungal pathogens (LeibundGut-Landmann et al., 2012; Garcia-Vidal et al., 2013).

Chitin is an important component of the fungal cell wall, yet a PRR for chitin has not been determined (Hardison & Brown, 2012). Chitin elicits an allergic response promoting C3a and CCL2 production in the lungs of mice (Roy et al., 2012; Roy et al., 2013). TLR2 and Dectin-1 appear to play a role in recognition of chitin (Da Silva et al., 2008; Da Silva et al., 2009). Other

putative receptors for chitin are FIBCD1 (fibrinogen C domain-containing 1) and galectins because the ligands for these receptors are acetylated structures such as N-acetylglucosamine, the repeating component of chitin (Roy & Klein, 2013). The down-stream activation of immune responses are greatly dependent on the size of chitin polymers. Very large chitin polymers have no immune effect; intermediate fragments activate inflammatory cytokine production; and small fragments promote anti-inflammatory responses (Da Silva et al., 2008; Da Silva et al., 2009). The fragmentation of chitin may serve as monitoring system for the immune system to determine whether or not fungal pathogens have been destroyed (Lee et al., 2008).

Once a fungus is recognized by PRRs, signaling induces cytokine production to promote innate responses and cue adaptive immune responses to promote pathogen clearance. Dectin-1 activation typically activates protective immune responses by inducing production of tumor necrosis factor α (TNFα) and pathogen-killing by phagocytes (Goodridge et al., 2009; Kerrigan & Brown, 2010). Dectin-1 signaling also up-regulates production of IL-1β, IL-12, IL-6, and IL-23, which promote protective inflammatory Th1 and Th17 responses (Wüthrich et al., 2012a). Many fungal pathogens evade Dectin-1 detection by masking β-glucans on their surface (Table 1-2). There is some variability in the role of Dectin-1 in resistance to fungal infection. For example, vaccine protection by Th17 in *B. dermatitidis* and similar endemic fungi is independent of Dectin-1 signaling (Wüthrich et al., 2011), and the protection given by Dectin-1 varies greatly among *C. albicans* strains (Marakalala et al., 2013). The role of Dectin-1 in protection is likely to vary depending on fungal cell wall composition and architecture and whether or not other PRR signals can convey protection independent of Dectin-1.

CLRs other than Dectin-1 have not been investigated as thoroughly, but they are important for activating protective immunity against fungi. Mincle and Dectin-2 bind to

mannose-containing PAMPs and require FcRγ for intracellular signaling (Hardison & Brown, 2012). Both Mincle and Dectin-2 have been shown to promote immune responses against *C. albicans* in mice (Wells et al., 2008; Saijo et al., 2010). Dectin-2 is also important in activating protective inflammation during *Candida glabrata* infection (Ifrim et al., 2013). The MR is another important CLR recognizing terminal mannose in fungal PAMPs that activates production of inflammatory cytokines (Hardison & Brown, 2012; Wüthrich et al., 2012a). The MR is important for inducing Th17 responses to *C. albicans* in human lymphocytes (van de Veerdonk et al., 2009).

TLR signaling plays an important role in activation of immune responses against fungal pathogens. Deficiency in MyD88, the down-stream adaptor protein for most TLRs, greatly increases fungal burden in mice, but it does not appear to factor into human fungal disease (Bellocchio et al., 2004; Brown, 2011). Single nucleotide polymorphisms in human TLRs 1, 4, 6 and 9 are linked with increased susceptibility to aspergillosis, and polymorphisms in TLRs 1 and 4 are linked with increased susceptibility to candidiasis (Romani, 2011; LeibundGut-Landmann et al., 2012). The majority of studies investigating the role of TLRs in mouse models of fungal disease have focused on TLR2, TLR4, and TLR9. These TLRs are important in Th1 immunity, but it is not well understood what role these or other TLRs play in activating or repressing Th17 responses (Bellocchio et al., 2004; Wüthrich et al., 2012a). Both TLR2 and TLR4 recognize mannan structures but have different ligands, typically phospholipomannan and O-linked mannans, respectively (Brown, 2011). TLR2 appears to have a contradictory role in inflammatory responses against fungi. In response to fungi, TLR2 can induce TNFα and IL-10; thus, TLR2 can either promote or dampen inflammatory responses (Bellocchio et al., 2004; Netea et al., 2004). TLR2 can promote regulatory T cell responses, which in some instances may

decrease resistance and in other cases may convey protection against immunopathology in fungal disease (Netea et al., 2004; Sutmuller et al., 2006). TLR4 has a more inflammatory and protective role in fungal diseases. TLR4 is important for resistance in mouse models of *C. albicans* and *A. fumigatus* (Bellocchio et al., 2004). Fungal PAMP activation of TLR4 induces production of TNFα (Tada et al., 2004) and appears to be necessary for Th1 immunity against these fungi (Bellocchio et al., 2004; Wüthrich et al., 2012a). TLR9 likely activates immune responses by recognizing fungal DNA. *A. fumigatus*, *Cryptococcus neoformans*, *C. albicans*, and *Saccharomyces cerevisieae* DNA activate TLR9 *in vitro* (Ramirez-Ortiz et al., 2008 Nakamura et al., 2008; Kasperkovitz et al., 2011). Although, fungal DNA promotes inflammatory responses via TLR9, TLR9 deficient mice do not have reduced resistance to *A. fumigatus* or *C. albicans* (Bellocchio et al., 2004). Differential TLR activation can be a mechanism used by the pathogen to evade immunity or by the host to differentiate different fungal forms. *A. fumigatus* and *C. albicans* have more surface expression of TLR2 ligands in the pathogenic filamentous form and more TLR4 ligands on the surface of conidia and yeast (d'Ostiani et al., 2000; Netea et al., 2003). TLR signaling is essential for activating immune responses against fungi, but much is still unknown about how TLR signaling promotes antifungal responses and how a combined signal from multiple PRRs cues downstream cytokine expression across fungal diseases.

Cell populations involved in fungal responses

Activation of immune responses is typically initiated by resident phagocytes or epithelial cells in infected tissue. However, other innate immune cells can detect fungi and activate immune responses. Induced natural killer T (iNKT) cells are activated by fungi and promote

immune responses through the production of IFN-γ. The glycolipids α-galactosylceramide and asperamide B from *C. neoformans* and *A. fumigatus*, respectively, are known to be recognized by iNKT cells to activate immune responses (Kawakami et al., 2001; Albacker et al., 2013). Antigen presenting cells (APCs) exposed to fungal β-glucan can also activate iNKT cells to produce IFN-γ (Cohen et al., 2011). Mast cells also may play a role in fungal detection (Urb & Sheppard, 2012). Dectin-1 activation on mast cells induces production of leukotriene C_4, an eicosanoid inflammatory mediator (Olynych et al., 2006), and *A. fumigatus* induces mast cell degranulation *in vitro* (Urb et al., 2009).

Activation of adaptive immunity against fungi

Activation of PRRs on APCs, particularly dendritic cells (DCs), promote T cell responses. Resident DCs, such as Langerhans cells (LCs) in the skin, recognize fungal PAMPs. After phagocytosis of fungal cells, DCs are activated and begin to move toward secondary lymphoid organs, typically lymph nodes (Roy & Klein, 2012). In the lymph node, DCs bring fungal antigens and present them to T cells. During presentation, signals from DCs polarize $CD4^+$ T cells to activate different Th responses. DCs can also present antigen to $CD8^+$ T cells to promote cytotoxic T lymphocyte (CTL) responses.

DCs can prime diverse T cell responses depending on their origin and which PRR signals have been activated in the DC. Activation of Dectin-1, Dectin-2, MR, TLR4 and TLR9 typically promote the production of Th1 or Th17 polarizing cytokines to promote inflammatory responses (Brown, 2011; Wüthrich et al., 2012a). In a model of cutaneous candidiasis, different DC subsets activate different T cell responses. For example, LCs prime Th17 but not Th1 or CTL responses,

and dermal DCs (Langerin^{+} subset) instead promote Th1 and CTL responses producing very little IL-6 and IL-23 that cue Th17 polarization (Igyártó et al., 2011).

Inflammatory monocytes, typically classified by high expression of Ly6C, are very important in immune responses against many fungi (Ersland et al., 2010; Wüthrich et al., 2012a). These cells express CCR2 and are recruited by chemokines CCL2 and CCL7 to sites of infection to promote inflammatory responses of T cells (Tsou et al., 2007; Jia et al., 2008). Loss of CCR2 by deficiency or fungal immunomodulation greatly impairs protective immune responses to fungal pathogens in the lungs (Osterholzer et al., 2008; Szymczak & Deepe, 2009; Wüthrich et al., 2012b). CCR2-deficient mice tend to have greater Th2 responses and decreased Th1 responses to fungal pathogens in the lungs suggesting that inflammatory monocytes are important in promoting Th1 activation (Hohl et al., 2009; Szymczak & Deepe, 2010). Inflammatory monocytes are important in the skin as well, but the activities of inflammatory monocyte-derived DCs can be replaced by other DCs in the skin of mouse infection models (Ersland et al., 2010).

Lymphocytes responses mediating protection

The most important determinant of protection from fungal pathogens is thought to be the activation of Th1-type immunity, which is characterized mainly by the production of IFNγ by T cells (Romani, 2011; LeibundGut-Landmann et al., 2012; Wüthrich et al., 2012a). Defects in Th1 cells or production of IL-12 or IFNγ increase susceptibility to fungal disease (Brown, 2011). Th1 cytokines activate phagocytes to promote pathogen killing. IFNγ is an important inducer of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that kill pathogens. Th1-type responses also promote “nutritional immunity”. For example, granulocyte macrophage-colony

stimulating factor (GM-CSF) activated during a Th1 response promotes zinc sequestration that can, in turn, inhibit a pathogen's defenses to ROS and RNS (Subramanian Vignesh et al., 2013). IFNγ promoting Th1-like responses can also come from $CD8^+$ T cells and from iNKT cells (Wüthrich et al., 2003; Lin et al., 2005; Cohen et al., 2011). Infiltration of inflammatory monocytes is an important determinant in promoting Th1-mediated fungal resistance particularly in the lungs (Blease et al., 2000; Szymczak & Deepe, 2009; Hohl et al., 2009; Ersland et al., 2010). CCR2 is necessary to recruit inflammatory monocytes to sites of infection and prime Th1; mice deficient in CCR2 are more susceptible to pulmonary change by fungal pathogens. Absence of Th1-promoting DCs, especially inflammatory monocytes, tends to allow for more Th2 responses and decrease resistance to fungi (Szymczak & Deepe, 2009; Hohl et al., 2009; Wüthrich et al., 2012b).

Th17 cells, mainly producing IL-17A, IL-17F and IL-22, are important in resistance to multiple fungal pathogens (LeibundGut-Landmann et al., 2012). IL-17A and IL-17F recruit and activate neutrophils and other phagocytic leukocytes to mediate fungal killing (Romani, 2011). For mouse models of *C. albicans*, *C. neoformans*, *B. dermatitidis*, *C. immitis*, *H. capsulatum*, and *Pneumocystis carinii* infection, IL-17 production and signaling are necessary for resistance (Conti et al., 2009; Wozniak et al., 2011; Wüthrich et al., 2011; Rudner et al., 2007). However, there are instances of fungal disease in which induction of Th17 is not protective (Zelante et al., 2007). In humans, deficiencies in receptors, signaling pathways, and cytokines involved in Th17-type immunity are linked with susceptibility to candidiasis, but not necessarily other fungal infections (Romani, 2011; Lilic, 2012; LeibundGut-Landmann et al., 2012). IL-17 canonically is produced by effector $CD4^+$ T cells but can be produced by myeloid cells, γδ T cells, $CD8^+$ T cells, and innate lymphocytes (LeibundGut-Landmann et al., 2012). In mice with helper T cell

defects, $CD8^+$ T cells can produce IL-17, promoting resistance to *H. capsulatum* and *B. dermatitidis* (Nanjappa et al., 2012). Innate lymphoid cells (ILCs) also play an important role in IL-17-mediated protection against fungi. For example, in a mouse model of oropharyngeal candidiasis, ILCs have been shown to be the main producers of IL-17 (Gladiator et al., 2012). In type-17 immune responses, IL-22 from T cells or neutrophils activates AMP production by leukocytes and epithelial cells to promote epithelial barrier function (Wüthrich et al., 2012a, Zindl, et al., 2013). IL-17 can also be inflammatory, often causing immunopathology, but in the instance of fungal infection, this inflammation tends to limit fungal growth and promote disease resolution.

Unlike Th1 and Th-17 immune responses, Th2 responses are not generally protective against fungal pathogens. Th2 cytokines, IL-4, IL-13, and IL-33, promote alternative activation of macrophages and antagonize Th1-type responses typically decreasing resistance to fungi (Szymczak & Deepe, 2009; Wüthrich et al., 2012a). Th2 responses can, however, promote resistance to *Pneumocystis murina*, potentially by driving production of protective antibodies to this fungus (Nelson et al., 2011). Alternative activation of macrophages down-regulates antimicrobials and nutritional sequestration necessary for fungal killing (Szymczak & Deepe, 2009; Subramanian Vignesh et al., 2013). Th2 responses typically impair Th1 responses and decrease resistance in *C. albicans* (Cenci et al., 1993), *A. fumigatus* (Cenci et al., 1999), *B. dermatitidis* (Wüthrich et al., 2007; Wüthrich et al., 2012), *H. capsulatum* (Szymczak & Deepe, 2009), and *C. neoformans* (Jain et al., 2009) infection models.

Regulatory T cells (Tregs) play an important role in preventing immunopathology by producing anti-inflammatory cytokines, IL-10 and transforming growth factor-β (TGF-β) (Dario et al., 2008). The general trend of inflammation-mediated resistance to fungi suggests that Tregs

would suppress protective immunity in most fungal diseases. TNF-α antagonism, used frequently to treat rheumatoid arthritis, puts human patients at risk for developing fungal diseases, particularly histoplasmosis (Wood et al., 2003). Antagonizing TNF-α elevates Treg populations which dampen protective immune responses to *H. capsulatum* (Deepe & Gibbons, 2008). CCR5 deficient mice have decreased recruitment of natural Tregs to the lungs and have increased resistance to histoplasmosis (Kroetz & Deepe, 2011). Deficiency of CCR5 in mice reverses the negative effects of TNF-α antagonism and increases resistance to histoplasmosis (Kroetz & Deepe, 2012). Tregs in TNF-α targeted therapy likely differ greatly from the normal Tregs present during an infection in the absence of anti-inflammatory therapies. Thus, Tregs may not necessarily play a negative role in resistance to fungal pathogens. In a natural infection, Tregs may play a coordinated role in immunity to fungi. For example, in *A. fumigatus* infection, Tregs can moderate inflammation and prevent allergy to limit pathology (Montagnoli et al. 2006). The role of Tregs in fungal disease is a delicate balance such that too great a Treg presence promotes fungal pathogenesis and an absence of Tregs leads to immunopathology.

$CD8^+$ T cells may play an important role in resistance to fungi. Pro-inflammatory cytokines can be produced by $CD8^+$ T cells to promote Th1 and Th17-like responses. In blastomycosis, mice deficient in $CD4^+$ T cells have protective responses due to production of IFNγ and IL-17 by $CD8^+$ T cells (Wüthrich et al., 2003; Nanjappa et al., 2012). In histoplasmosis, $CD8^+$ T cells also seem to play a protective role by producing IFNγ and targeting cells infected with *H. capsulatum* (Lin et al., 2005). DC phagocytosis of apoptotic cells infected with *H. capsulatum* engage in cross-presenting antigen to $CD8^+$ T cells and lead to increased resistance (Hsieh et al., 2011). The cytotoxic activities of $CD8^+$ T cells are important in viral immunity but may not be very important in fungal immunity. Most fungal pathogens can and do

persist outside of host cells, so for these fungi, killing the host cell would have little or no effect on the pathogen. Fungi with mainly intracellular niches may be more susceptible to cytotoxic killing by $CD8^+$ T cells. For example, perforin is important for some killing of *H. capsulatum* by T cells (Lin et al., 2005). However, lysing or killing host cells may have a more pathogenic effect by causing the release and dissemination of intracellular fungi.

Antibody responses have not been overwhelmingly tied to resistance to fungi. The trend of Th2 responses impairing antifungal immunity suggests that T-dependent antibody responses might be more harmful than protective, except in cases such as *P. murina* in which Th2 can be protective (Nelson et al., 2011). Resistance to *P. carinii* appears to require protective antibodies (Zheng et al., 2001), but resistance to most other fungal diseases are typically independent of antibody production. Antibodies directed to virulence features or surface structures can be protective. Such protective antibodies have been identified for candidiasis (Cassone & Casadevall, 2012). Two *C. albicans* proteins associated with virulence, Als3 and Sap2, have been selected for vaccine therapy because antibodies against these antigens have been shown to be protective (De Bernardis et al., 2012; Ibrahim et al., 2013). Some antibodies against the *Cryptococcus* capsule have been shown to confer protection (Mukherjee et al., 1995); such antibodies could act to promote opsonization and complement activation but may also promote mechanical changes in the capsule to inhibit yeast growth and dissemination (Taborda & Casadevall, 2002; Cordero et al., 2013). Antibodies bound to *C. neoformans* can change gene expression and metabolic activity of the yeast (McClelland et al., 2010). Protective antibodies against *H. capsulatum* have also been identified (Nosanchuk et al., 2003; Guimarães et al., 2011). In aspergillosis, however, antibodies have not been found to play a protective role (Diaz-Arevalo et al., 2011; Cassone & Casadevall, 2012). Despite the potential for antibody protection,

resistance to most fungal infections is more dependent on inflammatory T-cell response than antibodies.

The studies of adaptive immunity to fungi have led the way to developing vaccination strategies for fungal diseases. Successful fungal vaccines will require activating protective immune responses: mostly Th1- and Th17-type responses, potentially with antibody or $CD8^+$ protection as well. Mice have been immunized against various fungi, but the most effective methods require the use of attenuated fungal strains such as the BAD1 mutant of *B. dermatitidis*, but these would be incredibly difficult to develop as commercial vaccines (Brandhorst et al., 1999; Cassone & Casadevall, 2012; Wüthrich et al., 2013). Use of specific antigens or peptides that activate protective immunity would be more sensible because such immunizations would not require exposure to a live fungus. Two transgenic T cell lines have been discovered that have TCRs that recognize fungal epitopes. One of these recognizes a shared antigen for several endemic fungi and has been shown to be protective in mouse models of *B. dermatitidis*, *C. immitis*, and *H. capsulatum* (Wüthrich et al., 2003; Wüthrich et al., 2007). Another TCR has been identified that in transgenic mice protects against aspergillosis (Rivera et al., 2006; Rivera et al., 2011). The antigens recognized by these TCRs, once identified, may be used to immunize humans to multiple mycoses. Currently, no commercially-available vaccine exists for human mycoses. However, two *C. albicans* vaccines are currently undergoing clinical trials (Cassone & Casadevall, 2012). One of these, PEV7 is a virosome coated with Sap2, a secreted protease linked with transition to pathogenic hyphae (De Bernardis et al., 2012). The second is NDV-3 delivered with alum and the N-terminus of Als-3, a surface adhesin (Ibrahim et al., 2013).

Immunity to fungal infections varies greatly among pathogens, pathogen strains, hosts, and sites of infection. However, resistance to fungal pathogens requires proper coordination

between the innate and adaptive immune system to overcome fungal evasion mechanisms and remove the fungus from host tissue. General trends suggest that inflammatory processes promote the clearance of fungal pathogens by recruiting phagocytic cells and priming Th1 and Th17 immunity. Continued research in immunity to fungal pathogens will aid in future treatments and vaccinations to reduce fungal pathogenesis in humans and susceptible animal populations.

Research Aims

Batrachochytrium dendrobatidis is well adapted to living in keratinized epithelia of amphibians. As a successful pathogen, *B. dendrobatidis* must have acquired mechanisms to inhibit amphibian immunity. Amphibians have an arsenal of innate and adaptive immune defenses in and on the skin to protect against an epidermal pathogen (Rollins-Smith et al., 2009); however, *B. dendrobatidis* continues to infect even mostly resistant amphibian species (Mazzoni et al., 2003; Ramsey et al., 2010). The role of defenses against *B. dendrobatidis* in the skin mucus has been greatly investigated in recent years (Rollins-Smith, 2009; Bletz et al., 2013), but the role of immunity inside the skin where *B. dendrobatidis* causes pathology is still poorly understood. *Xenopus laevis* has been developed as a model of of amphibian immunology (Robert & Cohen, 2011). *Xenopus laevis* provides a model to understand immune responses against *B. dendrobatidis* at the molecular, cellular, and organismal level.

Some characterization of the immune responses to chytridiomycosis has been investigated in *X. laevis* that confirm the importance of adaptive immunity in chytridiomycosis (Ramsey et al., 2010). Despite adaptive immune responses playing a role, much evidence suggests that immune responses to chytridiomycosis are not robust enough to clear infection

(Pessier et al., 1999; Berger et al., 2005b; Rosenblum et al., 2009; Stice & Briggs, 2010; Rosenblum et al., 2012a; Cashins et al., 2013). Rollins-Smith et al. (2009) noted that *B. dendrobatidis*-immunized *X. laevis* lymphocytes do not proliferate in the presence of dead *B. dendrobatidis* cells. These studies combined suggest that *B. dendrobatidis* inhibits amphibian immune responses in the skin. Immune evasion strategies are necessary for *B. dendrobatidis* to persist in the skin without being cleared by the normally robust immune responses that amphibians possess.

The first goal of this study was to investigate why amphibian lymphocytes do not respond to *B. dendrobatidis in vitro*. *B. dendrobatidis* inhibits mitogen-induced lymphocyte proliferation *in vitro* suggesting that *B. dendrobatidis* can impair adaptive immune responses. This impairment is mediated by soluble factors collected in *B. dendrobatidis* supernatants. Soluble factors released by *B. dendrobatidis* induce apoptosis in amphibian lymphocytes and inhibit both B and T lymphocytes. *B. dendrobatidis* supernatants also impaired human and mouse lymphocytes suggesting that the mechanism of inhibition targets a conserved pathway in vertebrate lymphocytes.

The effects of *B. dendrobatidis* on innate phagocytic leukocytes were also investigated. Phagocytes are important components of immune responses to fungal pathogens (Brown, 2011) and are likely targets of *B. dendrobatidis* immune evasion. *Xenopus laevis* macrophages and neutrophils were obtained by peritoneal lavage. Phagocytes were incubated with *B. dendrobatidis* cells and supernatants. Despite the capacity of *B. dendrobatidis* to greatly impair lymphocytes, no impairment of amphibian phagocytes was observed.

To confirm the effects of *B. dendrobatidis* on amphibian leukocytes *in vitro*, immune responses were investigated *in vivo* in *X. laevis*. A modified protocol to induce inflammatory

swelling in *X. laevis* feet was developed to quantify the effects of *B. dendrobatidis* factors on innate and adaptive immune responses. Induction of innate immune responses was not decreased by *B. dendrobatidis* factors *in vivo*, but adaptive immune responses were decreased. Thus, the *in vitro* effects of *B. dendrobatidis* on immune responses were reproduced *in vivo* in *X. laevis*.

To fully understand the mechanism of lymphocyte impairment, the factors responsible for inhibition of adaptive immune responses must be identified. The lymphotoxins are likely associated with the *B. dendrobatidis* cells wall. *B. dendrobatidis* zoospores which lack cell walls (Loncore et al., 1999; Berger et al., 2005a) do not inhibit lymphocyte proliferation. Also, nikkomycin Z, a chitin synthase inhibitor (Cohen 1987; Hector 1993) which disrupts the *B. dendrobatidis* cell wall, decreases *B. dendrobatidis* impairment of lymphocytes. The lymphotoxic factors do not appear to be RNA, lipid, or protein due to the fact that they are water soluble and are resistant to heat, acid, RNase, and proteases. The inhibitory *B. dendrobatidis* factors separate into two size classes of less than 10 kDa and greater than 50 kDa. Therefore, the lymphotoxic factors are likely to be small molecules and polysaccharides, respectively.

The specific goal of this study was to investigate immune evasion by *B. dendrobatidis*. A mechanism was identified where *B. dendrobatidis* produces soluble molecules that impair lymphocyte responses. The primary focus of this study was to characterize the effects of these factors on host cells and to determine the nature of these factors.

CHAPTER II

BATRACHOCHYTRIUM DENDROBATIDIS PRODUCES FACTORS THAT IMPAIR AND KILL LYMPHOCYTES[1]

Abstract

Although amphibians have robust immune defenses, clearance of *Batrachochytrium dendrobatidis* is impaired. Because inhibition of host immunity is a common survival strategy of pathogenic fungi, it is likely that *B. dendrobatidis* evades clearance by inhibiting immune functions. *B. dendrobatidis* cells and supernatants impaired *Xenopus laevis* lymphocyte proliferation suggesting that *B. dendrobatidis* produces soluble factors to modulate host immunity. Lymphocytes from another anuran species, *Rana pipiens*, and from humans and mice also were impaired by *B. dendrobatidis* cells or supernatants. Epithelial cell lines of Chinese hamster ovary and HeLa cells were also inhibited in the presence of the *B. dendrobatidis* supernatant factors suggesting that the effects of the soluble mediators are not limited to lymphoid cells and may be responsible for some disease symptoms of chytridiomycosis. The mechanism of inhibition of amphibian lymphocytes is the induction of apoptosis, and soluble *B. dendrobatidis* factors activate both intrinsic and extrinsic caspases signaling pathways. A closely related non-pathogenic chytrid fungus, *Homolaphlyctis polyrhiza*, does not inhibit lymphocytes or epithelial cells suggesting that the toxic factors produced by *B. dendrobatidis* may have been important in the transition from a non-pathogenic saprobe or commensal to a pathogenic lifestyle. An immortal human T lymphocyte cell line (Jurkat), was also inhibited by *B. dendrobatidis* toxic factors providing a reproducible assay system for the characterization of the

[1] Most of the data shown in this chapter is published in Fites et al., 2013.

toxic factors. Evasion of host immunity may explain why *B. dendrobatidis* has devastated amphibian populations worldwide.

Introduction

Amphibians have several natural defenses against *B. dendrobatidis* that may affect survival from chytridiomycosis. Amphibian skin epithelium is covered in a layer of mucous which may contain chemical defenses that kill or inhibit invading zoospores (Fig. 1-1). Antimicrobial peptides (AMPs) are released from dermal granular glands into the mucous of many amphibian species (Gammill et al., 2012). Amphibians have also been shown to secreted *B. dendrobatidis*-specific antibodies into the mucous (Ramsey et al., 2010). Several amphibian species are also known to have symbiotic skin bacteria that produce antifungal metabolites with activity against *B. dendrobatidis* (Harris et al., 2006; Woodhams et al, 2007b; Lauer et al., 2008; Becker & Harris, 2010; Lam et al., 2010). Both AMPs and beneficial bacteria have been proposed as protective in amphibian populations that possess these defenses (reviewed in Rollins-Smith, 2009).

Once *B. dendrobatidis* forces itself into the skin via a germ tube (Van Rooij et al., 2012; Greenspan et al., 2012), the interaction between the host's immune system and the pathogen is not well understood. Amphibians have a functional immune system present in the skin that should be able to mount an adaptive immune response against *B. dendrobatidis* within the skin (Ramanayake et al., 2007; Robert & Ohta, 2009; Rollins-Smith et al., 2009). Inactivating adaptive immune responses with sub-lethal irradiation impairs clearance of *B. dendrobatidis* (Ramsey et al., 2010). Survival from chytridiomycosis is linked with MHC class II alleles (Savage & Zamudio, 2011), and multiple exposures to *B. dendrobatidis* infection limits pathogen

burden following a subsequent infection (Richmond et al., 2009; McMahon et al., submitted). Despite the capacity of amphibians to mount an immune response against *B. dendrobatidis*, complete clearance of the pathogen is rare (Mazzoni et al., 2003; Ramsey et al., 2010) suggesting that *B. dendrobatidis* inhibits immune responses in the skin epithelium.

Batrachochytrium dendrobatidis likely evades immune responses in a variety of ways. By quickly invading keratinocytes via a germ tube (Van Rooij et al., 2012; Greenspan et al., 2012), *B. dendrobatidis* can avoid detection by phagocytes. Also, *B. dendrobatidis* appears to inhibit leukocyte migration as suggested by early histological studies that report no significant leukocyte infiltration in infected skin (Pessier et al., 1999; Berger et al., 2005b). My study provides the first evidence of direct inhibition of immune responses by *B. dendrobatidis*.

Soluble factors released by *B. dendrobatidis* inhibit lymphocyte proliferation *in vitro*. The lack of robust immune responses observed during chytridiomycosis may explain why so many amphibians die from *B. dendrobatidis* infections.

Materials and Methods

Animals

Outbred *Xenopus laevis* ranging in size from 35 to 200 g were purchased from Xenopus I (Dexter, MI) or Nasco (Fort Atkinson, WI, USA) and held in polystyrene containers at a density of about 10 frogs per 16 liters of de-chlorinated tap water. Outbred *Rana* (*Lithobates*) *pipiens* ranging in size from approximately 20 to 40 g were purchased from Connecticut Valley Biological (Southampton, MA, USA) and held in polystyrene containers at a density of about 5 frogs per tank. Containers for *R. pipiens* were inclined to provide a wet or dry area. Room

temperature was maintained between 20 to 24°C. Sixty-one of the *X. laevis* used to obtain splenocytes were tested for the presence of *B. dendrobatidis* as described by Boyle et al. (2004) and Hyatt et al. (2007). Only 11 individuals (18%) tested positive for *B. dendrobatidis*; these *B. dendrobatidis* positive individuals had very low pathogen load of 17.84 ± 0.91 (± SEM) zoospore equivalents. BALB/c-J mice were bred in the Vanderbilt University animal facilities. Research using mice complied with all relevant institutional and federal guidelines and policies. All animal procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine.

Batrachochytrium dendrobatidis **and** ***Homolaphlyctis polyrhiza*** **culture**

Batrachochytrium dendrobatidis isolates JEL197 (Loncore et al., 1999) and JEL275 (Carey et al., 2006) were cultured and maintained as previously described in 1% tryptone broth (T-broth) (Rollins-Smith et al., 2006). The non-pathogenic chytrid fungus *Homolaphlyctis polyrhiza* (isolate JEL142) is closely related to *B. dendrobatidis* (Joneson et al., 2011) and was cultured in medium containing 0.1% peptonized milk, 0.1% tryptone, and 0.5% glucose. Cultures were incubated at 20-21°C and sub-cultured twice weekly. Mixed cultures (zoospores and maturing cells) or purified zoospores (Rollins-Smith et al., 2002) were counted using a hemocytometer slide. Unless specified, JEL197 was the *B. dendrobatidis* isolate used for all experiments. Unless specified otherwise, *B. dendrobatidis*, was killed by treatment at 60°C for 10 minutes (Rollins-Smith et al., 2006). Chytrid cells were centrifuged to remove growth medium and re-suspended in Leibovitz (L-15) medium before being incubated with leukocytes.

Batrachochytrium dendrobatidis and *H. polyrhiza* supernatant preparation

After 6-7 days of culture in T-broth, *B. dendrobatidis* or *H. polyrhiza* cells were centrifuged, washed with sterile glass distilled water, re-suspended at 10^7 matured cells (all cells beyond zoospore stage) per mL in sterile distilled water, and incubated at 21°C for 24 hours in large flasks. Cells were centrifuged, and supernatants were passed through 0.2 µm filters (Fisher, Waltham, MA USA) to remove any cells. Supernatants were then frozen and lyophilized. Lyophilized supernatants were re-suspended at 2/5 to 1/20 of the original volume to reconstitute at 2.5X to 20X concentration above the original concentration in either Leibovitz (L-15) medium or sterile glass-distilled water. Some *B. dendrobatidis* supernatants were re-suspended in L-15 and mixed 1:1 with splenocytes in L-15 for proliferation or apoptosis assays. Other supernatants were re-suspended in water and mixed 1:1 with splenocytes in 2X L-15. All re-suspended supernatants were filter-sterilized through 0.2 µm syringe filters (VWR, Radnor, PA, USA) just before being added to cell culture. Pellets containing *B. dendrobatidis* cells after removal of supernatants were tested for viability by re-culture in T-broth. Except when *B. dendrobatidis* was killed prior to supernatant preparation, *B. dendrobatidis* cells in the pellet were viable. *H. polyrhiza* supernatants were lyophilized and re-suspended in L-15 to be incubated with splenocytes or mammalian cell lines at concentrations ranging between 1.25X and 10X the original concentration.

Preparation of killed *B. dendrobatidis*

Batrachochytrium dendrobatidis cells were killed as described above by a single 60°C incubation or by four consecutive freeze-thaw cycles in which *B. dendrobatidis* cells were frozen at -20°C and then brought to room temperature until thawed before re-freezing. The procedure

for freezing and thawing *B. dendrobatidis* cells lasted four days. In experiments using dead *B. dendrobatidis* cells, live control cells from the same culture were incubated at 4°C for the duration of the killing procedure. After treatments, a subset of *B. dendrobatidis* cells was incubated in 1% T-broth; lack of growth confirmed that cells were no longer viable. Supernatants from dead *B. dendrobatidis* were prepared as described above at 21°C for 24 hours.

Splenocyte culture

Spleens from *X. laevis* or *R. pipiens* were dissociated between two frosted glass slides that were sterilized with 70% ethanol, and lymphocytes were enriched by centrifugation over a Ficoll-Hypaque cushion (ρ = 1.119; Sigma, St. Louis, MO, USA). This enriched lymphocyte fraction was used for all splenocyte culture assays. Splenocytes were cultured as previously described (Rollins-Smith et al., 1984) in L-15 culture medium (Sigma, St. Louis, MO, USA) diluted to amphibian tonicity and supplemented with 100 I.U./mL penicillin, 100 μg/mL streptomycin, 12.5 mM sodium bicarbonate, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 1% heat-inactivated fetal calf serum (Life Technologies, Grand Island, NY, USA). *X. laevis* splenocytes were cultured at a density of 5 x 10^5/mL (10^5 per well) in proliferation assays. *R. pipiens* splenocytes were cultured at 3.75-5.0 x 10^5/mL (0.75-1.0 x 10^5 per well). Phytohemagglutinin (PHA) was used at a final concentration of 2 μg/mL to induce T-cell proliferation. Phorbol-12-myristate 13-acetate (PMA) (Hsu et al., 1985) was used at a final concentration of 5 ng/mL to induce lymphocyte proliferation. Heat-killed *Aeromonas hydrophila* originally isolated from a diseased Wyoming toad (Taylor et al., 1999) was used at a concentration of 10^6 bacteria per well (5 x 10^6/mL) to induce B-cell proliferation (Morales et al., 2003). Allogeneic lymphocytes from unrelated outbred frogs were also used for induction of a

mixed leukocyte reaction (MLR) (Du Pasquier & Horton, 1976). *B. dendrobatidis* or *H. polyrhiza* cultures containing both sporangia and zoospores, purified zoospores alone (Rollins-Smith et al., 2002), or supernatants prepared as described above were added to the splenocyte population simultaneously with the lymphocyte activator in all experiments unless described otherwise. For assays in which proliferation was induced by PHA, PMA, or in MLR, splenocytes were incubated at 26°C in an atmosphere of 5% CO_2 and 95% air for three days before harvesting as previously described (Rollins-Smith et al., 1984). All wells were pulsed with 0.5 µCi ^{3}H-thymidine (5 µCi/mL, specific activity 2 Ci/mmole) (Perkin Elmer, Waltham, MA, USA) during the last 24 hours prior to harvesting. Splenocytes stimulated with *A. hydrophila* were incubated an extra day (four days in total) and pulsed with ^{3}H-thymidine 24 hours prior to harvesting. Overall proliferation, measured as ^{3}H-thymidine uptake, was quantified as counts per minute (CPM) using a Wallac 1205 Betaplate Beta Liquid Scintillation Counter (Perkin Elmer, Waltham, MA, USA). Data shown for representative proliferation assays were the mean CPM ± SEM of at least five replicates for each treatment using splenocytes from a single individual. Experiments were repeated using splenocytes obtained from different individuals to verify the reproducibility of the results.

Transwell cell culture of splenocytes and *B. dendrobatidis*

Splenocytes from *X. laevis* (5×10^5 cells per well) were cultured in a 24-well plate with 0.4 µm pore-size transwell inserts (Corning, Corning, NY, USA) at a final density of 8.3×10^5 cells per mL. PHA was added to achieve a final concentration of 2 µg/mL. Mixed cultures of *B. dendrobatidis* sporangia and zoospores (5×10^5 cells per well) or zoospores alone (2×10^6 zoospores per well) were added above the transwell inserts. For co-culture, *B. dendrobatidis*

cells were added directly to splenocytes, and medium was added above the insert. In one experiment, 5 x 10^5 splenocytes were cultured adjacent to zoospores above the insert to determine whether cell contact was necessary for zoospore production of an inhibitory factor. Plates were incubated at 26°C for 3 days and pulsed with ^{3}H-thymidine during the last 24 hours of culture before harvesting. Immediately before harvesting, transwell inserts were removed, and lymphocytes were mixed and transferred to multiple wells of a 96-well plate to allow for harvesting and scintillation counting, as described above.

Flow cytometry of apoptotic splenocytes

Amphibian splenocytes prepared as described were analyzed by flow cytometry in the Vanderbilt University Flow Cytometry Core using a 5-laser BD LSRII flow cytometer (BD, San Jose, CA, USA). Flow cytometry events were analyzed and gated using BD FACSDiva Software (BD, San Jose, CA, USA). For analysis of apoptosis, splenocytes from *X. laevis* were cultured in 24-well plates at 10^6 cells per well (1.7 x 10^7/mL). When *B. dendrobatidis* cells were added, the fungal cells were separated by a transwell insert as described above at 2 x 10^7 cells per well. When *B. dendrobatidis* supernatants were added, splenocytes were cultured with a 10X concentrated *B. dendrobatidis* supernatant. Cultures were incubated for 24, 48, 72, or 96 hours at 26°C before analysis. To quantify apoptosis, cells were stained with the fluorescein isothiocyanate (FITC)-conjugated Annexin V Apoptosis Detection Kit according to manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Briefly, lymphocytes were washed with amphibian phosphate buffered saline (APBS), re-suspended in binding buffer (modified for amphibian tonicity) at 10^6 cells/mL, and stained with 5 µL Annexin V-FITC and 1 µL propidium iodide (PI) (50 µg/mL). Control preparations of cells were left unstained, stained

with Annexin V only, or stained with PI only. Cells were gated based on PI and FITC positivity determined by the staining of untreated control preparations. Cells that were PI^- /$FITC^+$ were considered to be undergoing early events of apoptosis (Vermes et al., 1995; Vermes et al., 2000). For each sample, 10,000 events fitting within normal forward and side scatter parameters for lymphocyte populations were collected. From these events, relative percentages were determined for cell populations positive or negative for PI and FITC. Mean percent of PI^- /Annexin V^+ cells were reported for multiple experiments using splenocytes from different individuals. Each experiment used splenocytes from a single individual, and therefore paired Student's *t*-tests were used to compare treatments.

Cell death inhibitors

All inhibitors were mixed with splenocytes and activators of proliferation or inducers of apoptosis at the initiation of culture. The pan-caspase inhibitor Z-VAD-FMK (BD Pharmingen, San Diego, CA, USA) was incubated with splenocytes at a concentration of 100 µM. This optimal concentration was determined by titration of the ability of Z-VAD-FMK to decrease apoptosis induced by anti-Fas (CD95) mAb (Southern Biotech, Birmingham, AL, USA) (Mangurian et al., 1998). Necrostatin-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an inhibitor of programmed necrosis (Degterev, 2005), was incubated with lymphocytes at concentrations between 20 to 60 µM. Cells incubated with these inhibitors were assayed for interference with induction of cell death using flow cytometry and/or proliferation assays as described above.

Caspase activity assay

Caspase-Glo® luminescence assay kits (Promega, Madison, WI, USA) for caspase-3/7, -8, and -9 were used to quantify caspase activation by *B. dendrobatidis* supernatants. Splenocytes (5 x 10^4 cells per well = 10^6/mL) were incubated for 24 hours at 26°C in sterile, white-walled, 96-well plates (BD Falcon, Franklin Lakes, NJ, USA) with L-15 alone, *B. dendrobatidis* supernatant, anti-Fas mAb, or corticosterone. The anti-Fas (CD95) mAb from clone DX2 (Southern Biotech, Birmingham, AL, USA) was added to cells at a final concentration of 2.5 µg/mL (Mangurian et al., 1998). Corticosterone (Sigma, St. Louis, MO, USA) was added to cells at a final concentration of 10 nM (Rollins-Smith et al., 1997). After the initial incubation, Caspase-Glo® reagents were added to each well according to the manufacturer's instructions and incubated at room temperature. Luminescence was recorded every 30 minutes over three hours using a Synergy HT Multi-Mode Microplate Reader (Bio Tek, Winooski, VT, USA), and luminescence sensitivity was set to positive controls. Relative light units (RLU) for samples did not vary greatly between 30 and 120 minutes of incubation with luminescence reagents. Therefore, 90 minutes of incubation was chosen as a representative endpoint for all assays. Anti-Fas mAb was used to induce apoptosis in assays detecting caspase-3/7 and caspase-8 activity because this antibody triggers extrinsic apoptosis pathways (Mangurian et al., 1998; Rathmell & Thompson, 2002). Corticosterone (10 nM) was used to induce apoptosis in assays detecting caspase-9 activity because corticosterone triggers intrinsic apoptosis pathways (Garvey et al., 1993; Laakko & Fraker, 2002). Lyophilized *B. dendrobatidis* supernatants were mixed into splenocyte cultures at 10X concentration. Each treatment was replicated in at least four wells for each experiment, and experiments were repeated four times using lymphocytes from different individuals.

Magnetic separation of lymphocytes

B or T lymphocytes were isolated from enriched *X. laevis* spleen populations using magnetic anti-mouse IgG1-coated microbeads (Miltenyi, Auburn, CA, USA). To label B cells, anti-IgM mAb 6.16 (Bleicher & Cohen, 1981) was used at a concentration of 10 μg/mL in APBS containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA. The anti-CD5 mAb 2B1 (Jürgens et al., 1995) used to label T cells was a hybridoma supernatant, and it was used at an approximate concentration of 100 μg/mL. Anti-*Xenopus* antibodies were obtained from the *Xenopus laevis* Research Resource for Immunobiology at the University of Rochester Medical Center. The respective antibodies were incubated with enriched splenic lymphocytes for 10 minutes at 4°C and washed with APBS/BSA/EDTA buffer. Labeled T and B lymphocytes were then incubated with magnetic anti-IgG1 microbeads in APBS/BSA/EDTA buffer for 15 minutes at 4° C and washed. Lymphocytes were re-suspended in 500 μL APBS/BSA/EDTA buffer and separated using an AutoMACS® magnetic cell sorter (Miltenyi, Auburn, CA, USA) in the Vanderbilt Flow Cytometry Core. After cell sorting, isolated B or T cells were counted and re-suspended at a known concentration in L-15. Cells from sorted populations were cultured with PHA or heat-killed *A. hydrophila* in a proliferation assay, as described above, in order to confirm whether *B. dendrobatidis* inhibits B- or T-cell proliferation. The B-cell isolation protocol selected about 20% of the cells in the splenocyte population. The T-cell isolation protocol selected about 65% of the cells in the splenocyte population.

Mouse splenocyte culture

Splenocytes from 6 to 8 week-old BALB/c-J mice were obtained from Dr. Thomas Aune's laboratory at Vanderbilt University. Splenocytes were isolated and cultured, as described previously (Collier et al., 2012). Briefly, splenocytes were isolated by mechanical disruption, and erythrocytes were removed by standard water lysis. Total splenocytes were re-suspended at 5.6×10^5 cells per mL in complete RPMI medium with 50 ng of PMA per mL and 1 µM of ionomycin (iono). Control cultures were left unstimulated. In a 96-well plate format, 180 µL of splenocyte suspensions with or without PMA/iono were mixed with 20 µL of phosphate buffered saline (PBS) or lyophilized *B. dendrobatidis* supernatants, re-suspended in PBS in increasing doses to achieve a final concentration of 1.25 to 10 times the original concentration. Supernatants from either *B. dendrobatidis* (JEL 197) or *H. polyrhiza* (JEL 142) were prepared as described above and filter sterilized by passing through 0.2 µm syringe filters (VWR, Radnor, PA, USA) before addition to the splenocyte culture. Splenocytes were incubated for 48 hours before being pulsed with 0.5 µCi ^{3}H-thymidine (5 µCi/mL, specific activity 2 Ci/mmole) (Perkin Elmer, Waltham, MA, USA). Six hours after being pulsed, splenocytes were harvested and proliferation was quantified as described above.

Human $CD4^+$ T cell isolation and culture

Peripheral blood was processed as previously described (Oswald-Richter et al., 2009; Oswald-Richter, 2010). Resting $CD4^+$ T cells were purified from fresh or cryopreserved peripheral blood mononuclear cells (PBMC) by magnetic separation using a Dynal CD4 Positive Isolation Kit (Invitrogen, Carlsbad, CA, USA). Purified resting $CD4^+$ T cells were incubated with increasing doses of *B. dendrobatidis* supernatant for 30 minutes. After this

incubation, cells were maintained in media containing *B. dendrobatidis* supernatant and were also activated by cross-linking with plate-bound anti-CD3 antibody (OKT-3; American Type Culture Collection) and soluble anti-CD28 antibody at 1 μg/mL (BD Biosciences, San Jose, CA, USA) as previously described (Oswald-Richter et al., 2010).

To analyze activation markers on T cells, cells were stained with the relevant antibody on ice for 30 minutes in PBS containing 2% fetal calf serum and 0.1% sodium azide. Cells were then washed twice, fixed with 1% paraformaldehyde, and analyzed with a LSR-II flow cytometer (BD Biosciences, San Jose, CA, USA). Live cells were gated based on forward- and side-scatter properties, and analysis was performed using FlowJo software (Tree Star, Ashland, Oregon, USA). The following anti-human antibodies were used for surface staining: CD3, CD4, CD62L, and HLA-DR, all obtained from BD Biosciences. A minimum of 30,000 events were acquired per sample. To determine proliferation and quantify cell division, purified $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Purified cells were first washed and re-suspended in PBS. While vortexing the cells, CFSE was added at a final concentration of 5 μM. The mixture was vortexed for an additional 15 seconds and incubated at 37°C for 3 minutes. Labeling was quenched by the addition of 50% FCS in PBS. Cells were washed once more with 50% serum PBS, followed by two washes with RPMI supplemented medium. CFSE-labeled $CD4^+$ T cells were TCR-stimulated in RPMI supplemented medium, using anti-CD3 and anti-CD28 antibodies. At day 5 post-activation, cells were fixed and analyzed for CFSE expression and cell size by flow cytometry as described above in this section.

Supernatants were collected from T cell receptor (TCR) stimulated cells at 24 hours and analyzed for extracellular IL-2 and IFN-γ by cytokine bead array (CBA) according to the

manufacturer's instructions (BD Biosciences, San Jose, CA, USA). For TCR stimulation, 2 x 10^5 $CD4^+$ T cells were stimulated through the TCR by plate-bound anti-CD3 and soluble anti-CD28 antibodies.

Pre-treatment of frog lymphocytes with *B. dendrobatidis* supernatant

Splenocytes (10^5 cells per well = 5 x 10^5 cells per mL) were pre-treated with *B. dendrobatidis* supernatant re-suspended in L-15 for 48 hours without stimulation. Control splenocytes were incubated in L-15 alone. Plates were centrifuged to pellet cells, and media was removed. After washing once with fresh L-15, cells on these plates were re-suspended in 200 µL L-15 with or without 2 µg/mL PHA. The splenocytes were cultured for another 3 days before harvesting (pulsed with ^{3}H-thymidine 24 hours prior to harvesting). This experiment was repeated three times using splenocytes from different *X. laevis* individuals.

Delayed addition of *B. dendrobatidis* supernatant after frog lymphocyte stimulation

Splenocytes (10^5 cells per well) were incubated in 100 µL of L-15 medium containing 2 µg/mL PHA for 24 hours. After the initial 24 hour incubation, 100 µL of L-15 or *B. dendrobatidis* supernatants (re-suspended in L-15 at 2.5X to 20X the original supernatant concentration) were added to wells and incubated for two more days. Splenocytes were pulsed with ^{3}H-thymidine 24 hours after the addition of *B. dendrobatidis* supernatant and harvested 24 hours later. This experiment was repeated three times using splenocytes from three different *X. laevis*.

Chinese hamster ovary and HeLa cell culture

Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium supplemented to contain 10% fetal calf serum, 2 mM L-glutamine, 100 µg/mL streptomycin, 100 I.U./mL penicillin (Invitrogen, Carlsbad, CA, USA), and 25 ng/mL of amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). HeLa-CCL2 cells (obtained from Carolyn Coyne, University of Pittsburgh) were grown in Dulbecco's Modified Eagle Medium (Invitrogen) with the same supplementation. CHO and HeLa cells were seeded in 12-well plates (Costar, Tewksbury, MA, USA) at 10^4 cells per well in a 1 mL volume. CHO cells were seeded 13 hours pre-treatment (h.p.t), and HeLa cells were seeded 5 h.p.t. Wells were treated in triplicate with either 100 µL of PBS, G418 sulfate (Mediatech, Manassas, VA, USA) diluted in PBS, or lyophilized supernatant from *B. dendrobatidis* (JEL197) or *H. polyrhiza* (JEL142) re-suspended in PBS. *Batrachochytrium dendrobatidis* supernatants used to treat CHO or HeLa cells were incubated in a 100°C water bath for 30 minutes before lyophilization to denature any potentially active proteins. All treatments were filter-sterilized before addition by passage through 0.2 µm syringe filters (VWR, Radnor, PA, USA). G418 sulfate is generally toxic to eukaryotic cells (Mingeot-Leclercq et al., 1999) and was used as a cell proliferation control at a final concentration of 500 µg/mL. The approximate final concentrations of supernatants in cell culture wells were 10X, 5X, 2.5X, and 1.25X the pre-lyophilization concentration. CHO and HeLa cells were treated for 0, 24, 48, or 72 hours before being harvested for counting. Cells were rinsed with PBS and dissociated using 0.5% trypsin-EDTA (Invitrogen). Trypsin-treated wells were neutralized with an equal volume of complete Ham's F-12 medium, and the total volume was transferred to microcentrifuge tubes. Cells were centrifuged at 2000 RPM for 5 minutes in a Beckman TJ-6 Centrifuge (Bria, CA, USA). Media was removed from the cell pellets, and cells were re-

suspended in a known volume (50-500 µl) of PBS supplemented with 2% fetal calf serum. A small volume of re-suspended cells was mixed with an equal volume of 0.4% trypan-blue viability stain (Invitrogen). Cells were counted either using a hemocytometer slide or using a Countess® Automated Cell Counter (Invitrogen). The numbers of both living and dead cells were recorded.

Flow cytometric analysis of apoptosis in B and T lymphocytes incubated with *B. dendrobatidis* cells

Splenocytes were plated in 24-well plates at 1.7 x 10^6 cells per well. *Batrachochytrium dendrobatidis* cells were heat-killed and mixed with splenocytes at 1.7 x 10^7 cells per well. Plates were incubated for 48 hours at 26°C before use in flow cytometry. The mixed cell population was stained with anti-*Xenopus* monoclonal antibodies specific for MHC class II (14A2) (Flajnik et al., 1990), followed by polyvalent allophycocyanin (APC)-conjugated goat anti-mouse IgG2a antibodies (0.5 mg/mL). [All lymphocytes are MHC class II positive in adults of this species (Flajnik et al., 1990; Du Pasquier & Flajnik, 1990; Rollins-Smith & Blair, 1990).] This allowed for the selection of splenocytes while gating out *B. dendrobatidis* cells. After washing, the cells were divided and stained either with monoclonal antibodies specific for IgM (6.16) (Bleicher & Cohen, 1981) to identify IgM^+ B cells or with monoclonal antibodies specific for CD5 (2B1) (Jürgens et al., 1995) to identify T cells. Both populations were further stained with polyvalent phycoerythrin (PE)-conjugated goat anti-mouse IgG1 antibodies (0.5 mg/mL) and 7-Aminoactinomycin D (7-AAD, Sigma, St. Louis, MO, USA) (24 µg/mL). The IgM^+ fraction and the $CD5^+$ fraction were washed and stained with FITC-conjugated Annexin V as described above. Control populations of splenocytes were unstained, stained with 7-AAD only

(24 µg/mL), with APC only (0.5 mg/mL), with PE only (0.5 mg/mL), or with Annexin-FITC only (5 µL). Cells were analyzed by flow cytometry with the assistance of the Vanderbilt Flow Cytometry core. Splenocytes were gated by MHC class II (APC) positivity. The fraction of cells stained with anti-IgM (6.16) was gated to determine the IgM-PE positive population of B cells. The fraction of cells stained with anti-CD5 (2B1) was gated to determine the CD5-PE positive population of T cells. Both B and T cells were then analyzed for dual staining for Annexin V-FITC and 7-AAD, and those cells that were $7AAD^-$/Annexin V^+ were considered to be undergoing early events of apoptosis (Vermes et al., 1995; Vermes et al., 2000). To determine the extent of apoptosis in all lymphoid cells, the cell population was also gated on MHC II^+ cells before being analyzed for Annexin V-FITC and 7-AAD fluorescence.

Flow cytometric analysis of apoptosis by enumeration of cells with hypodiploid DNA content

Splenocytes or peritoneal leukocytes (PLs) from *X. laevis* were cultured in 24-well plates at 10^6 cells/well (1.0-1.7 x 10^6 cells per mL) with *B. dendrobatidis* supernatants, *H. polyrhiza* supernatants, or known inducers of apoptosis [10 nM corticosterone (Sigma, St. Louis, MO) (Rollins-Smith et al., 1997) or 2.5 µg/mL anti-Fas mAb (Southern Biotech, Birmingham, AL) (Mangurian et al., 1998)]. Lyophilized *B. dendrobatidis* and *H. polyrhiza* supernatants, at a final concentration of 10X, were mixed with splenocytes or PLs in L-15. Cultures were incubated for 24 or 48 hours at 26°C before fixation and nuclear staining with PI as described by Hotz et al. (1994) and modified for use in amphibian lymphocytes as described by Rollins-Smith et al. (1997). Ten thousand events were collected, gated within normal forward and side scatter

parameters for the population. Those displaying less than a normal diploid DNA content were considered to be undergoing cell death (Hotz et al., 1994).

Culture of Jurkat cells

The Jurkat cell line used was the Jurkat-E6 T-lymphoid-cell line obtained from the lab of Christopher Aiken at Vanderbilt University. This strain was donated by A. Weiss and obtained from the AIDS Research and Reference Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Jurkat cells were cultured in RPMI with 100 I.U./mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% heat-inactivated FCS. When Jurkat cells were cultured with *B. dendrobatidis* or *H. polyrhiza* supernatants (prepared as described above), lyophilized supernatants were re-suspended in complete RPMI and filter sterilized. Supernatants were diluted to final concentrations as indicated, in cell culture with Jurkat cells.

For proliferation assays 10^4 Jurkat cells were incubated with chytrid supernatants for three days at 37° C. When Jurkat cells were cultured at 26° C, they were seeded at 10^5 cells per well. Proliferation assays using ^{3}H -thymine uptake assays were completed as described above for amphibian lymphocytes except ^{3}H-thymidine was diluted in PBS instead of APBS.

The effects of chytrid supernatants on Jurkat cells were also assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay (van Meerloo et al., 2011). MTT (Sigma, St. Louis, MO, USA) was re-suspended to 500 μg/ml in PBS and added at an equal volume to cultures of Jurkat after the culture incubation was completed. The MTT reagent was incubated with Jurkat cells for approximately three hours at 37°C. Volumes were removed

from cells after incubation, and cells were lysed in DMSO to release the reduced formazan crystal. Absorbance of plate wells was quantified at 570 nm.

Results

Batrachochytrium dendrobatidis produces soluble factors that impair *X. laevis* lymphocytes.

A previous study showed that lymphocytes from *X. laevis* immunized with killed *B. dendrobatidis* did not respond to killed *B. dendrobatidis in vitro* suggesting that *B. dendrobatidis* inhibits lymphocyte activation (Rollins-Smith et al., 2009). To test this hypothesis, both living and heat killed *B. dendrobatidis* were co-cultured with *X. laevis* splenocytes in the presence of PHA, a T-cell mitogen. The cellular proliferation induced by PHA was significantly decreased as both living and killed *B. dendrobatidis* cells were titrated into the lymphocyte culture (Fig. 2-1 A, B). To determine if this was mediated by soluble factors, *B. dendrobatidis* cells were separated from lymphocytes by a cell-impermeable membrane in a transwell culture system. *B. dendrobatidis* cells were not as inhibitory when separated in a transwell, but they could still inhibit lymphocyte proliferation suggesting that *B. dendrobatidis* produces soluble factors that impair lymphocytes (Fig. 2-1 C). Supernatants from *B. dendrobatidis* cultures placed in sterile water for 24 hours were added to lymphocyte culture as well, and concentrated *B. dendrobatidis* supernatants inhibited PHA-induced lymphocyte proliferation in a dose-dependent manner (Fig 2-1 D). Supernatants from *B. dendrobatidis* that was killed either by heat or consecutive freeze-thaw cycles were also inhibitory to lymphocyte proliferation (Fig. 2-2). The fact that dead *B. dendrobatidis* can inhibit lymphocyte proliferation suggests that the soluble factors are shed from *B. dendrobatidis*. The decrease in inhibition by dead *B. dendrobatidis* cells and supernatants

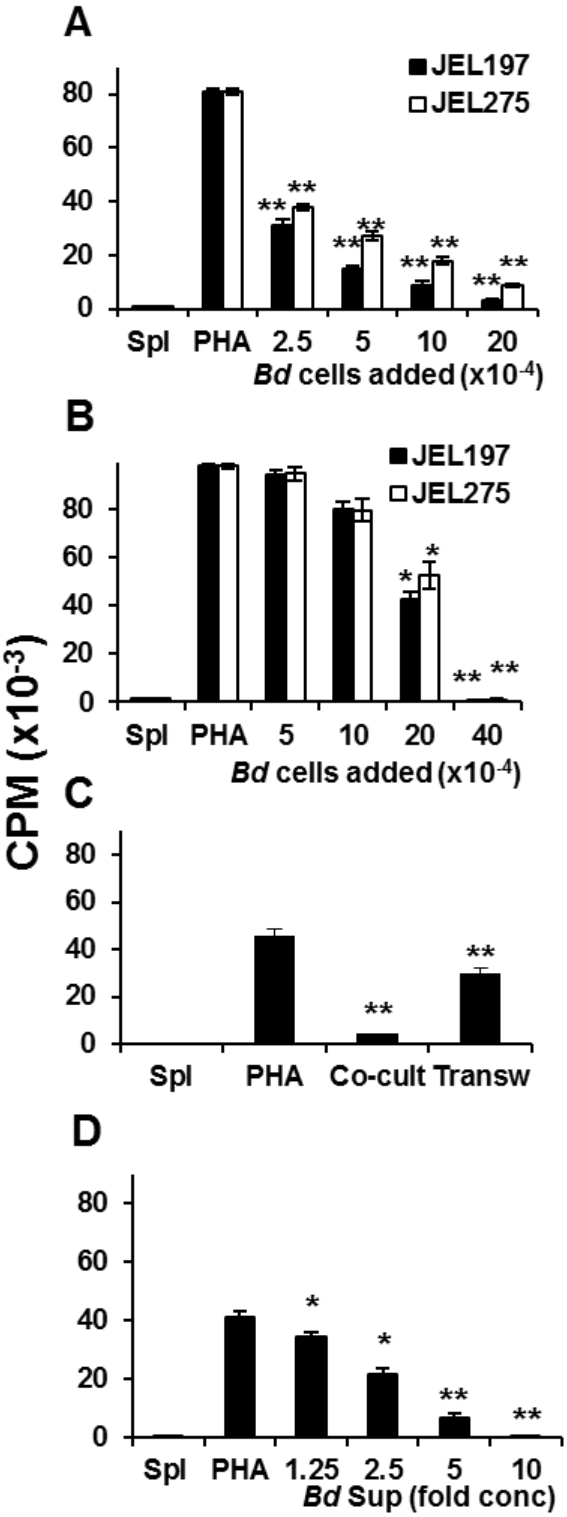

Fig. 2-1. Inhibition of lymphocyte proliferation by *B. dendrobatidis (Bd)*. Splenocytes (Spl) from *X. laevis* were cultured alone or with phytohemagglutinin (PHA). PHA-stimulated Spl were cultured alone or with increasing numbers of live (**A**) or heat-killed (**B**) *Bd* cells from two pathogenic isolates JEL197 or JEL275. (**C**) Spl were cultured as in A, and PHA-stimulated Spl were co-cultured (Co-cult) with or separated from live *Bd* cells by a 0.4 µm pore filter in transwell (Transw). (**D**) Lymphocytes were cultured as in A except that live *Bd* cells were replaced by *Bd* supernatants (Sup) at increasing concentrations. Significantly reduced ^{3}H-thymidine uptake detected as counts per minute (CPM) using a scintillation counter compared to the control treatment, *$p<0.05$, **$p<0.01$ (ANOVA with post hoc test). CPM data in each panel are averages ± SEM of five or more replicate wells and represent three or more similar experiments. Unless noted, JEL197 was the *Bd* isolate used for all cell culture and supernatant experiments. (Panel A completed with Laura Reinert; panel C completed by Jeremy Ramsey.)

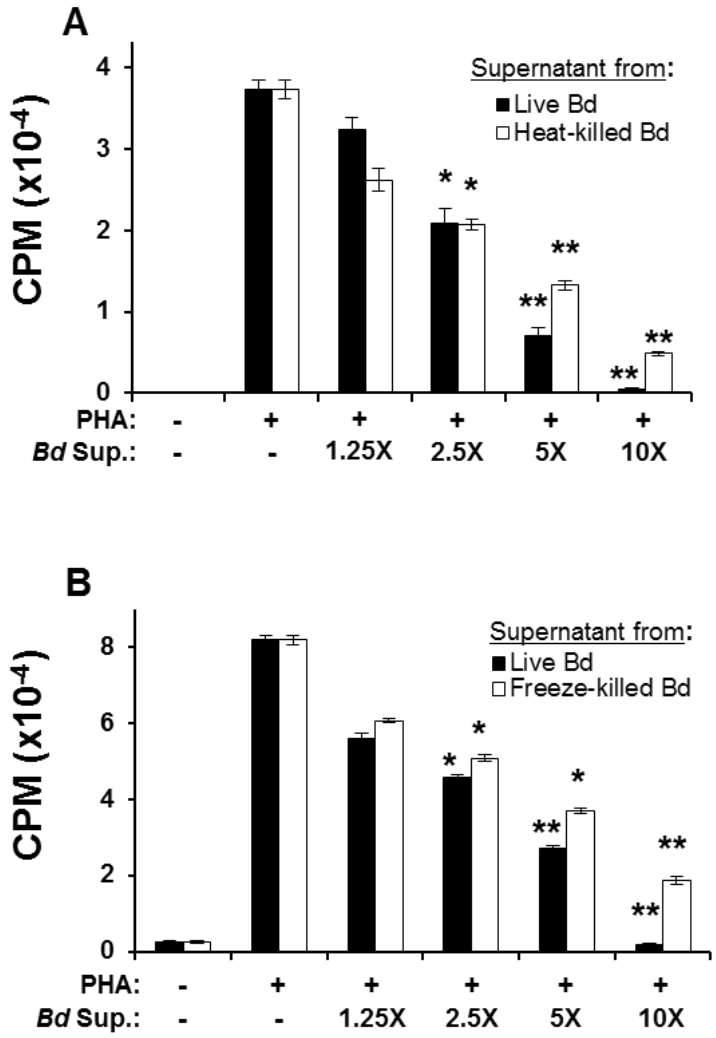

Fig. 2-2. Effects of by *B. dendrobatidis (Bd)* supernatants prepared from heat-killed or freeze-killed cultures. *Bd* isolate JEL197 was killed by treatment at 60°C for 10 min (**A**) or by four consecutive freeze-thaw cycles (**B**). An aliquot of the treated *Bd* cells was placed in 1% tryptone broth to confirm the loss of viability. Supernatants (Sup) from these killed *Bd* cells were prepared as described in the methods. Live *Bd* cells used for preparation of control supernatants were incubated at 4°C while the heat or freeze-thaw treatment occurred. For A-B, splenocytes were cultured with or without PHA, and PHA-stimulated cells were incubated with increasing concentrations of each supernatant in L-15 medium. *Significantly reduced ^{3}H-thymidine uptake compared to the control treatment by ANOVA with post hoc test, *$p<0.05$, **$p<0.01$. CPM data are averages ± SEM of six replicates. The panels are representative of four similar experiments.

compared to living *B. dendrobatidis* may be due to the decrease in production once *B. dendrobatidis* cells are killed. The *B. dendrobatidis* cell population used for supernatants and co-culture contained cells of all stages (zoospore, encysted cells, germlings, thalli, and zoosporangia), and killing *B. dendrobatidis* may prevent transition events that occur during culture with splenocytes or during supernatant preparation.

Proliferation induced by other mitogenic stimulators was also inhibited by both *B. dendrobatidis* cells in co-culture and *B. dendrobatidis* supernatants (Fig 2-3). Killed bacteria induce proliferation of amphibian B cells (Morales et al., 2003), so killed *A. hydrophila* was used to induce B lymphocyte proliferation which was also inhibited by *B. dendrobatidis* cells and supernatants (Fig. 2-3 A, B). Phorbol-12-myristate 13-acetate (PMA) induces lymphocyte proliferation by activating PKCθ, an important signaling kinase downstream of the TCR and BCR (Niedel et al., 1983). PMA-induced proliferation of *X. laevis* lymphocytes was inhibited by *B. dendrobatidis* cells and supernatants (Fig. 2-3 C, D). Lymphocyte proliferation can also be induced by a mixed leukocyte reaction (MLR) where splenocyte from allogenic individuals are mixed in culture (Du Pasquier & Horton, 1976). When splenocytes from different *X. laevis* were combined in a MLR, the cellular proliferation was significantly greater than when splenocytes were not mixed; MLR proliferation was significantly inhibited by *B. dendrobatidis* cells in co-culture and by *B. dendrobatidis* supernatants (Fig 2-3 E, F).

The closest non-pathogenic relative of *B. dendrobatidis* is *H. polyrhiza* (Joneson et al., 2011). Because *H. polyrhiza* is a saprobe and does not infect amphibians, it should lack virulence features that *B. dendrobatidis* has adapted to infect and persist in host tissue. *H. polyrhiza* cells and supernatants only inhibit lymphocytes at very high concentrations, and relative to *B. dendrobatidis*, *H. polyrhiza* does not inhibit lymphocyte proliferation (Fig. 2-4).

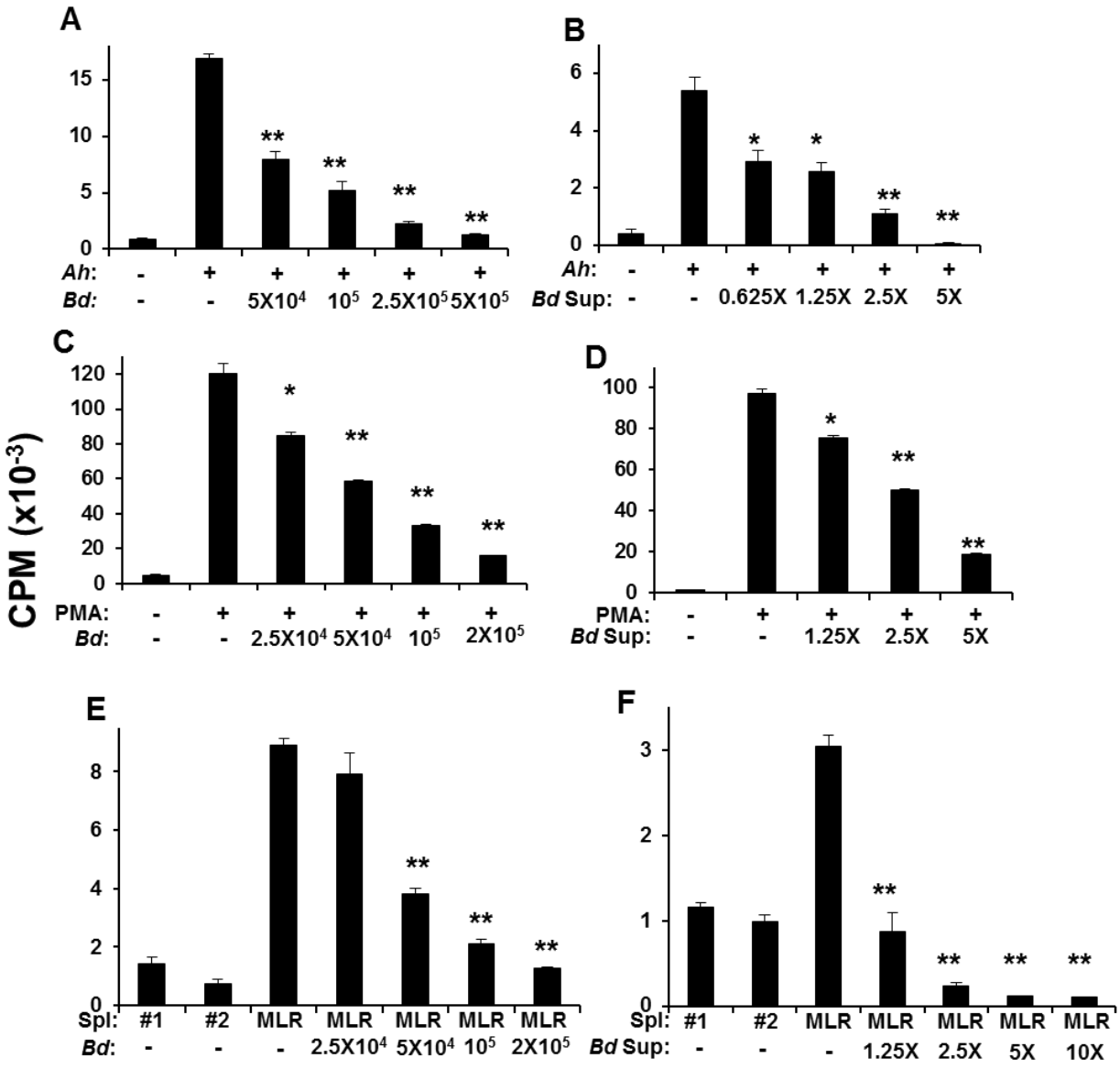

Fig. 2-3. Effects by *B. dendrobatidis (Bd)* cells or supernatants (Sup) on T- or B-cell proliferation. Splenocytes (Spl) from *X. laevis*, stimulated with heat-killed *A. hydrophila (Ah)* (**A**, **B**) or with phorbol-12-myristate 13-acetate (PMA) (**C**, **D**) or by allogeneic cells in a mixed-leukocyte reaction (MLR) (**E**, **F**), were incubated with increasing numbers of live *Bd* cells (A, C, E) or *Bd* Sup (B, D, F). For MLR, a total of 10^5 Spl from two unrelated frogs were mixed 1:1 with each other. Controls for the MLR experiments contained 10^5 Spl from each individual which contributed cells to the MLR (#1, #2). Significantly reduced ^{3}H-thymidine uptake compared to the control, *$p<0.05$, **$p<0.01$ (ANOVA with post hoc test). CPM data in each panel are averages ± SEM of five or more replicate wells. Panels are representative of at least three similar experiments. (Panel A completed by Whitney Holden.)

The inhibition by *H. polyrhiza* at very high concentrations may be due to the presence of proteases or other molecules that may have negative effects on lymphocytes but are not virulence factors. Alternatively, some or all of the *B. dendrobatidis* virulence factors may be present in chytrids as a structural components or metabolites, and *B. dendrobatidis* may have evolved to produce more potent or greater amounts of these factors.

Toxic factors from *B. dendrobatidis* inhibit lymphocytes from other species including humans.

To confirm that lymphocyte inhibition driven by *B. dendrobatidis* was not limited to *X. laevis* splenocytes, splenocytes from another frog, *R. pipiens*, were incubated in co-culture with *B. dendrobatidis* cells. The inhibition of B- and T-cell proliferation was replicated with *R. pipiens* splenocytes (Fig. 2-5). Although killed *A. hydrophila* did not induce strong proliferation response in *R. pipiens* lymphocytes, the ^{3}H-thymidine incorporation of lymphocytes was significantly increased in the presence of killed bacteria and significantly reduced in the presence of *B. dendrobatidis*. *X. laevis* and *R. pipiens* are distantly related frogs (Hay et al., 1995), so the molecular or cellular target of *B. dendrobatidis* factors is shared at least among anurans and likely among amphibians. Because the lymphocyte responses of another amphibian are impaired by *B. dendrobatidis*, this immune evasion strategy is likely important for *B. dendrobatidis* infection across amphibians.

In a similar investigation of mouse lymphocytes, mouse splenocytes were stimulated with PMA and ionomycin and mixed with *B. dendrobatidis* or *H. polyrhiza* supernatants. Mouse lymphocyte proliferation was inhibited by *B. dendrobatidis* supernatants at even lower

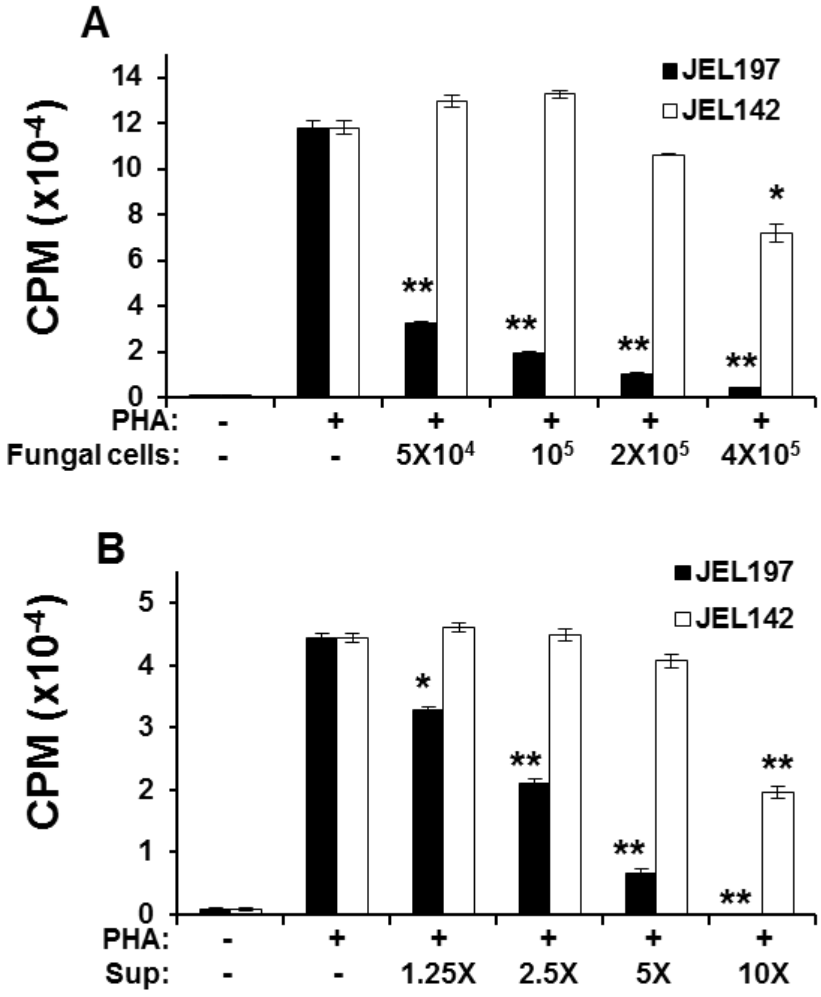

Fig. 2-4. Effects of non-pathogenic *Homolaphlyctis polyrhiza* (isolate JEL142) and pathogenic *B. dendrobatidis (Bd)* (isolate JEL197) on PHA-induced splenocyte proliferation. *X. laevis* splenocytes were cultured with or without PHA, and PHA-stimulated splenocytes were cultured alone or with living cells (**A**) or with concentrated supernatants (Sup) (**B**) from *Bd* (JEL197) or *H. polyrhiza* (JEL142). *Significantly reduced ^{3}H-thymidine uptake compared to the control treatment by ANOVA with post hoc test, *$p<0.05$, **$p<0.01$. CPM data are averages ± SEM of six replicates. Panels are representative of four similar experiments.

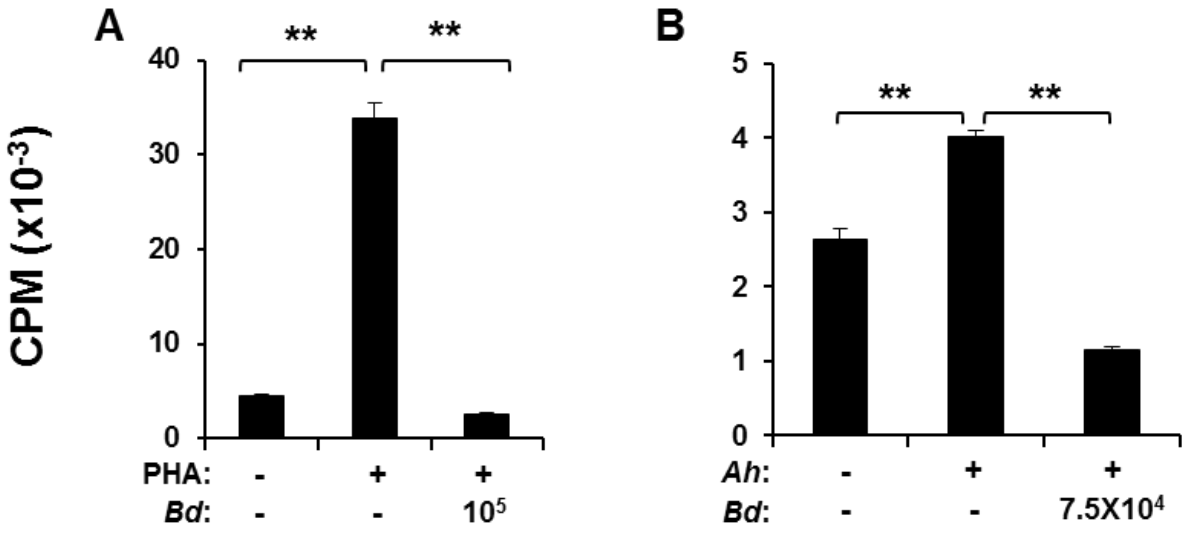

Fig. 2-5. Effects of *B. dendrobatidis (Bd)* cells on proliferation of splenocytes from a second frog species (*Rana pipiens*). (**A**) Splenocytes (10^5/well; 5 x 10^5/mL) were cultured alone or with PHA, and PHA-stimulated cells were co-cultured with or without live *Bd* cells. (**B**) Splenocytes (7.5 x 10^4/well; 3.75 x 10^5/mL) were cultured alone or with heat-killed *A. hydrophila* (*Ah*), and *Ah*-stimulated populations were co-cultured with or without live *Bd* cells. **Significantly different ^{3}H-thymidine uptake between treatments by Student's *t*-test with correction for multiple tests, $p<0.01$. Panels are representative of two similar experiments showing the average CPM ± SEM of four or more replicate wells. (Experiments completed with Whitney Holden.)

concentrations than those that inhibited *X. laevis* splenocytes (Fig 2-6 A). Higher concentrations of *H. polyrhiza* supernatants actually increased the amount of proliferation. Viability of resting mouse splenocytes was also quantified using an Annexin V and PI flow cytometric assay. Unstimulated mouse splenocytes were induced to die very rapidly in the presence of *B. dendrobatidis* supernatants (Fig 2-6 B). *B. dendrobatidis* supernatants induced a significant decrease in splenocyte viability by six hours post-treatment and killed the entire population after 16 hours. Because *B. dendrobatidis* supernatants also reduce the viability of mouse lymphocytes, the mechanism of inhibition is likely to target a conserved signaling or metabolic pathway shared among vertebrate, or at least tetrapod, lymphocytes.

Batrachochytrium dendrobatidis supernatants were also tested on purified $CD4^+$ T cell population obtained from human PBMCs (Fig. 2-7). Proliferation of human $CD4^+$ T cells stimulated by anti-CD3 and anti-CD28 was inhibited in a dose-dependent manner by *B. dendrobatidis* supernatants (Fig. 2-7 A). During activation, human T cells increased expression of CD25 (IL-2 receptor) and CD62L (L-selectin) and decreased expression of HLR-DR (Caruso et al., 1997). The expression of these markers by T cells treated with *B. dendrobatidis* supernatants show that *B. dendrobatidis* factors inhibit activation of $CD4^+$ T cells (Fig. 2-7 B). *B. dendrobatidis* supernatants also inhibited production of IL-2 and IFNγ by T cells (Fig. 2-7 C, D). The lack of cytokine production is likely a result of the lack of activation by T cells which is necessary to promote production of IL-2, IFNγ, and other cytokines. The results from human $CD4^+$ T cells show that factors can directly impair T cells and suggests the target of the factors is shared by amphibian and mammalian lymphocytes.

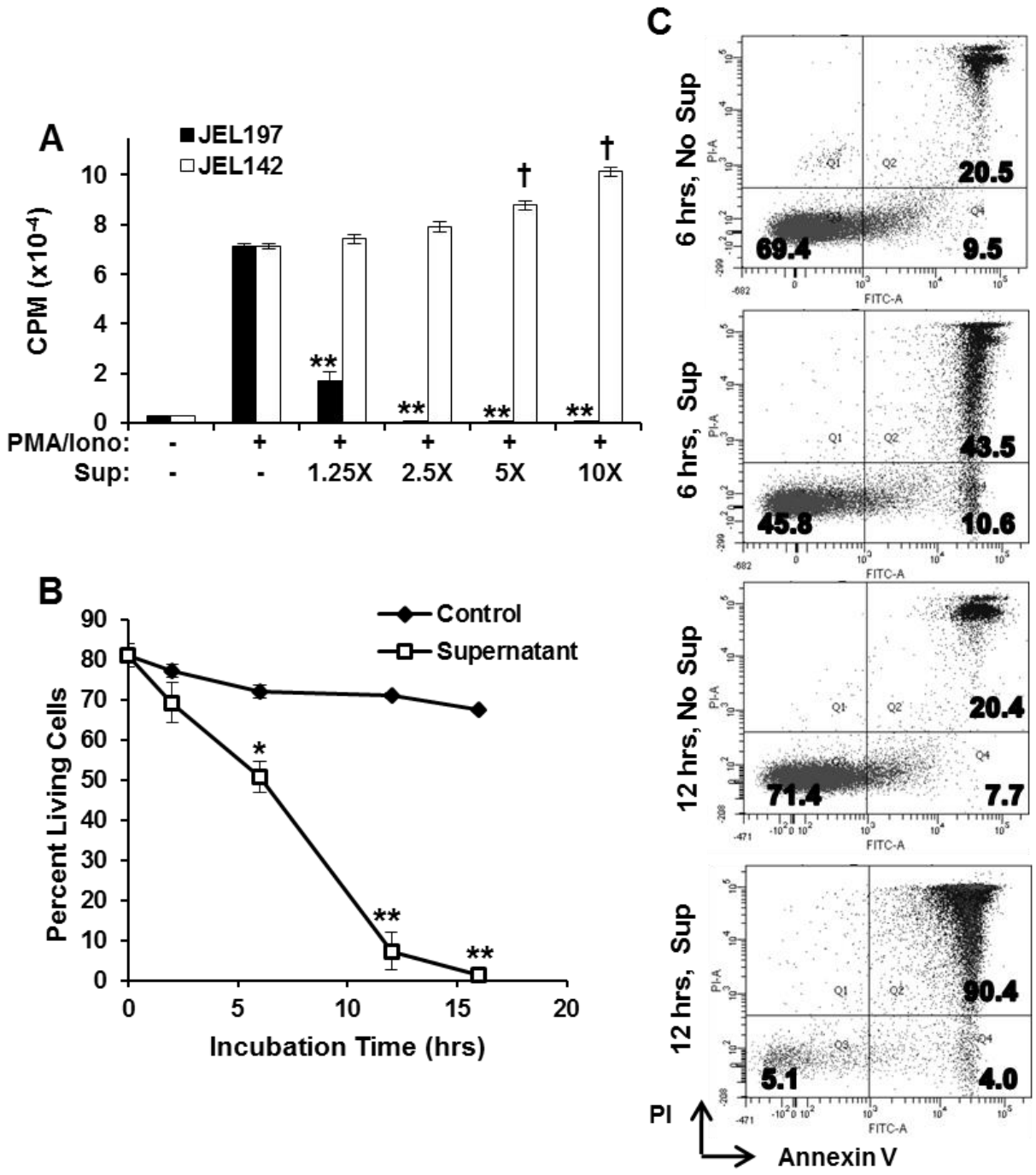

Fig. 2-6. *B. dendrobatidis (Bd)* supernatants inhibit mouse splenocytes; see next page for details.

Fig. 2-6. *B. dendrobatidis (Bd)* supernatants inhibit mouse splenocytes. (**A**) Mouse splenocytes were cultured alone or with PMA/iono. Stimulated cells were cultured alone or with increasing concentrations of *Bd* (JEL197) or *H. polyrhiza* (JEL142) supernatants (Sup). Significance was determined by a single-factor ANOVA with Tukey post-hoc: **$p < 0.01$ significantly less proliferation compared to the supernatant-free control; $^{\dagger}p < 0.01$ significantly greater proliferation compared to the supernatant-free control. CPM data are averages ± SEM of at least seven replicates. This experiment is representative of three similar experiments. (**B-C**) Resting mouse splenocytes were cultured alone (Control) or with *Bd* supernatant (Supernatant) and were assayed for cell death by flow cytometry after staining with Annexin V and PI at 0, 2, 6, 12, and 16 h.p.t. Cells negative for Annexin V and PI staining were considered to be living. Data show mean (±SEM) percentage of living cells ($Annexin\ V^-/PI^-$) (B) or representative flow cytometry plots at 6 and 12 h.p.t. (C) of splenocytes from three mice. Significance was determined by a two-tailed, paired *t*-test comparing supernatant and control treatments at each time point: *$p<0.05$, **$p<0.01$. Inset numbers on plots are percentage of cells in each quadrant. (Experiments completed with Sarah Parker Collier.)

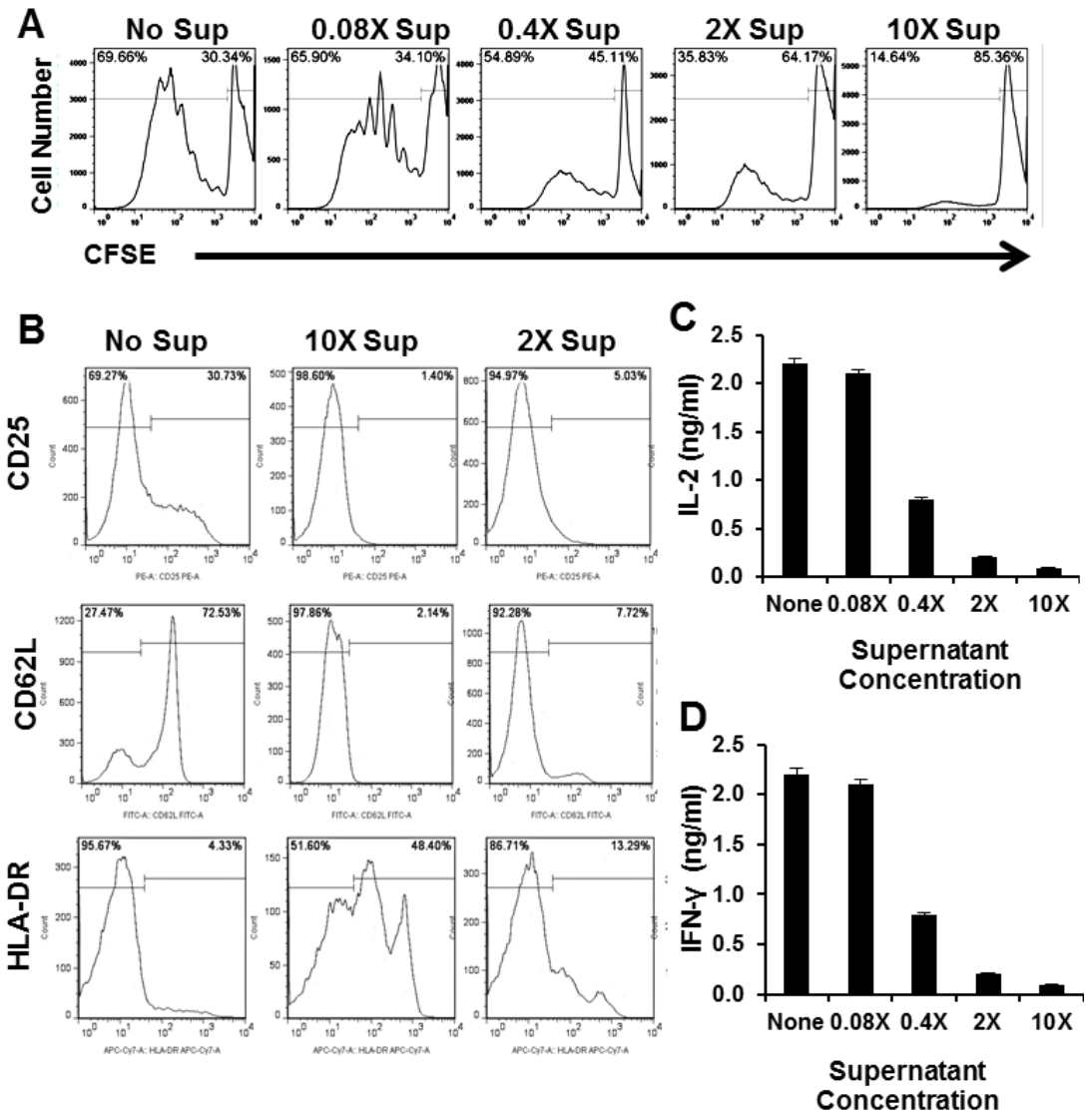

Fig. 2-7. Human helper T cells are inhibited by *B. dendrobatidis (Bd)* supernatants. (**A**) Proliferation of human $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 (after 5 days) was impaired by increasing concentrations of *Bd* supernatants as shown by reduced CFSE dilution. (**B**) Up-regulated markers in anti-CD3/anti-CD28-activated T cells (CD25 and CD62L) were decreased in cells treated with *Bd* supernatant (Sup); HLA-DR is a down-regulated marker in activated human T cells, but was up-regulated in cells treated with *Bd* supernatants. (**C-D**) Supernatants from *Bd* cultures inhibited secretion of IL-2 (C) and IFN-γ (D) by purified human $CD4^+$ T cells. $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 and treated with *Bd* supernatants at the fold concentrations shown for 24 hours. Culture medium was assayed for cytokines by CBA. These experiments are representative of two replicate experiments. (Experiments completed by Kyra Oswald-Richter.)

***Batrachochytrium dendrobatidis* inhibits the growth of non-lymphoid cell lines.**

To determine if soluble factors may have negative impacts on non-lymphoid cells, mammalian cell lines with epithelial origin were incubated with supernatants from *B. dendrobatidis* and *H. polyrhiza*. Chinese hamster ovary cells and HeLa cells (human cervical cancer cells) were incubated in culture with or without chytrid supernatants for up to three days, and cells were harvested and counted each day. CHO cells were greatly impaired by *B. dendrobatidis* supernatants but not *H. polyrhiza* supernatants (Fig. 2-8 A, B). Lower concentrations of *B. dendrobatidis* supernatant inhibited proliferation of CHO cells, and higher concentrations appeared to kill CHO cells. Proliferation of HeLa cells were inhibited by *B. dendrobatidis* supernatants and not affected by *H. polyrhiza* supernatants (Fig. 2-8 C, D). Due to the crude preparation of supernatants containing many different molecules, the factors responsible for CHO and HeLa cell inhibition may be different from the factors that inhibit lymphocytes. Supernatants used to treat CHO and HeLa cells were boiled before lyophilizing, decreasing the likelihood that proteins are responsible for this inhibition. The factors responsible for lymphocyte inhibition are also heat resistant, so soluble factors produced by *B. dendrobatidis* may impair all rapidly dividing cells.

***Batrachochytrium dendrobatidis* induces apoptosis in lymphocytes.**

To determine the effect of *B. dendrobatidis* on *X. laevis* lymphocytes, splenocytes were treated with *B. dendrobatidis* supernatants both before and after PHA stimulation. Splenocytes were treated with *B. dendrobatidis* supernatants for 48 hours in the absence of mitogenic stimulation and were then centrifuged and re-suspended in fresh media with PHA and without *B. dendrobatidis* supernatant. Pre-treatment of splenocytes with *B. dendrobatidis* factors caused a significant decrease in PHA-induced proliferation (Fig. 2-9 A). *B. dendrobatidis* factors can also

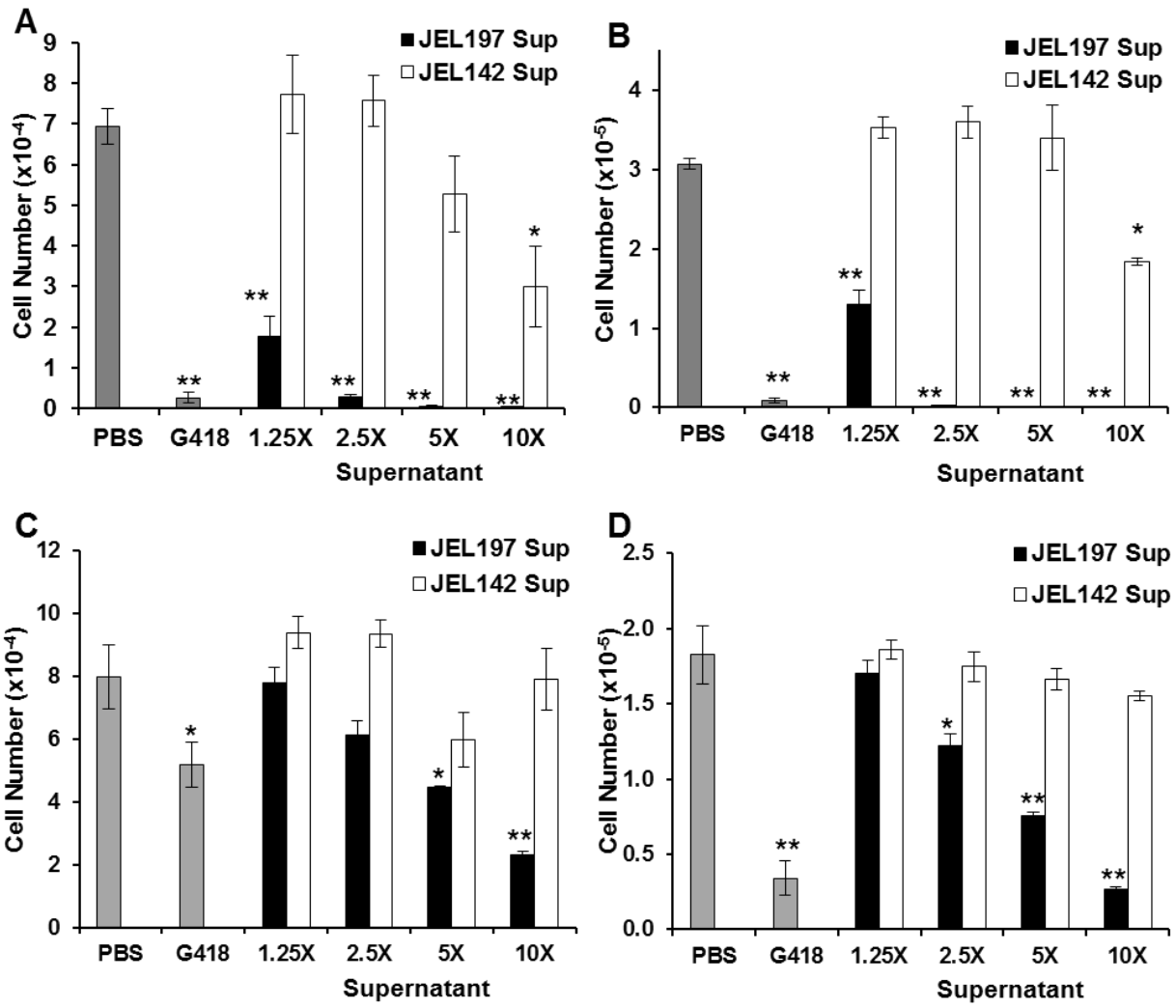

Fig. 2-8. *Batrachochytrium dendrobatidis (Bd)* supernatants (boiled prior to lyophilization) inhibit proliferation of Chinese hamster ovary (CHO) and HeLa cells, while *H. polyrhiza* supernatants weakly inhibit CHO cells but do not inhibit HeLa cells. CHO (**A**, **B**) or HeLa (**C**, **D**) cells were incubated with different concentrations of supernatant (Sup) from *Bd* (JEL197, black filled columns) or *H. polyrhiza* (JEL142, open columns) or 500 µg/mL G418 or PBS (gray filled columns). Cells were counted at 48 (**A**, **C**) or 72 (**B**, **D**) h.p.t. (total cell numbers in each well were multiplied by 10^{-4} or 10^{-5}). At the time of the treatment (0 h.p.t., 24 hrs after seeding wells), CHO cells numbered $6.0 \times 10^3 \pm 1.0 \times 10^3$ (± SEM) cells in each well, and HeLa cells numbered $1.9 \times 10^4 \pm 1.9 \times 10^3$ (± SEM) cells in each well. Data show means ± standard error of three treatment wells. Significantly fewer cells than the PBS control by a single-factor ANOVA with Tukey post-hoc test, *$p < 0.05$, **$p < 0.01$. Panels are representative of two separate experiments. (Experiments completed with Danica Sutherland.)

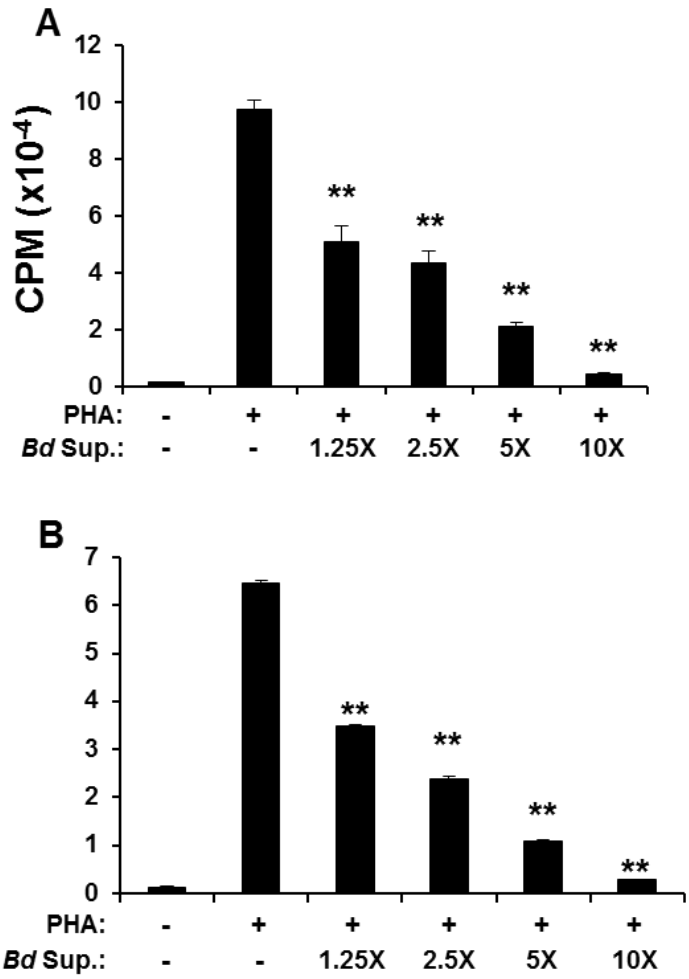

Fig. 2-9. Effects of pre-treatment or delayed addition of *B. dendrobatidis (Bd)* supernatants on splenocyte proliferation induced by PHA. (**A**) Unstimulated *X. laevis* splenocytes were cultured with increasing concentrations of *Bd* supernatants (Sup) or no Sup (-) for 48 hours. Plates were centrifuged to pellet cells, and the media was removed and replaced with fresh L-15. Splenocytes not treated with *Bd* Sup were cultured with or without PHA, and splenocytes previously treated with *Bd* Sup were stimulated with PHA. (**B**) Splenocytes were cultured with or without PHA, and after 24 hours, *B. dendrobatidis* supernatants were added to the PHA-stimulated cells at increasing concentrations. **Significantly reduced ^{3}H-thymidine uptake compared to the control treatment, $p<0.01$ (ANOVA with Tukey post hoc test). CPM data are averages ± SEM of six replicates. Panels are representative of three similar experiments.

inhibit lymphocyte proliferation when added to splenocytes after 24 hours of PHA-stimulation (Fig. 2-9 B). These results indicate that the effect of *B. dendrobatidis* on lymphocytes is not reversible once factors are removed from culture, and the factors can still impair activated lymphocytes.

Because *B. dendrobatidis* supernatant can inhibit lymphocytes both before and after stimulation, a likely mechanism of inhibition is induction of cell death in lymphocytes. Resting mouse splenocytes and CHO had a decreased viability when incubated with *B. dendrobatidis* supernatants (Figs. 2-6, 2-8). Viability of *X. laevis* splenocytes in the presence of *B. dendrobatidis* supernatants was determined by nuclear staining with PI following the degradation of DNA which occurs during cell death (Hotz et al., 1994). *Batrachochytrium dendrobatidis* supernatant treatment significantly increased the number of hypodiploid splenocytes (undergoing cell death) within 24 hours with an even greater increase in cell death at 48 hours (Fig. 2-10). At 24 hours, the amount of cell death induced by *B. dendrobatidis* supernatants was comparable to other known inducers of amphibian lymphocyte apoptosis, corticosterone and anti-Fas antibodies (Rollins-Smith et al., 1997; Mangurian et al., 1998). At 48 hours, *B. dendrobatidis* supernatants induced much greater cell death than both corticosterone and anti-Fas antibodies.

I hypothesized that one mechanism of cell death induced by *B. dendrobatidis* factors is would be apoptosis. During the early stages of apoptosis, the composition of the outer leaflet of the cellular membrane changes increasing the amount of phosphatidylserine (PS). PS is recognized by receptors on macrophages to engulf and clear apoptotic cells (Fadok et al., 1992). Annexin V binds to PS and can be used to determine cells undergoing the early stages of apoptosis with flow cytometry (Vermes et al., 1995; Vermes et al., 2000). In such a flow

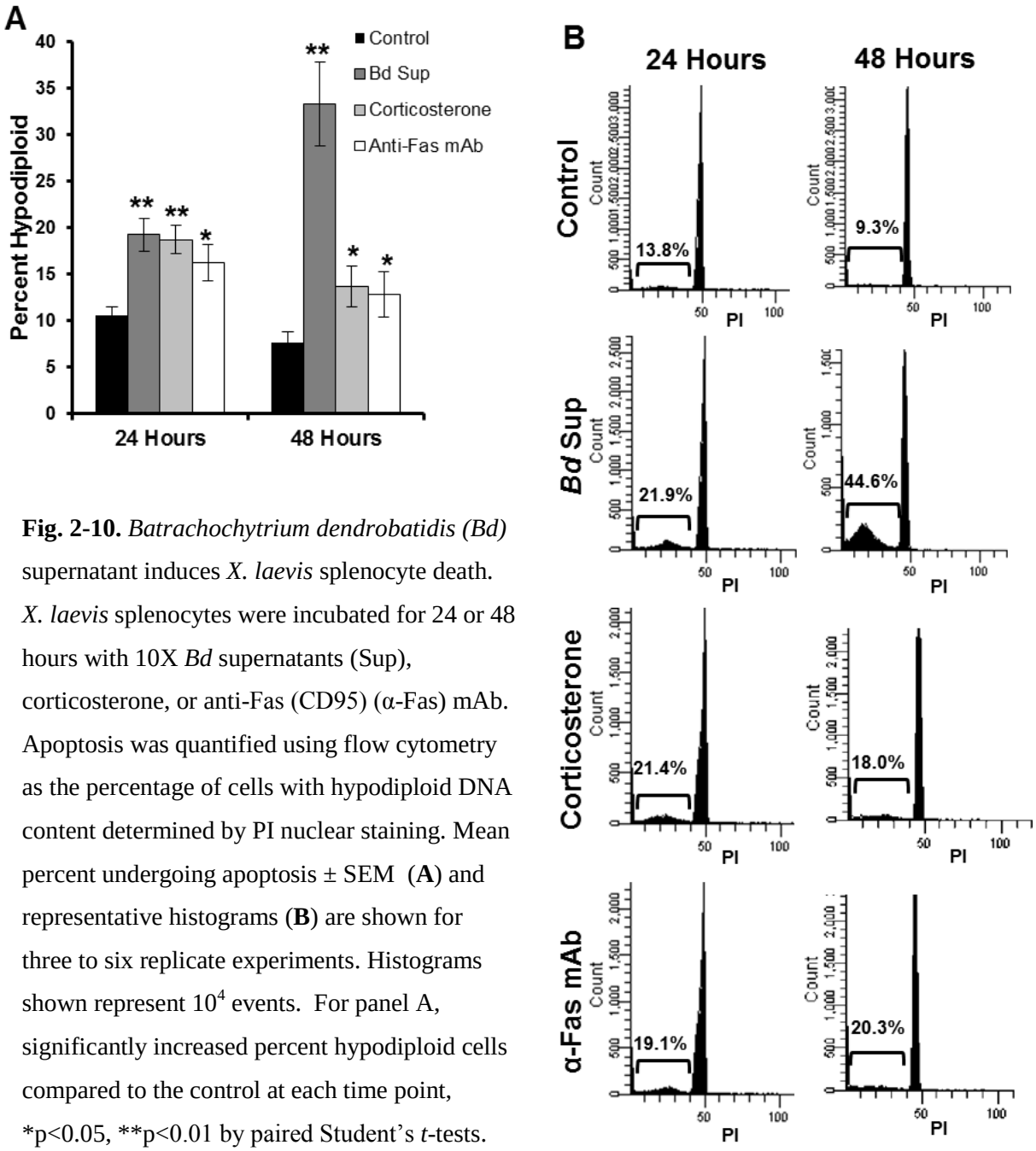

Fig. 2-10. *Batrachochytrium dendrobatidis (Bd)* supernatant induces *X. laevis* splenocyte death. *X. laevis* splenocytes were incubated for 24 or 48 hours with 10X *Bd* supernatants (Sup), corticosterone, or anti-Fas (CD95) (α-Fas) mAb. Apoptosis was quantified using flow cytometry as the percentage of cells with hypodiploid DNA content determined by PI nuclear staining. Mean percent undergoing apoptosis ± SEM (**A**) and representative histograms (**B**) are shown for three to six replicate experiments. Histograms shown represent 10^4 events. For panel A, significantly increased percent hypodiploid cells compared to the control at each time point, *p<0.05, **p<0.01 by paired Student's *t*-tests.

cytometric assay, Annexin V can be used in conjunction with PI, as a viability stain, to differentiate the stages and types of cell death. Cells positive for PI have lost cell membrane integrity either late in apoptosis or during necrotic cell death. In following a population undergoing cell death, the population only staining positive for Annexin V are most likely undergoing early stages of apoptosis, so the Annexin V^+/PI^- population was chosen to quantify apoptosis in this assay. A caveat to this assay is that apoptosis can only be detected at its early stages; if apoptosis death is rapid or if cells in the population are undergoing apoptosis at different times, then the early stages of apoptosis may be entirely missed.

Apoptosis of resting *X. laevis* splenocytes cultured across a transwell from *B. dendrobatidis* cells was determined with Annexin V and PI staining by flow cytometry over 96 hours. By 48 hours, there was a significant increase in Annexin V^+/PI^- cells in the splenocyte population cultured in transwell with *B. dendrobatidis* (Fig. 2-11). *Batrachochytrium dendrobatidis* induced an elevated amount of apoptosis through 72 hours, but the background apoptosis in control cells increased at 96 hours indicating that the resting splenocyte were naturally dying in the absence of survival or stimulation factors. *B. dendrobatidis* supernatants also induced apoptosis, a significant increase in Annexin V^+/PI^- cells, in resting *X. laevis* splenocytes (Fig. 2-12). Because factors were immediately mixed with splenocytes it appears that the kinetics of apoptosis was a little faster and more robust. At 24 hours, *B. dendrobatidis* supernatants had increased apoptosis nearly significantly ($p=0.07$) compared to no difference yet in the transwell assay. *B. dendrobatidis* supernatants also induced apoptosis in many more cells indicated by a greater loss of viability after 48 hours.

The Annexin V/PI assay is beneficial for following cell death in cells across a population but does not necessarily distinguish apoptosis from other forms of cell death. Necroptosis is a

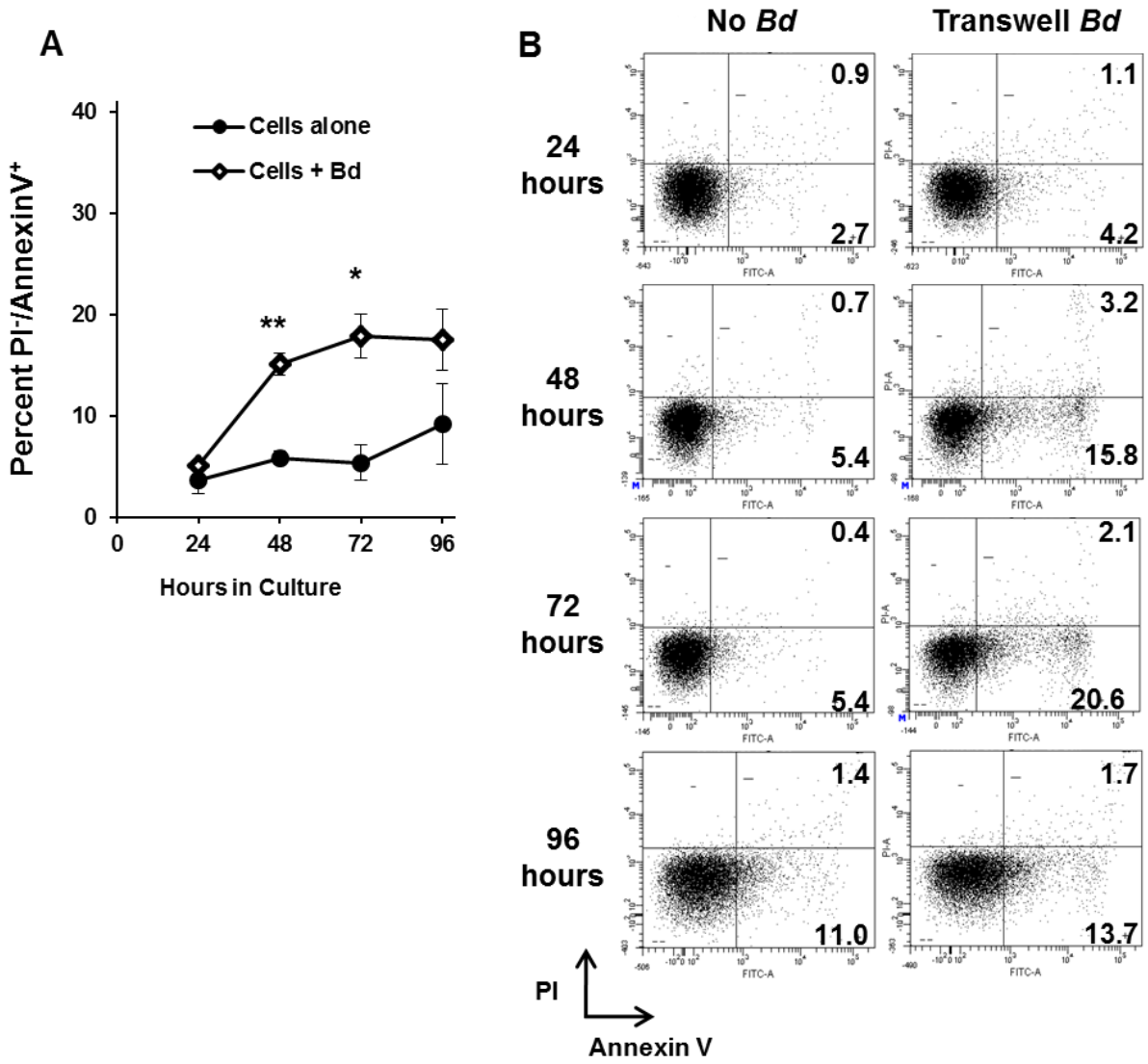

Fig. 2-11. *Batrachochytrium dendrobatidis (Bd)* cells release factors that induce apoptosis in *X. laevis* splenocytes. (**A**) Average percent apoptosis ± SEM of splenocytes cultured with or without *Bd* cells separated by a 0.4 µm filter (20:1 *Bd* to splenocytes) in transwell. (B) Representative flow cytometry plots from the experiments show 10^4 events and inset percentages in quadrants. Data show the mean or representative plots from three experiment. Percent apoptosis in the presence of *Bd* cells was significantly greater than that observed for splenocytes alone by a paired Student's *t*-test; *p<0.05, **p<0.01. (Experiments completed by Jeremy Ramsey.)

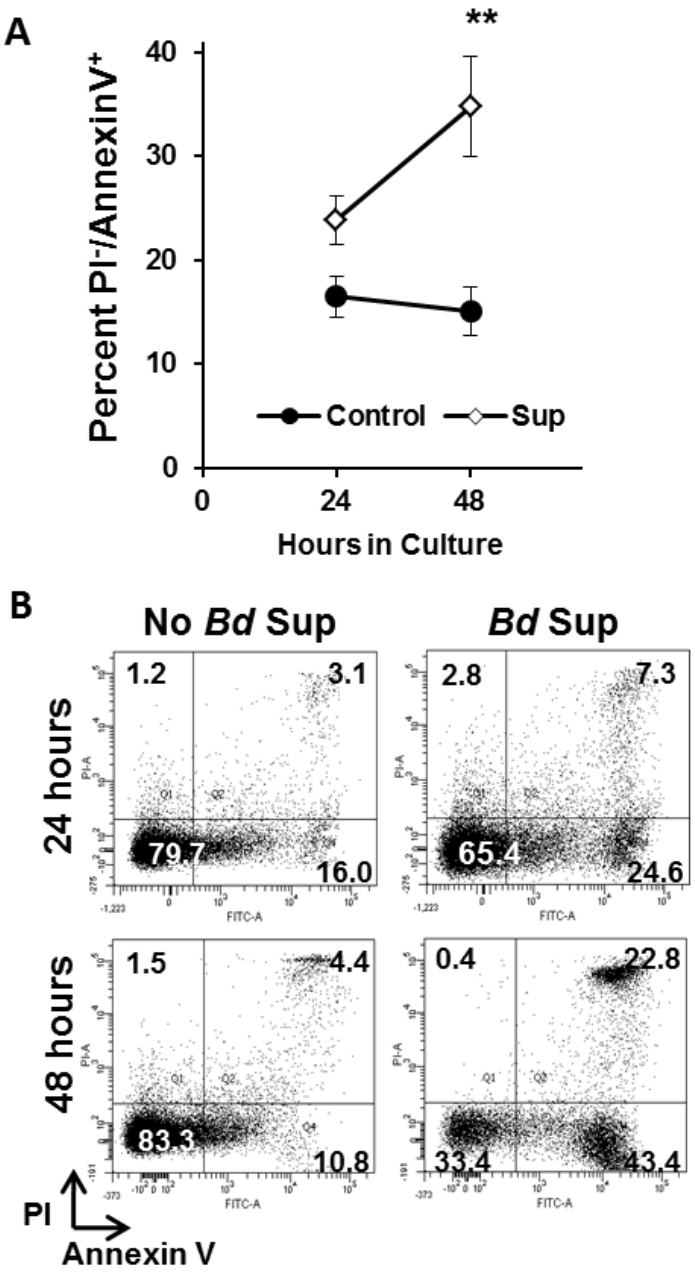

Fig. 2-12. *Batrachochytrium dendrobatidis (Bd)* supernatants (Sup) induce apoptosis in resting *X. laevis* splenocytes. (**A**) Average percent apoptosis ± SEM of splenocytes cultured with or without *Bd* supernatants (10X concentration). (B) Representative flow cytometry plots from the experiments show 2 x 10^4 events and inset percentages in quadrants. Data show the mean or representative plots from four experiments. Percent apoptosis in the presence of *Bd* Sup was significantly greater than that observed for splenocytes alone by a paired Student's *t*-test; **$p<0.01$.

form of programmed necrosis mediated by signaling pathways which cannot always be distinguished from apoptosis (Han et al., 2011). Two kinases, RIP1 and RIP3 mediate programmed necrosis at TNF receptors. Necrostatin-1 (Nec-1) inhibits RIP signaling to prevent necroptosis (Degterev, 2005). To determine if the cell death was mediated through programmed necrosis, *X. laevis* splenocytes were cultured with Nec-1 in the presence and absence of *B. dendrobatidis* supernatants. Nec-1 did not reverse the effects of *B. dendrobatidis* factors and actually appeared to enhance killing (Fig. 2-13). The presence of Nec-1 slightly decreased lymphocyte proliferation in the absence of *B. dendrobatidis* supernatants and caused greater inhibition by *B. dendrobatidis* factors when supernatants were present (Fig. 2-13 A). Nec-1 had no impact on the viability of resting splenocytes but had a synergistic effect on induction of cell death when lymphocytes were treated with both Nec-1 and *B. dendrobatidis* supernatants (Fig. 2-13 B). The RIP1/3 complex that induces necrosis contains several components of apoptosis signaling pathways, and the necroptosis and apoptosis signaling pathways are interlinked (Han et al., 2011). When the RIP1/3 complex is inhibited by Nec-1, cells are more susceptible to apoptotic cell death. The results suggest that *B. dendrobatidis* factors induce apoptosis, and when Nec-1 is present, apoptosis signaling is more effective at killing splenocytes.

Apoptosis is mediated through the cleavage and activation of caspases. Typically, apoptosis is either induced extrinsically through TNF receptors such as Fas or intrinsically activated by the release of cytochrome c from the mitochondrion (Rathmell & Thompson, 2002). TNF receptors canonically activate caspase-8 by forming activation complexes at the intracellular domain, and cytochrome c promotes formation of the apoptosome complex which activates caspase-9 (Siegel, 2006). Caspases recognize and cleave specific peptide sequences which can be inhibited by

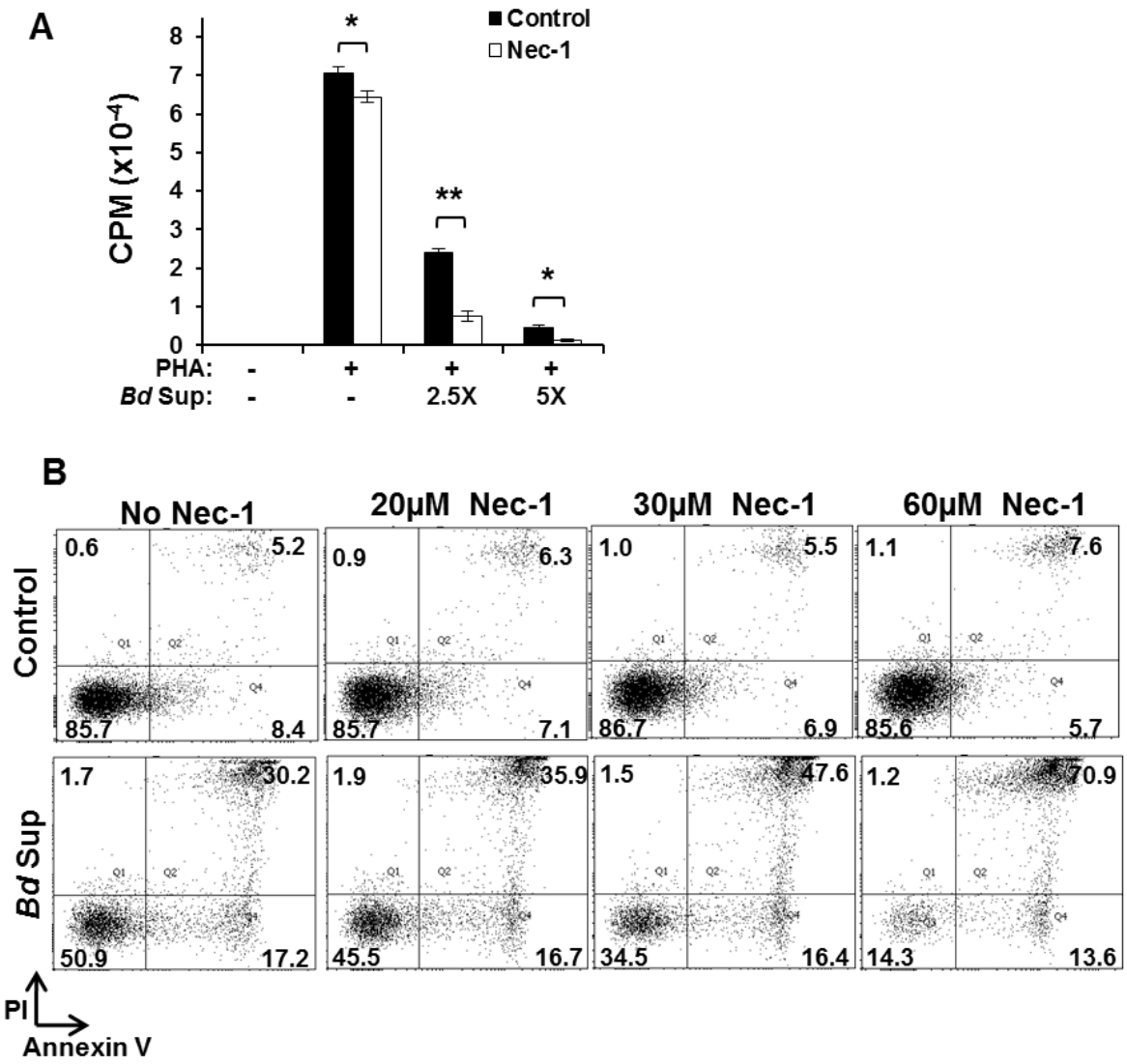

Fig. 2-13. Necrostatin-1 (Nec-1) does not reduce the cell death induced by *B. dendrobatidis (Bd)* upernatants. (**A**) *X. laevis* splenocytes were cultured for three days in the presence or absence of PHA, *Bd* Sup, and 30 µM Nec-1. Significantly decreased CPM for PHA-induced cells treated with *Bd* Sup alone in comparison with *Bd* Sup with Nec-1, *p<0.05, **p<0.01 by Student's *t*-test with a correction for multiple *t*-tests. CPM are averages ± SEM of six replicate wells and represent two similar experiments. (**B**) Lymphocytes were cultured for 48 hours in the presence or absence of *Bd* Sup and Nec-1; cell death was quantified by flow cytometry (PI and Annexin V-FITC). Splenocytes that were PI^- /Annexin V^+ were considered to be undergoing early apoptosis. Inset numbers indicate percent of cells in each quadrant. Each panel shows 10^4 events analyzed and represents one experiment two replicated experiments.

peptides which cannot be cleaved. One such inhibitor Z-VAD is recognized by all caspases and is a pan-caspase inhibitor. Due to the conservation of the active site and recognition sequence of caspases, peptide inhibitors used to inhibit mammalian caspases also inhibit amphibian caspases (Du Pasquier et al., 2006). Z-VAD treatment of *X. laevis* splenocytes significantly reduced the amount of apoptosis induced by *B. dendrobatidis* supernatants (Fig. 2-14 A, B) suggesting that cell death is mediated through caspases. Caspase activity of upstream caspase-8 and caspase-9 and downstream caspase-3 and caspase-7 was quantified using a luminescence detection assay. Caspase activity of extrinsic, intrinsic, and downstream caspases was significantly increased in splenocyte treated with *B. dendrobatidis* supernatant (Fig. 2-14 C-E). *Batrachochytrium dendrobatidis* supernatants induced significantly greater proliferation in splenocytes than known inducers of amphibian lymphocyte apoptosis (Rollins-Smith et al., 1997; Mangurian et al., 1998). Fas-specific antibodies activate the Fas receptor which induces apoptosis through the extrinsic pathway (Mangurian et al., 1998; Rathmell & Thompson, 2002). Corticosterone induces lymphocyte death by promoting intrinsic apoptotic pathway induction (Garvey et al., 1993; Rollins-Smith et al., 1997; Laakko & Fraker, 2002). *Batrachochytrium dendrobatidis* supernatants activated both caspase-8 and caspase-9 greater than no treatment and positive controls suggesting that factors induce both extrinsic and intrinsic apoptotic pathways. Caspase-8 can activate the intrinsic pathway by cleaving BID which then promotes cytochrome c release from the mitochondrion (Du Pasquier et al., 2006; Siegel, 2006). One factor present in *B. dendrobatidis* supernatants may activate caspase-8 in splenocytes which then can activate the intrinsic pathway and caspase-9 activation. Alternatively, multiple targets of either a single factor or multiple factors may induce apoptosis through both the intrinsic and extrinsic pathways.

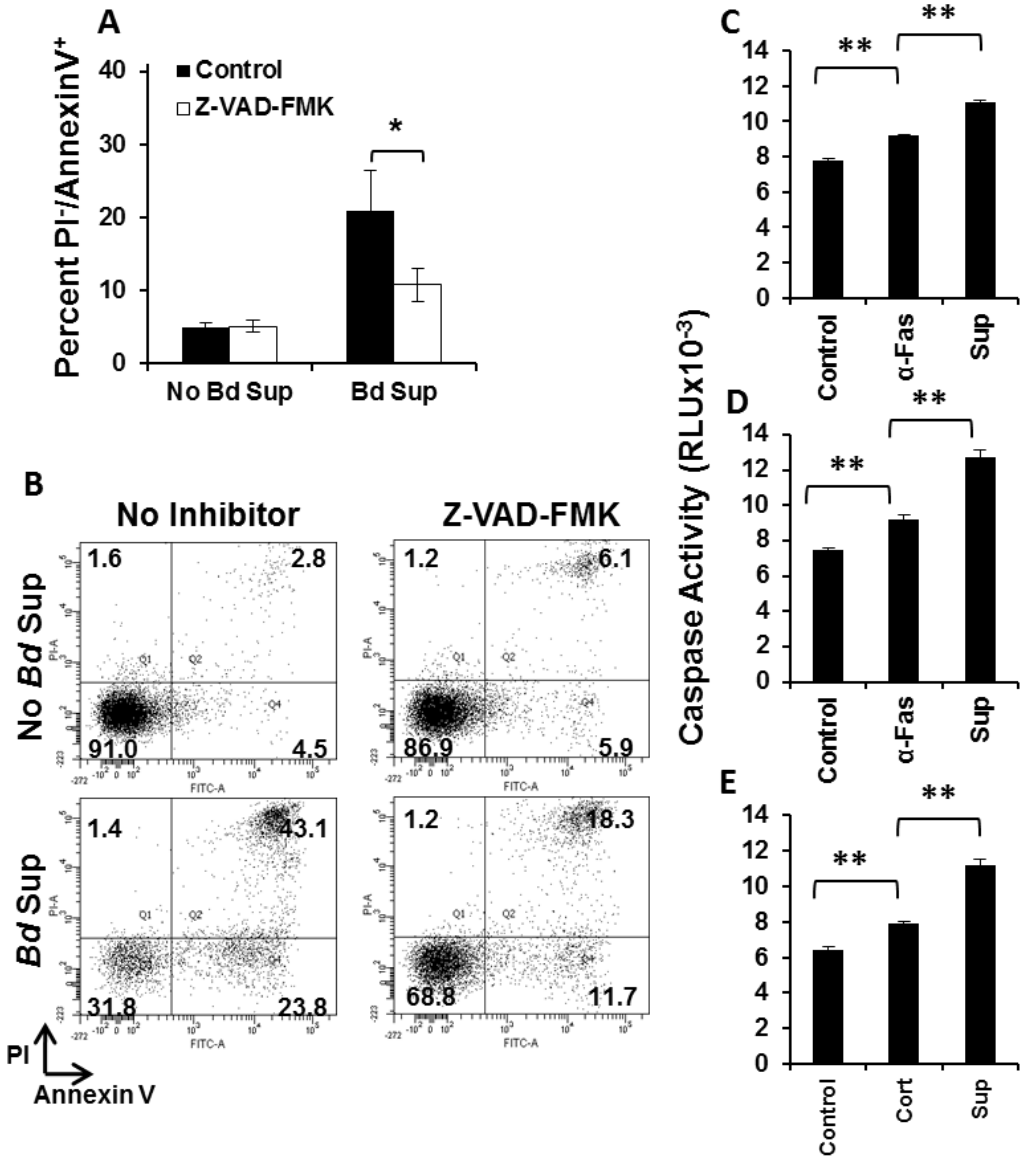

Fig. 2-14. *Batrachochytrium dendrobatidis (Bd)* supernatants activate caspases to induce apoptosis in splenocytes. (**A-B**) *X. laevis* splenocytes were cultured for 48 hours with or without *Bd* supernatant (Sup) and with or without Z-VAD-FMK (five experiments) and apoptosis was quantified by flow cytometry. Splenocytes that were PI^- /Annexin V^+ were considered to be undergoing early apoptosis. Mean percentage of apopototic cells (A) and representative flow plots from five experiments are shown. The apoptosis induced by *Bd* supernatant was significantly reduced by Z-VAD-FMK, *p< 0.05 by a paired Student's *t*-test. (**C-E**) Caspase activity assays for caspase-3/7 (D), caspase-8 (E), and caspase-9 (F) induced by *Bd* Sup, anti-Fas (α-Fas) mAb, or corticosterone (Cort) (representative of four experiments). *Bd* Sup treatments induced significantly greater caspase activity than that of splenocytes alone and of positive controls, **p<0.01 (ANOVA with post hoc test).

Both B and T cells are impaired by *B. dendrobatidis*.

In all lymphocyte proliferation assays except for the human $CD4^+$ T cell experiments, *B. dendrobatidis* cells or supernatants were cultured with a splenocyte population which mostly consisted of B and T lymphocytes but also contaieds accessory myeloid cells. Interactions among splenocytes may be responsible for the inhibition of B- and T-cell proliferation. To determine if *B. dendrobatidis* factors directly impact lymphocytes, B and T cells were isolated using magnetic beads coated in anti-IgM to bind to B cells or anti-CD5 to bind to T cells. Enriched B cells were stimulated with killed *A. hydrophila*, and enriched T cells were stimulated with PHA. The enriched B cell population did not proliferate in the presence of PHA, and the enriched T cell population did not proliferate in the presence of killed *A. hydrophila* (data not shown). When *B. dendrobatidis* cells were placed in co-culture with enriched B cells, there was a significant decrease in cellular proliferation (Fig. 2-15 A). The enrichment of B cells via the BCR induced B cell proliferation in the absence of killed *A. hydrophila*, and this activation before culture with *B. dendrobatidis* cells was significantly inhibited by *B. dendrobatidis*. Enriched T cells alone did not proliferate but had great proliferation in the presence of PHA; the PHA-induced proliferation of T cells was significantly inhibited by *B. dendrobatidis* in co-culture (Fig. 2-15 B). These data suggest that *B. dendrobatidis* factors directly interact with lymphocytes to inhibit activation.
To confirm whether *B. dendrobatidis* induces apoptosis in lymphocytes and determine differential effects on B and T cells, B and T cells were gated before quantifying apoptosis with flow cytometry. *Batrachochytrium dendrobatidis* cells in co-culture induced apoptosis in both B and T cells at 48 hours (Fig. 2-15 C). A large portion of T cells (CD5 positive splenocytes) underwent apoptosis in the presence of *B. dendrobatidis* cells. *Batrachochytrium dendrobatidis* induced an increased number of B cells to undergo apoptosis, but to a lesser degree

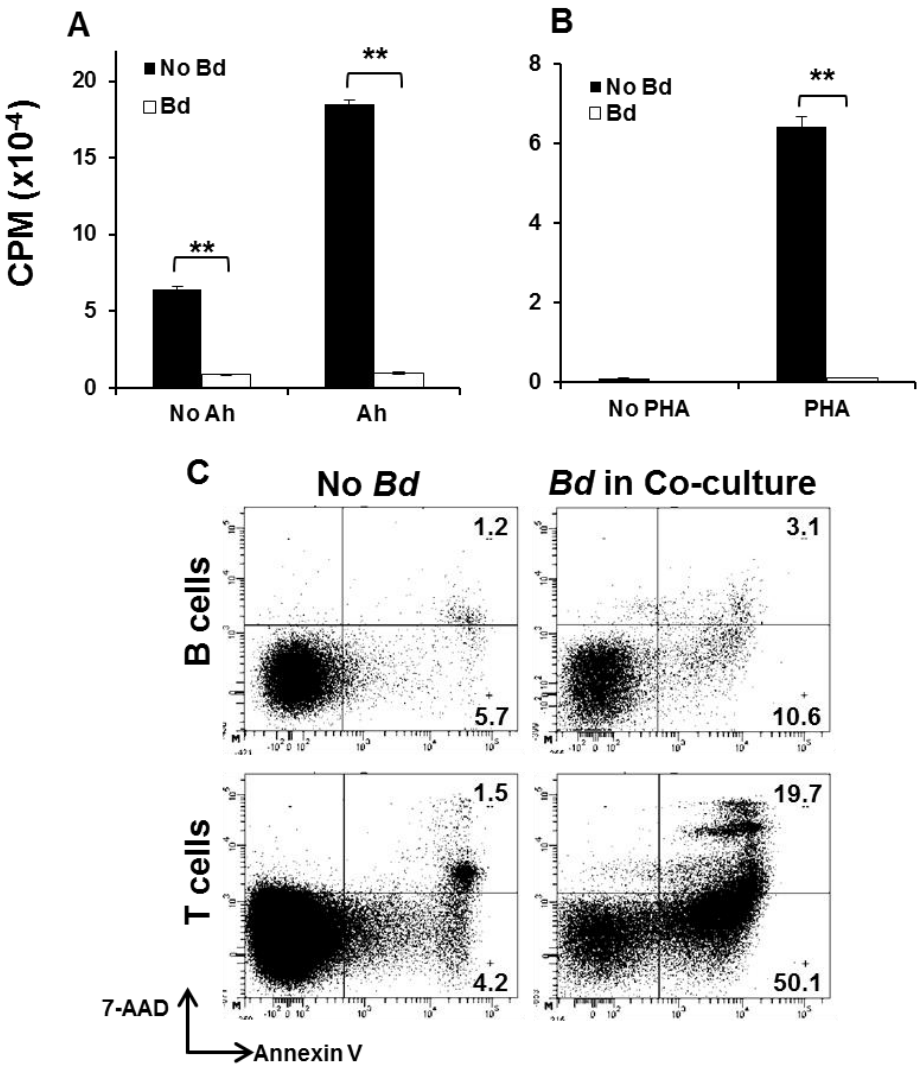

Fig. 2-15. *Batrachochytrium dendrobatidis (Bd)* inhibits B and T cells. (**A**) Enriched B cells were incubated with or without *A. hydrophila* (*Ah*) and with or without *Bd* cells. Note that B-cell proliferation without *A. hydrophila* was likely a result of activation due to engagement of the IgM receptors during purification. (**B**) Enriched T cells were incubated with or without PHA and with or without *Bd* cells. (A-B) Significant differences by Student's *t*-test comparing treatments grouped under brackets, **p<0.0001; p-values were multiplied by two to correct for multiple tests within the same experiment. CPM data are averages ± SEM of five or more replicate wells. (**C**) Splenocytes were cultured alone or co-cultured for 48 hours with heat-killed *Bd*. B and T cells were gated based on MHC class II, IgM, and CD5 positivity. Cells were also stained with 7-AAD, a viability stain, and Annexin V-FITC to determine apoptotic cells. Lymphocytes that were 7-AAD$^-$ /Annexin V$^+$ were considered to be apoptotic. Inset numbers indicate percent of cells in each quadrant. All panels are representative of two independent experiments. (Panel C completed by Jeremy Ramsey.)

compared to T cells. The target of *B. dendrobatidis* factors to induce B and T cell apoptosis may be different, or T cells may simply be more susceptible to the *B. dendrobatidis* factors.

Jurkat cells provide an *in vitro* system for studying *B. dendrobatidis* effects on lymphocytes.

Jurkat cells are a cell line derived from a human T lymphoma and act much like activated T cells (Schneider et al., 1977). The effect of chytrid supernatants on Jurkat cells was very similar to what was observed for amphibian and mouse lymphocytes (Fig. 2-16). *Batrachochytrium dendrobatidis* supernatants greatly inhibited Jurkat cell proliferation at even lower concentrations than those that inhibited amphibian lymphocytes (Fig. 2-16 A). *H. polyrhiza* supernatants only inhibited Jurkat cells at higher concentrations needing to be concentrated about 8 to 10-fold compared to *B. dendrobatidis* supernatants to see the same effect (Fig. 2-16 B). Jurkat cells offer a faster and cheaper way to assay the virulence of *B. dendrobatidis* on lymphocytes that does not require obtaining lymphocytes from a live animal.

An important application of using Jurkat cells to assay *B. dendrobatidis* virulence is to compare the relative virulence of different *B. dendrobatidis* isolates. Infection studies have demonstrated that *B. dendrobatidis* virulence is highly variable among isolates (Berger et al., 2005c, Retallick & Miera, 2007; Fisher et al., 2009). Much of the variation can be caused by genetic and species differences in the host, but there also appear to be differences in ability of different isolates to cause disease. Some of the variability in isolate virulence may be attributed to the capacity of an isolate to produce lymphotoxic factors. Two isolates that have been cultured in lab extensively, JEL197 and JEL275, show similar inhibition of *X. laevis* lymphocytes (Fig. 2-1). Determining the relative potency of this virulence feature of *B. dendrobatidis* will require many lymphocyte assays, too many to use amphibian splenocytes, so Jurkat cells would provide

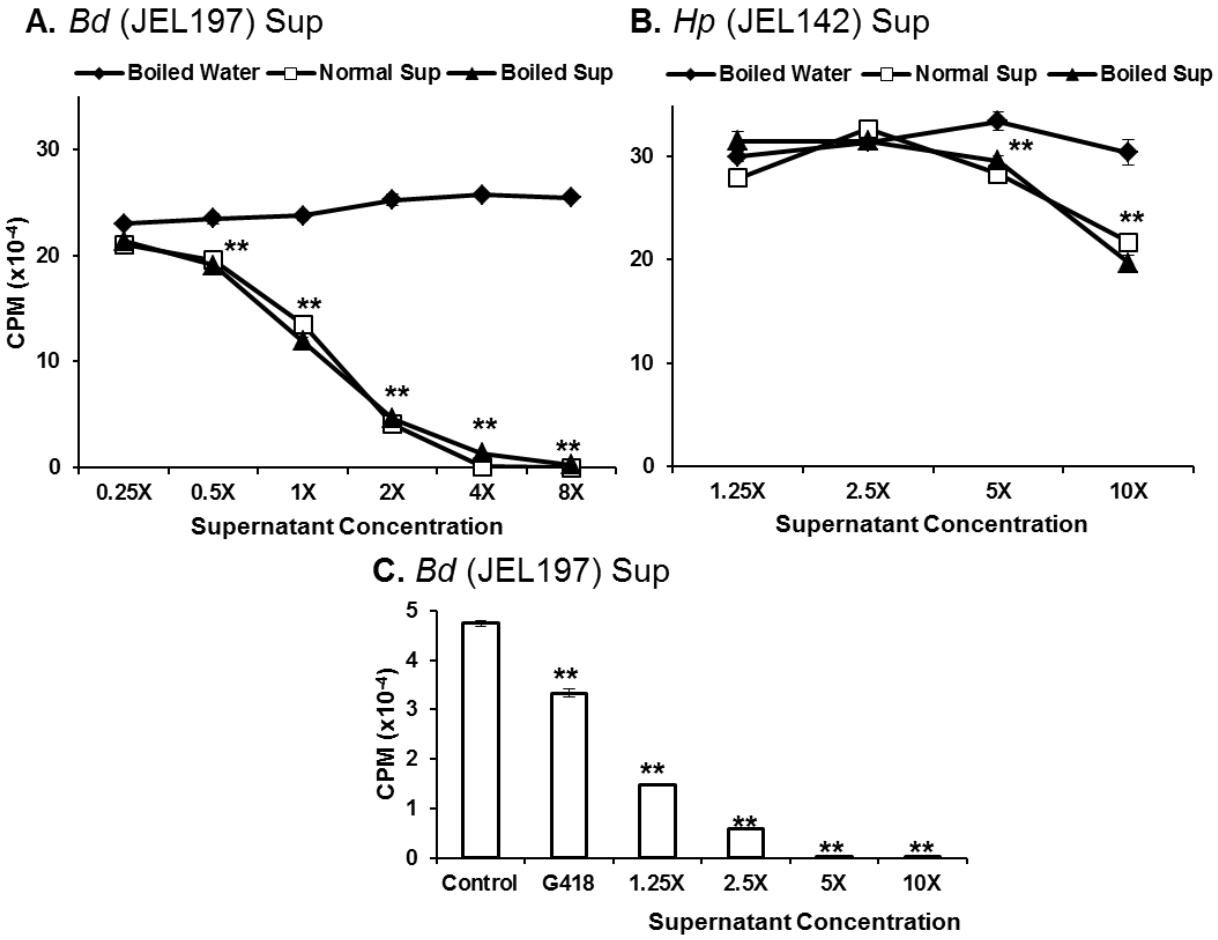

Fig. 2-16. Jurkat cells are inhibited by *B. dendrobatidis (Bd)* supernatants but not *H. polyrhiza* (*Hp*) supernatants. Jurkat cells were cultured for three days or without *Bd* (JEL197) or *Hp* (JEL142) supernatants (Sup) in a ^{3}H-thymidined proliferation assay at 37°C (A-B) or 26°C (C). (**A**) *Bd* Sup or water controls were either boiled or not, lyophilized, and re-suspended in RPMI to be mixed with Jurkat cells in a proliferation assay. (**B**) *Hp* Sup or water controls were either boiled or not, lyophilized, and re-suspended in RPMI to be mixed with Jurkat cells in a proliferation assay. (**C**) Jurkat cells were cultured at 26°C either alone (control) or in the presence of *Bd* Sup (not boiled) or 2 mg/ml G418. (A-B) There was no significant difference between boiled and not boiled supernatants, and significant inhibition of proliferation compared to the boiled water control is indicated, **$p<0.01$. (C) All treatments had significantly decreased proliferation compared to the untreated control, **$p<0.01$. For all panels, a one-way ANOVA was used for statistical comparisons, individual treatment comparisons were analyzed by Tukey post-hoc tests. Each panel is representative of three similar experiments.

a way to assay multiple isolates. Supernatants from various isolates can be obtained, but this is time consuming and may not fully reflect the ability of each isolate. To determine relative inhibition, *B. dendrobatidis* cells may need to be co-cultured with Jurkat cells. Despite the low thermal maximum of *B. dendrobatidis* (Woodhams et al., 2003), co-culture assays can be conducted with Jurkat and living *B. dendrobatidis* cells at 26° C. Proliferation of Jurkat cells and inhibition by *B. dendrobatidis* supernatants at 26° C reflected the results at 37° C (Fig. 2-16 C) suggesting that assays co-culturing Jurkat with living *B. dendrobatidis* cells are possible. Some work has begun to test the effects of various *B. dendrobatidis* isolates on Jurkat cells, and we have observed significant variation in the capacity of isolates to impair lymphocytes (data not shown).

If a high through-put assay were developed to analyze inhibition by various *B. dendrobatidis* isolates, treatments of *B. dendrobatidis*, or even *B. dendrobatidis* mutants, it would need to be consistent, fast, and inexpensive. Jurkat cells provide a good system for high through-put analysis, but the proliferation assay using ^{3}H-thymidine can be expensive. An alternative assay is the MTT assay which uses the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) that is reduced to a purple crystal by reducing enzymes present in the mitochondria of active cells (van Meerloo et al., 2011). The MTT assay is a simple and inexpensive method for quantifying the number of viable cells. Inhibition of Jurkat cells by *B. dendrobatidis* and *H. polyrhiza* supernatants was quantified using the MTT assay, and a nearly identical dose response was observed in the MTT assay as was observed with the ^{3}H-thymidine proliferation assay (Fig. 2-17).

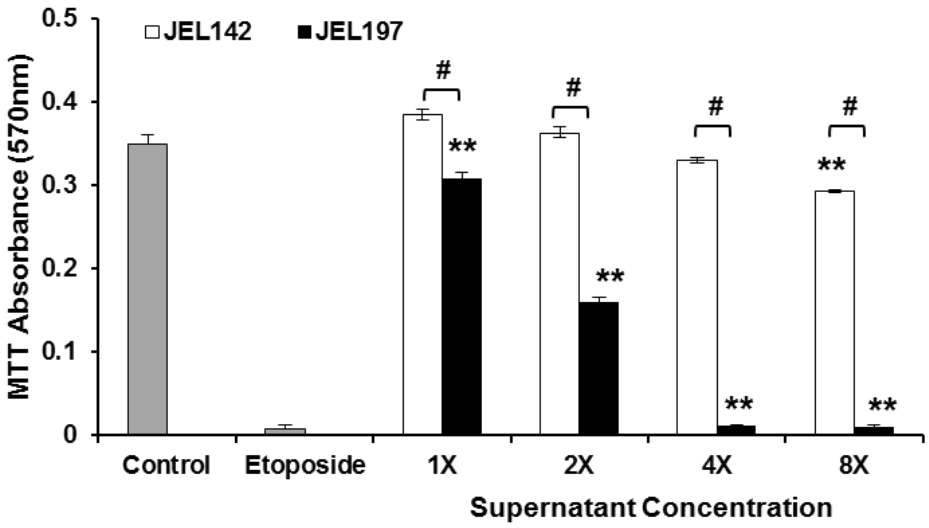

Fig. 2-17. The MTT assay functions to assay inhibition of Jurkat cells by chytrid supernatants. Supernatants (not boiled) from *Batrachochytrium dendrobatidis (Bd*, JEL197) or *Hp* (JEL142) were re-suspended at different concentrations and incubated with Jurkat cells. After three days of incubation at 37°C Viability of Jurkat was quantified by the MTT assay reading absorbance at 570 nm. Wells not receiving supernatant (gray bars) either received RPMI alone (Control) or 12.5 µg/ml etoposide (as a negative control). *Bd* supernatants at all concentrations were significantly more inhibitory than *Hp* supernatants, $^{\#}p<0.01$. Supernatants that significantly inhibited Jurkat cells are indicated, $^{**}p<0.01$, one-way ANOVA with Tukey post-hoc tests for individual comparisons. Data are representative three similar experiments.

Discussion

Batrachochytrium dendrobatidis has evolved a mechanism to inhibit host lymphocyte responses

Many studies have observed poor immune responses against *B. dendrobatidis* by amphibians (Pessier et al., 1999; Berger et al., 2005b; Rosenblum et al., 2009; Rollins-Smith et al., 2009; Stice & Briggs, 2010; Rosenblum et al., 2012a). Because amphibians have a well-equipped adaptive immune system that is also present in the skin (Ramanayake et al., 2007; Robert & Ohta, 2009; Rollins-Smith et al., 2009), *B. dendrobatidis* seems to have developed ways to inhibit protective immune responses in the skin. Lymphocyte responses, particularly T cell responses, are essential for clearance of fungal infections (Wüthrich et al., 2012a). An evasion strategy that impairs and kills lymphocytes would promote colonization and replication of a fungal pathogen. Data in this chapter characterize such a mechanism by *B. dendrobatidis* to inhibit host adaptive immune responses by inhibiting proliferation and inducing apoptosis in lymphocytes. Defects in lymphocyte function are mediated by soluble factors released by *B. dendrobatidis* which may be shed by *B. dendrobatidis* during infection to prevent robust immune clearance.

Several fungi are known to produce molecules that inhibit lymphocyte responses (see Table 1-2). Gliotoxin is released by *Aspergillus fumigatus* and kills lymphocytes by activating the intrinsic apoptosis pathway (Fox & Howlett, 2008; Ben-Ami et al., 2010). *Cryptococcus* species have two immunomodulatory polysaccharides in the capsule: glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (De Jesus et al., 2010). Phagocytosis of GXM by macrophages and dendritic cells impairs inflammatory responses and promotes expression of Fas ligand which can induce apoptosis in lymphocytes (Monari et al., 2005; Piccioni et al., 2011).

GalXM directly induces apoptosis in lymphocytes (De Jesus et al., 2009; Pericolini et al., 2009). The *Blastomyces dermatitidis* adhesion factor, BAD-1, necessary for much of the virulence of the fungus has recently been shown to also impair T cell activation (Brandhorst et al., 2013). Some evidence also suggests that *Paracoccidioides brasiliensis* can suppress lymphocytes (Campanelli et al., 2003; Bordon et al., 2007).

Like other pathogenic fungi, *B. dendrobatidis* has evolved mechanisms to inhibit lymphocytes. This adaptation was probably important in the transition to be an amphibian pathogen. *B. dendrobatidis* infects an entire class of vertebrates. It is known to infect several hundred species of amphibians including frogs, salamander, and caecelians (Olson et al., 2013; Doherty-Bone et al., 2013). *B. dendrobatidis* has the capacity to infect many hosts because the shared adaptive defenses are impaired during infection. The inhibition of lymphocytes by *B. dendrobatidis* explains why resistant species still do not clear infection (Ramsey et al., 2010; Mazzoni et al., 2003) and why certain innate immune defense are so important in resistance to chytridiomycosis (Rollins-Smith et al. 2011).

The presence of inhibitory factors even on killed *B. dendrobatidis* cells may explain the absence of protection in amphibians immunized with killed *B. dendrobatidis* (Rollins-Smith et al., 2009; Stice & Briggs, 2010). During these immunizations there may be some adaptive response to *B. dendrobatidis* antigens, but the responses may be dampened by the lymphotoxic factors released by *B. dendrobatidis*. Immunization may be a useful strategy to protect highly susceptible amphibian populations, but a successful vaccine must be developed. Killed fungi have not been successful vaccines in mouse models (Cassone & Casadevall, 2012), but this may be overcome with appropriate adjuvants (Wüthrich et al., 2013). An attenuated *B. dendrobatidis*

that does not produce these lymphotoxic factors could be used to promote long-lasting immunity in amphibians without causing disease.

The use of Jurkat cells will simplify and hasten the investigation of the *B. dendrobatidis* inhibitory factors. Analysis of various *B. dendrobatidis* isolates from around the world is currently proceeding. Isolates from the CH (or Swiss) strain of *B. dendrobatidis* will be tested on Jurkat cells to determine if this strain can inhibit lymphocytes as robustly as *B. dendrobatidis* from the global panzootic lineage (GPL). The GPL *B. dendrobatidis* has been proposed to be highly virulent compared to other isolates declared as enzootic that have been found in Switzerland, Brazil, South Africa, and east Asia (Farrer et al. 2011; Bai et al., 2012; Farrer et al., 2013; Rosenblum et al., 2013). Falling outside of the GPL, CH *B. dendrobatidis* isolates are proposed to lack much virulence; therefore, these isolates may have decreased production of lymphotoxic factors. If isolates outside of the GPL had decreases capacity to inhibit lymphocytes, then genetic analysis may provide insight to the genes responsible for synthesis and release of these factors. Enzootic isolates are also good candidates for attenuated vaccination strategies.

Possible use of *B. dendrobatidis* factors for human therapies and medical research

I observed that *B. dendrobatidis* factors impair mammalian lymphocytes and immortal cell lines. Although the factors responsible have not been identified yet and there may be different factors responsible for different aspects of cellular inhibition, these factors appear to inhibit and potentially kill proliferating cells. If proliferating cells are the target, then these factors would be beneficial for treating or at least studying diseases of cellular proliferation such as cancer and autoimmunity. Because the *B. dendrobatidis* supernatants are a crude mixture of

anything shed from *B. dendrobatidis* in a 24-hour time period, there are probably multiple factors that may have differential effects separately inhibiting cellular proliferation, targeting lymphocytes, and inducing apoptosis. If specific factors only target lymphocytes inhibiting activation or inducing apoptosis, then these factors would definitely be useful for preventing transplant rejection and limiting autoimmunity. Polysaccharide factors from *Cryptococcus*, GXM and GalXM, have been proposed for use in treating autoimmune disorders (Vecchiarelli & Monari, 2012). GalXM, in particular, appears to have great potential in treating rheumatoid arthritis (Pericolini et al., 2013). Once the *B. dendrobatidis* factors are identified and the mechanism of action clarified, they may be important drugs used to study or treat human diseases.

CHAPTER III

FACTORS FROM *BATRACHOCHYTRIUM DENDROBATIDIS* IMPAIR ADAPTIVE IMMUNITY BUT NOT INNATE IMMUNITY *IN VITRO* AND *IN VIVO*[2]

Abstract

Innate immune cells, especially phagocytes, play an important role in detection, antigen presentation, and pathogen clearance during a fungal infection. In order for *Batrachochytrium dendrobatidis* to avoid being cleared, it may evade phagocyte recognition. *B. dendrobatidis* produces soluble factors that impairing lymphocytes *in vitro* (Chapter II of this thesis). Inhibition of lymphocytes would significantly decrease the robust phagocyte response mediated mostly by T cell cytokines but would not prevent early innate recognition by phagocytes. If the *B. dendrobatidis* inhibitory factors broadly impair leukocytes, then *B. dendrobatidis* would essentially prevent any protective immune response inside the skin. *B. dendrobatidis*, however, does not appear to impair phagocytosis, viability, or accessory activities of amphibian macrophages and neutrophils *in vitro*. This suggests that the soluble factors released by *B. dendrobatidis* are directed to inhibiting lymphocyte-mediated responses and do not have an effect on phagocytes. To confirm this hypothesis, *B. dendrobatidis* supernatants were injected into *Xenopus laevis* to determine the effects of the soluble factors on immune responses. Inducers of inflammation such as killed bacteria and phytohemagglutinin (PHA) can be used to follow inflammatory responses *in vivo*. Here, a modified protocol for injection of PHA or killed bacteria was developed employing intramuscular injection into the foot region of *X. laevis*. This

[2] Some of the data in this chapter is published in Fites et al., 2013.

protocol induced much greater swelling than previous studies using subcutaneous injection of PHA into amphibians. Using this new protocol, we tested whether *B. dendrobatidis* supernatants would alter innate and adaptive immune responses induced by injection of killed bacteria or PHA. As previously described *in vitro*, factors from *B. dendrobatidis* inhibited lymphocyte-mediated swelling (a delayed-type hypersensitivity response) induced by PHA but not swelling caused by killed bacteria. Investigation of the systemic effects of *B. dendrobatidis* suggest that infection does not cause general immune suppression but rather inhibits lymphocytes in a localized fashion to prevent adaptive immune responses in the skin.

Introduction

Adaptive immune responses, particularly mediated by T lymphocytes, are essential for the clearance of fungal pathogens (Wüthrich et al., 2012a). Immunity to the lethal amphibian-infecting chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), also appears to require lymphocyte responses (Ramsey et al., 2010; Savage and Zamudio, 2011). *B. dendrobatidis* appears to overcome host immunity in the skin by producing inhibitory factors that impair lymphocytes (Chpater II). This immune evasion strategy employed allows *B. dendrobatidis* to infect immunocompetent hosts and explains why species lacking innate mucosal immune defenses are so susceptible to chytridiomycosis (Rollins-Smith et al., 2011).

Innate leukocytes play an important role in immunity to fungi (Brown, 2011; Roy & Klein, 2012). Dendritic cells (DCs) are essential for presenting antigen to activate lymphocyte responses. Macrophages and neutrophils play an important role in killing fungi to clear infection. Other innate leukocytes play important roles in activating inflammatory responses and promoting

immune responses by producing cytokines (Kawakami et al., 2001; Cohen et al., 2011; Urb & Sheppard, 2012), but phagocytes are the most important leukocytes to both promote inflammation and clear fungal infection. In mammals, protective T cell responses (typically Th1 and Th17) require priming by DCs and then activate and recruit macrophages and neutrophils to the sites of infection to resolve fungal infection (Wüthrich et al., 2012a).

During fungal infections of the skin, DCs play an essential role in promoting immune responses and presenting antigen to T cells (Ersland et al., 2010; Igyártó et al., 2011). The presence of certain populations of DCs also determines the type of response against the fungal infection. In particularly monocyte-derived DCs classified as 'inflammatory' play an essential role in promoting inflammatory immune responses in infected tissues (Hohl et al., 2009; Wüthrich et al., 2012b).

Macrophages and neutrophils are typically very important in clearing fungal infections. When supported by helper T cell responses, these phagocytes are heavily recruited and are activated to kill fungal pathogens. Th1 responses promote classical activation of macrophages which promote antimicrobial killing inside of phagosomes and "nutritional immunity" (Brown, 2011; Subramanian Vignesh et al., 2013). Th17 responses promote recruitment of neutrophils and macrophages and activate production of antimicrobials by both phagocytes and epithelial cells (Romani, 2011; LeibundGut-Landmann et al., 2012; Wüthrich et al., 2012a).

Unlike lymphocytes, phagocytic immune cells are present in animals outside of jawed vertebrates and are important components of arthropod immunity (Stuart & Ezekowitz, 2008; Grigorian & Hartenstein, 2013). Amphibians are known to possess cells morphologically and functionally identical to the macrophages, neutrophils, and DCs present in the mammalian immune system (Robert & Ohta, 2009; Rollins-Smith et al., 2009). Phagocytes are also known to

be an important component of amphibian skin immunity. Langerhans cells (LCs) are a DC subset important in skin immunity and present in amphibian skin (Carrillo-Farga et al., 1990; Castel-Rodriguez et al. 1999; Mescher et al., 2007). Also, histological analysis of immune responses in amphibian skin has identified infiltration of cells with phagocyte features (Ramanayake et al., 2007). The role of phagocytic leukocytes in chytridiomycosis is poorly understood. Very little infiltration of leukocytes occurs into the skin during *B. dendrobatidis* infection (Pessier et al., 1999; Berger et al., 2005b). The absence of phagocytic cells likely decreases clearance of *B. dendrobatidis*, but *B. dendrobatidis* also avoids phagocytic detection by direct infection of keratinocytes (Berger et al., 2005b). *B. dendrobatidis* enters host cell via germination tubes (Van Rooij et al., 2012; Greenspan et al., 2012) which likely minimize recognition by phagocytes in the skin. Phagocytes may play important roles in recognizing and clearing *B. dendrobatidis*, but it is possible that *B. dendrobatidis* completely avoids phagocytes inside of host epithelial cells.

Due to the current lack of molecular tools in amphibian immunology and the absence of genetic manipulation in *B. dendrobatidis*, the *in vivo* interactions between *B. dendrobatidis* and host cells are difficult to investigate. *Xenopus laevis* is one of the best vertebrate models of immunology yet lacks many of the antibodies to follow cell populations and cytokines that are likely to be important in immunity to chytridiomycosis (Robert & Cohen, 2011). Therefore, investigations of amphibian immunity, particularly outside of the model *Xenopus* species, must rely on primitive immunological techniques. Amphibian leukocytes respond to chemical and cellular reagents similar to other vertebrates. Intraperitoneal injection of killed *Escherichia coli* into *X. laevis* induces infiltration of phagocytes into the peritoneum (Nedelkovska et al., 2010). Phytohemagglutinin, phorbol-12-myristate 13-acetate, concanavalin A, and killed bacteria also

have mitogenic effects on amphibian B and T cells (Rollins-Smith et al., 1984; Hsu et al., 1985; Morales et al., 2003; Chapter II of this thesis).

Phytohemagglutinin (PHA) injection is a common method used to investigate immunocompetence in non-model vertebrates. This technique is frequently used in birds. PHA is injected subcutaneously in avian patagia (wing-webs), and inflammation is measured by swelling at the site of injection (Martin et al., 2006). PHA induces robust T cell proliferation *in vitro* in amphibians (Rollins-Smith et al., 1984; Chapter II), and can also induce *in vivo* inflammatory swelling in adults, metamorphs, and tadpoles (Gilbertson et al., 2003; Gervasi & Foufopoulos, 2008; Brown et al., 2011; Venesky et al., 2012). *In vivo*, PHA induces swelling through the recruitment of many different cell types including granulocytes, phagocytes, thrombocytes, and lymphocytes (Martin et al., 2006; Brown et al., 2011). The kinetics of leukocyte recruitment typically begins with infiltration of neutrophil/heterophils and macrophages and is later followed by lymphocyte infiltration. Significant lymphocyte, primarily T cell, recruitment and activation induced by PHA typically requires a second injection of PHA (Tella et al., 2008; Brown et al., 2011). Evaluation of T cell responses using PHA injection likely requires priming because the inflammation of the primary response is overwhelmingly comprised of innate phagocytes and granulocytes.

In amphibians, studies have used different PHA injection sites including the toe (Gilbertson et al., 2003), the thigh (Gervasi & Foufopoulos, 2008), and toe webbing (Brown et al., 2011). Subcutaneous injection of PHA into amphibians induces less than a millimeter of swelling compared to buffer controls with most recording differences of near 0.1 mm. Such measurements require very precise tools and handling and may not represent a biologically significant response. In pilot experiments with large *X. laevis*, no noticeable or measurable

difference in swelling occurred after PHA injection, so a new technique using PHA injected intramuscularly into the foot was adopted. The middle of the foot was chosen for injection because it was easiest to measure with a caliper and showed the greatest inflammatory swelling compared to sites on the toes and ankle.

To determine the effects of *B. dendrobatidis* on amphibian immune responses *in vivo*, the foot injection method was developed to follow immune responses. Primarily innate responses were induced with killed *E. coli* which is known to induce phagocyte infiltration (Nedelkovska et al., 2010). PHA was used to follow a mixed response characterized by induction of a primarily phagocyte response following a single injection and induction of a mixed lymphocyte and phagocyte response following a second injection (Tella et al., 2008; Brown et al., 2011). *B. dendrobatidis* supernatants were injected into *X. laevis* with PHA or killed *E. coli* to determine if inhibition of amphibian immunity also occurred *in vivo*.

Data in this chapter show that *B. dendrobatidis* does not inhibit amphibian phagocytes obtained from the peritoneum of *X. laevis*. The viability and function of theses phagocytes, determined to be mostly macrophages and neutrophils, were not impaired by *B. dendrobatidis* cells or supernatant. This suggests that the *B. dendrobatidis* factors responsible for lymphocyte impairment have no negative effect on innate immunity. The observation of inhibition of adaptive but not innate immunity *in vitro* was replicated *in vivo* by injecting PHA or killed bacteria to induce inflammatory swelling. The *in vivo* experiments suggest that *B. dendrobatidis* inhibits adaptive immune responses in a local manner. To determine the systemic effects of *B. dendrobatidis* on immunity, the effect of infection or intraperitoneal injection of *B. dendrobatidis* supernatant was investigated on splenocyte cell numbers. At least in these preliminary experiments in *X. laevis*, *B. dendrobatidis* does not appear to impair immunity systemically.

Materials and Methods

Obtaining leukocytes from the *X. laevis* peritoneum

Peritoneal leukocytes (PLs) were obtained via peritoneal lavage as described previously (Morales et al., 2010; Nedelkovska et al., 2010). Briefly, *Escherichia coli* (strain DH 5α) was grown to a concentration of approximately 5.7 x 10^8 colony forming units (CFUs)/mL. *E. coli* was boiled for one hour in a water bath, washed twice, and re-suspended in sterile APBS to 3.2 x 10^{10} killed CFUs/mL. Re-suspended *E. coli* (300 μL, 9.7 x 10^9 killed CFUs) were injected intraperitoneally (i.p.) into *X. laevis* individuals ranging in mass between 35-70 g. Three days later, the same individuals were anesthetized, and 10 mL of APBS were injected into the peritoneum. This volume was drained from the peritoneum using an 18G needle. PLs were centrifuged and re-suspended at 2.0 to 4.0 x 10^6 in complete L-15. To determine relative numbers of leukocytes present, subsets of PLs were cytocentrifuged onto glass slides and stained with either Hema 3 manual staining kit (Fisher, Waltham, MA, USA) or Accustain® Wright-Giemsa stain (Sigma, St. Louis, MO, USA). Leukocytes were viewed and photographed with a Nikon Eclipse 90i microscope with a Nikon DS-Fi camera (Nikon Instruments, Melville, NY, USA).

Treating PLs with supernatants from *B. dendrobatidis* and *H. polyrhiza*

PLs were incubated overnight at 26°C in 24-well plates at 10^6 cells/mL (in volumes of 1.0 to 1.4 mL) in L-15 alone or L-15 containing *B. dendrobatidis* (JEL197) or *H. polyrhiza* (JEL142) supernatant at 10X concentration (see Chapter II Materials and Methods). After incubation PLs were either prepared for mixing with lymphocytes or for flow cytometry to quantify cell death by PI nuclear staining as described in Chapter II Materials and Methods.

Mixing supernatant-treated PLs with enriched lymphocytes

One day following peritoneal lavage, the same *X. laevis* individuals were euthanized in order to obtain splenocytes as described above. Accessory splenocytes (macrophages or dendritic cells) were removed from the splenocyte population by glass adherence. Briefly, splenocytes in warm L-15 were incubated on pre-warmed glass Petri dishes for one hour at 26°C. Cells not adhering to glass were pipetted off and saved. PLs treated with *B. dendrobatidis* supernatant or cultured in L-15 alone overnight were washed in L-15 and mixed with enriched lymphocytes in 96-well plates. Wells contained 5 to 7.5 x 10^4 lymphocytes alone or lymphocytes plus 2.5 x 10^4 PLs from the same *X. laevis* individual. Wells receiving PHA to stimulate lymphocyte proliferation contained a sub-optimal concentration of 100 ng/mL PHA. Lymphocytes and PLs were incubated for three days at 26° C and pulsed with ^{3}H-thymidine one day before harvesting as described in Chapter II Materials and Methods.

Measuring phagocytosis in PLs treated with supernatant

Phagocytosis in PLs was followed using pHrodo Green™ Zymosan Bioparticles® (Molecular Probes, Life Technologies Corporation, Carlsbad, CA, USA). Zymosan is a particulate form of the cell wall of *Saccharomyces cerevisiae* and mimics a fungal cell for phagocytic recognition (Goodridge et al., 2011; Elsori et al., 2011). Zymosan was conjugated to pHrodo™ green fluorescent dye which has peak fluorescence at about pH 4.0 and thus only fluoresces within phagosomes/endosomes (Neaga et al., 2013). PLs at 2.0 x 10^6 cells/mL (50µl, 1.0 x 10^5 cells/well) were incubated at 26° C with or without *B. dendrobatidis* supernatant at 10X concentration for 2 or 24 hours. After incubation, pHrodo Green™ Zymosan Bioparticles® (50 µl of 500 µg/mL in APBS) were added to PLs or cultured in L-15 alone and incubated for

two hours at 26°C. Fluorescence in wells was quantified using a Synergy 2 Multi-Mode Micoroplate Reader (BioTek, Winooski, VT, USA) with excitation wavelength of 485 nm and emission spectrum measured at 528 nm. Control wells receiving APBS alone were used to subtract out background fluorescence of L-15 media and autofluorescence of cells. After quantifying fluorescence, PLs were allowed to adhere to glass slides, fixed with 4% formaldehyde in APBS, and stained with Hoechst 33342 (Molecular Probes, Life Technologies Corporation) (King & Hillyer, 2013). Cells were viewed using a Nikon Eclipse 90i microscope (Nikon Instruments, Melville, NY, USA) and photographed using a CoolSNAP HQ^2 camera (Photometrics, Tucson, AZ, USA).

In vitro phagocytosis of *B. dendrobatidis* cells by *X. laevis* PLs

B. dendrobatidis was cultured for 3-4 days at 21°C on 1% tryptone agar plates. These plates were flooded with 1% tryptone broth for 20 minutes to promote the release of zoospores from sporangia adherent to the agar (Rollins-Smith et al., 2002). Liquid broth was removed from plates, and *B. dendrobatidis* cells were re-suspended at 5.0×10^5 cells per mL (this population had about 70% zoospore purity with many germlings and some thalli, see Appendix) and moved to culture flasks containing tryptone broth for three days at 21°C. The *B. dendrobatidis* obtained after this second incubation contained a balanced mixture of *B. dendrobatidis* cell types containing zoospores, germlings, thalli, and sporangia (Longcore et al., 1999; Berger et al., 2005a). These cells were centrifuged and re-suspended at a known concentration in L-15; a subset of *B. dendrobatidis* was incubated at 60°C to kill the cells. *X. laevis* PLs were obtained as described above and mixed with *B. dendrobatidis* cells at a ratio of either 2:1 or 1:1 PLs to *B. dendrobatidis* cells in L-15. PLs were incubated with *B. dendrobatidis* for either 2 or 24 hours at

26°C. To visualize phagocytosis, PLs mixed with *B. dendrobatidis* were cytocentrifuged onto glass slides and stained with either Hema 3 (Fisher, Waltham, MA, USA) or Wright-Giemsa (Sigma, St. Louis, MO) stains and examined by microscopy.

Foot injection and measurement

Female *X. laevis* ranging in size between 110-225 g were used for foot injection experiments. During injection experiments, frogs were housed individually in polystyrene containers so that the foot swelling could be measured in a blinded fashion. Before foot injection and measurements, frogs were anesthetized in ethyl-m-aminobenzoate methanesulfonate salt (MS-222) (Biomedicals, Solon, OH, USA) at 5 g/L until all movement ceased and then immediately placed in de-chlorinated water to wash off anesthetic. After measurements were taken, frogs were carefully observed in fresh de-chlorinated water until able to make voluntary motion. Due to the toxic effects of prolonged exposure to MS-222 causing skin peeling and discoloration and eventually death, individuals were only anesthetized up to three times. Animal procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine. The site and depth of injection were determined based on pilot injections of PHA and killed bacteria into sites in the foot from the ankle to the toes. Subcutaneous injection yielded insignificant swelling and intramuscular injection of the foot caused more swelling than injection in to the ankle or toes. Following this optimization, injections were made intramuscularly into the feet of *X. laevis* in the middle of the plantar side of the foot (Fig. 3-1) using 1 cc syringes with 25 or 30 gauge needles. Dimensions of the foot were

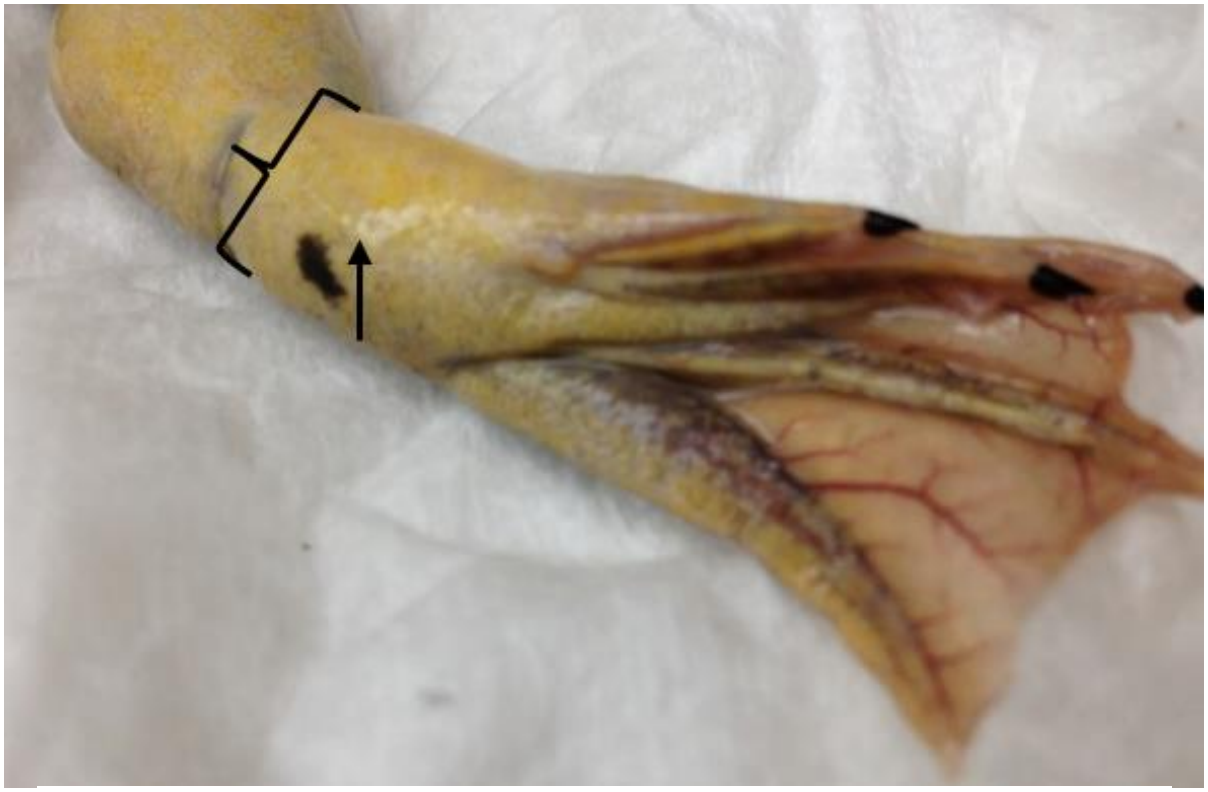

Fig. 3-1. *Xenopus laevis* feet were injected intramuscularly in the middle of the foot on the plantar side (arrow). Measurements of the foot thickness (bracket) and width (perpendicular to the thickness) were recorded with a caliper.

measured to the nearest 0.1 mm using a Mitutoyo plastic digital , model 4LB11 (Mitutoyo USA, Morgan Precision Tools, Aurora, IL, USA). Foot width (in the plane of the foot) and foot thickness (perpendicular to the plane of the foot) were measured (Fig 3-1). The peak time of inflammatory swelling induced by PHA in amphibians is typically around 24 hours (Gilbertson et al., 2003; Gervasi & Foufopoulos, 2008; Brown et al., 2011); therefore, foot dimensions were measured before injection and 24 hours after injection for all experiments. Before injection, the average foot width was 11.69 ± 0.08 mm (mean ± SEM), and the average foot thickness was 11.24 ± 0.08 mm (mean ± SEM). Foot dimensions were also measured at 48 hours to follow the kinetics of PHA-induced swelling.

Blinded, randomized experimental design for foot injections

Injection treatments were randomly assigned by a coin flip to the right or left foot after the initial measurements were taken. Based on the coin flip, one foot received the control treatment and the other received the experimental treatment. Before measurements were taken at 24 and 48 hours post-injection, frogs were randomly reassigned new identification so that the person measuring the foot dimensions was blinded to the injection treatment.

Injection of PHA into feet

To determine if PHA induces inflammatory swelling in feet, 100 µL PHA-P (Sigma, St. Louis, MO, USA) at a concentration of 2 mg/mL in APBS or APBS alone was injected into the right or left foot of six individual *X. laevis*. Seven days before foot injection with PHA and *B. dendrobatidis* supernatant, *X. laevis* were or were not primed with 100 µL of 1 mg/mL PHA in APBS by intra-peritoneal (i.p.) injection. When PHA (1 mg/mL) was injected with *B.*

dendrobatidis supernatants (10X), 200 µL of either PHA alone or PHA with *B. dendrobatidis* supernatant (10X) diluted in APBS were injected into the feet of 6 (no PHA priming) or 12 (PHA priming) individual *X. laevis*.

Injection of killed *Escherichia coli* into feet

E. coli was prepared as previously described above. Bacteria were grown to a known concentration overnight, boiled for one hour, washed and re-suspended in APBS. To verify that injection of heat-killed *E. coli* induces inflammatory swelling, 200 µL of either APBS or *E. coli* in APBS were injected into the feet of six individual *X. laevis*. When heat-killed *E. coli* was injected with *B. dendrobatidis* supernatants, 200 µL of either killed *E. coli* alone or killed *E. coli* with *B. dendrobatidis* supernatant were injected into feet of 12 individual *X. laevis*. The concentration of *E. coli* in injections was 10^{10} killed colony forming units per mL. The concentration of *B. dendrobatidis* supernatant in the injections was ten times the concentration before lyophilization, 10X.

Correlating *B. dendrobatidis* infection with spleen size

Spleens were harvested from *X. laevis* to obtain a lymphocyte population to investigate the effects of *B. dendrobatidis* on amphibian lymphocytes (see Chapter II). The number of splenocytes from each individual's spleen was counted and recorded after spleens were dissociated and splenocytes were enriched by centrifugation over a Ficoll gradient. To normalize the number of splenocytes among frogs of different sizes, the total number of splenocytes was divided by the mass, in mg, of the frog.

To keep track of the *B. dendrobatidis* infection status in the colony, a number of these frogs were swabbed before euthanasia (Ramsey et al., 2010). DNA was extracted from these

swabs to determine the number of zoospore equivalents on the skin according to the methods of Boyle et al. (2004) and Hyatt et al. (2007) by quantitative PCR. PCR conditions and standard curves were as described by Ramsey et al. (2010).

Intraperitoneal injection of *B. dendrobatidis* supernatants

To determine whether or not *B. dendrobatidis* can systemically inhibit amphibian immunity, a pilot experiment was developed in which *B. dendrobatidis* supernatant suspended in APBS or APBS alone was injected i.p. into *X. laevis*. For this pilot, each experimental treatment group had three individuals ranging in size between 33.9 and 76.1 g. The treatment groups each had two males and one female of approximately the same size. Each individual was either injected i.p. with 10 μL per gram body mass of APBS or 5X *B. dendrobatidis* supernatant in APBS at day 0, 3, and 5. The supernatants were boiled before lyophilization (see Chapter II Materials and Methods) and filter sterilized after re-suspending in APBS. Individuals were fed and then weighed before each injection. At day 7, individuals were weighed and then euthanized to obtain spleens.

Before euthanizing, frogs were randomly assigned new identification so that all measurements of the spleen were done in a blinded fashion as to treatment. The diameter of spleens was recorded before spleens were dissociated (see Chapter II Materials and Methods). A subset of dissociated spleens was placed on a hemocytometer to count splenocytes and erythrocytes.

Equations

Relative proliferation (proliferation index) was calculated from ^{3}H-thymidine uptake (CPM) of splenocytes. The proliferation of stimulated splenocytes was divided by the value for unstimulated splenocytes:

$$\text{Relative proliferation} = \frac{\text{Mean CPM of stimulated}}{\text{Mean CPM of unstimulated}}$$

Foot perimeter (*P*) was calculated using the width and thickness measurements to calculate the perimeter of and ellipse where *a* is one-half the foot width and *b* is one-half the foot thickness.

$$P = 2\pi\sqrt{\frac{a^2 + b^2}{2}}$$

Zoospore equivalents (z) determined by qPCR were Log transformed to calculate *B. dendrobatidis* infection load (*B. dendrobatidis* Load) using the equation:

$$Bd \text{ Load} = Log_{10}[z + 1]$$

X. laevis spleens are spherical so spleen volumes were calculated by measuring the diameter (*d*) to the nearest mm using the equation for the volume (*V*) of a sphere (*X. laevis* spleens are spherical):

$$V = \frac{4}{3}\pi(\frac{1}{2}d)^3$$

Statistics

For experiments with PLs, statistical comparisons were made using a single factor ANOVA. Individual comparisons among treatments were analyzed post-hoc using a Tukey test. The limit for statistical significance was $p < 0.05$.

The increase in size due to inflammatory swelling from the two compared treatments in foot injection experiments was analyzed using a two-tailed, paired Student's *t*-test because each individual received both treatments but in different feet. A Bonferroni correction was made because each experiment contained three measurements from the feet, so the alpha for statistical significance was set to 0.017. All statistical comparisons with p-values under 0.05 are noted; comparisons without asterisks were not significant. Comparisons were made between left and right feet irrespective of treatment, and there was no significant difference between the left feet and right feet for any experiment at any time point. Also, foot sizes were not significantly different before injection.

To determine if there was a correlation between infection load and spleen size (relative number of splenocytes) a regression analysis was used of the Log transformed zoospore equivalents and the normalized splenocyte number. A p-value less than 0.05 was considered statistically significant. For comparisons made in the pilot experiment between *X. laevis* injected with *B. dendrobatidis* supernatant or APBS, Student's *t*-tests were used to compare each measurement; due to the low number of individuals of this experiment and to determine which measurements were relevant, no Bonferoni correction was applied for multiple measures within the same experiment.

Results

B. dendrobatidis supernatants do not affect the viability of peritoneal leukocytes (PLs)

A population of mostly phagocytic leukocytes can be obtained from the peritoneum of *X. laevis* by a lavage of the peritoneum. To obtain a larger population of phagocytes, individuals can be primed by i.p. injection of killed bacteria three days before lavage (Nedelkovska et al., 2010). The population obtained from the peritoneum after bacterial stimulation is composed almost entirely of macrophages and neutrophils. Cells were characterized by morphology and staining in PL populations obtained from *X. laevis*. Macrophages composed about 71% of the PL population, and about 26% of cells were neutrophils (Fig. 3-2 A). Lymphocytes made up most of the remaining population, and a few granulocytes (basophils and eosinophils) were present in some samples.

This population of PLs was treated with *B. dendrobatidis* or *H. polyrhiza* supernatants for 24 hours and the cell viability was determined by either counting cells after trypan-blue staining or by flow cytometry quantifying PI nuclear staining. The number of PLs decreased somewhat even when not treated with supernatant likely due to the short lifespan of neutrophils especially outside the host (Pillay et al., 2010; Tofts et al., 2011), but no difference was observed among treatments, suggesting that *B. dendrobatidis* soluble factors have no effect on phagocyte viability after 24 hours (Fig. 3-2 B). *B. dendrobatidis* supernatant also had no significant effect on the number of PLs undergoing cell death (Fig. 3-2 C-F).

Batrachochytrium dendrobatidis does not appear to impair phagocytosis

PLs were co-cultured with living and killed *B. dendrobatidis* cells to determine whether PLs could engulf *B. dendrobatidis* cells at different stages. Both living and killed *B.*

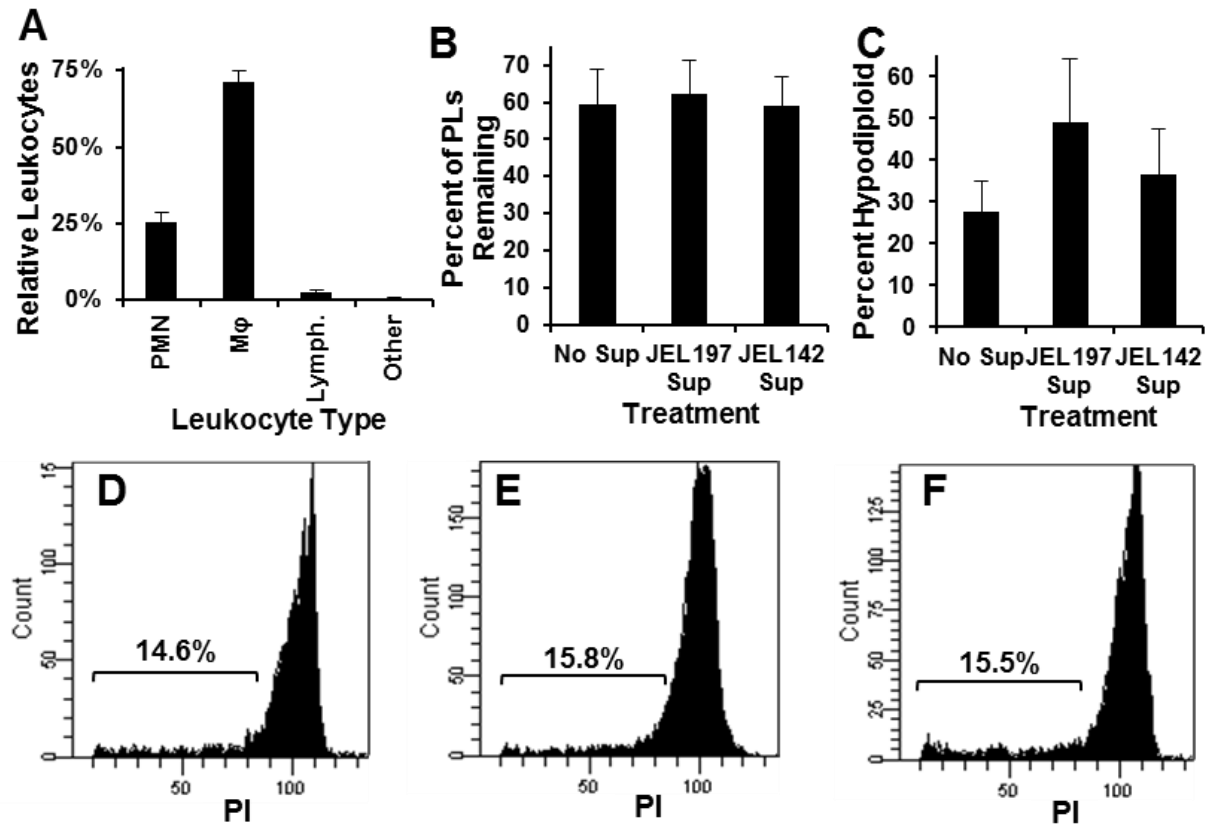

Fig. 3-2. *Batrachochytrium dendrobatidis (Bd)* supernatants (Sup) do not impair PL viability. (**A**) The PL population obtained from *X. laevis* was primarily macrophages (Mφ) and neutrophils (PMN); lymphocytes (Lymph.) and other granulocytes (Other) comprised a small minority of cells. Shown are the mean relative percentages (± SEM) of total PLs from nine frogs. **(B-F)** PLs were incubated with or without *Bd* (isolate JEL197) or *H. polyrhiza* (isolate JEL142) Sup. These cells were either counted manually (B), or nuclei were stained with propidium iodide (PI) and analyzed by flow cytometry (C-F). (**B**) Relative number of PLs remaining after an overnight treatment with or without each Sup and stained with trypan-blue to determine numbers and viability. The number of leukocytes counted after incubation was compared to the number of leukocytes originally added to wells for each treatment. There was no significant difference among treatments (single factor ANOVA with repeated measures, $p = 0.5$). Data shown represent the mean percent of PLs recovered from wells (± SEM) in replicate experiments using PLs from six individuals. (**C**) The mean percentage of hypodiploid PLs (cells undergoing apoptosis) (± SEM) from five individual frogs treated with or without *Bd* or *H. polyrhiza* Sup for 24 hours. There was no significant difference among treatments (single factor ANOVA with repeated measures, $p = 0.16$). (**D-F**) Data from a single experiment showing percent of hypodiploid cells (inset numbers) of untreated cells (D), *B. dendrobatidis* (JEL197) Sup-treated cells (E), or *H. polyrhiza* (JEL142) Sup-treated cells (F). The diploid peak was

dendrobatidis cells were visualized inside of phagosomes inside of both macrophages and neutrophils after two hours of co-incubation (Fig. 3-3). To determine if *B. dendrobatidis* may have mechanisms to escape phagosomes, live *B. dendrobatidis* cells were incubated with PLs for 24 hours. At 24 hours, *B. dendrobatidis* cells were still visible inside of phagosomes and no visual evidence suggested that *B. dendrobatidis* was escaping after phagocytosis (Fig. 3-4). The *B. dendrobatidis* cells phagocytized by PLs appeared to be mostly germlings and thalli. Zoospores may be phagocytized as well, but are either quickly degraded or indistinguishable from small germlings inside of phagosomes. Zoosporangia appear to be too large for phagocytes to engulf (Fig. 3-4 C); some clumps of PLs were noted to surround but not phagocytize zoosporangia (data not shown). These data suggest that *B. dendrobatidis* does not resist phagocytosis and may not have any evasion strategies to prevent being phagocytized or to escape killing inside the phagosome. The intracellular niche of *B. dendrobatidis* inside of epithelia cells may limit phagocytosis during infection, but phagocytes are likely to contribute to a protective immune response against *B. dendrobatidis*. To determine if *B. dendrobatidis* might inhibit phagocytosis, PLs were exposed to *B. dendrobatidis* supernatants for 2 or 24 hours and phagocytosis of pHrodo Green™ zymosan Bioparticles® was quantified. Zymosan is a particulate form of fungal PAMPs derived from yeast cell walls and mimics a fungal cell (Elsori et al., 2010; Goodridge et al., 2011). The zymosan used for to quantify phagocytosis was conjugated to a pH-sensitive fluorophore, pHrodo Green™, which fluoresces at pH 4 inside of endosomes and phagosomes but not outside of cells (Neaga et al., 2013). The uptake of zymosan by *X. laevis* PLs, quantified by fluorescence, was minimally affected by treatment with *B. dendrobatidis* supernatants (Fig. 3-5). When PLs were treated with *B. dendrobatidis* supernatants

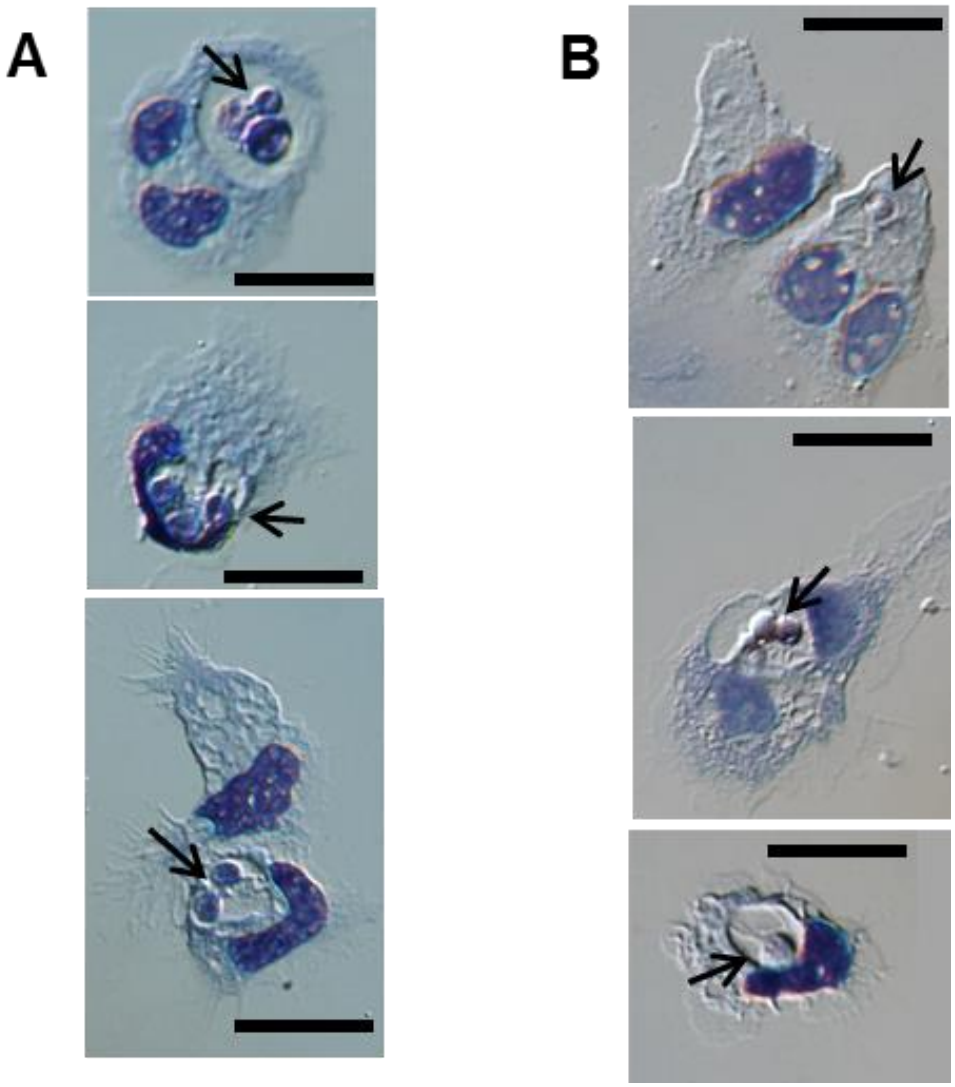

Fig. 3-3. *Batrachochytrium dendrobatidis (Bd)* cells are engulfed by amphibian phagocytes. Live (**A**) or heat-killed (**B**) *Bd* cells were mixed with PLs at a ratio of 1 or 2 fungal cells per phagocyte and incubated for 2 hours at 26° C. After incubation, cells were cytocentrifuged onto glass slides, stained with Hema 3 stain, and viewed with a Nikon Eclipse 90i microscope under differential interference contrast at 100X magnification. *Bd* cells inside of phagosomes are marked with arrows. Scale bars indicate 10 μm. Images are representative of slides prepared from two experiments using PLs from two individual frogs.

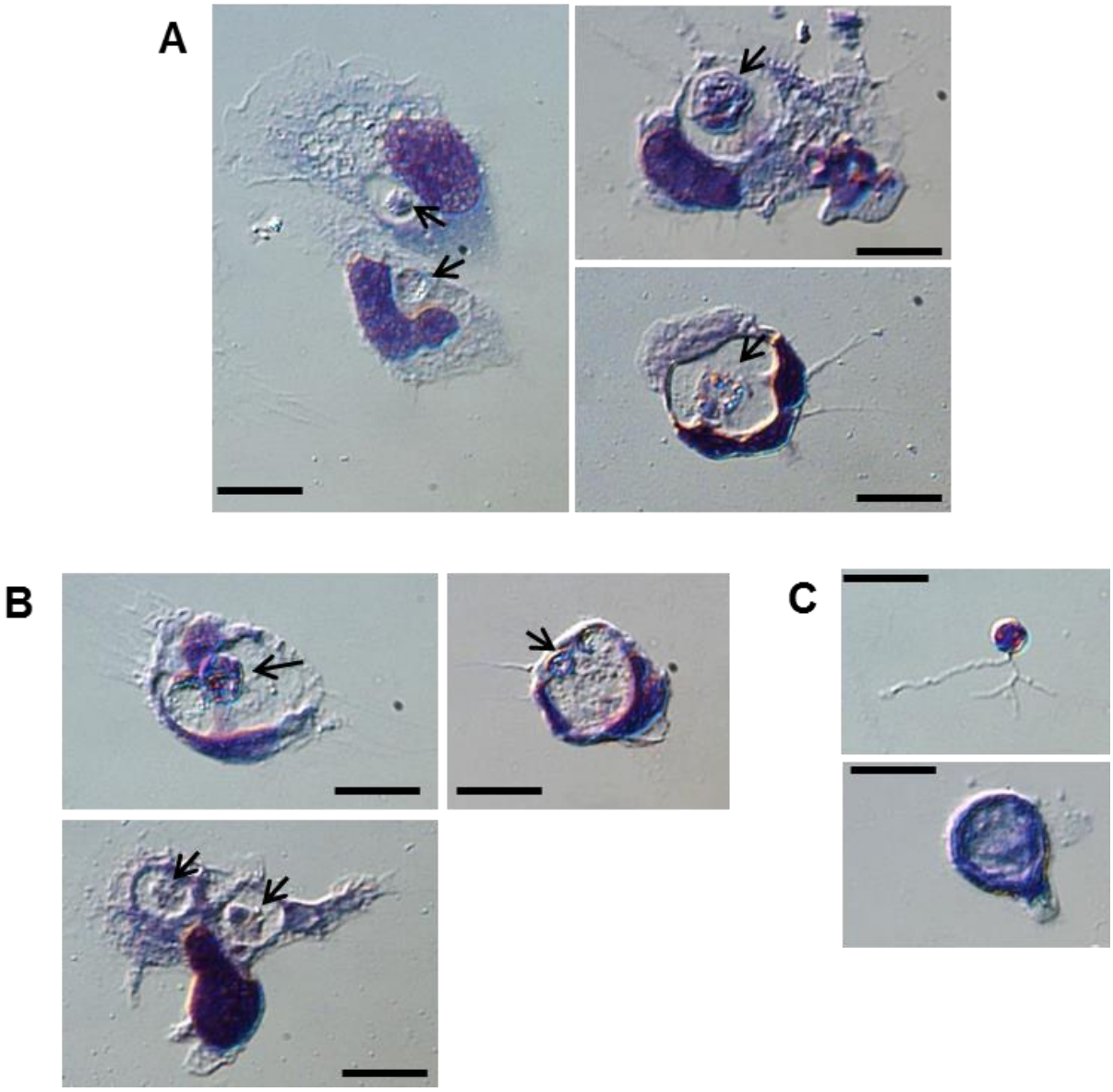

Fig. 3-4. *Batrachochytrium dendrobatidis (Bd)* cells do not appear to escape from phagosomes. Live *Bd* cells were mixed with PLs at at a ratio of 0.5 or 1 fungal cells per phagocyte and were incubated for 2 (**A**) or 24 (**B**) hours at 26° C. After incubation, cells were cytocentrifuged onto glass slides and stained with Wright-Giesma. Arrows indicate *Bd* cells inside phagosomes of *X. laevis* macrophages. (**C**) Representative *Bd* cells not engulfed by PLs and stained with Wright-Giemsa are shown; the top panel shows a germling stage with well-defined rhizoids, and the bottom panel shows a zoosporangium with a visible discharge tube. Scale bars indicate 10 μm. Images are representative of slides prepared from four experiments (using PLs from four different *X. laevis*).

for two hours before incubation with zymosan, a *B. dendrobatidis* supernatant caused a decrease in phagocytosis in four out of five experiments (Fig. 3-5 A-D). However, when PLs were treated with *B. dendrobatidis* supernatant for 24 hours before incubation with zymosan, the supernatant-treated PLs had an increase in phagocytosis in all experiments (Fig. 3-5 A, B, E, F). Although a significant difference in phagocytosis (fluorescence) between treatments occurred in most experiments, there was no significant effect in relative phagocytosis (compared to the untreated control) across experiments for either incubation period (Fig. 3-5 B). PLs were observed with differential interference contrast (DIC) and fluorescence microscopy (Fig. 3-5 C-F). Zymosan particles were visible under DIC (left panels) both inside and outside of PLs but only fluoresced inside of cells (right panels). Hoechst 33342 was used to stain nuclei. Zymosan particle fluorescence was localized outside of nuclei and inside of what appeared to be phagosomes. Nuclear staining also allowed for some morphological distinction of PLs. Both neutrophils and macrophages phagocytized zymosan particles. A few lymphocytes were also visible on slides but did not engulf zymosan (data not shown).

Phagocytes exposed to *B. dendrobatidis* supernatants can promote adaptive immune responses

Phagocytes play an important role in killing of fungal pathogens and also interact with the adaptive immune system to promote immune responses (Brown, 2011; Roy & Klein, 2012; Wüthrich et al., 2012a). After pathogen phagocytosis, phagocytes are induced to produce cytokines which have effects on other leukocytes including lymphocytes. Another important task of phagocytes is to present antigen to lymphocytes. Phagocytes loaded with antigen can activate lymphocytes through both antigen-presentation and secretion of cytokines.

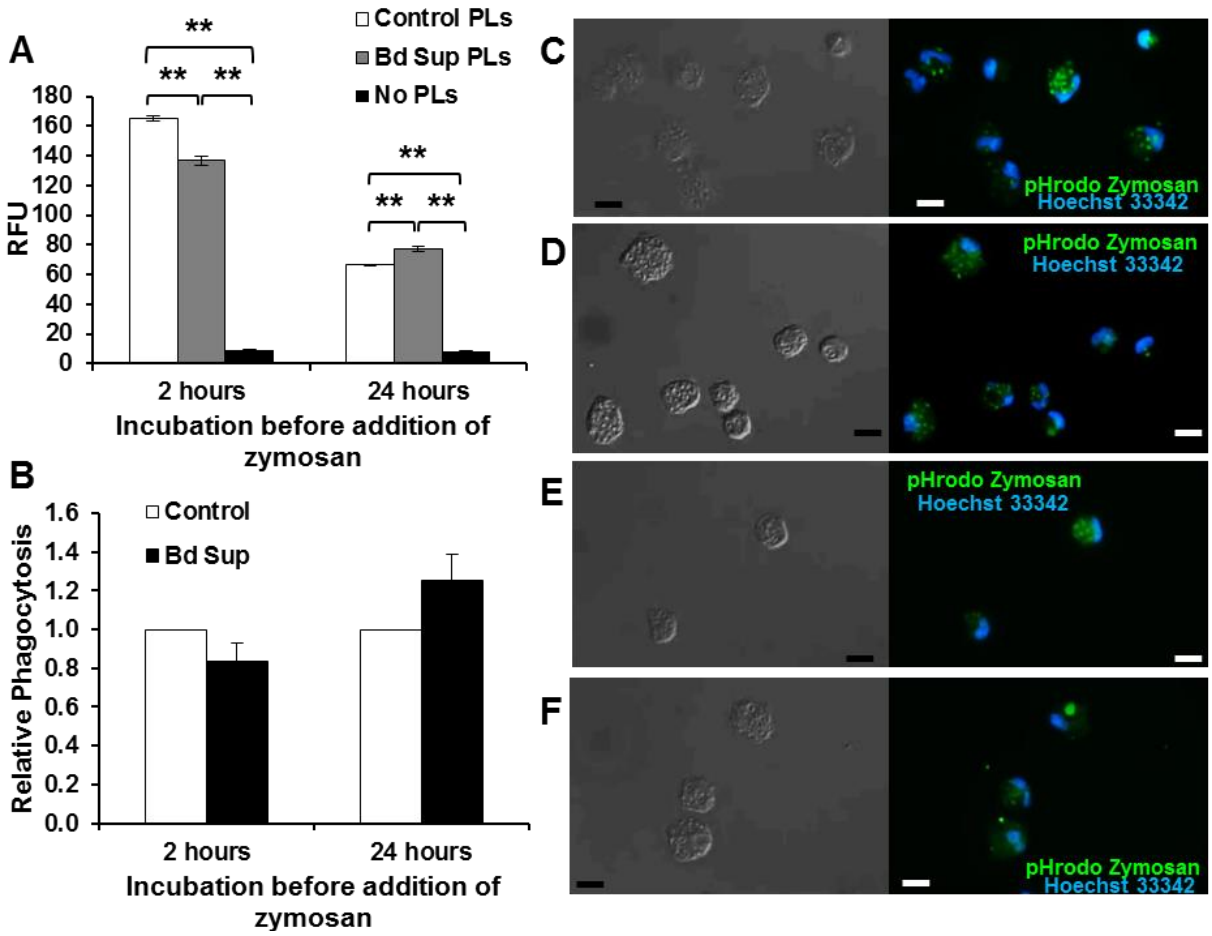

Fig. 3-5. *Batrachochytrium dendrobatidis (Bd)* supernatants (Sup) have minimal impact on the capacity of PLs to engulf zymosan. PLs were incubated with *Bd* Sup for either 2 or 24 hours at 26°C and then pHrodo Green™ Zymosan Bioparticles® were added to PLs and incubated another two hours (**A**) A representative experiment investigating phagocytosis by differentially treated PLs from a single *X. laevis* individual. Phagocytosis was quantified as relative fluorescence units (RFU) of zymosan Bioparticles inside PLs. Zymosan Bioparticles without PLs were used as a control to show background fluorescence of non-phagocytized particles (No PLs). RFU was significantly different in treatment groups, **p<0.01 single-factor ANOVA with Tukey test. (**B**) Combined results from multiple experiments where PLs from four (24 hrs) or five (2 hrs) frogs where phagocytosis was normalized among experiments to the fluorescence of control (no *Bd* Sup) treated PLs. There was no significant effect of *Bd* Sup across experiments, p>0.05 by paired *t*-test. (**D-G**) Images of PL phagocytosis of pHrodo zymosan from a single representative individual. PLs were pre-incubated without (D, F) or with (E, G) *Bd* Sup for either 2 (D, E) or 24 hrs (F, G) before zymosan Bioparticles were added to PLs. Nuclei were labeled with Hoechst 33342. Images were obtained by DIC microscopy (left) or by merging fluorescence of pHrodo zymosan and Hoechst 33342 (right). Scale bars indicate 10 μm.

To investigate the ability of phagocytes exposed to *B. dendrobatidis* antigens and soluble inhibitory factors, PLs were exposed to *B. dendrobatidis* supernatant, washed, and then incubated with enriched lymphocytes from the same individual *X. laevis*. Splenocytes were depleted of macrophages and dendritic cells by glass adherence to limit accessory functions of these cells to promote lymphocyte proliferation. PLs were incubated alone or with *B. dendrobatidis* supernatant overnight and then were washed to remove supernatant before being mixed with enriched lymphocytes to aid in cell proliferation. These cell mixtures received no stimulus or a sub-optimal concentration of PHA. Although control PLs added to lymphocytes did not promote greater proliferation than lymphocytes cultured alone, PLs exposed to *B. dendrobatidis* supernatants induced significantly greater lymphocyte proliferation with and without PHA stimulation (Fig. 3-6). The lack of increase in proliferation induced by the addition of untreated PLs was probably because the PLs were placed in an environment free of stimulation before being added to lymphocytes; when PLs were freshly added to lymphocytes, there was an augmentation of proliferation (data not shown). The increase in proliferation when *B. dendrobatidis* supernatant-treated PLs were added suggests that components of *B. dendrobatidis* supernatant activate phagocytes to produce cytokines and potentially engage in antigen-presentation. The proliferation quantified was completely from lymphocytes because PLs had background CPM levels no greater than empty wells. These data suggests that the soluble factors from *B. dendrobatidis* do not inhibit accessory functions of phagocytes for lymphocyte proliferation and actually appear to activate phagocytes to support lymphocyte proliferation.

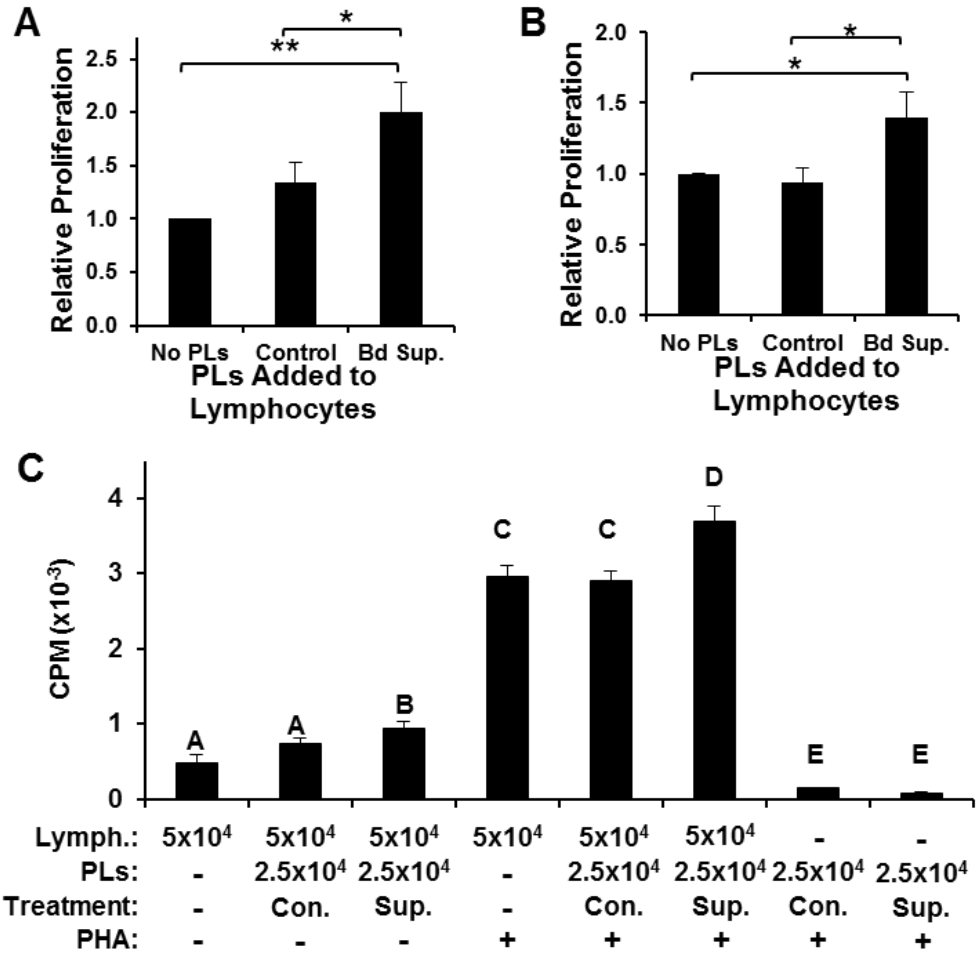

Fig. 3-6. *Batrachochytrium dendrobatidis (Bd)* supernatants (Sup) induce PLs accessory function to promote lymphocyte proliferation. PLs were treated overnight with or without *Bd* Sup and then washed and re-suspended in fresh L-15. An enriched lymphocyte population from the same frog was incubated alone, or with untreated PLs (Control), or with *Bd* Sup-treated PLs. (**A-B**) Combined proliferation data for cells treated with no PHA (A) or a sub-optimal concentration (100 ng/mL) of PHA (B). Proliferation was normalized by dividing the mean CPM of each treatment by the mean CPM of lymphocytes without PLs. Data show mean proliferation index (± SEM) from nine (A) or seven (B) experiments. Lymphocytes mixed with PLs that had been treated with *Bd* Sup showed significantly greater proliferation than lymphocytes alone or lymphocytes mixed with control PLs; *$p < 0.05$, **$p < 0.01$ by repeated measures ANOVA with Tukey post hoc test. (**C**) One representative experiment showing mean ^{3}H-thymidine uptake (± SEM) of enriched lymphocytes (Lymph.) incubated alone or with untreated PLs (Con) or with *Bd* Sup-treated PLs (Sup). PLs by themselves showed very little ^{3}H-thymidine uptake, suggesting that the increase in proliferation was due to PL support and not PL mitosis. Treatments with significantly different ^{3}H-thymidine uptake, $p < 0.05$, are indicated with different letters determined by ANOVA with post hoc tests.

Injection of PHA or killed *E. coli* induces inflammatory swelling in the foot

Intramuscular PHA injection into the foot area caused significantly greater swelling in feet in all dimensions compared with injection of APBS (Fig. 3-7 A, C). Swelling induced by PHA increased the width of the foot by about 5% and the thickness by about 20% (Fig. 3-7 B, C). PHA induced a large increase in the foot size 24 hours after injection (Fig. 3-7 A-B) which was not significantly reduce after 48 hours (Fig. 3-7 C-D); however the difference in swelling between PHA and APBS treatments were more significant at 48 hours than at 24 hours. Feet injected with PHA were visibly larger than feet injected with APBS for all individuals (Fig. 3-7 E-F).

X. laevis feet injected with heat-killed *E. coli* also had profound swelling 24 hours after injection, but control feet injected with APBS buffer did not have a significant change in foot size (Fig. 3-8). Injection of killed *E. coli* caused a significant increase in the size of both foot dimensions leading to a large increase in the overall perimeter of the foot (Fig. 3-8 A). This increase represented an approximate 10-20% increase in the foot size after *E. coli* injection (Fig. 3-8 B). As with injection with PHA, *E. coli* induced a visible increase in foot size compared to the APBS control (Fig. 3-8 C).

Batrachochytrium dendrobatidis* supernatants diminish lymphocyte-mediated inflammation *in vivo

Batrachochytrium dendrobatidis supernatant was also injected with PHA to determine if inhibitory factors decreased the swelling caused by innate and adaptive leukocytes activated by PHA. Presence of *B. dendrobatidis* supernatant had no significant impact on the increase of foot size for a single PHA injection (Fig.3-9). A single PHA injection typically promotes a more

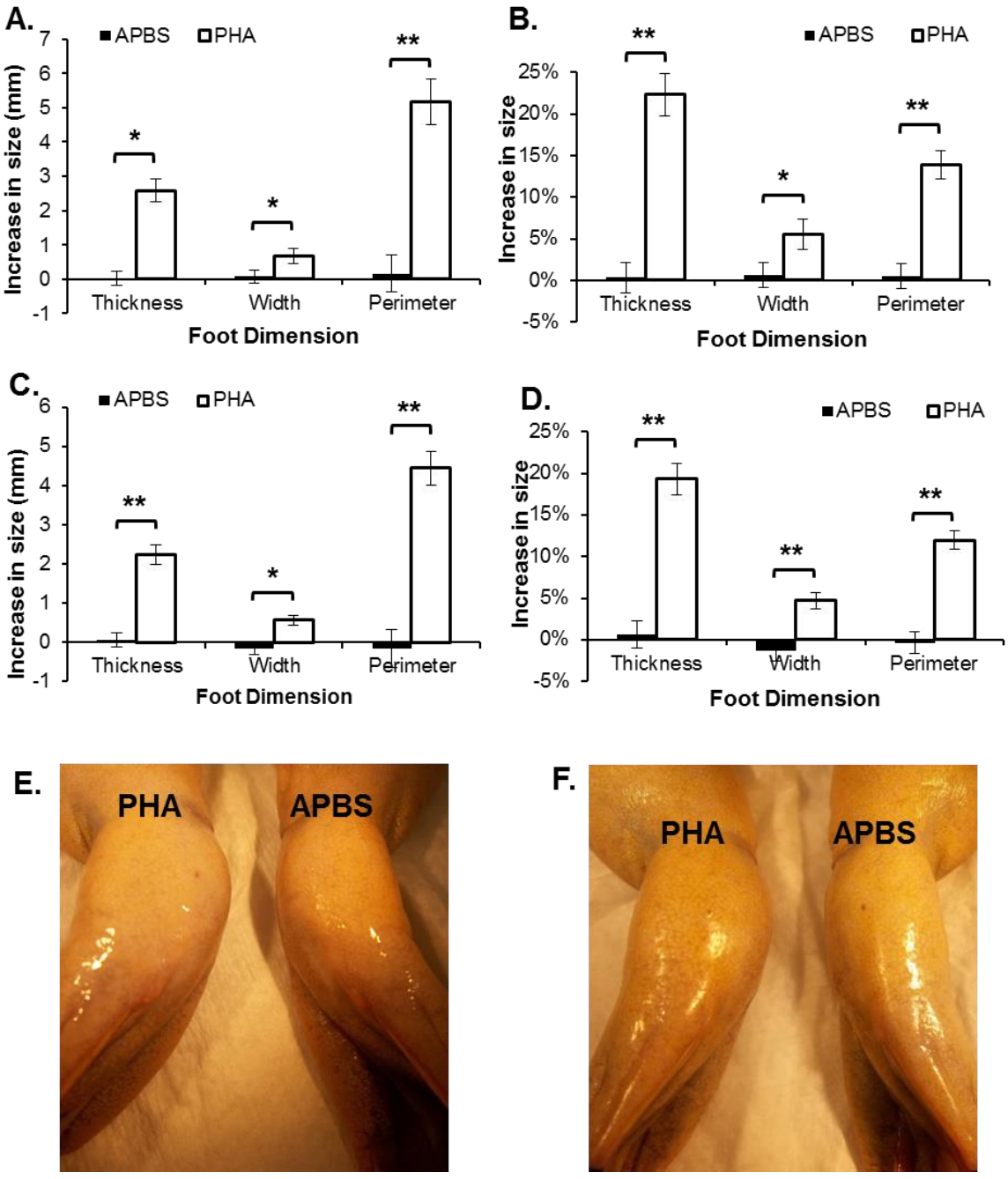

Fig. 3-7. Intramuscular injection of phytohemagglutinin (PHA) into *X. laevis* feet induces inflammatory swelling after 24 hours (**A**, **B**, **E**) and 48 hours (**C**, **D**, **F**). PHA injections induced significantly greater swelling than buffer (APBS) controls *p<0.01, **p<0.001, paired Student's *t*-test (alpha set to 0.017 for multiple tests). Data show mean (±SEM) increase in actual size (A, C) or percent increase (B, D) in foot size compared to each foot's measurement before injection from both feet of six frogs. (E-F) Representative photographs of individuals 24 hours (E) or 48 hours (F) after injection of APBS in the left foot and PHA into the left foot (pictures shows ventral side of frog).

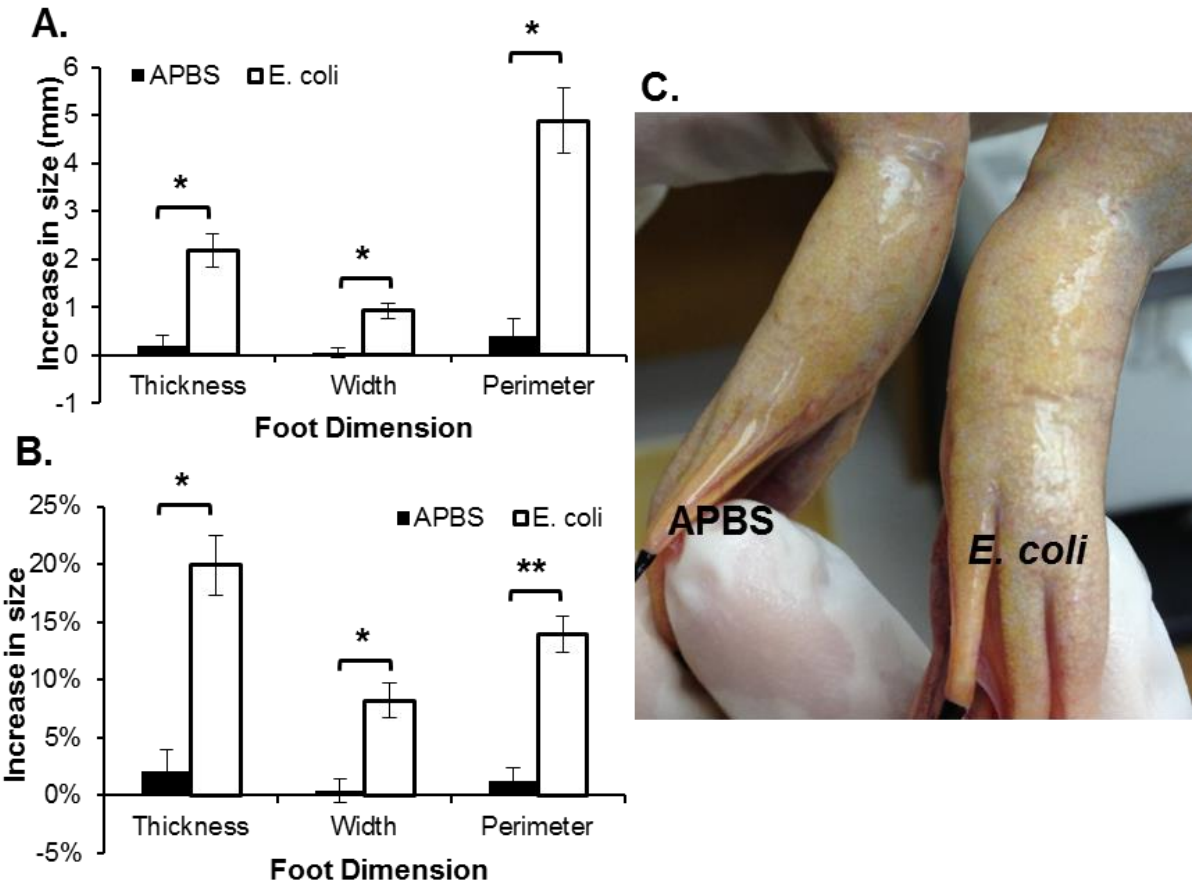

Fig. 3-8. Intramuscular injection of killed *E.* coli into *X. laevis* feet induces inflammatory swelling after 24 hours. *E. coli* injections induced significantly greater swelling than buffer (APBS) controls *p<0.01, **p<0.001, paired Student's *t*-test (alpha set to 0.017 for multiple tests). Data show mean (±SEM) increase in actual size (**A**) or percent increase (**B**) in foot size compared to each foot's measurement before injection from both feet of six frogs. (**C**) Representative photograph of an individual 24 hours after injection of APBS in the right foot and killed *E. coli* into the left foot (picture shows ventral side of frog).

robust innate leukocyte response, and a second PHA injection promotes recruitment of phagocytes again, but with greater activation and presence of lymphocytes (Tella et al., 2008; Brown et al., 2011). To investigate whether *B. dendrobatidis* factors impair adaptive immune responses *in vivo*, *X. laevis* were primed with a single intraperitoneal PHA injection before receiving injections of PHA alone or PHA and *B. dendrobatidis* supernatants in the feet. After PHA priming, *B. dendrobatidis* supernatants did cause a significant reduction in foot swelling caused by PHA (Fig. 3-10). At 24 hours after injection, the difference in swelling was substantial but not significant (Fig. 3-10 A-B). By 48 hours, the swelling of feet receiving *B. dendrobatidis* supernatants with PHA was significantly diminished compared to the feet receiving PHA alone (Fig. 3-10 C-D). In some individuals, the decrease in swelling caused by *B. dendrobatidis* supernatants was visible at both 24 and 48 hours (Fig. 3-10 E-F).

B. dendrobatidis supernatant was injected with heat-killed *E. coli* to determine if factors present in the supernatant decreased inflammatory swelling caused by infiltrating phagocytes activated by dead bacteria. Injection with *B. dendrobatidis* supernatant had no significant effect on the increase in foot size caused by injection with killed *E. coli*, and foot sizes were not different between feet receiving *E. coli* alone and those receiving both *E. coli* and *B. dendrobatidis* supernatant (Fig. 3-11). The increase in foot size in this experiment was comparable to what was observed when only one foot received killed *E. coli* injection. A single injection of PHA or killed *E. coli* induces infiltration of innate leukocytes to cause swelling, and a second injection of PHA induces a mixed response of innate and adaptive immune response (Tella et al., 2008; Nedelkovska et al., 2010; Brown et al., 2011). When innate immune responses are activated (by the first injection of PHA), co-injection with *B. dendrobatidis*

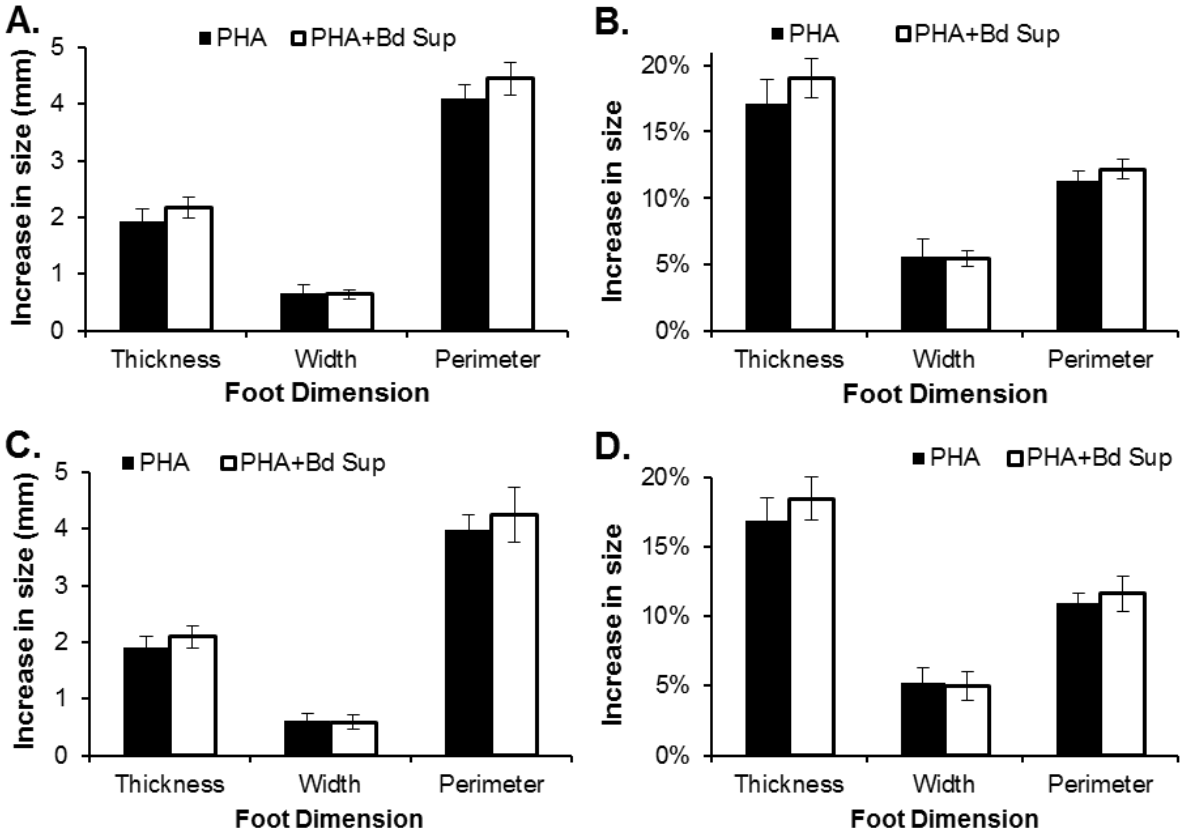

Fig. 3-8. *Bd* supernatant (Sup) does not impair inflammatory swelling induced by a single PHA injection (frogs were not primed with PHA). *X. laevis* feet were injected with PHA alone or with PHA and *Bd* supernatant. Feet were measured before injection, 24 hours (**A**, **B**), and 48 hours (**C**, **D**) after injection. Data show mean (±SEM) increase in actual size (A, C) or percent increase (B, D) in foot size compared to each foot's measurement before injection from both feet of six frogs. Swelling was not significantly different between treatments (paired Student's *t*-test).

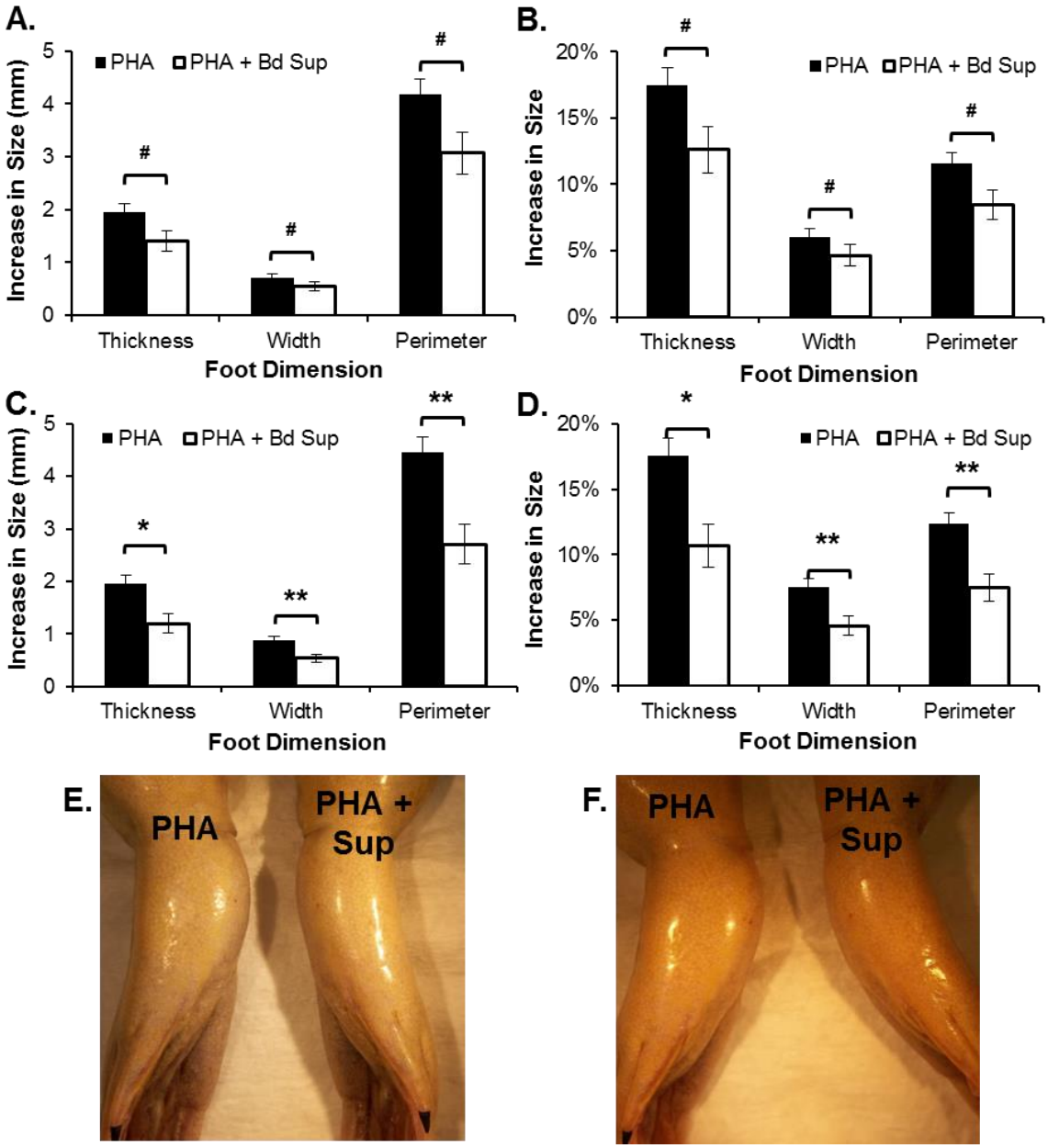

Fig. 3-10. *Bd* supernatant (Sup) reduces inflammatory swelling induced by a second PHA injection. *X. laevis* feet were injected with PHA alone or with PHA and *Bd* supernatant 7 days after priming with i.p. injection of PHA. Feet were measured before injection, 24 hours (**A**, **B**, **E**), and 48 hours (**C**, **D**, **F**) after injection. Data show mean (±SEM) increase in actual size (A, C) or percent increase (B, D) in foot size compared to each foot's measurement before injection from both feet 12 frogs. Swelling was only significantly different between treatments in feet 48 hours after injection, $^{\#}p<0.05$, $*p<0.01$, $**p<0.001$, paired Student's *t*-test (alpha set to 0.017 for multiple tests). (E, F) Representative photographs of an individual 24 hours (E) and 48 hours (F) after injection of PHA and *Bd* Sup in the left foot and PHA alone into the left foot (pictures shows ventral side of frog).

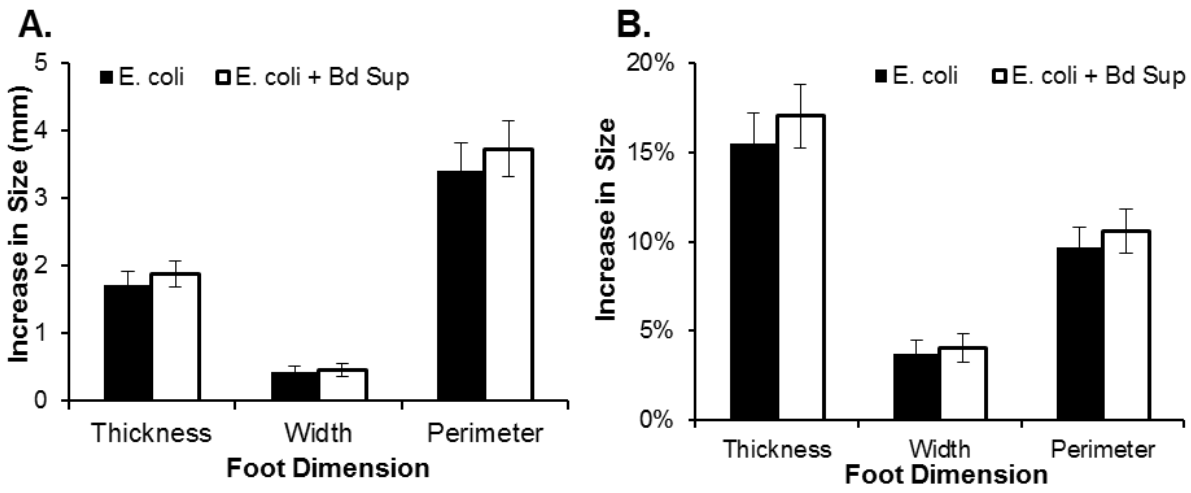

Fig. 3-11. *Bd* supernatant (Sup) does not impair inflammatory swelling induced by killed *E. coli*. *X. laevis* feet were injected with either killed *E. coli* alone or *E. coli* with *Bd* Sup. Feet were measured before injection and 24 hours after injection. Data show mean (±SEM) increase in actual size (**A**) or percent increase (**B**) in foot size compared to each foot's measurement before injection from both feet of 12 frogs. Swelling was not significantly different between treatments (paired Student's *t*-test).

supernatants had no effect on foot swelling (Figs. 3-9, 3-11). However, when more lymphocytes were recruited by a second PHA injection, *B. dendrobatidis* supernatants significantly decreased swelling (Fig 3-10). These data replicate, *in vivo*, the observed results seen *in vitro* where *B. dendrobatidis* factors impair lymphocytes but do not appear to inhibit innate phagocyte functions.

Batrachochytrium dendrobatidis appears to impair adaptive immunity in a local area but not systemically

High levels of *B. dendrobatidis* infection are lethal for most amphibians. The skin loses complete integrity, and amphibians die of complications due to loss of electrolyte balance and dehydration (Berger et al., 2005a; Voyles et al., 2009; Marcum et al., 2010; Voyles et al., 2012). The release of toxins from *B. dendrobatidis* may also contribute to death, although some evidence suggests that no great accumulation of toxins occurs in the blood (Voyles et al., 2009). The lymphotoxic factors may be shed from the skin in sufficient concentrations to inflict systemic immune impairment. A couple of infection studies have noted that infected frogs tend to have smaller spleens (Rosenblum et al., 2009; Rosenblum et al., 2012) suggesting that *B. dendrobatidis* can induce systemic effects on the host immune system. If enough factors were released during infection in the skin and these factors entered the blood at a high enough concentration, then *B. dendrobatidis* infection could impair amphibian immunity systemically.

To begin to examine this question, *B. dendrobatidis* infection loads were quantified on several *X. laevis* individuals used to obtain splenocytes for other experiments (Chapter II). The data obtained from these frogs was used to look for a relationship between the total splenocyte number and the infection load on *X. laevis*. The majority of frogs tested, 82%, did not have

detectable *B. dendrobatidis* infections. The mean infection load (±SEM) for infected individuals was 17.8 ± 0.9 zoospore equivalents. All but one individual had infection loads of less than 40 zoospore equivalents. This one individual had an infection load of 1524 zoospore equivalents. The *B. dendrobatidis* infection status and zoospore burden of *X. laevis* were used to determine whether there was an effect on splenocyte number. No significant correlation exists between infection load and relative splenocyte number in either infected individuals or all individuals (Fig 3-12A). The infection status of *X. laevis* (infected or not) also had no significant effect on the relative number of splenocytes (Fig 3-12B).

To investigate directly whether the soluble factors released by *B. dendrobatidis* can actually have a negative impact on the spleen and splenic lymphocytes, *B. dendrobatidis* supernatants were injected i.p. into *X. laevis* in attempt to mimic release of factors during infection. Because *in vitro* and *in vivo* the peak of inhibition of lymphocytes was about 48 hours, injections were given two to three days apart. Control individuals received an APBS buffer control while experimental individuals received *B. dendrobatidis* supernatant containing factors from approximately 5×10^7 mature *B. dendrobatidis* cells per mL injected. The amount of cells from which the supernatants were prepared was equal to the number of killed cells injected in a previous study used to immunize *X. laevis* (Ramsey et al., 2010). Some studies have defined the lethal threshold of *B. dendrobatidis* as 10^5 zoospore equivalents (Briggs et al., 2010; Vredenburg et al., 2010; Kinney et al., 2011), so the amount of *B. dendrobatidis* factors injected i.p. likely represents as much or more of the factor present outside of the skin during a lethal infection. Amphibians do not tend to survive long after *B. dendrobatidis* loads exceed the lethal maximum. *Xenopus* (*Silurana*) *tropicalis* show symptoms of severe chytridiomycosis 10-21 days post-

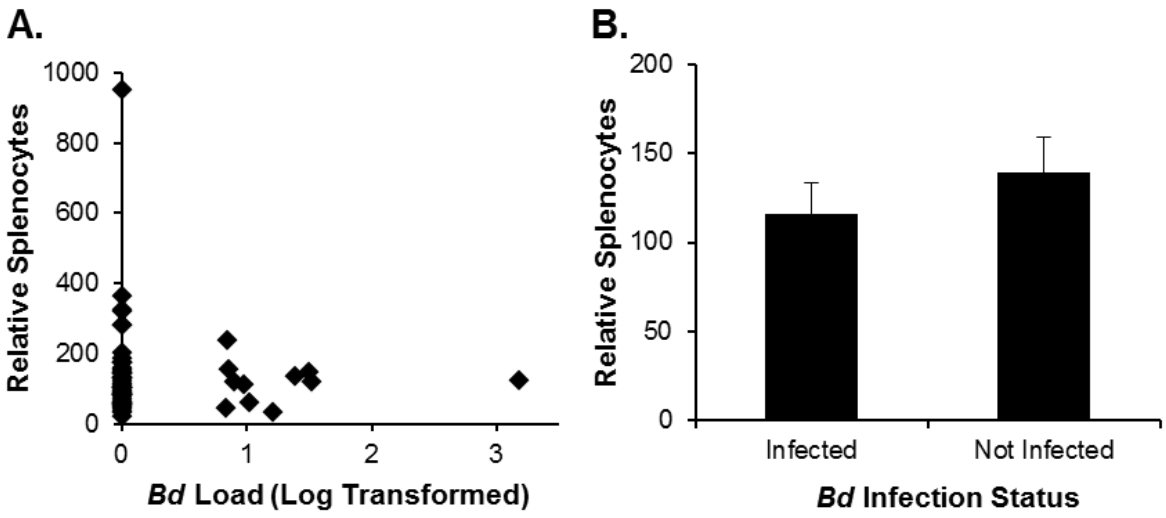

Fig. 3-12. *Batrachochytrium dendrobatidis* (*Bd*) infection does not affect the number of leukocytes present in the spleen (splenocytes) of *X. laevis.* (**A**) No significant relationship exists between the infection load and relative number of splenocytes by regression analysis (all: p=0.64; infected: p=0.99). (**B**) There is also no significant difference in the mean number of splenocytes (±SEM) between individuals that were or were not infected with *B. dendrobatidis* by a two-tailed Student's *t*-test (p=0.37). The number of splenocytes was normalized to the size of each individual by dividing the total splenocytes by the mass in mg of the frog. *Bd* load was determined by qPCR using zoospore equivalent standards and Log transformed. N=50 for individuals that were not infected. N=11 for infected individuals. (Experiment completed with Laura

infection when individuals have average infection loads of 50,000 zoospore equivalents (Rosenblum et al., 2009), so a week of *B. dendrobatidis* supernatant injections probably is the longest length of time an amphibian would survive during exposure to similar levels of *B. dendrobatidis* factors.

During the pilot experiment using intraperitoneal injection of *B. dendrobatidis* supernatants, no significant differences were observed between *B. dendrobatidis* supernatant-treated and APBS-treated control frogs in any trait measured (Fig. 3-13). All individuals lost weight over the course of the experiment, and the weight loss of supernatant-treated frogs was not significantly greater than that of control frogs (Fig. 3-13 A). However, the weight loss was not significant in the APBS controls, but the mass of frogs treated with *B. dendrobatidis* supernatant had significantly decreased by Day 5. Spleen size was measured, and no significant difference was observed between spleen volumes in relation to body size between treatments (Fig. 3-13 B). The number of splenocytes both in relation to body size (Fig. 3-13 C) and number of splenic erythrocytes (Fig 3-13 D) also was not significantly different between treatments. In this was a pilot experiment, significant differences were not observed, and the only visible trend was weight loss. Weight loss is a symptom of chytridiomycosis (Retallick & Miera, 1997; Ramsey et al., 2010), and injection of *B. dendrobatidis* supernatants may have an impact on the general health of individuals. The absence of any apparent effect on splenic leukocytes suggests that the soluble factors from *B. dendrobatidis* supernatants do not impair lymphocytes in the spleen during infection.

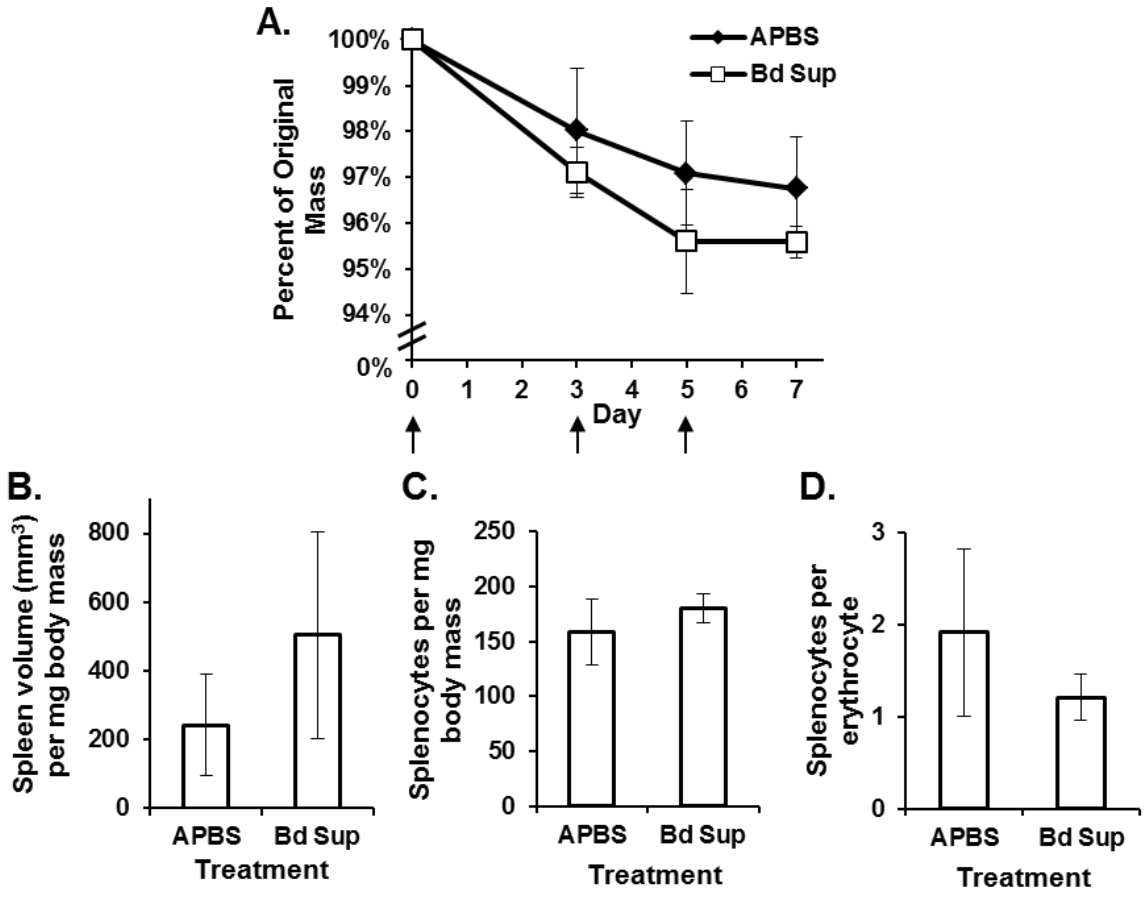

Fig. 3-13. Multiple injections of *B. dendrobatidis* supernatants (Bd Sup) into the peritoneum of *X. laevis* have no effect on splenocytes. *Bd* supernatants or APBS were injected three times (arrows) over the course of a week. (**A**) The relative decrease in mass of individuals was not significantly different between APBS and *Bd* supernatant treatments at any time point, $p>0.05$; however, at Day 5 and 7 the relative mass had significantly decrease compared to Day 0, $p<0.01$, single factor ANOVA with Tukey post-hoc test. (**B**) The spleen volume relative to the total mass of the individual also did not significantly different between treatments, $p=0.61$ by two-tailed Student's *t*-test. (**C**-**D**) The number of splenic leukocytes relative to body mass and splenic erythrocytes was not significantly different between treatment groups, $p=0.54$ and $p=0.49$, respectively, by two-tailed Student's *t*-test. Data are from a pilot experiment where with three individuals in each treatment group.

Discussion

Amphibian and mammalian lymphocytes are impaired by factors released by *B. dendrobatidis* (Chapter II). Although soluble factors from *B. dendrobatidis* also impair dividing epithelial cells, the effect on the immune system appears to be limited to lymphocytes. Phagocyte viability and function was not decreased in the presence of *B. dendrobatidis* supernatants. *In vivo*, *B. dendrobatidis* supernatants only inhibited local swelling mediated by lymphocytes and not innate leukocytes which supports my *in vitro* observations. Investigation of the effects of *B. dendrobatidis* infection and supernatant on splenocytes *in vivo* suggest that the impairment of is not systemic.

X. laevis phagocytes are not impaired by *B. dendrobatidis*

Amphibian lymphocytes undergo apoptosis in the presence of *B. dendrobatidis* factors, but phagocytes obtained from the peritoneum of *X. laevis* did not have decreased viability when treated with *B. dendrobatidis* supernatants. There was a non-significant increase in cell death determined by PI staining suggesting that the factors may not induce apoptosis in phagocytes. If *B. dendrobatidis* does induce apoptosis in phagocytes, the kinetics of cell death may be different than observed with lymphocytes. Neutrophils are relatively short lived cells (Pillay et al., 2010; Tofts et al., 2011). The neutrophils in the PL population likely die within hours in culture, so any death mediated by *B. dendrobatidis* supernatant on neutrophils may be difficult to determine. *Aspergillus fumigatus* produces an exopolysaccharide that induced apoptosis in mouse neutrophils (Fontaine et al., 2011), and factors released from *B. dendrobatidis*, potentially exopolysaccharides (see Chapter IV), could induce apoptosis in amphibian neutrophils as well. Macrophages may be induced to die, but because there was no significant effect *in vitro*, *B. dendrobatidis* is not likely to kill macrophages *in vivo*.

Phagocytosis by amphibian macrophages and neutrophils was not impaired by *B. dendrobatidis*. When PLs and *B. dendrobatidis* cells were mixed *in vitro*, *B. dendrobatidis* was readily engulfed by phagocytes. *B. dendrobatidis* also did not appear to be capable of exiting phagocytes. Soluble factors in *B. dendrobatidis* supernatants do not impair amphibian phagocytes from engulfing zymosan, a fungal particle. Together these data suggest that *B. dendrobatidis* lacks the ability to evade phagocytosis and phagocytic killing that most other pathogenic fungi possess (see Table 1-2). Because *B. dendrobatidis* is an intracellular pathogen of epithelial cells (Berger et al. 2005a), phagocytes may not encounter *B. dendrobatidis*. If phagocytes never encounter *B. dendrobatidis* free from a host cell, then *B. dendrobatidis* would not need any strategies to evade phagocytosis.

Prior to my studies, we hypothesized that *B. dendrobatidis* factors may impair the accessory functions of phagocytes to dampen inflammation. Antigen presentation is very important for cueing the correct adaptive immune responses that promote fungal clearance in mammals (Roy & Klein, 2012; Wüthrich et al., 2012a). Appropriate antigen presentation also appears to be important for resistance to chytridiomycosis as well (Savage & Zamudio, 2011). Soluble *B. dendrobatidis* factors could prevent appropriate antigen presentation to diminish immune responses. This would be accomplished by simply preventing activation of phagocytes or by inducing inappropriate responses. For example, the capsular polysaccharide GXM from *Cryptococcus neoformans* induces expression of Fas ligand on macrophages and DCs to induce apoptosis in T cells (Monari et al., 2005; Piccioni et al., 2011). Another evasion mechanism used by *Candida albicans* and *A. fumigatus* is to express more TLR2 ligands on the surface of invasive hyphae which typically promotes more of a Th2 response over a protective Th1 response (d'Ostiani et al., 2000; Netea et al., 2003). Unlike these pathogenic fungi, *B.*

dendrobatidis does not appear to inhibit accessory functions of amphibian phagocytes. PLs exposed to *B. dendrobatidis* supernatant actually promote greater lymphocyte proliferation than PLs in medium alone. The results from this experiment suggest that amphibian phagocytes are not impaired by *B. dendrobatidis* factors but instead are activated by *B. dendrobatidis* PAMPs present in supernatants.

A caveat to this experimental design was that in order to obtain enough PLs to complete experiments *ex vivo*, *X. laevis* were primed with killed bacteria. The PLs obtained from *X. laevis* were activated by Gram negative bacterial PAMPs and thus were already activated against a pathogen. In *B. dendrobatidis*-infected skin, phagocytes need to be activated by *B. dendrobatidis* PAMPs or inflammatory signals from epithelial cells. If *B. dendrobatidis* prevents this initial activation or activates inappropriate immune responses, these effects may not occur with phagocytes that were already activated with bacterial PAMPs.

Because the PL population obtained from *X. laevis* was primarily macrophages and neutrophils, the effect on other innate leukocytes was not determined. Innate lymphoid cells (ILCs) are likely to be inhibited by *B. dendrobatidis* factors, and because there are antibodies to NK cell and T cell markers, some populations of amphibian ILCs may be obtained to be treated with *B. dendrobatidis* factors (Robert & Cohen, 2011; Edholm et al., 2013) in future experiments. Analysis of the any effects of *B. dendrobatidis* factors on innate leukocytes would likely be more successful using mouse models that have better defined cell populations.

A revised method for eliciting immune responses in amphibians

PHA injection is a common method to measure immune responses in vertebrates. In birds a consensus protocol of injecting patagia has been developed (Martin et al., 2006), but no such

site has been determined for amphibians or anurans. In amphibians, many of the sites chosen for injection have a very small amount of swelling induced by PHA (Gervasi & Foufopoulos, 2008; Brown et al., 2011). Such small differences in sizes require very precise tools and a large number of replicates to obtain statistical significance. For our experimental design in which factors from *B. dermatitidis* were expected to decrease swelling, more inflammatory swelling was necessary to determine significant inhibition of inflammatory processes. Therefore, a modified protocol in which PHA was injected intramuscularly into the feet of *X. laevis* was developed. The amount of swelling observed was between 3 to 20 times greater than previously recorded with subcutaneous injection in frogs (Gilbertson et al., 2003; Gervasi & Foufopoulos, 2008; Brown et al., 2011; Venesky et al., 2012). The amount of inflammatory swelling induced by intramuscular injection was so great that a noticeable difference could be seen between PHA and buffer injections and feet could be measured with a caliper. This method was also suitable for measuring swelling induced by killed bacteria.

Intramuscular foot injection of PHA or other inducers of inflammation may be a better method for investigating immune responses in amphibians. Injection into the foot requires less precision decreasing experimental error and decreasing the need for many experimental replicates. The simplicity of the method may also make it preferable for complex studies. The increase in swelling did not appear to negatively affect *X. laevis*, but greater foot swelling may be restricting for non-aquatic anurans.

B. dendrobatidis inhibition of adaptive immunity *in vivo*

Early studies of chytridiomycosis noted minimal leukocyte infiltration into *B. dendrobatidis*-infected skin (Pessier et al., 1999; Berger et al., 2005). Transcriptional studies

also found little activation of immune gene expression during chytridiomycosis (Rosenblum et al., 2009; Rosenblum et al., 2012). The absence of robust immune responses to *B. dendrobatidis* is not likely due to the incapacity of amphibian immunity; adaptive immunity appears to be capable of promoting resistance to chytridiomycosis (Richmond et al., 2009; Ramsey et al., 2010; Savage & Zamudio, 2011). The ineffective clearance of *B. dendrobatidis* is most likely due to immune evasion. *B. dendrobatidis* impairs lymphocytes *in vitro*, which may explain the lack of robust immunity (Chapter II).

The factors that impair and kill amphibian lymphocytes *in vitro* do not appear to inhibit amphibian phagocytes suggesting that the target of evasion is the adaptive immune response. The current study reproduces these *in vitro* observations *in vivo* using a modified protocol to induce inflammatory swelling in the feet of *X. laevis*. Primary injection with PHA or killed *E. coli* induce a primarily innate immune response causing a major infiltration of phagocytic leukocytes (Tella et al., 2008; Nedelkovska et al., 2010; Brown et al., 2011). The inflammatory swelling induced by these injections was not significantly affected when *B. dendrobatidis* supernatant was simultaneously injected suggesting no impairment of innate immune responses. Amphibian macrophages and neutrophils are not impaired *in vitro* by *B. dendrobatidis* supernatants, and it appears that soluble *B. dendrobatidis* factors also neither kill nor impair recruitment of innate leukocytes *in vivo*.

A single injection of PHA does appear to activate a small amount of lymphocyte recruitment in amphibians, but this is likely to be a minor component of the inflammatory response causing swelling (Brown et al., 2011). A second PHA injection promotes a more robust lymphocyte response in birds (Tella et al. 2008) and induces greater swelling in amphibians (Brown et al., 2011). In our experimental design, *X. laevis* was primed with an intraperitoneal

injection of PHA a week before feet were injected with either PHA alone or PHA and *B. dendrobatidis* supernatant. The priming injection of PHA allowed for a greater lymphocyte response during the second injection. Unlike the single PHA injection, the inflammatory swelling induced by the second injection was significantly reduced by factors present in *B. dendrobatidis* supernatants suggesting that lymphocytes are impaired by *B. dendrobatidis* factors *in vivo* as previously characterized *in vitro*. Swelling still occurred when *B. dendrobatidis* supernatants were injected with PHA, but the swelling that had occurred was probably caused by infiltration of innate leukocytes. The full effect of *B. dendrobatidis* supernatant did not become significant until 48 hours after injection. This correlates well with the observed peak of *B. dendrobatidis* induced amphibian lymphocyte apoptosis (Fites et al., 2013). The delayed effect of *B. dendrobatidis* supernatant may be also explained by the later recruitment of lymphocytes during PHA injection compared to innate leukocytes (Brown et al., 2011).

B. dendrobatidis likely evades many components of the amphibian host immune response. Because *B. dendrobatidis* factors inhibit activation and induce apoptosis in lymphocytes, the mechanism of decreased swelling induced by a secondary PHA injection is likely also mediate by lack of lymphocyte proliferation and induction of lymphocyte apoptosis after recruitment. However, *B. dendrobatidis* supernatants may also inhibit recruitment of lymphocytes by disrupting or damping chemokine signaling.

A localized effect of *B. dendrobatidis* lymphotoxic factors

The *in vivo* experiments demonstrated that soluble factors from *B. dendrobatidis* inhibited adaptive immune responses at the sites of lymphocyte recruitment and activation in living *X. laevis*. The decrease in inflammation caused by *B. dendrobatidis* factors only appeared

to affect the site where supernatants were injected; the amount of inflammation in feet injected with PHA resembled the amount of inflammation caused by PHA when no *B. dendrobatidis* supernatant was injected. However, a systemic inhibition of immunity may be possible during an infection which typically last longer than the duration of the foot injection experiment. The lymphotoxic factors released by *B. dendrobatidis* in the skin may be released into the blood stream at a high enough concentration to have an impact on lymphocytes away from the sites of infection.

The spleen is the only organized secondary lymphoid organ in amphibians, and the leukocyte population in the spleen is largely composed of lymphocytes (Manning & Horton, 1982). Therefore, the spleen is the best location to investigate the systemic impairment of adaptive immunity. Some infection studies have noted that the spleen is negatively affected during chytridiomycosis and that infection appears to shrink the size of the spleen (Rosenblum et al., 2009; Rosenblum et al., 2012). To further investigate whether *B. dendrobatidis* may impair adaptive immunity systemically, infection loads were compared to the number of leukocytes present in the spleen. This study found no correlation between *B. dendrobatidis* infection and number of splenocytes in *X. laevis* suggesting that immune impairment is localized near the sites of infection. However, *X. laevis* show very little susceptibility to chytridiomycosis and infection loads present on *X. laevis* in this study were very low. An infection threshold may exist where only high *B. dendrobatidis* loads produce enough of the lymphotoxic factor to actually impair and kill lymphocytes in the spleen.

To test whether lymphotoxic factors could induce systemic lymphocyte impairment, *B. dendrobatidis* supernatants were injected into the peritoneum of *X. laevis*. The amount of factors shed from the skin into the blood or lymph would not likely be any greater than the amount

injected. Also, intraperitoneal injection would allow the factors to be spread throughout the frog, and more of the factors would enter the spleen. If the soluble factors shown to impair lymphocytes *in vitro* and adaptive immunity *in vivo* do cause a systemic impairment of lymphocytes, then the number of splenocytes would decrease after treatment with *B. dendrobatidis* supernatants. In a pilot experiment, injection of *B. dendrobatidis* supernatants had no effect on spleen size or the number of splenic leukocytes present in the spleen. The lack of correlation between infection load and splenocytes in *X. laevis* could be explained by low infection intensities, but the amount of *B. dendrobatidis* supernatant injected probably is equal to or greater than the concentration of factors entering the blood during severe infection.

Although injection of *B. dendrobatidis* supernatants appeared to have no effect on spleens, the results suggest that *B. dendrobatidis* supernatants do negatively impact the health of *X. laevis*. Treatment with *B. dendrobatidis* supernatants caused a trend toward greater weight loss which is a symptom of chytridiomycosis in some amphibians (Retallick & Miera, 1997; Ramsey et al., 2010). *B. dendrobatidis* supernatants have negative effects on some non-lymphoid cell lines (Chapter II); factors released by *B. dendrobatidis*, possibly different from the lymphotoxic factors, are likely to cause pathogenic damage to tissues which can lead to morbidity.

At this time, evidence only supports a local inhibition of adaptive immunity during chytridiomycosis and does not suggest that amphibians infected with *B. dendrobatidis* can become immunocompromised. The observed decrease in spleen size could as easily be explained by disease progression and not a systemic release of toxic factors. The skin destruction caused by *B. dendrobatidis* infection causes dehydration and disruption of electrolyte homeostasis in later stages of severe chytridiomycosis (Voyles et al., 2009; Marcum et al., 2010; Voyles et al., 2012).

Dehydration and general dysregulation caused by heavy *B. dendrobatidis* infection may decrease spleen size more than toxic factors that originating from the skin.

CHAPTER IV

PARTIAL CHARACTERIZATION OF *BATRACHOCHYTRIUM DENDROBATIDIS* INHIBITORY FACTORS[3]

Abstract

The soluble factors released by *Batrachochytrium dendrobatidis* impair adaptive immune responses *in vitro* and *in vivo*. The supernatant from *B. dendrobatidis* used to characterize this inhibition of immune cells is a complex mixture of all the molecules released by the fungus over a 24-hour period. To understand the evasion of adaptive immune responses by *B. dendrobatidis,* the inhibitory components of supernatants must be identified. The inhibitory factors appear to be associated with the *B. dendrobatidis* cell wall because: 1) only cells with cell walls produce inhibitory factors; 2) cells with cell walls continue to release inhibitory factor after being killed; 3) treatment of *B. dendrobatidis* cells with nikkomycin Z, a cell-wall synthesis inhibitor, decreases the inhibitory effects on lymphocytes by *B. dendrobatidis*. The inhibitory factors which appear to be associated with the *B. dendrobatidis* cell wall are resistant to heat, acid, proteases, and RNase suggesting that the factors are not protein or RNA. The factors are soluble in water and separate into two main size classes: less than 10 kDa and greater than 50 kDa, suggesting that the factors may be small molecules and/or carbohydrates. Small molecule analysis of *B. dendrobatidis* supernatants provides a short list of candidate compounds based on mass spectrometry or HPLC analysis. Several of the identified small metabolites produced by *B. dendrobatidis* were assayed on lymphocytes, but none were shown to be responsible for lymphocyte inhibition. The large molecule factors are most likely carbohydrate based on process

[3] Some of data shown in this chapter is published in Fites et al., 2013. Data present in two figures were also published in Holden et al., 2014.

of elimination. Fractionation of *B. dendrobatidis* supernatants over solid-phase extraction matrix enriched carbohydrate into certain fraction that also had greater inhibition of lymphocytes. This correlative data suggest that carbohydrates are likely responsible for lymphocyte inhibition by *B. dendrobatidis*. Fungal cell walls are primarily composed of carbohydrates, and several polysaccharides associated with fungal cell walls are known to impair leukocyte functions and induce apoptosis. Polysaccharides may also be active when broken into smaller components suggesting that both large and small molecule inhibitors could be part of the same carbohydrate complex. The data in this chapter characterize the nature of the *B. dendrobatidis* lymphotoxic factors; these data suggest that *B. dendrobatidis* has carbohydrates associated with its cell wall that act as immunomodulators to impair host lymphocytes.

Introduction

The two previous chapters (Chapters II and III) characterized the effects of the soluble factors released by *B. dendrobatidis* on hosts and host cells. *Batrachochytrium dendrobatidis* supernatants impaired lymphocyte proliferation and induced apoptosis in lymphocytes. Factors in *B. dendrobatidis* supernatants also reduced proliferation of non-lymphoid cell lines. However, *B. dendrobatidis* did not inhibit function of phagocytes and did not kill phagocytic leukocytes. The *in vitro* experiments suggested that *B. dendrobatidis* produces soluble factors that impair adaptive immune responses but not innate leukocyte responses. These data were confirmed *in vivo* by injecting *B. dendrobatidis* supernatants into *Xenopus laevis* feet, which inhibited induction of inflammation.

All of the characterization of the effects of *B. dendrobatidis* on host cells was completed either with *B. dendrobatidis* cells or supernatants. *Batrachochytrium dendrobatidis* supernatants contain all of the molecules that are secreted, shed, or discharged by *B. dendrobatidis* into water over 24 hours. Data in Chapter II showed that dead *B. dendrobatidis* cells continued to release factors even after being killed and washed, suggesting that the factors did not need to be actively secreted to be released. The soluble factors in *B. dendrobatidis* supernatants are likely to be a complex mixture of macromolecules and metabolites that may have different effects on host cells. Therefore, the components most responsible for impairing lymphocytes need to be isolated and identified to tease apart the contributions of each component. Also, identification of the lymphotoxic factors would allow for more direct investigation of the mechanism of inhibition and induction of apoptosis.

Depending on the nature and effects of the identified factor or factors, these molecules may be developed into drugs to treat human disease. *Batrachochytrium dendrobatidis* supernatants effectively inhibit human and mouse lymphocytes (Chapter II). If the isolated factors are specific to lymphocytes, these molecules could be used to treat autoimmune disorders or graft-versus-host disease that are typically mediated by lymphocyte responses (Kahan, 2003; Penn, 2006; Vecchiarelli & Monari, 2010). *Batrachochytrium dendrobatidis* supernatants also inhibited proliferation of CHO and HeLa cells (Chapter II) which are continuously proliferating, epithelial-derived cells. If more than one factor produced by *B. dendrobatidis* is responsible for inhibition of epithelial-like cells distinct from the effects on lymphoid cells, a separate investigation should determine what these independent factors are. However, if the factors responsible for lymphocyte impairment also inhibit cell proliferation in other cells, these factors likely kill or slow proliferation of dividing cells. This effect is desirable to treat diseases of

cellular proliferation. Autoimmune diseases may be treated by such a drug, but the broader effect would make it more suitable for killing tumorigenic cells.

To characterize the inhibitory factors present in *B. dendrobatidis* supernatants, different approaches were taken to understand how *B. dendrobatidis* produces these factors and what the chemical nature of the factors may be. *Batrachochytrium dendrobatidis* cells were incubated at different temperatures to determine how and when *B. dendrobatidis* might release the factors. A chitin synthesis-inhibiting drug, nikkomycin Z, was used to treat *B. dendrobatidis* cells to determine the importance of the cell wall on the release of inhibitory factors. *Batrachochytrium dendrobatidis* supernatants were treated under different conditions or with lysing enzymes to understand the general chemical properties of inhibitory factors. Treatments of heat, acid, protease, RNase, and a cocktail of cell wall-digesting enzymes were applied to *B. dendrobatidis* supernatants. *Batrachochytrium dendrobatidis* supernatants were also fractionated using several techniques. Some spectrometric analysis was also used to attempt to identify components of *B. dendrobatidis* supernatants. The results from these analyses suggests that *B. dendrobatidis* sheds soluble lymphotoxic factors from its cell wall that are likely to be carbohydrates and small molecule metabolites.

Materials and Methods

Zoospore isolation and preparation of zoospore supernatants

Batrachochytrium dendrobatidis isolate JEL197 was cultured as described in Chapter II. Zoospores were purified as previously described (Rollins-Smith et al ., 2002) by twice flooding the agar surface of seven-day old cultures of *B. dendrobatidis* growing on 1% tryptone agar

using 3 to 5 mL of sterile 1% T-broth. The broth containing zoospores was passed over sterile nylon spectra/mesh filters (Spectrum Laboratories, Rancho Dominguez, CA, USA) of several mesh opening sizes to remove mature cells. Zoospores used for co-culture experiments were enriched by passage through 3 µm mesh opening filters (about 99% purity, see Appendix A). Zoospores used for transwell experiments were enriched by passage through 20 µm mesh opening filters (about 80% purity). Zoospores used for supernatant experiments were enriched by passage through 5 µm mesh opening filters (about 93% purity). To prepare supernatants, zoospores were isolated, centrifuged to remove tryptone growth medium, washed with sterile distilled water, re-suspended at 2 x 10^7 zoospores per mL (twice the cellular concentration compared to supernatants prepared with mixed cultures of mature cells) in sterile distilled water, and incubated at 21°C for 24 or 48 hours. After incubation, zoospores were centrifuged, and supernatants were passed through 0.2 µm filters (Fisher, Waltham, MA, USA) to remove any cells, frozen, and lyophilized. Lyophilized supernatants were re-suspended in L-15 and mixed with *X. laevis* splenocytes (as described in Chapter II Materials and Methods) at 1.25X to 10X concentration.

Calcofluor White staining of *B. dendrobatidis* cell walls

Calcofluor White Stain (Sigma, St. Louis, MO) was used to stain *B. dendrobatidis* cells walls. Calcofluor white is a non-specific fluorochrome that binds to cell wall cellulose and chitin (Herth & Schnepf, 1980; Monheit et al. 1984). *Batrachochytrium dendrobatidis* cells were added to a clean glass slide and mixed with one drop of calcofluor white stain followed by a drop of 10% potassium hydroxide. Photographs were obtained using an excitation wavelength of 365 nm with an Olympus BX41 microscope and an Olympus DP71 camera with DP Controller

software, v.3.1.1.267 (Olympus Corporation). Excitation of calcofluor white at this wavelength results in an emission wavelength of 435 nm, by calcofluor white associated with chitin producing a blue color (Herman 2001; Henry-Stanley et al. 2004).

Nikkomycin Z treatments

Nikkomycin Z-HCl (NZ) was obtained for research purposes from Dr. John Galgiani, Director of the Valley Fever Center for Excellence of the University of Arizona. For experiments in which *B. dendrobatidis* was pretreated with NZ before addition to lymphocytes, mixed zoospores and sporangia were split, and half of the cells were treated with 20 µM NZ in 1% T-broth. After incubation at 21°C for 3 days, *B. dendrobatidis* was centrifuged to remove NZ, washed, and re-suspended in complete L-15 medium. *Batrachochytrium dendrobatidis* cells treated or untreated with NZ were incubated with splenocytes at 10^5 cells per mL. For experiments in which *B. dendrobatidis* supernatants were prepared from NZ-treated *B. dendrobatidis*, *B. dendrobatidis* cells were treated with 10 µM NZ for 24 hours. The NZ was removed and washed of the cells, and supernatants were prepared as previously described. Supernatants from NZ-treated *B. dendrobatidis* were incubated with *X. laevis* splenocytes at 5X concentration.

The effect of NZ on *B. dendrobatidis* growth and replication was quantified by an optical density as described by Rollins-Smith et al. (2002). Zoospores (5×10^4 per well) in tryptone broth were added to the wells of a 96-well flat-bottom microtiter plate (BD Falcon, Franklin Lakes, NJ) along with serial dilutions of NZ in HPLC-grade water (Fisher Scientific, Pittsburgh, PA) to achieve final concentrations of 0.02 to 2000 µM. Positive and negative control wells received HPLC-grade water alone. Zoospores in negative control wells were heat-killed as previously

described (Chapter II). The total volume of every well was 100 µl. The assay plates were incubated for seven days at 21°C. Growth of *B. dendrobatidis* during this time period was measured as the change in optical density measured at 490 nm (OD_{490}) with an MRS Microplate Reader (Dynex Technologies, Inc., Chantilly, VA).

Maturation of enriched zoospores treated with NZ was determined morphologically under a light microscope. Cells were either characterized as zoospore or mature cell. Mature cells were grouped as *B. dendrobatidis* cells that have a cell wall; mature cells include the stages of encysted zoospore, germling, thallus, and zoosporangium (Berger et al., 2005a). The total number of each cell type was divided by the original number of zoospores to assess the ability of zoospores to mature in the presence of NZ.

Batrachochytrium dendrobatidis cells were stained with calcofluor white to determine the cell diameter of mature cells. *B. dendrobatidis* zoospores were incubated for three or seven days in 1% tryptone broth with or without NZ and then stained, as described above, with calcofluor white. Cell diameters of mature cells were measured to the nearest µm in images using a computer-calibrated scale bar.

Treating supernatants with Glucanex™

Batrachochytrium dendrobatidis supernatants were treated with a cocktail of lysing enzymes from *Trichoderma harzianum* (Sigma, St. Louis, MO, USA), also known as Glucanex™, which contains β-glucanases, chitinases, cellulases, and proteases. *Batrachochytrium dendrobatidis* supernatants boiled prior to lyophilization were re-suspended in 50 mM sodium citrate, pH 5.5, at 100 times the original concentration before lyophilization. Supernatants and buffer controls were incubated with 0 or 2 mg/mL Glucanex at 37°C for 24

hours. After this digest, enzymes were inactivated by incubation in 100°C water bath for 30 minutes. All treatments were lyophilized and re-suspended in L-15 to be incubated with splenocytes in a proliferation assay as described above. Because the buffer used for the Glucanex treatment had a lower pH than L-15, a small amount of 0.5 M NaOH was added to re-suspended samples to achieve a pH of about 7.5. To determine if carbohydrates (β-glucans and potentially chitin) were being hydrolyzed by enzymes in the Glucanex cocktail, reducing sugar was quantified before and after incubation using the Dinitrosalicylic Acid (DNSA) method with a glucose standard (Miller, 1959; Rana et al., 2003). Briefly, a DNSA reagent [10 mg/mL 3,5-dinitrosalicylic acid (Sigma), 300 mg/mL sodium potassium tartrate tetrahydrate (Sigma), 0.5 M NaOH] was added to an equal volume of sample or glucose standard (ranging in concentration from 0.1 to 8 mg/mL) and boiled for 10 minutes. After boiling with the DNSA reagent, samples were diluted ten-fold in water and absorbance was read at 530 nm. As a positive control, laminarin (Sigma) at a concentration of 10 mg/mL was also treated with 2 mg/mL Glucanex and assayed using the DNSA method. Because Glucanex produced a background color change with the DNSA reagent, absorbance at 530 nm was measured for samples at the beginning and end of incubation with Glucanex.

Treating splenocytes with laminarin

Laminarin, a soluble form of β-1,3-glucan with some β-1,6 branching (Rioux et al., 2010; Elsori et al., 2011), from *Laminarina digitata* (Sigma, St. Louis, MO, USA) was used to determine if one of the soluble factors that inhibit lymphocytes could be β-1,3 or β-1,6-glucan, major cell wall components in higher fungi (Klis et al., 2002). *X. laevis* splenocytes, prepared as described previously (Chapter II) and stimulated with 2 μg/mL PHA, were incubated with

laminarin at concentrations between 25 and 400 μg/mL [the approximate concentrations at which soluble β-1,3-glucan blocks the pattern recognition receptor Dectin-1 in mammalian phagocytes (Elsori et al., 2011; Goodridge et al., 2011)]. Proliferation of lymphocytes was quantified by ^{3}H-thymidine uptake as described in Chapter II.

Heat treatment of supernatants

Batrachochytrium dendrobatidis supernatants were prepared as described in Chapter II. Heat treatment of *B. dendrobatidis* supernatants occurred either before or after lyophilization. When heating occurred before lyophilization, freshly prepared supernatants were placed in glass tubes and incubated for 30 minutes in a 100°C water bath. When heating occurred after lyophilization, lyophilized supernatants were re-suspended at one-tenth their original volume in distilled water and placed in glass vessels in 100°C water baths for 30 minutes. Distilled water controls were also boiled to ascertain that there were no artifacts from the heating process. If concentrated supernatants were boiled right before adding to lymphocytes, the boiled samples were cooled to 4°C before being mixed with *X. laevis* splenocytes in 2X L-15. Untreated *B. dendrobatidis* supernatants or water controls were incubated at 4°C during the period of heat-treatment.

Trifluoroacetic acid treatment of supernatants

Lyophilized *B. dendrobatidis* supernatants were re-suspended in sterile distilled water, mixed with an equal volume of 4% trifluoroacetic acid (TFA), and incubated at 37°C for 30 minutes. A control containing water with TFA only and a control containing untreated *B. dendrobatidis* supernatant were also incubated at 37°C for 30 minutes. The TFA-treated *B.*

dendrobatidis supernatant and the TFA-treated water control were diluted with an equal volume of sterile distilled water and placed in dialysis tubes (8 kDa cut-off) (BioDesign, Carmel, NY, USA) and dialyzed against distilled water for 24 hours at 4°C to remove TFA. After dialysis, the samples were lyophilized again and added to *X. laevis* splenocyte cultures at 10X concentration.

RNase A treatment of supernatants

Lyophilized *B. dendrobatidis* supernatants were re-suspended to 100X concentration in PBS. RNase A stock was prepared at 4.39 mg/mL (250 Kunitz units/mL) in 1.12% sodium citrate at pH 8.2 (see PI nuclear staining method in Chapter II). Supernatant at 100X or PBS control (160 μL) was added to 40 μL of RNase A stock or sodium citrate buffer control. These treatments were incubated overnight at room temperature. After incubation, all samples were diluted ten-fold in complete RPMI medium and serially diluted with equal volumes of RPMI. Samples were incubated with Jurkat cells in a 96-well plate, and Jurkat cells were assayed with the MTT method viability as previously described (Chapter II). The final concentrations of supernatant in Jurkat cell cultures was 4X, 2X, and 1X. RNase A was diluted out in Jurkat cell cultures to 0.25, 0.125, and 0.0625 Kunitz units per well; at these concentrations RNase A had no effect on Jurkat cells.

Protein concentration of *B. dendrobatidis* supernatants

The protein concentration of *B. dendrobatidis* supernatants was determined by the colorimetric MicroBCA^{TM} (bicinchoninic acid) using bovine serum albumin protein standards, as previously described (Smith et al., 1985; Gammill et al., 2012). Fresh and lyophilized

supernatants were diluted to various concentrations in HPLC-grade water before BCA reagents were added to determine protein concentration.

Proteinase K treatment of *B. dendrobatidis* supernatants

To test whether inhibitory factors were protein in nature, *B. dendrobatidis* supernatants were digested with proteinase K-conjugated to agarose beads (Sigma, St. Louis, MO, USA). Because this insoluble proteinase K only efficiently digests protein in a buffered calcium solution, supernatants were re-suspended at 100X in a solution containing 40 mM Tris-HCl and 4 mM $CaCl_2$ at pH 7.8. Controls lacking supernatants consisted of this buffer alone. *Batrachochytrium dendrobatidis* supernatant or buffer alone, as a control, was mixed with 10 mg of proteinase K-agarose beads and incubated at 37°C overnight. Supernatants and buffer controls, with and without proteinase K, were also incubated at 37°C overnight. Proteinase K was removed by centrifuging the agarose beads and collecting the remaining liquid. The remaining liquid from each treatment was diluted ten-fold in distilled water to decrease the concentration of the calcium buffer. Supernatants and buffer controls were mixed 1:1 with lymphocytes in 2X L-15 so the final concentration of supernatants was 5X. Supernatants treated or not with proteinase K beads and buffer-only controls were resolved on a 12% polyacrylamide electrophoresis gel which was silver stained (Blum et al., 1987) to visualize the proteins.

Crude size separation of *B. dendrobatidis* supernatants

Freshly prepared supernatants were size fractionated by molecular weight using Amicon Ultra centrifugal filter columns (Millipore, Billerica, MA, USA) with size cut-offs at 10, 30, 50, and 100 kDa. Fractions at the top or bottom of columns were mixed into *X. laevis* splenocyte

culture with an equal volume of 2X L-15. In some instances fractions were lyophilized to be tested on lymphocytes. To investigate whether small or large molecular components are heat sensitive, fractions greater than and less than 10 kDa were boiled after size separation.

Mass spectrometry analysis of supernatants

Chytrid supernatant samples were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) in the Vanderbilt University Mass Spectrometry Core by Dr. Michelle Reyzer. Matrices employed for MALDI/MS were α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB). Data was acquired in positive and negative ion mode. Mass-to-charge ratios (m/z) were determined by time-of-flight (TOF), linear trap quadrupole (LTQ), and Fourier transform (FT) ion cyclotron resonance. Accurate masses were obtained when possible by (FT). Fragmentation of peaks was noted when available. Three different supernatants were tested: *B. dendrobatidis* supernatants, *B. dendrobatidis* supernatants boiled before lyophilization, and *H. polyrhiza* supernatants. Lyophilized supernatants were diluted to 100X, 10X, and 1X before mixing with an equal volume of matrix. Better spectra were obtained from the 10X concentrated supernatants, so this concentration was used for all MALDI/MS.

Analysis of *B. dendrobatidis* supernatants for the presence of lipids was completed by Dr. Pavlina Ivanova in the laboratory of Dr. Alex Brown at Vanderbilt University. Lyophilized *B. dendrobatidis* and *H. polyrhiza* supernatants were re-suspended to 100X in HPLC-grade water (100X concentration had better spectra than 10X and 1X). Supernatants were also re-suspended in 90% methanol, 10% chloroform. Samples were analyzed by liquid chromatography mass spectrometry (LC/MS). Unique peaks present in *B. dendrobatidis* supernatants and absent or

diminished in *H. polyrhiza* supernatants were analyzed with tandem mass spectrometry to determine if these components had any fragments corresponding to lipid components.

Small molecule analysis of supernatants

Small molecule analysis of *B. dendrobatidis* samples was completed by Dr. Thomas Umile in the laboratory of Dr. Kevin Minbiole at Villanova University. *Batrachochytrium dendrobatidis* supernatants or ethyl acetate extractions from *B. dendrobatidis* were analyzed by HPLC through a C18 column with a UV detector. Fractions with peak UV absorbance were analyzed by ^{1}H-NMR at Princeton University by Dr. Thomas Umile to identify the structures of metabolites.

Testing small molecule candidates on lymphocytes

Several peaks were visible on spectra from *B. dendrobatidis* supernatants and ethyl acetate extracts. Some of these compounds were isolated for further analysis by ^{1}H-NMR by Dr. Thomas Umile. The structure of a few of these compounds was identified. Identified compounds available for purchase were tested on *X. laevis* splenocytes to determine inhibition of lymphocyte proliferation.

Cyclo-L-phenylanalanyl-L-proline (cyclo[Phe-Pro]) is a metabolite produced by bacteria and fungi (Trigos et al., 1997; Stöm et al., 2002). Cyclo(Phe-Pro) (Chem-Impex International, Wood Dale, IL, USA) was re-suspended in isopropanol and added to splenocytes culture at final concentrations ranging between 0.5 and 20 mM. Isopropanol controls were also tested on splenocytes.

Cyclo-L-histidyl-L-proline (cyclo[His-Pro]) is an endogenous diketopiperazine present in various human tissues (Prasad, 1988). Cyclo(His-Pro) (Chem-Impex International, Wood Dale, IL, USA) was re-suspended in DMSO and added to splenocytes culture at final concentrations ranging between 50 μM and 10 mM. DMSO controls were also tested on splenocytes.

L-kynurenine is an oxidation product of L-tryptophan (Mellor, 2004; Belladonna et al. 2007). In humans and mice serum concentrations of L-kynurenine range between 2-8 μM, but can reach 1 mM concentration in the coronary lumen (Widner et al., 1997; Wang et al., 2010). A large concentration range of L-kynurenine (Sigma, St. Louis, MO, USA) was tested on Jurkat cells from 10 nM to 500 μM to determine the concentrations at which lymphocyte proliferation may be affected. L-kynurenine was later mixed in *X. laevis* splenocyte culture at final concentrations ranging between 1-100 μM.

Phenol sulfuric acid

The phenol-sulfuric acid (P-S) method was used to determine the presence of carbohydrate in *B. dendrobatidis* supernatants and fractions of *B. dendrobatidis* supernatant obtained by solid phase extraction. The P-S method is a colorimetric assay that quantifies the number of hexose and pentose carbohydrates present in a sample (Nielsen, 2010). In the P-S method, concentrated sulfuric acid (18 M) was added to sample at a ratio of 1:4, sample:sulfuric acid. Immediately after adding sulfuric acid, 5% phenol was added at one-fifth the volume of sulfuric acid. When the assay was completed in wells of a 96-well plate, 50 μL of sample was added to wells, followed by 150 μL of 18M sulfuric acid, and finally 30 μL of 5% phenol. Heating of samples, often present in protocols of this method, was not necessary for a color change. Absorbance of samples was quantified at 490 nm. To determine the relative amount of

hexose present in *B. dendrobatidis* supernatant samples, glucose standards were used to generate a standard curve.

Solid phase extraction

Solid phase extraction (SPE) of *B. dendrobatidis* supernatants was completed with C18, SAX, and CN columns. C18 separation was completed at Vanderbilt University by Scott Fites using C18 Sep-Pak cartridges (Waters Corporation, Milford, MA, USA): supernatant volumes were pushed through columns using a peristaltic pump. C18 columns were eluted with increasing concentrations of acetonitrile (ACN), whereas the flow through fraction contained no ACN. SPE with SAX and CN was completed at James Cook University, Townsville, Australia by Dr. Alexandra Roberts. SAX was eluted with decreasing pH starting with 10 mM NaOH and then eluting with increasing concentrations of HCl. CN was eluted with increasing concentrations of methanol or ACN. Fractions obtained by SPE were dried to remove solvent. Dry samples were re-suspended in L-15 or RPMI media and added to *X. laevis* splenocytes (C18) or Jurkat cell (SAX and CN). Concentrations of samples were determined based on the volume of the original supernatant sample loaded into columns. Final concentrations of samples were between 5X and 10X indicating that the volume that fractions were re-suspended in was one-fifth or one-tenth the volume of the 1X *B. dendrobatidis* supernatant used to load columns.

Results

The *B. dendrobatidis* lymphotoxic factors are produced by cells after maturing past the zoospore stage

Batrachochytrium dendrobatidis is generally defined as having two developmental stages: a free-living motile zoospore and a host-associated zoosporangium (Longcore et al., 1999). Several morphological changes occur during the transition from the zoospore to a large zoosporangium filled with zoospores. The first transition occurs as the zoospore first settles on the surface of the skin of amphibian hosts. At this point zoospores encyst, absorbing their flagella and forming a cell wall (Berger et al., 2005a). At this stage, the encysted cell forms a germination tube to infect host keratinocytes deep inside the host epithelium (Van Rooij et al., 2012; Greenspan et al., 2012). Observation of germination tubes developing from *B. dendrobatidis* zoospores in culture has not been published, but electron microscopy of *Batrachochytrium salamandrivorans* suggests that this chytrid can form germination tubes in culture media (Martel et al., 2013). I have observed that *B. dendrobatidis* does form germination tubes in culture, and these germination tubes appear to be covered with a cell wall because they stain with calcofluor-white, a chitin stain (Fig. 4-1). These structures are likely germination tubes because they are too large to be flagella and are not branched like rhizoids.

Soon after the encystment stage, *B. dendrobatidis* cells develop into a form called a germling, which are not much larger than zoospores but have a cell wall and rhizoid structures (Berger et al., 2005a). Germlings develop into a stage called a thallus which eventually becomes a mature zoosporangium filled with zoospores. The mature stages of *B. dendrobatidis* are somewhat difficult to distinguish among each other and even more difficult to isolate, but zoospores can be enriched by passing over mesh filters (Rollins-Smith et al., 2002; also see

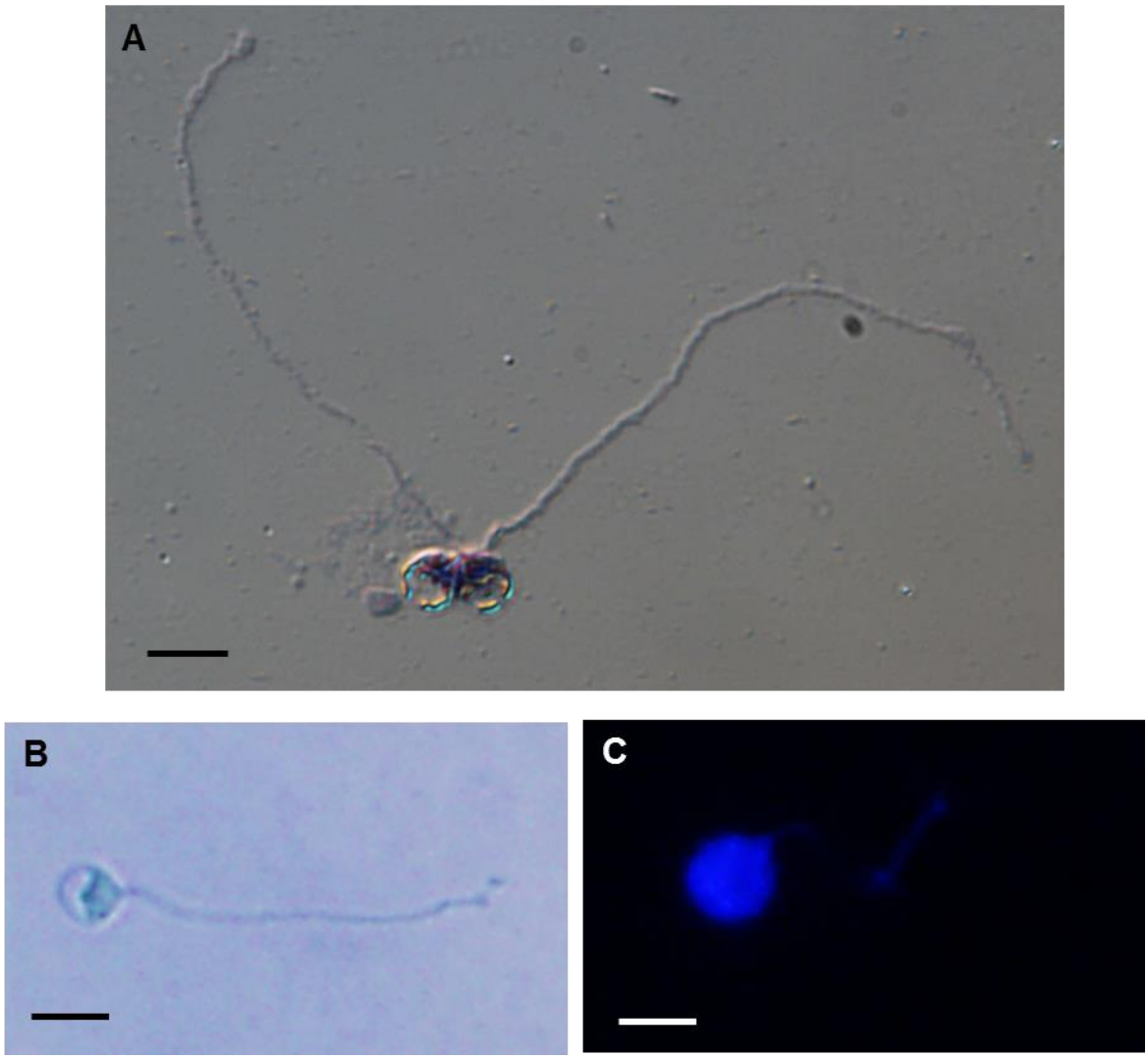

Fig. 4-1. *Batrachochytrium dendrobatidis* (*Bd*) cells produce germination tubes *in vitro*. *Bd* cells from cultures were stained with (**A**) Wright-Giemsa, (**B**) trypan-blue, or (**C**) calcofluor white. Projections off of cells are most likely germination tubes because they are larger in diameter and, in most cases, longer than flagella. Projections are not branched like rhizoids. Projections stain with calcofluor white suggesting that they are coated with cell wall. Cells in panels A and B were incubated in L-15 for 1-2 days and cells in panel A were in culture with *X. laevis* peritoneal leukocytes during this time. Panel C was an image captured of one of the cells treated in 1% tryptone alone (no nikkomycin Z) from the experiment shown in Fig. 4-5.

Appendix A). Enriched zoospores were obtained to investigate when *B. dendrobatidis* produces inhibitory factors.

To identify the life stages that inhibit lymphocytes, zoospores were purified and mixed directly with PHA-stimulated splenocytes. Living zoospores from two *B. dendrobatidis* isolates inhibited the PHA-induced proliferation of *X. laevis* lymphocytes (Fig. 4-2 A). Because zoospores can mature rapidly at 26°C, we tested whether heat-killed zoospores also inhibit splenocytes (Fig. 4-2 B). During three days of incubation, zoospores matured as indicated by the development of rhizoid structures (Fig. 4-2 C). The living zoospores that had matured into germlings inhibited lymphocyte proliferation (Fig. 4-2 B, solid bars). Heat-killed zoospores did not mature (Fig. 4-2 D). In contrast to live zoospores or freshly killed mature cells (Chapter II), heat-killed zoospores were incapable of inhibiting lymphocyte proliferation (Fig. 4-2 B, open bars). Live zoospores did not inhibit lymphocyte proliferation when physically separated from lymphocytes in a transwell, but reduced proliferation when in direct co-culture with lymphocytes (Fig. 4-2 E). Even when zoospores were placed with splenocytes in the top chamber of a transwell, proliferation was not inhibited in the bottom chamber (Fig. 4-2 F) suggesting that release of inhibitory factors is not induced by the presence of host cells.

Supernatants were also prepared from *B. dendrobatidis* zoospores. Unlike the supernatants from mixed cultures containing mature *B. dendrobatidis* cells, 24 hour supernatants from zoospores highly enriched over 5 μm pore-sized filters (93% zoospore purity, Appendix A) did not inhibit *X. laevis* lymphocyte proliferation (Fig. 4-3 A). If zoospores were enriched with a filter with lager pores (20 μm), the purity of zoospores was greatly decreased (83% purity, Appendix A). Supernatants from populations of *B. dendrobatidis* zoospores that were not as highly enriched did inhibit lymphocyte proliferation at higher supernatant concentrations

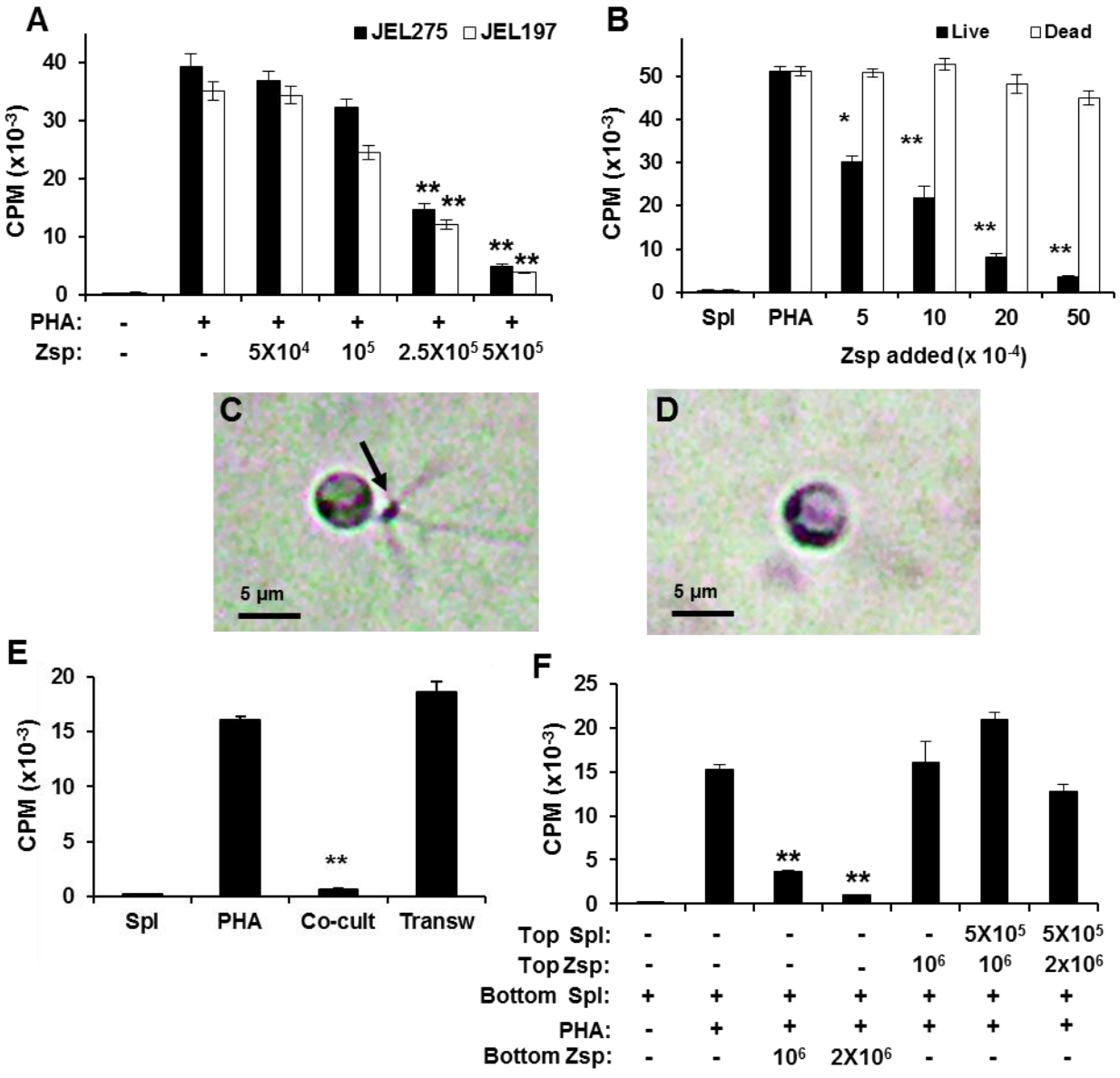

Fig. 4-2. *Batrachochytrium dendrobatidis* (*Bd*) zoospores do not inhibit lymphocyte proliferation of *X. laevis* splenocytes until they mature; see following page for details.

Fig. 4-2. *Batrachochytrium dendrobatidis* (*Bd*) zoospores (Zsp) do not inhibit lymphocyte proliferation of *X. laevis* splenocytes until they mature. (**A**) *X. laevis* splenocytes were cultured alone or with PHA, and PHA-stimulated cells were cultured with increasing numbers of living zoospores of *Bd* isolate JEL197 or JEL275. (**B**) Splenocytes (Spl) were cultured alone or with PHA. PHA-stimulated Spl were incubated with increasing numbers of live or heat-killed *Bd* (JEL197) zoospores. Live zoospores (**C**) developed into germlings during the 3-day culture as shown by the formation of rhizoids (arrow); dead zoopores (**D**) failed to develop (bar: 5 μm). (**E**) Splenocytes were cultured as in (A), and PHA-stimulated splenocytes were co-cultured (Co-cult) with or separated from live zoospores by a 0.4-mm filter in transwell (Transw). (**F**) Splenocytes were cultured as in (D) in the bottom chambers of a transwell plate at 5×10^5. PHA-stimulated Spl were cultured directly with Zsp (bottom chamber), or Zsp were placed in the top chamber of a transwell culture separate from Spl stimulated with PHA. In some wells, Spl were cultured along with Zsp in the upper chamber to determine whether the interaction would result in inhibition of PHA-stimulated lymphocyte proliferation in the bottom chamber. Significantly reduced ^{3}H-thymidine uptake compared to the control treatment, *$p<0.05$, **$p<0.01$ (ANOVA with Tukey post hoc test). CPM are averages ± SEM of at least three replicate wells and are representative at least two similar experiments. (Experiments in panels E and F were completed by Jeremy Ramsey and Sophia Gayek.)

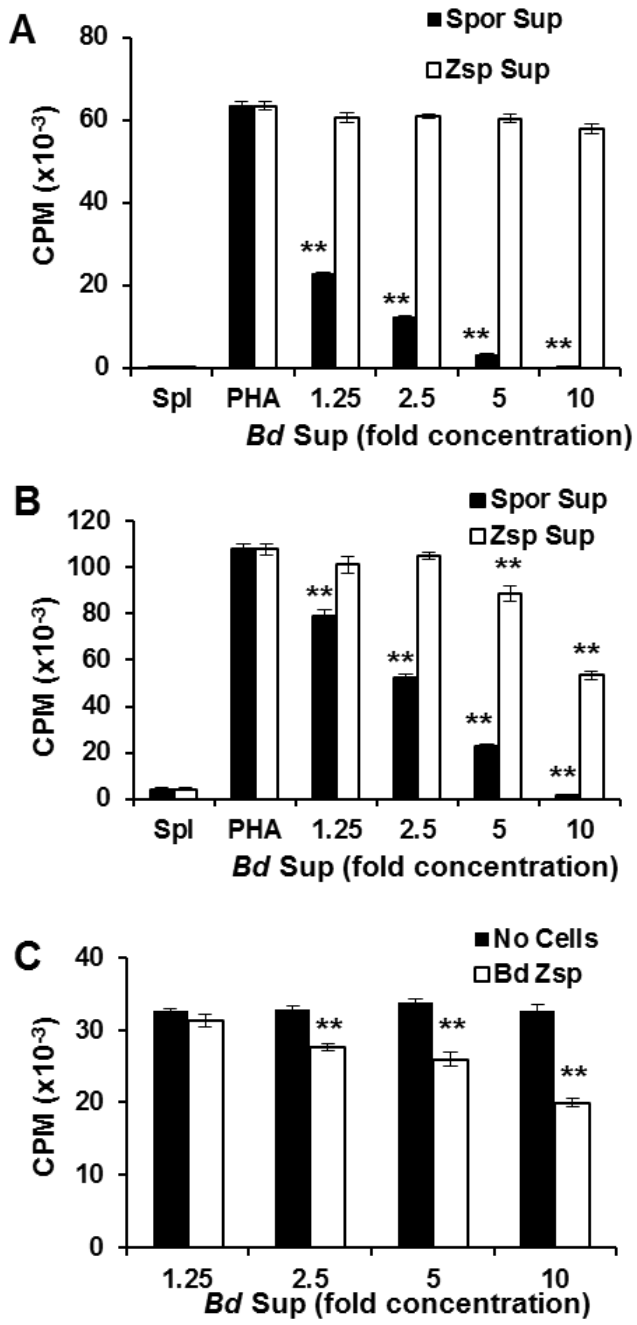

Fig. 4-3. *Batrachochytrium dendrobatidis* (*Bd*) zoospores do not release inhibitory factors until they begin to mature and form a cell wall. Supernatants from *Bd* populations were lyophilized and re-suspended into culture with PHA-stimulated *X. laevis* splenocytes (Spl) (**A-B**) Supernatant from whole *Bd* cultures containing both zoospores and sporangia (Spor Sup), from enriched zoospores (Zsp Sup) were incubated with splenocytes. Zoospores were either more enriched (A, 93% zoospores) or less enriched (B, 83% zoospores) before incubated in water for 24 hours. (**C**) Zoospores were highly enriched as in (A) but incubated in water for 48 hours to allow cells to mature; supernatants were lyophilized and re-suspended into splenocyte culture. Significantly reduced ^{3}H-thymidine uptake compared to the no supernatant (or no cell) control treatment, **$p<0.01$ (ANOVA with post hoc test). CPM in each panel are averages ± SEM of five or more replicate wells and represent three similar experiments.

(Fig. 4-3 B). Also when the highly enriched zoospore population was incubated an extra 24 hours to allow for maturation of zoospores, the supernatants were inhibitory to lymphocyte proliferation (Fig. 4-3 C). The inhibition by 20 μm-pore filter enriched zoospores and by 2-day zoospore supernatants was still greatly reduced compared to mixed *B. dendrobatidis* cultures containing many matured *B. dendrobatidis* cells. These data suggest that *B. dendrobatidis* begins to release factors soon after maturation and cell wall development, but the peak of the release of the lymphotoxic factors is delayed until later maturation steps.

The lymphotoxic factors appear to be associated with the *B. dendrobatidis* cell wall.

One major difference between *B. dendrobatidis* zoospores and mature *B. dendrobatidis* cells is that zoospores lack a cell wall (Longcore, et al. 1999; Berger et al., 2005a). Because zoospores do not produce lymphotoxic factors but appear to begin to produce them during early stages of maturation, I hypothesized that the lymphotoxic factors are associated with the *B. dendrobatidis* cell wall. Several other pathogenic fungi have immunomodulatory molecules associated with their cell walls (Rappleye et al., 2007; De Jesus et al., 2010; Gravelat et al., 2013). The cell wall is at the interface of host and pathogen encounters; therefore, possessing immunomodulatory factors on the cell wall would be beneficial for *B. dendrobatidis* to resist immune clearance.

To test whether the lymphotoxic factors were associated with the *B. dendrobatidis* cell wall, nikkomycin Z (NZ) was used to inhibit the *B. dendrobatidis* cell wall development. NZ is a competitive inhibitor of chitin synthase (Cohen 1987; Hector 1993). Chitin is an important component of the *B. dendrobatidis* cell wall, especially because *B. dendrobatidis* cell walls appear to lack the β-1,3-glucan component that is present in the cell walls of higher fungi (Ruiz-

Herrera & Ortiz-Castellanos, 2010). Treatment of *B. dendrobatidis* with NZ significantly impaired the *B. dendrobatidis* cell wall (Holden et al., 2014). *Batrachochytrium dendrobatidis* growth was inhibited by NZ at concentrations above 300 nM (Fig. 4-4 A). The decrease in growth is likely due to a decrease in *B. dendrobatidis* proliferation (Fig. 4-4 B) and a decrease in the number of cells that survive to maturity (Fig. 4-4 C). To investigate the effects on *B. dendrobatidis* cell morphology, enriched zoospore populations were treated with different concentrations of NZ for three or seven days. *Batrachochytrium dendrobatidis* cells treated with NZ were stained with calcofluor white and viewed by fluorescent microscopy. After three days, NZ treatment appeared to cause swelling in *B. dendrobatidis* cells (Fig. 4-5 A, B). Compared to untreated cells, the diameters of cells treated with 20 μM of NZ had significantly increased the diameter of the maturing cells, most of which were in the germling or thallus stage (Fig. 4-5 C, D). The population of NZ- treated cells appear to fall into three main populations based on size: a population with the same diameter as the mode of the control (6 μm), a population with a diameter of about 10 μm, and a population with a diameter of about 14 μm (Fig. 4-5 C). The distribution of *B. dendrobatidis* cell sizes suggests that the effect of NZ on *B. dendrobatidis* cells was variable among *B. dendrobatidis* cells.

After seven days, there was a much greater effect on the cell diameter of *B. dendrobatidis* cells treated with NZ; even cells treated with 3 μM NZ had significantly greater cell diameters than untreated control cells (Fig. 4-6). Cell diameters were visibly larger in NZ-treated populations (Fig. 4-6 A-E). Cell viability was greatly reduced in NZ-treated cells, and an extra experiment had to be completed to obtain a substantial number of cells treated with 200 μM NZ. The cells treated with NZ for seven days were very heterogeneous in cell size as they were at

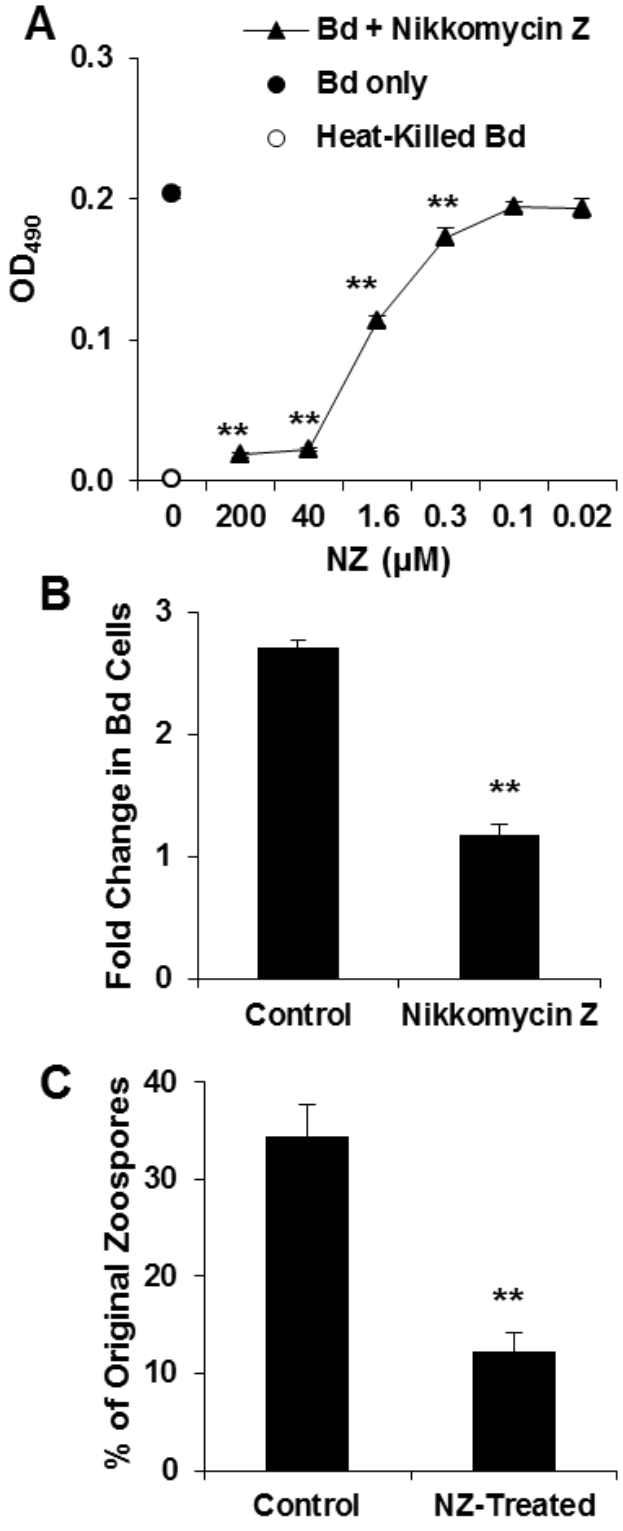

Fig. 4-4. Nikkomycin Z (NZ) inhibits *B. dendrobatidis* (*Bd*) in tryptone culture. (**A**) *Bd* was cultured with different concentrations of NZ ranging from 20 nM to 200 μM (Bd+Nikkomycin Z). Living (Bd only) and killed (Heat-Killed Bd) *Bd* were cultured in the absence of NZ. Symbols indicate the mean OD_{490} (±SEM) of five replicate wells in a representative experiment. NZ concentrations at or greater than 300 nM significantly decrease *Bd* growth, **p<0.01, one-way ANOVA with post-hoc test. (**B**) Whole cultures of *Bd* cells were treated for three days without NZ or with 20 μM NZ. The total cell number of *Bd* cells in culture was determined before and after incubation. NZ significantly reduce the proliferation of *Bd* cells (fold change), **p<0.001 by a two-tailed paired *t*-test. Mean fold change (±SEM) is shown from three experimental repeats. (**C**) Enriched zoospores were incubated with or without 20 μM NZ for three days. After incubation the relative number of zoospores that had matured was determined by microscopy. The relative number of cells that had matured significantly decreased when *Bd* was treated with NZ, **p=0.017 by a two-tailed paired *t*-test. Mean percent (±SEM) of the original zoospores that had matured is shown from four experimental repeats. (The experiment in panel A was completed by Whitney Holden.) Data published in Holden et al., 2014.

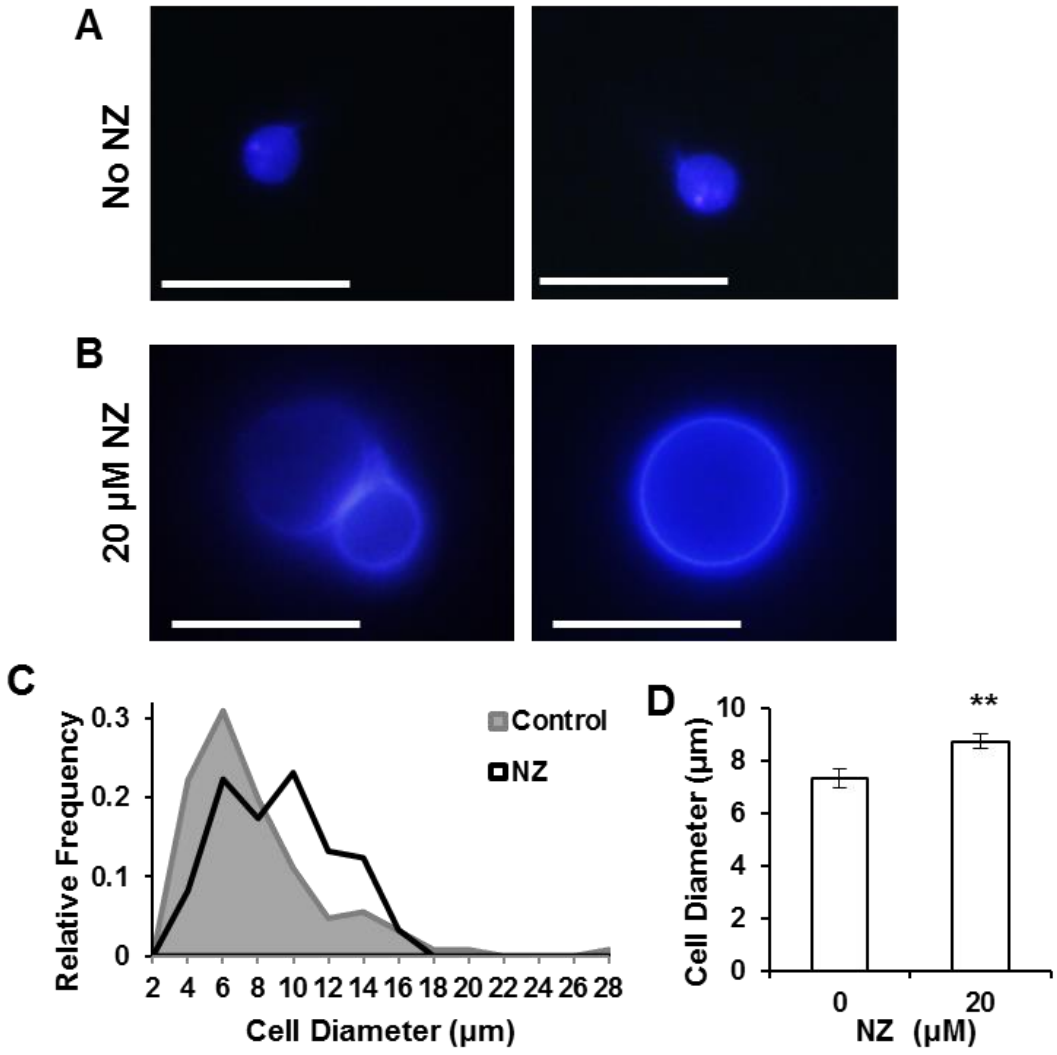

Fig. 4-5. NZ causes *B. dendrobatidis* (*Bd*) cells to swell, but the effect appears to be variable among *Bd* cells after three days. An enriched zoospore population was treated with 20 µM NZ for three days and cells were analyzed by fluorescent microscopy after being stained with calcofluor white. Representative images of cells not treated (**A**) or treated (**B**) with NZ are shown, scale bars indicate 20 µm. (**C**) The distribution of the size of *Bd* cells between treated and untreated was different. (**D**) The diameter of *Bd* cells were significantly greater in NZ-treated cells, **p=0.002 by two-tailed Student's *t*-test. Mean diameters (±SEM) are shown. N=126 for the no NZ control, N=121 for the 20 µM NZ treatment.

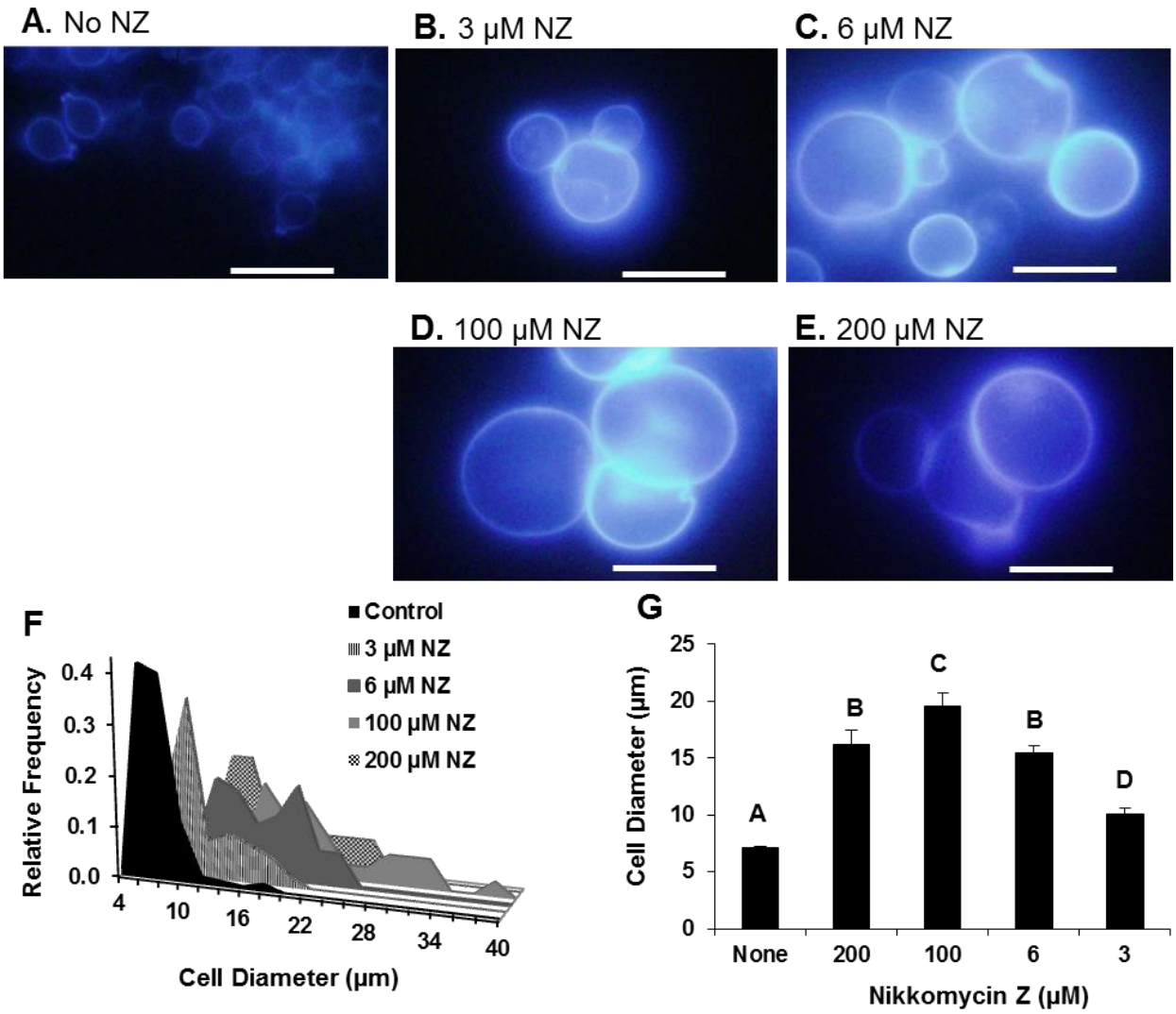

Fig. 4-6. NZ has causes *B. dendrobatidis* (*Bd*) cells to swell, and the effect still is variable among *Bd* cells after seven days. An enriched zoospore population was treated with 3-200 µM NZ for seven days and cells were analyzed by fluorescent microscopy after being stained with calcofluor white. Representative images show cells not treated with NZ (**A**) or treated with NZ at 3 µM (**B**), 6 µM (**C**), 100 µM (**D**), and 3 µM (**E**), scale bars indicate 20 µm. (**F**) The distribution of the size of *Bd* cells in NZ treated population was heterogeneous. (**D**) The diameter of *Bd* cells were significantly greater in NZ-treated cells than untreated cells, and the 100 µM NZ treatment had the greatest effect on size; ANOVA with Tukey post-hoc test, different letters indicate groups that with significantly different cell diameters: $p<0.01$. Mean diameters (±SEM) are shown. N=365 for the no NZ control, N=54 for the 200 µM NZ treatment, N=34 for the 100 µM NZ treatment, N=48 for the 6 µM NZ treatment, N=54 for the 3 µM NZ treatment. (*Bd* cells came from assays to determine the effects NZ on *Bd* growth as in Fig. 4-4A run by Whitney Holden.) Data published in Holden et al., 2014.

three days, but the sizes of the cells became much larger reaching up to 40 μm in diameter (Fig. 4-6 F). The cell diameters were the largest in the 100 μM NZ treatment (Fig. 4-6 G), but this was likely due to increased cell lysis at the highest concentration.

Batrachochytrium dendrobatidis possesses at least 8 chitin synthases (Rosenblum et al., 2008) which probably help to limit the effects of NZ (Gaughran et al., 1994). Despite being able to still synthesize some chitin, *B. dendrobatidis* growth is inhibited by NZ. NZ-treatment causes *B. dendrobatidis* cells to enlarge suggesting that cell-wall integrity is lost allowing osmotic pressure to cause cells to swell. NZ-treated *B. dendrobatidis* cells are more susceptible to osmotic lysis (Holden et al., 2014). *Batrachochytrium dendrobatidis* cells in a culture with NZ appear to have different sensitivities to the drug suggesting that *B. dendrobatidis* has some resistance to NZ. Some of this resistance may come from expressing chitin synthases with less NZ sensitivity (Gaughran et al., 1994). *Batrachochytrium dendrobatidis* cells treated with NZ also tended to clump together in culture. The colonial formations of *B. dendrobatidis* may help resist NZ by providing greater structural support and potentially limiting local concentrations of NZ.

To test the hypothesis that lymphotoxic factors are components of the *B. dendrobatidis* cell wall, NZ was used to disrupt cell walls, and the inhibition of lymphocytes by NZ-treated *B. dendrobatidis* was assayed. *Batrachochytrium dendrobatidis* cells were treated with sub-lethal concentrations of NZ (10 and 20 μM) before mixing with *X. laevis* splenocytes or preparing supernatants. *Batrachochytrium dendrobatidis* cells pre-treated with NZ before co-culturing with splenocytes were much less inhibitory than *B. dendrobatidis* cells not treated with NZ (Fig. 4-7 A). Supernatants from *B. dendrobatidis* that had been pre-treated with NZ were also significantly less inhibitory than supernatants from *B. dendrobatidis* that had not received NZ treatment (Fig.

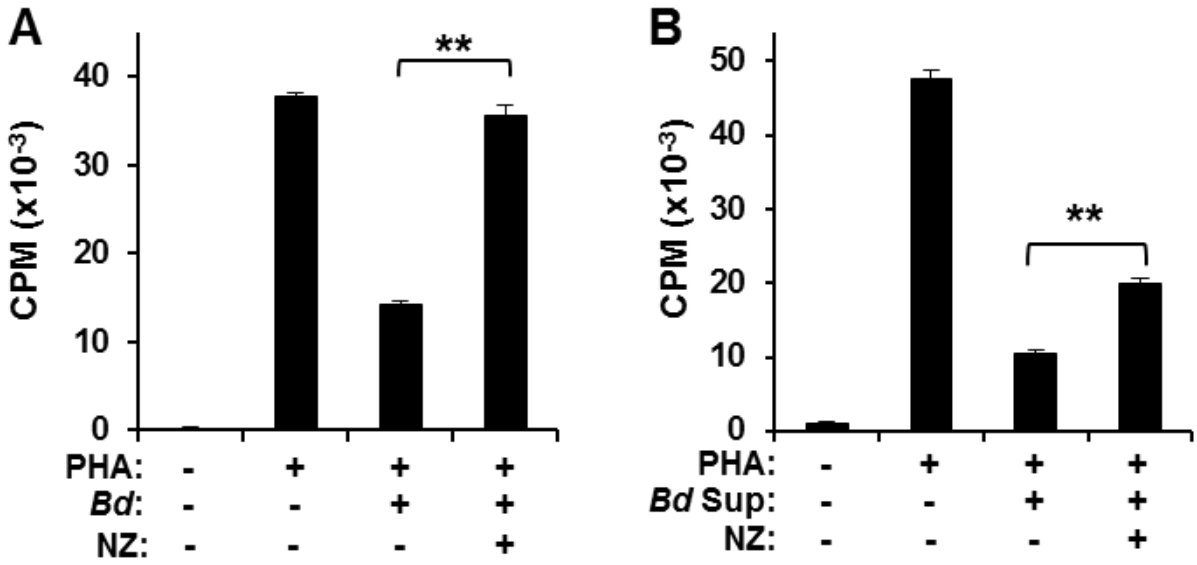

Fig. 4-7. NZ treatment of *B. dendrobatidis* (*Bd*) cells reduces the capacity of *Bd* to inhibit lymphocyte proliferation. Splenocytes were cultured alone or stimulated with PHA. PHA-stimulated splenocytes were mixed with (+) or without (-) 10^5 *Bd* cells (**A**) or 5X *Bd* Sup (**B**) that had (+) or had not (-) been pre-treated with 20 µM (A) or 5 µM (B) NZ. Proliferation was quantified by ^{3}H-thymidine uptake and CPM were averages ± SEM of five or more replicate wells per treatment. Significant differences between treatments grouped by bars using a Student's *t*-test (with correction for multiple tests in the same experiment), **$p<0.01$. Panels are representative of three similar experiments.

4-7 B). The significant decrease in impairment by NZ-treated *B. dendrobatidis* cells in co-culture compared to supernatants from NZ-treated supernatants was likely due to differences in NZ treatment. *Batrachochytrium dendrobatidis* cells that were co-cultured with splenocytes were pre-treated for three days with 20 μM NZ. Because of the large volumes necessary for supernatant preparation, *B. dendrobatidis* cells were treated with 10 μM NZ and only for 24 hours. The higher concentration and duration of treatment on *B. dendrobatidis* cells co-cultured with splenocytes explains the difference in the lymphocyte effects between *B. dendrobatidis* cells and *B. dendrobatidis* supernatants. Because NZ disrupted the cell wall and reduced the capacity of *B. dendrobatidis* to inhibit lymphocytes, these data further suggest that the lymphotoxic factors are associated with the *B. dendrobatidis* cell wall.

The major components of most fungal cell walls are carbohydrates (Klis et al., 2002; Hardinson & Brown, 2012). The base of the fungal cell wall is chitin. Most fungi have an intermediate layer of β-glucan composed of β-1,3-glucan with β-1,6 branches. Covering this β-glucan layer is a complex network of highly mannosylated proteins. Some fungi even have a polysaccharide coating to this classical cell wall. *Aspergillus* species have exopolysaccharides on the surface of cell walls (Latgé, 2012). Several *Cryptococcus* species secrete a capsule which surrounds the pathogenic yeasts (De Jesus et al., 2010). *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* can coat their cell walls with α-glucan to mask β-glucan PAMPs (Rappleye et al., 2007; Puccia et al., 2011).

To investigate whether common components of fungal cell walls do contribute to the inhibition of lymphocyte proliferation, *B. dendrobatidis* supernatants were digested with a cocktail of lysing enzymes from *Trichoderma harzianum*, called Glucanex™. Glucanex™ is frequently used to digest yeast cell walls and contains β-glucanases, chitinases, and proteases to

digest the major components of fungal cell walls (Lorito et al., 1993; Rana et al., 2003). Digestion of carbohydrates was quantified by the DNSA method (Miller, 1959) using laminarin, a soluble β-glucan (Rioux et al., 2010), as a positive control for digestion with Glucanex™. Digestion of laminarin caused a very large increase in the reducing sugars (digested from the polysaccharide), but Glucanex™ did not digest much carbohydrate in *B. dendrobatidis* supernatants (Fig. 4-8 A). Not surprisingly, Glucanex™-treated supernatants were still very inhibitory to lymphocyte proliferation (Fig. 4-8 B, C). Because Glucanex™ digestion had little to no effect on *B. dendrobatidis* supernatants, this suggests that the common carbohydrate components of fungal cell walls, chitin and β-glucan, are not responsible for lymphocyte toxicity. Chitin and β-glucan are probably not even present in *B. dendrobatidis* supernatants because there was so little reducing sugar after digestion. Also, β-glucans are not predicted to be present in *B. dendrobatidis* cell walls because the *B. dendrobatidis* genome lacks genes with homology to fungal β-1,3 and β-1,6-glucan synthases (Ruiz-Herrera & Ortiz-Castellanos, 2010). Also, chitin is not likely to be present in supernatants because chitin is very insoluble in water (Pillai et al., 2009), and the components of the *B. dendrobatidis* supernatants are quite soluble.

Glucanex™ also contains proteases, suggesting that the inhibitory factors present in *B. dendrobatidis* supernatants are not proteins. The protease activity of Glucanex™ was not tested, and the exact protease composition of Glucanex™ is not defined. Therefore, this is not definitive evidence that the factors are not protein, but this question is addressed later in this chapter.

Even though *B. dendrobatidis* lacks genes with homology to β-glucan synthases in higher fungi (Ruiz-Herrera & Ortiz-Castellanos, 2010), β-glucans may still be present in the *B. dendrobatidis* cell wall. If β-glucans were present in the *B. dendrobatidis* cell wall, they would likely activate immune responses via the recognition by Dectin-1 (Hardinson & Brown, 2012).

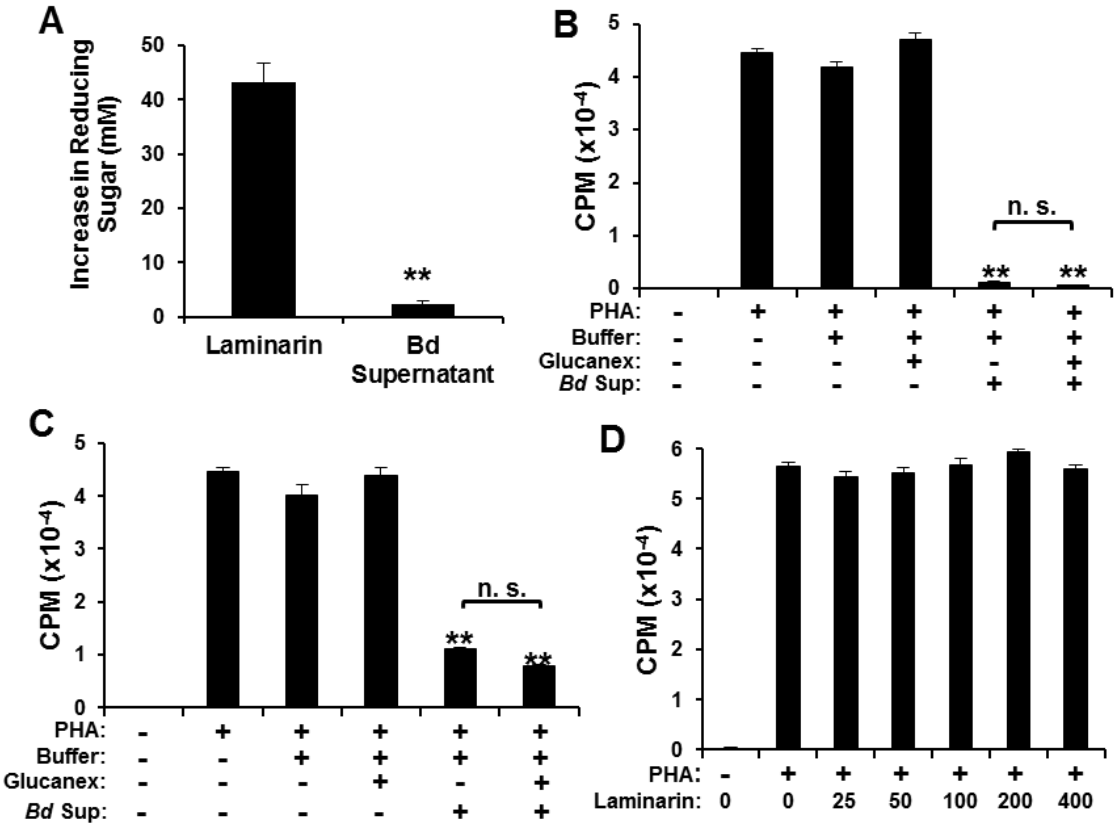

Fig. 4-8. Treating *B. dendrobatidis* (*Bd*) supernatants (Sup) with lysing enzymes (β-glucanases, chitinases, and other enzymes) from *Trichoderma harzianum* (Glucanex™) does not reduce inhibition of lymphocyte proliferation; laminarin also does not reduce lymphocyte proliferation. (**A**) *Bd* Sup (100X) or the soluble β-glucan laminarin (10 mg/mL) were treated with 2 mg/mL of Glucanex™ for 24 hours and reducing sugars were quantified using the DNSA method. **Significantly less reducing sugar by two-tailed, paired *t*-test $p<0.01$. Data show mean reducing sugar (± SEM) from four independent experiments. (**B**-**C**) *Bd* Sup or control buffer was incubated with or without Glucanex™ for 24 hours, boiled, and lyophilized. These samples were re-suspended in L-15 to achieve a final concentration of 5X (B) or 2.5X (C) and mixed with *X. laevis* lymphocytes stimulated with PHA. **Significantly reduced ^{3}H-thymidine uptake compared to the corresponding control (no supernatant) treatment by $p<0.01$ (ANOVA with Tukey test). There was no significant difference in lymphocyte proliferation (n. s.) between the Glucanex™ and no Glucanex™ treatments. (**D**) Laminarin does not inhibit *X. laevis* lymphocytes. Laminarin (0-400 μg/ml) was re-suspended and mixed with *X. laevis* splenocytes stimulated with PHA. None of the laminarin treatments showed significantly different ^{3}H-thymidine uptake from the no laminarin control (ANOVA with Tukey test). (B-D)Proliferation was quantified by ^{3}H-thymidine uptake, and CPM are averages ± SEM of a least five replicate wells and representative of three (D) or four (B-C) replicate experiments.

However, Dectin-1 requires recognition of particulate β-glucan for downstream signaling, and soluble β-glucan blocks Dectin-1 receptors (Goodridge et al., 2011). Although Dectin-1 is primarily expressed on phagocytes (Goodridge et al., 2009), soluble β-glucan could possibly interfere with amphibian lymphocyte proliferation. To test whether soluble β-glucan does interfere with lymphocyte proliferation, laminarin was incubated with PHA-stimulated *X. laevis* splenocytes. Laminarin is a soluble β-1,3-glucan with some β-1,6 branching produced by the algae *Laminarina digitata* (Rioux et al., 2010). Laminarin does not inhibit proliferation of *X. laevis* lymphocytes (Fig. 4-8 D). The β-glucans in laminarin are very similar to the β-glucans present in fungal cell walls (Klis et al., 2002), so this potential, but unlikely, component of the *B. dendrobatidis* cell wall does not appear to inhibit lymphocyte responses. Components of *B. dendrobatidis* supernatants appear to be water soluble, so chitin which is not water soluble (Pillai et al., 2009) was not investigated in a similar manner.

Batrachochytrium dendrobatidis **factors are resistant to high heat and strong acid conditions.**

In order to better define the nature of the lymphotoxic *B. dendrobatidis* factors, *B. dendrobatidis* supernatants were treated in ways designed to inactivate the factors. *B. dendrobatidis* supernatants were incubated at 100° C for 30 minutes. Boiling of *B. dendrobatidis* supernatants did not reduce the inhibition of lymphocyte proliferation (Fig. 4-9 A, B). Heating the supernatant either after (Fig. 4-9 A) or before (Fig. 4-9 B) lyophilization appeared to have the same effect. There was a slight increase in inhibition by boiled supernatants potentially indicating that heating may induce a conformational or chemical change in the inhibitory factors to become better inhibitors. This effect, however, was not seen when boiled supernatants were

exposed to Jurkat cells (see Fig. 2-16 in Chapter II). Supernatants were also treated with a strong acid (2% TFA). After the acid was dialyzed out of samples, the supernatants still inhibited lymphocyte proliferation (Fig. 4-9 C). These experiments show that the lymphotoxic factors are resistant to high heat and acid, suggesting that they are very stable and not likely to be proteins, which typically are denatured by heat and acid.

Batrachochytrium dendrobatidis cells were also treated under various temperature conditions to better understand the nature of the lymphotoxic factors and how they might be released. *Batrachochytrium dendrobatidis* supernatants were prepared at 21° (optimal growth temperature of *B. dendrobatidis*), 4°, 26°, and 37° C. Viability of *B. dendrobatidis* was determined by culturing a subset of cells after incubation, and *B. dendrobatidis* cells survived all treatments except for 37° C. *Batrachochytrium dendrobatidis* has a low thermal maximum temperature and can be cleared from amphibians at 37° C (Woodhams et al., 2003), so this temperature was the highest temperature at which *B. dendrobatidis* was incubated. Incubation at 4° C greatly decreased the amount of factor shed from *B. dendrobatidis* cells into supernatants (Fig. 4-10 A). Supernatants incubated at 26° C, the optimal temperature for *X. laevis* splenocytes, were significantly more inhibitory than supernatants incubated at 21° C (Fig. 4-10 A, B). Incubating *B. dendrobatidis* cells at 37° C killed the *B. dendrobatidis* (determined by culturing after supernatant preparation) and still caused greater release of inhibitory factors compared to incubation at 21° C (Fig. 4-10 A). These results indicate that release of factors from *B. dendrobatidis* is temperature dependent but not necessarily dependent on viable cells. If the lymphotoxic factors are associated with the *B. dendrobatidis* cell wall, as hypothesized, then higher temperatures may promote the deterioration of the cell wall causing more lymphotoxic factors to shed from the wall.

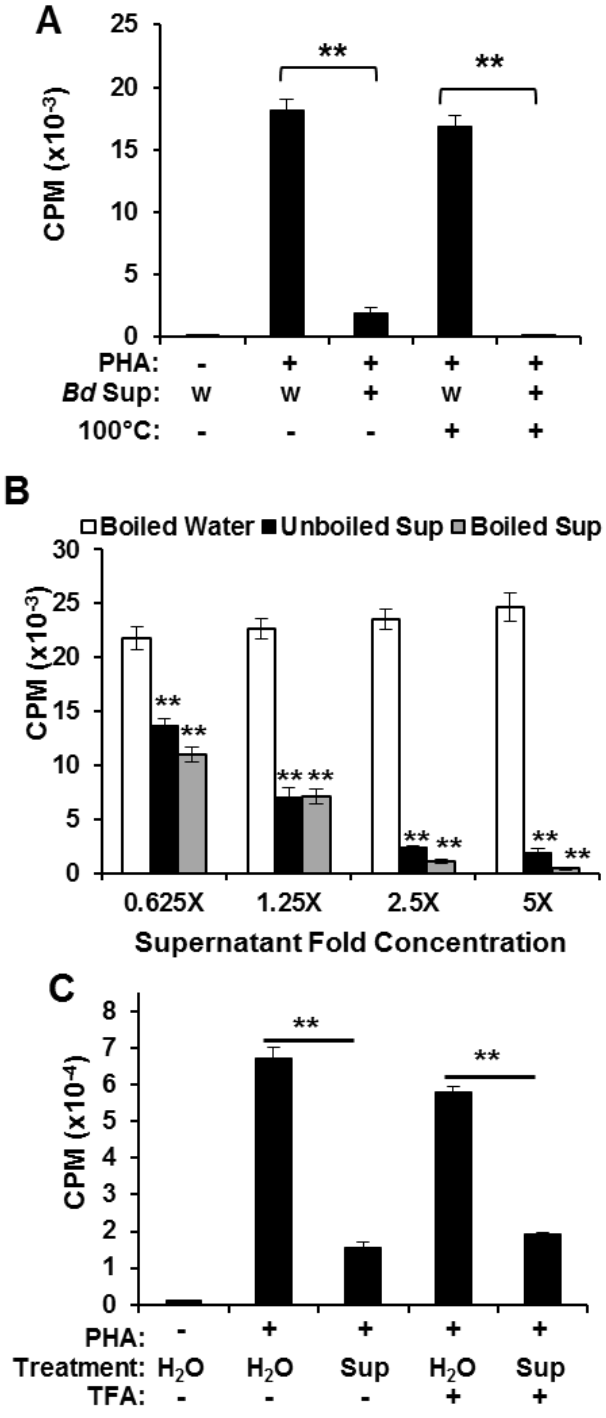

Fig. 4-9. *Bd* factors are resistant to heat and acid. *Bd* supernatants (Sup) were incubated at 100° C for 30 minutes either after (**A**) or before (**B**) lyophilization. *X. laevis* splenocytes were cultured alone or stimulated with PHA. (A) Splenocytes were exposed to water only (w) or concentrated *Bd* Sup (5X) that had (+) or had not (-) been boiled. Significant differences between supernatant and control treatment by Student's *t*-test (with correction for multiple tests in the same experiment), **p<0.01. (B) Sup that had or had not been boiled and a water control that had been boiled were re-suspended into splenocyte culture at different concentration. Sup significantly decreased proliferation, but heat treatment did not significantly affect the capacity of Sup to inhibit proliferation. (**C**) Lyophilized *Bd* Sup and a water control (H_2O) containing no *Bd* factors were mixed with 2% TFA and incubated at 37°C for 30 minutes. Control water and supernatant lacking TFA were also incubated at 37°C for 30 minutes. Following this treatment, the TFA-treated Sup and the TFA-treated water control were dialyzed to remove TFA. Samples were lyophilized again and re-suspended in L-15 before being mixed with splenocytes stimulated with PHA. Both treated and untreated *Bd* Sup decreased cell proliferation significantly compared to controls, **p<0.001 by a Student's *t*-test with correction for multiple tests.

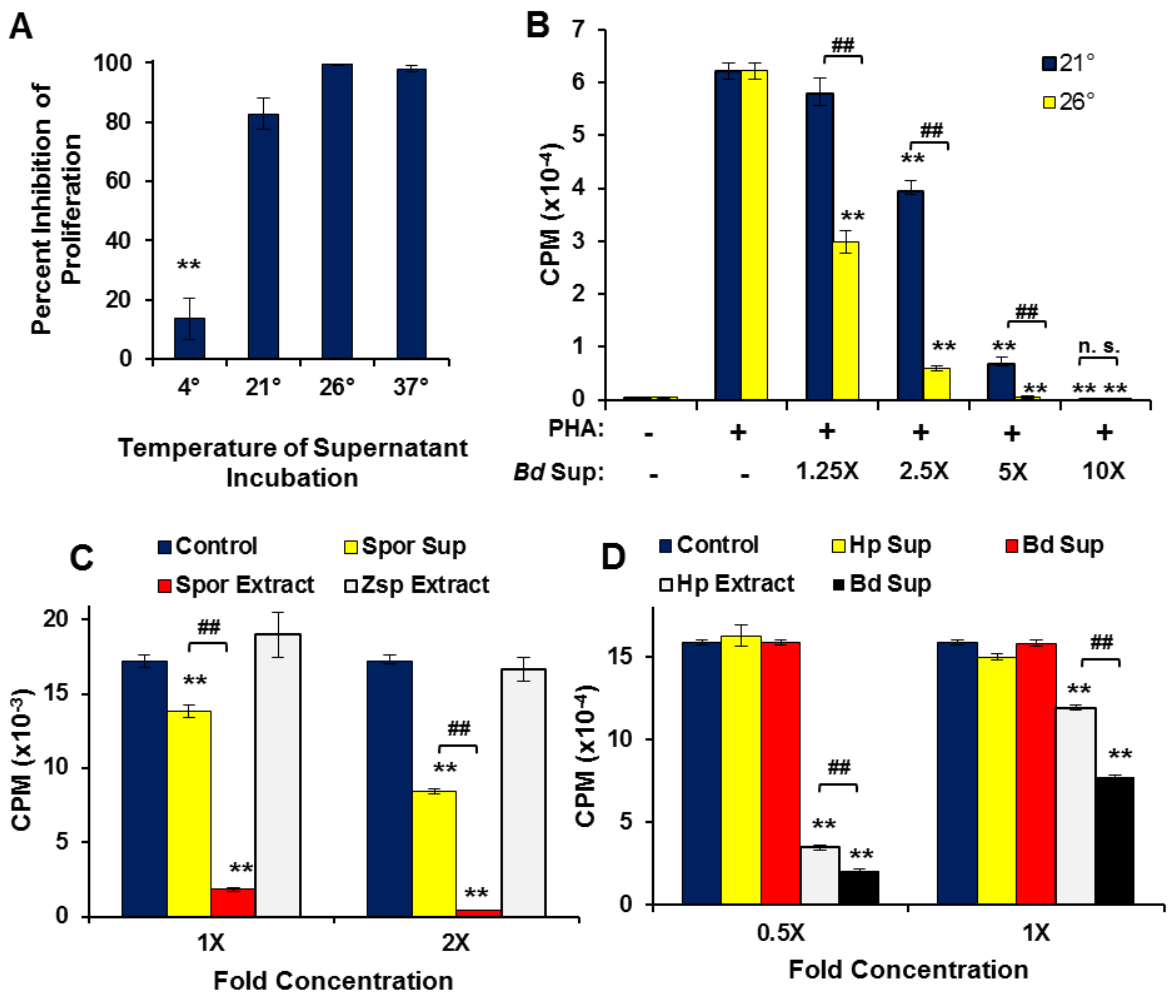

Fig. 4-10. Temperature greatly impacts the release of lymphotoxic factors from *B. dendrobatidis* (*Bd*). (**A**) *Bd* supernatants (Sup) were prepared at different temperatures and tested on PHA-stimulated *X. laevis* splenocytes at 5X. Mean relative inhibition (±SEM) of proliferation from three or more experiments is shown. The 4° C incubated Sup was significantly less inhibitory than the supernatants prepared at warmer temperatures. (**B**) A representative experiment from (A) comparing supernatants prepared at 21° or 26°. (**C**) Boiled extracts of mixed *Bd* culutures (Spor Extract) are much more inhibitory on *X. laevis* lymphocyte proliferation than supernatants prepared from the same culture (Spor Sup). Extracts from zoospores (Zsp) did not significantly inhibit proliferation. Control treatment was a boiled water control. (**D**) Extracts and Sup from *Bd* or *H. polyrhiza* (*Hp*) were mixed in culture with Jurkat cells in a proliferation assay. The control treatment was Jurkat cells in RPMI. (B-D) Statistical comparisons analyzed with ANOVA with Tukey post-hoc test: **p<0.01, significantly decreased proliferation compared to the control (no chytrid Sup or extract); $^{\#\#}$p<0.01, significant difference between treatments under brackets.

An alternative hypothesis to explain why higher temperatures increase the release of inhibitory factors is that the lymphotoxic factors are produced under stress conditions. To test this hypothesis, *B. dendrobatidis* cells were washed, re-suspended in water, and boiled for two hours. Placing *B. dendrobatidis* cells into a boiling water bath would limit the capacity of a stress response to activate the production or release of lymphotoxic factors. The cell-free factors from this boiled extraction of *B. dendrobatidis* cells was collected, lyophilized, and incubated with lymphocytes. Boiled extracts from mature *B. dendrobatidis* cells were significantly better inhibitors of *X. laevis* lymphocyte proliferation than the 21° C supernatants (Fig. 4-10 C). Boiled *B. dendrobatidis* extracts also inhibited Jurkat cell proliferation better than *B. dendrobatidis* supernatants (Fig. 4-10 D). These data provide further evidence to suggest that the lymphotoxic factors are components of the *B. dendrobatidis* cell wall and that they are very stable. *H. polyrhiza* cells were also boiled to prepare extracts. The extracts from *H. polyrhiza* were not as inhibitory as *B. dendrobatidis* extracts but were still very inhibitory (Fig. 4-10 D). Boiling cells may release more than just the lymphotoxic factors from *B. dendrobatidis* and *H. polyrhiza* cells causing both to be inhibitory to lymphocytes. *H. polyrhiza* may also produce the same lymphotoxic factors as common cell wall components, but these factors do not appear to be released unless the cell wall is completely dissociated by heat treatment.

Because the factors responsible for inhibition of lymphocytes are very stable, they are not likely to be RNA. RNA is typically very unstable (Grunbert-Manago, 1999). To confirm that RNA is not responsible for lymphocyte inhibition, *B. dendrobatidis* supernatants were digested with RNase A, an endoribonuclease (Raines, 1998). As expected, RNase A had no effect on the capacity of *B. dendrobatidis* supernatants to inhibit Jurkat cells (Fig. 4-11); therefore, the inhibitory factors are not RNA.

The lymphotoxic factors do not appear to be proteins.

The lymphotoxic factors are resistant to up to two hours of high heat and are resistant to acid treatment. Most proteins would be denatured under these conditions of extreme heat and acid, but very stable proteins might be able to resist these conditions. *Batrachochytrium dendrobatidis* supernatants contain a substantial amount of protein. A *B. dendrobatidis* supernatant from JEL197 has about 265 (±9.3, SEM) μg/mL of protein at 1X concentration, as determined by MicroBCA. Resolution of *B. dendrobatidis* supernatants by polyacrylamide gel electrophoresis (PAGE) indicated that there are many proteins of various sizes (Fig. 4-12 A). To determine whether stable proteins could be the lymphotoxic factors, *B. dendrobatidis* supernatants were digested with proteinase K conjugated to agarose beads (Fig. 4-12). By conjugating proteinase K to beads, the protease can be removed before incubating supernatants with *X. laevis* splenocytes. Proteinase K digested all the proteins visible by silver stain of a PAGE gel (Fig. 4-12 A), yet the supernatants retained inhibitory activity (Fig. 4-12 B). Oddly, both the supernatants and buffer controls treated with proteinase K beads increased in their capacity to inhibit lymphocyte proliferation. Because the relative inhibition by *B. dendrobatidis* supernatants was the same this does not suggest that there is a protein component of the inhibitory factors. The best explanation for this effect was that the calcium buffer needed for proteinase K to digest protein was somewhat inhibitory to lymphocyte proliferation. Proteinase K uses calcium as a cofactor, so it is likely that when the beads were removed from samples calcium was also removed from the medium. When supernatants were later digested at higher concentrations and lyophilized, this effect was no longer present probably due to the dilution effect of the buffer.

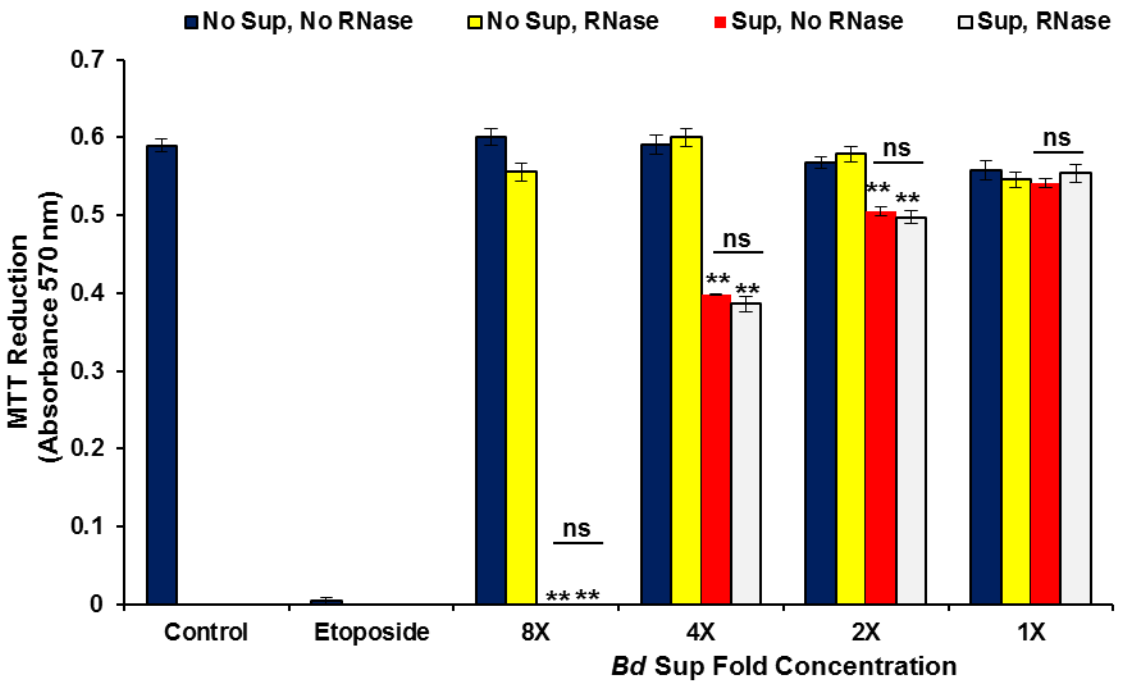

Fig. 4-11. The *B. dendrobatidis* (*Bd*) lymphotoxic factors are not RNA. *Bd* supernatants (Sup) or buffer controls (No Sup) either received RNase A or buffer (no RNase) treatment overnight. Samples were diluted in complete RPMI in Jurkat cell culture at final concentration between 1X and 8X. After three days in culture, Jurkat cell growth and viability was assayed by the MTT assay. "Control" treatment was Jurkat cells without any treatment in RPMI. A negative control treatment of 12.5 µg/ml etoposide was used to kill Jurkat cells. *Bd* Sup significantly inhibited Jurkat cells compared to the "No Sup" control, **$p<0.01$. There was no significant effect of RNase A on the inhibition of Sup. ANOVA with Tukey post-hoc. Data show the mean absorbance reading at 570 nm (±SEM) of six replicate wells. The data shown is representative of three experiments.

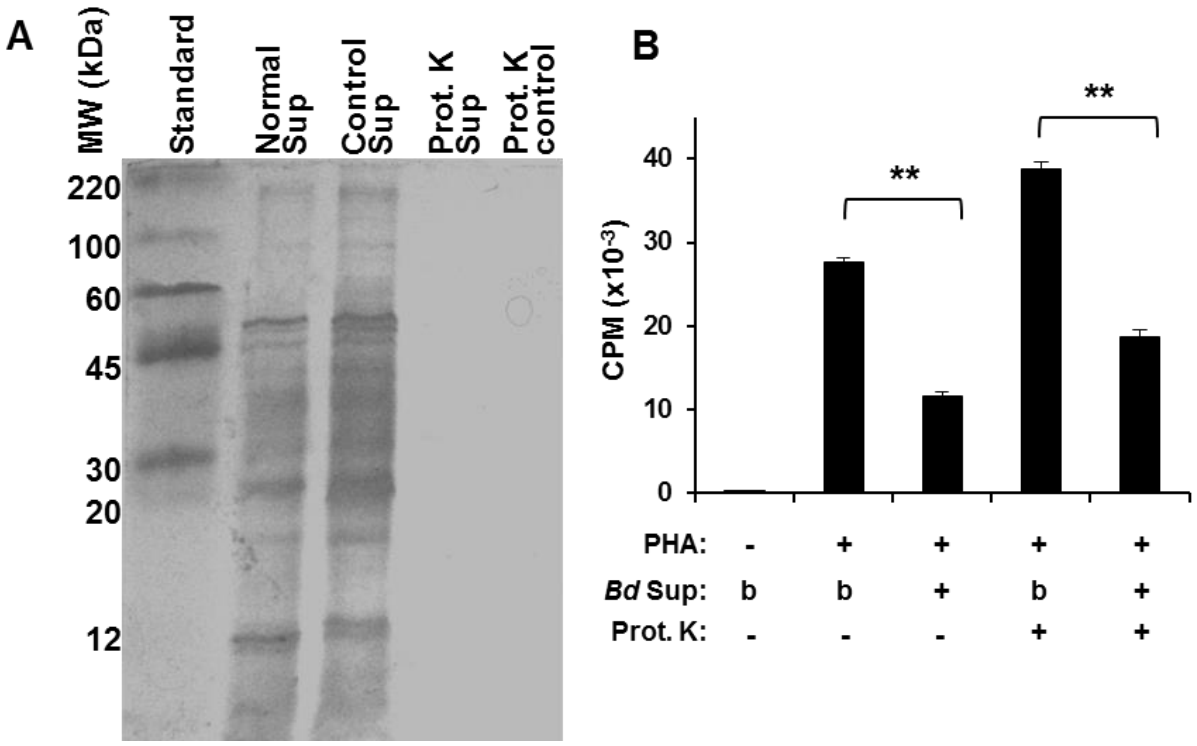

Fig. 4-12. *B. dendrobatidis* (*Bd*) lymphotoxic factors are resistant to proteases. (**A**) *Bd* supernatants were incubated at 4°C alone (Normal Sup), 37°C alone (Control Sup), or 37°C with proteinase K conjugated to agarose beads (Prot. K Sup). Proteinase K-conjugated beads were also incubated in buffer as an added control (Prot. K control). Beads were removed by centrifugation. Supernatant treatments and controls were resolved by SDS-PAGE and visualized by silver stain. Molecular weight standards are indicated on the left side of the gel image. The silver stain is representative of three independent experiments. (**B**) PHA-stimulated *X. laevis* splenocytes were incubated with control buffer (b) or with *Bd* Sup (5X) previously treated with (+) or without (-) proteinase K-conjugated agarose beads (Prot. K).

Size fractionation of *B. dendrobatidis* supernatants

Crude size separation using centrifugal columns was used to determine the approximate molecular size of *B. dendrobatidis* factors. This technique was originally used to concentrate *B. dendrobatidis* supernatants and investigate the approximate size of factors. Using columns with differential size cut-offs of 10, 30, 50, and 100 kDa, the inhibitory activity of supernatants was found in two size classes: less than 10 kDa and greater than 50 kDa (Fig. 4-13 A). Both fractions 50-100 kDa in size and >100 kDa in size were very inhibitory to lymphocyte proliferation. This result could indicate that a single factor is present near 100 kDa and was split between fractions or that there are multiple factors in the two fractions. Because the fractions were concentrated differently, the relative inhibition could not be compared easily among fractions. The large and small fractions (using a 10 kDa separation) were lyophilized and re-suspended in volumes relative to the original volume sent through the column to compare the relative inhibition of the smaller and larger molecules. *X. laevis* lymphocytes were inhibited by both the large and small molecular weight fractions, but the large molecular weight fraction showed greater inhibition (Fig. 4-13 B). The factors present in the small fraction may be fragments of the same inhibitory factors present in the larger molecular weight fraction. The factors could exist in a large complex and be inhibitory whether or not the complex is still intact. A large molecule inhibitory factor may also be fragmented into smaller molecules that have the same effect. Whether there are two different groups of inhibitory factors or there is a single factor remains to be determined.

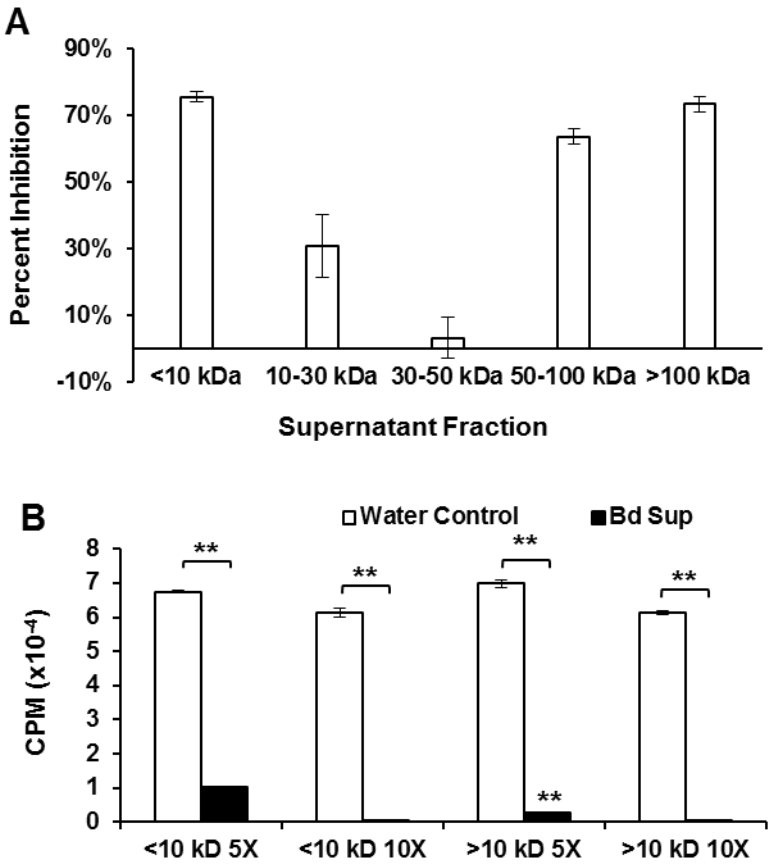

Fig. 4-13. Crude size separation of *B. dendrobatidis* (*Bd*) supernatants (Sup) with centrifugal columns indicates two size classes of inhibitory factors. (**A**) *Bd* Sup was passed through centrifugal columns to separate into fractions of different molecular size. Components in each size fraction were concentrated, so these samples were not lyophilized before mixing with 2X L-15 into culture with PHA-stimulated *X. laevis* splenocytes. Relative inhibition (±SEM) of lymphocyte proliferation from each fraction is shown. (**B**) *Bd* Sup was passed through a 10 kDa cut-off centrifugal column, and fractions were lyophilized. Fractions were re-suspended in L-15 in one-tenth or one-fifth of the volume of the Sup originally placed in the column for final concentration of 10X and 5X in *X. laevis* splenocyte culture (with PHA stimulation). Data show mean CPM (±SEM). Supernatant fractions at both concentrations were significantly more inhibitory to lymphocyte proliferation than water controls fractionated with 10 kDa cut-off columns, **$p<0.01$. At 5X concentration, the greater than (>) 10 kDa fraction was significantly more inhibitory than the less than (<) 10 kDa fraction, **$p<0.01$. Statistical comparisons among treatments were made by a single-factor ANOVA with Tukey post-hoc test.

Mass spectrometry analysis of supernatants

Batrachochytrium dendrobatidis and *H. polyrhiza* supernatants were analyzed by MALDI/MS. Unique peaks within the *B. dendrobatidis* spectra were noted as potential candidates for the lymphotoxic factor. Both boiled and control *B. dendrobatidis* supernatants were analyzed by MALDI/MS. Thirty-one unique peaks for *B. dendrobatidis* supernatants were identified by MALDI/MS. In positive ion mode, 19 unique peaks to *B. dendrobatidis* were identified (Table 4-1). Fourteen of these were in both boiled and unboiled *B. dendrobatidis* supernatants, but three were unique to the normal supernatant, and two were unique to the boiled supernatant. In negative ion mode, 11 unique peaks for *B. dendrobatidis* supernatants were identified (Table 4-2). Six of these peaks were in both *B. dendrobatidis* supernatants, and five were unique to the *B. dendrobatidis* supernatant that had not been boiled. Fragmentation spectra were obtained from four of the unique peaks. The fragmentation patterns could only predict identity of one of the peaks. In negative ion mode, one of the peaks (*m/z* 506.2) most likely represents ATP (Horai et al., 2010). These spectra may help identify the lymphotoxic factors, but also would be beneficial for understanding unique molecules released by *B. dendrobatidis*.

Supernatants were also analyzed by LC/MS to identify unique components from *B. dendrobatidis* supernatants, and to attempt to discern whether or not unique lipid components are present in *B. dendrobatidis* supernatant. LC/MS identified three unique peaks in positive ion mode and two unique peaks in negative ion mode present in *B. dendrobatidis* supernatants and absent or diminished in *H. polyrhiza* supernatants (Fig. 4-14). Of the unique peaks, only one (*m/z* 345) was found by MALDI/MS. Certain components of *B. dendrobatidis* supernatants may be only detectable with different mass spectrometry techniques. More unique *B. dendrobatidis*

Table 4-1. Unique peaks to *B. dendrobatidis* (*Bd*) supernatant spectra from MALDI/MS in positive ion mode. *Bd* and *H. polyrhiza* supernatants were analyzed with time-of-flight (TOF), linear trap quadrupole (LTQ), or Fourier transformation ion cyclotron resonance (FT) MALDI/MS. Accurate masses were obtained with FT. Fragmentation spectra obtained are noted. (MALDI/MS was completed by Michelle Reyzer in the Vanderbilt Mass Spectrometry core.)

	Unique to unboiled *Bd* Sup spectra			Unique to boiled *Bd* Sup spectra			Unique to *Bd* Sup spectra (boiled or not)				
m/z	TOF	LTQ	FT	TOF	LTQ	FT	TOF	LTQ	FT	accurate mass	fragment ions
172.6									x	172.63965	
250.3	x	x	x							250.09115	
254.3							x	x	x	254.15880	
294.1									x	294.16567	
298.3		x	x							298.09529	
300.2									x	300.22661	
302.2									x	302.24228	
304.2									x	304.17533	
313.2									x	313.20820	
314.1						x				314.09055	
345.7							x	x	x	345.28536	189, 286, 130, 175, 314
399.3	x	x	x							399.14506	250, 298
443.2						x				443.20547	
473.4									x	473.38299	
542.1									x	542.07145	
738.8							x	x			474, 721, 456, 500, 482, 584, 679, 708
3538.1								x			
3812.8							x	x			
8542.3							x				

Table 4-2. Unique peaks to *B. dendrobatidis* (*Bd*) supernatant spectra from MALDI/MS in negative ion mode. *Bd* and *H. polyrhiza* supernatants were analyzed with linear trap quadrupole (LTQ) or Fourier transformation ion cyclotron resonance (FT) MALDI/MS. Accurate masses were obtained with FT. Fragmentation spectra obtained are noted. (MALDI/MS was completed by Michelle Reyzer in the Vanderbilt Mass Spectrometry core.)

	Unique to unboiled *Bd* Sup spectra		Unique to boiled *Bd* Sup spectra		Unique to *Bd* Sup spectra (boiled or not)			
m/z	LTQ	FT	LTQ	FT	LTQ	FT	accurate mass	fragment ions
286.1					x			
426.0		x					426.01976	
465.5					x			
506.2					x			408
540.0		x					540.04995	
648.2					x			
650.0		x					650.10013	
707.3					x			
729.1		x					729.09261	
783.0		x					783.01101	
784.1						x	784.14594	

supernatant peaks were found with MALDI/MS than LC/MS probably because additional techniques were used to analyze supernatants with MALDI/MS in comparison with LC/MS. The major peak unique to *B. dendrobatidis* spectra and absent from *H. polyrhiza* spectra in LC/MS analysis was *m/z* 489. It was suggested that this peak may correspond to the T2 mycotoxin (Lattanzio et al., 2012). The T2 mycotoxin is a lipid toxin produced by *Fusarium* species that induces apoptosis of leukocytes (Li et al., 2011). To determine if the *m/z* 489 component was T2, it was subjected to tandem mass spectrometry (Fig. 4-15). Fragmentation analysis of *m/z* 489 did not correspond to the fragmentation of T2, and the fragmentation spectra did not reveal the chemical structure of this compound. LC/MS analysis and tandem mass spectrometry did not indicate presence of any lipid fragments, suggesting an absence of lipids from *B. dendrobatidis* supernatants.

Small molecule analysis of *B. dendrobatidis* supernatants

Thomas Umile and Kevin Minbiole at Villanova University identified several small metabolite components present in *B. dendrobatidis* cultures extracted with ethyl acetate (Fig. 4-16 A). Two of these metabolites were identified by NMR as cyclo-L-phenylanalanyl-L-proline [cyclo(Phe-Pro)] and cyclo-L-histidyl-L-proline [cyclo(His-Pro)]. Both of these are in a class of molecules called diketopiperazines which are cyclized dipeptides (Martins & Carvalho, 2007).

Cyclo(Phe-Pro) is a metabolite produced by fungi and bacteria (Trigos et al., 1997; Stöm et al., 2002) and has been shown to have antibiotic effects on various bacteria. Cyclo (Phe-Pro) induces apoptosis in human colon cancer cells at 5-10 mM (Brauns et al., 2005). Cyclo(Phe-Pro) inhibited *X. laevis* splenocyte proliferation at 50 μM and completely inhibited proliferation at 10mM (Fig. 4-16 B). Because the inhibitory concentrations of cyclo(Phe-Pro) are near

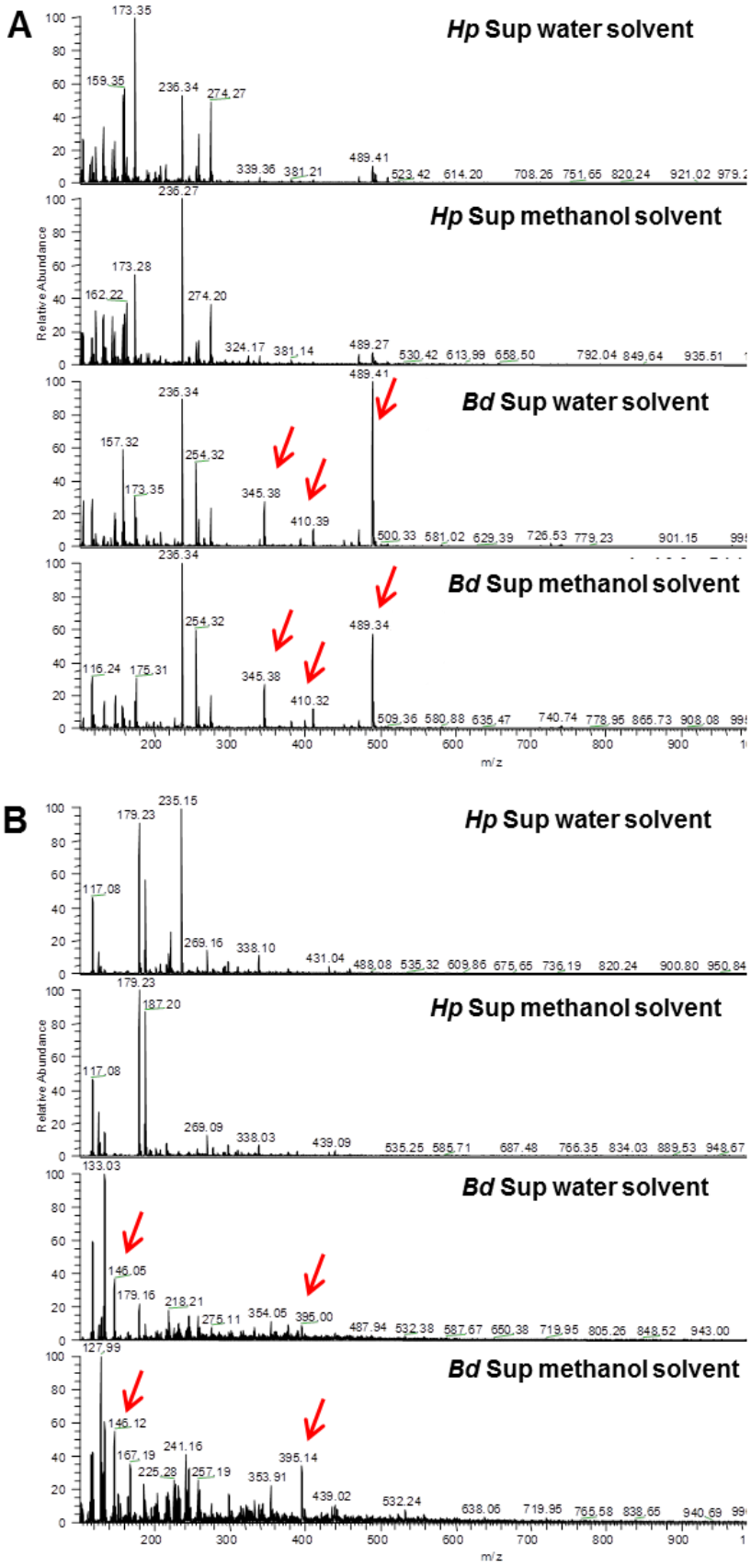

Fig. 4-14. LC/MS analysis of *H. polyrhiza* (*Hp*) and *B. dendrobatidis* (*Bd*) supernatants (Sup). Lyophilized *Hp* and *Bd* Sups were re-suspend in water or 90% methanol/10% chloroform and analyzed by LC/MS in positive (**A**) or negative (**B**) ion modes. Peaks present *Bd* spectra in both solvents and absent or greatly diminished in *Hp* spectra are indicated with red arrows. (Analysis completed by Pavlina Ivanova.)

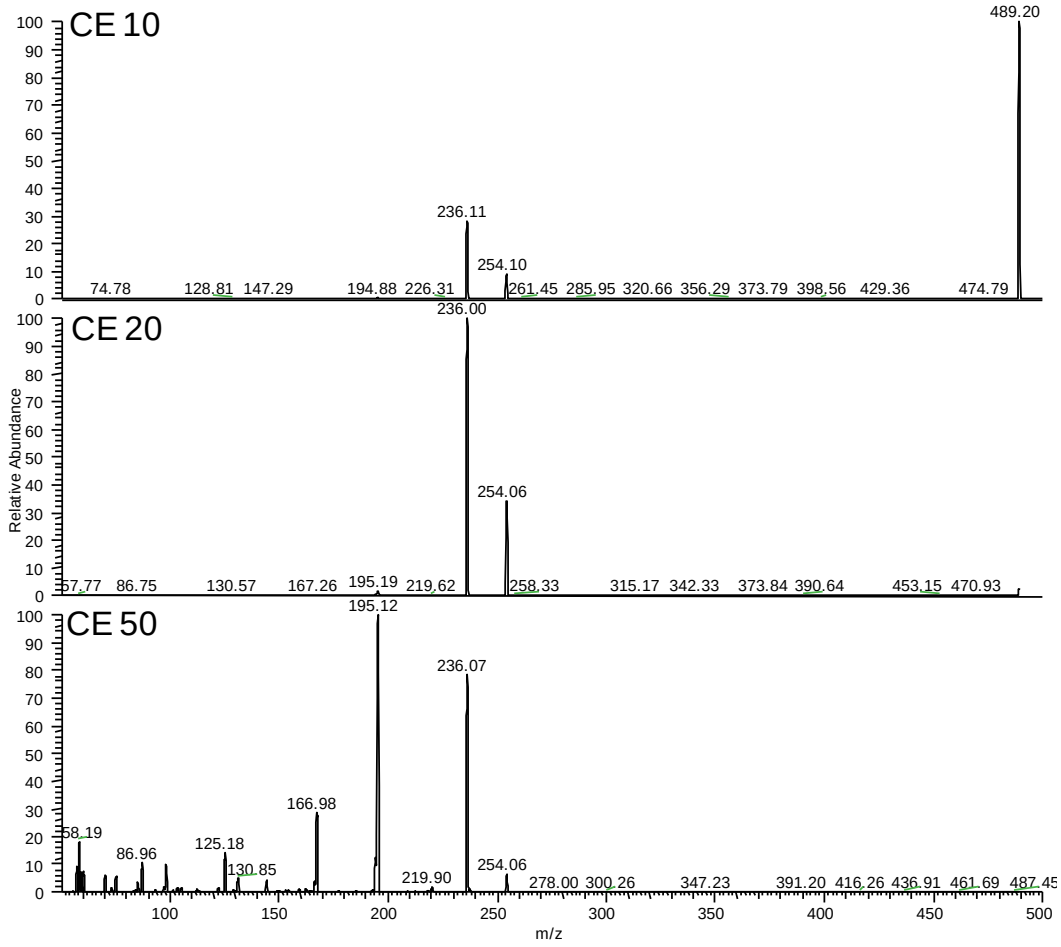

Fig. 4-15. Tandem mass spectrometry analysis of *m/z* 489 present in *B. dendrobatidis* (*Bd*) supernatants. The compound corresponding to *m/z* 489 on LC/MS spectra of *Bd* supernatants (Fig. 4-14) was exposed to different collision energies (CE, in electron volts) to view the fragmentation. (Analysis completed by Pavlina Ivanova.)

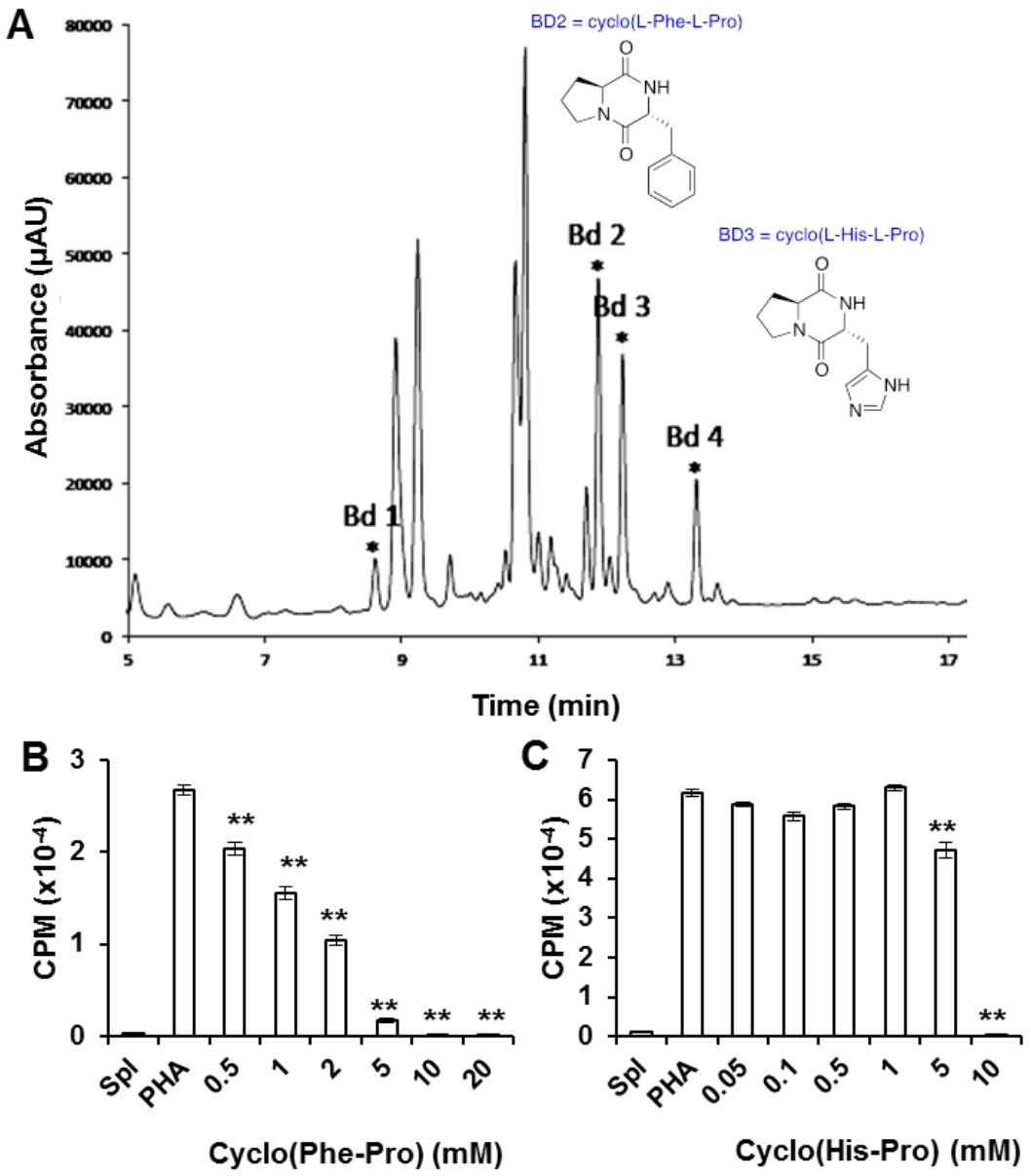

Fig. 4-16. Small molecule analysis of *B. dendrobatidis* (*Bd*). (**A**) HPLC analysis by C18 of ethyl acetate *Bd* extracts. Two metabolites isolated by HPLC were identified structurally by NMR as cyclo(Phe-Pro) and cyclo(His-Pro). (**B-C**) *X. laevis* splenocytes (Spl) were cultured alone or with PHA. PHA-stimulated Spl were incubated with increasing numbers of cyclo(Phe-Pro) (B) or cyclo(His-Pro) (C). Data show mean CPM (±SEM) of at least five replicate wells and are representative of at least two experiments. Significantly decreased proliferation compared to the PHA-stimulated splenocytes without treatment, **p<0.01 by ANOVA with Tukey post-hoc test. (Data in panel A was obtained from Thomas Umile at Villanova University.)

concentrations known to inhibit cancer cells, it is a candidate to be the molecule responsible for lymphocyte impairment in *B. dendrobatidis* supernatants.

Cyclo(His-Pro) is a small molecule found to be present in many tissues in humans and mice (Prosada, 1988; Minelli et al., 2012). Some evidence suggests that cyclo(His-Pro) is protective to oxidative stress by activating Nrf2 which promotes antioxidant responses (Minelli et al., 2009; Ma, 2013). Cyclo(His-Pro) may have effects on immune responses as well because Nrf2 activation by cyclo(His-Pro) inhibits NF-κB (Minelli et al., 2012). The physiological concentration of cyclo(His-Pro) is near 50 μM in human tissue (Prasad, 1988), and this concentration of cyclo(His-Pro) inhibited NF-κB nuclear accumulation and decreased inflammation in mice (Minelli et al., 2012). Due to the importance of NF- κB to lymphocyte activation (Sun & Andersson, 2002), cyclo(His-Pro) was also a candidate as the factor that impaired lymphocytes. Cyclo(His-Pro), however, did not have a significant impact on *X. laevis* lymphocyte proliferation at relevant concentrations (Fig. 4-16 C). Cyclo(His-Pro) did inhibit lymphocyte proliferation at concentrations greater than 5 mM, but these concentration are too high to implicate cyclo(His-Pro) as one of the *B. dendrobatidis* lymphotoxic factors.

A similar C18 HPLC analysis was performed on supernatants from *H. polyrhiza*, *B. dendrobatidis* whole cultures, and *B. dendrobatidis* zoospores. The *B. dendrobatidis* supernatant only appeared to share one of the components found in the ethyl acetate extracts (Fig. 4-17 A). Neither cyclo(Phe-Pro) nor cyclo(His-Pro) appear to be present in *B. dendrobatidis* supernatants. The single peak shared between *B. dendrobatidis* supernatant and ethyl acetate extract was eluted at 8.8 minutes and was called Bd1. This molecule was present in *H. polyrhiza* supernatants as well, but at about one-fourth the intensity as indicated by lower UV absorbance (Fig. 4-17 B). Some evidence indicates that *H. polyrhiza* releases some inhibitory components which need to be

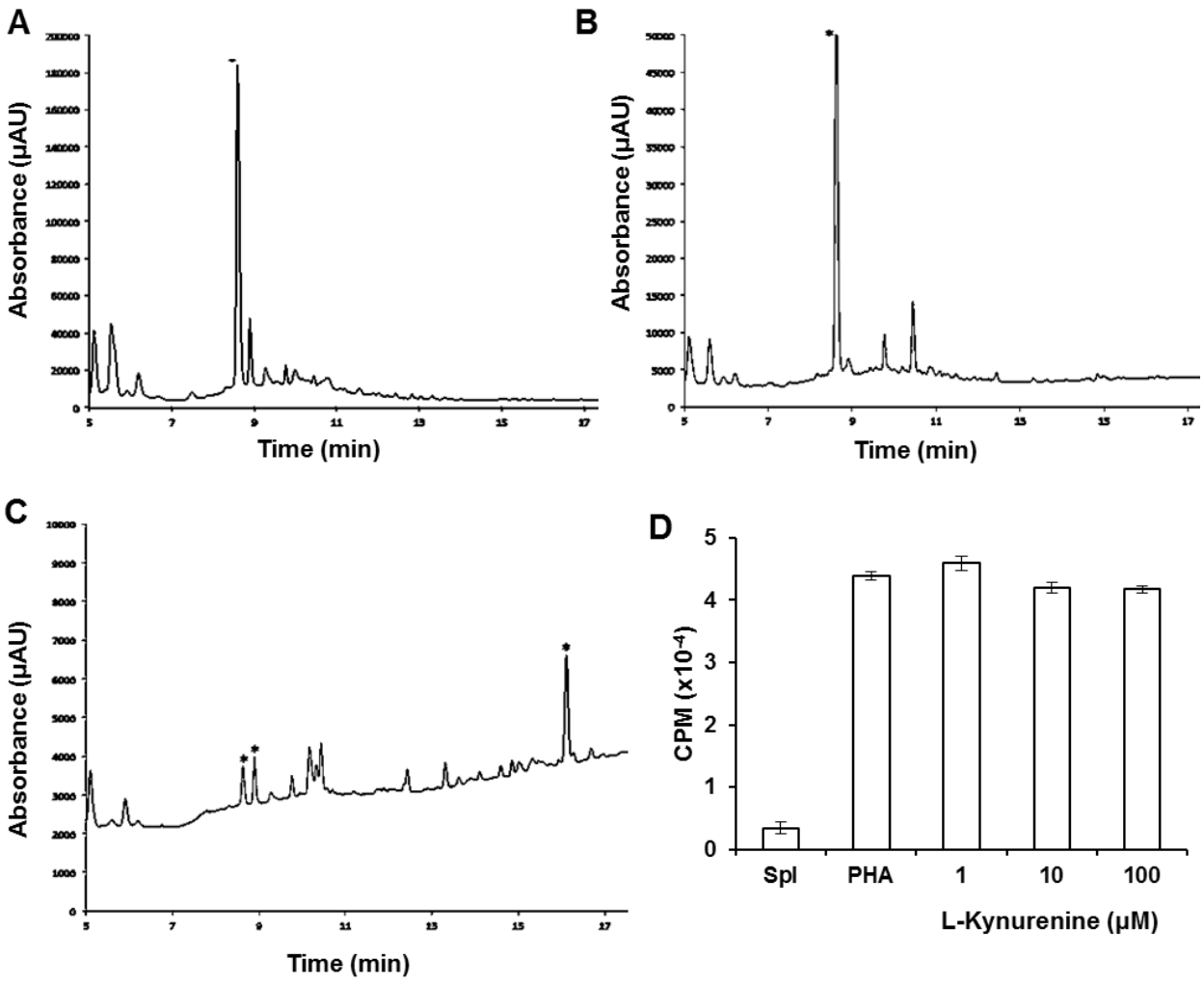

Fig. 4-17. Small molecule analysis of *B. dendrobatidis* (*Bd*) supernatants. (**A-C**) HPLC analysis by C18 of supernatants from *Bd* whole cultures (A), *H. polyrhiza* whole cultures (B), or enriched *Bd* zoospores. The largest peak in (A) and (B) at 8.8 minutes, Bd1, was identified as L-tryptophan. The next peak at 8.9 minutes was named Bd1'. (**D**) One of the components of *Bd* supernatant is an oxidized form of tryptophan, L-kynurenine. *X. laevis* splenocytes (Spl) were cultured alone or with PHA. PHA-stimulated splenocytes were incubated with physiologically relevant concentrations of L-kynurenine. Data show mean CPM (±SEM) of at least five replicate wells and are representative of at three experiments. L-kynurenine did not inhibit PHA-induced lymphocyte proliferation, $p>0.05$, single factor ANOVA. (Experiments in panels A-C were completed by Thomas Umile.)

concentrated between four and five-fold to inhibit lymphocytes to the same degree as *B. dendrobatidis* supernatants. Therefore, *H. polyrhiza* may produce lymphotoxic factors as well but at a much lower concentration. A second component eluting nearly immediately after Bd1, at about 8.9 minutes, was present in *B. dendrobatidis* supernatant with a very small peak in the *H. polyrhiza* supernatant (Fig. 4-17 A, B). This molecule was named Bd1'. Supernatants from *B. dendrobatidis* zoospores were also analyzed (Fig. 4-18 C). Two peaks with elution times similar to Bd1 and Bd1' were present in the zoospore supernatant but the UV-visual spectra of these components did not match Bd1 or Bd1'. The zoospore supernatant did have a component elute at 16.1 minutes that was not present in supernatants from mature *B. dendrobatidis* or *H. polyrhiza*.

The component of the *B. dendrobatidis* and *H. polyrhiza* supernatants termed Bd1 was identified as the amino acid L-tryptophan. Tryptophan by itself is not likely to impair lymphocytes, but different oxidation and metabolic products of tryptophan are known to decrease inflammatory responses (Cheng et al., 2010). Interestingly, boiling *B. dendrobatidis* supernatants causes a shift in the relative abundance of Bd1 (tryptophan) and Bd1' suggesting that Bd1' is a modified form of tryptophan. Work is continuing to attempt to identify Bd1'.

One of the metabolic products in tryptophan metabolism is L-kynurenine. L-kynurenine is an oxidation product of L-tryptophan that is naturally produced in eukaryotes from tryptophan by the enzyme indoleamine 2,3-dioxygenase (Mellor, 2004; Belladonna et al. 2007). L-kynurenine is an important inflammatory suppressor and may contribute to tolerance of fungi along mucosal surfaces to prevent immunopathology (Romani, 2011). *Batrachochytrium dendrobatidis* may have adapted to use tryptophan metabolism to dampen amphibian immune responses. L-kynurenine was identified as one of the small molecule metabolites present in *B. dendrobatidis* supernatants but is not Bd1'. The concentration of L-kynurenine in *B.*

dendrobatidis supernatant was low, about 60 ppb in 1X supernatant, but was about 10-fold more concentrated in *B. dendrobatidis* supernatants than *H. polyrhiza* supernatants. In human serum, L-kynurenine concentrations range between 2.1 and 7.6 μM (Widner et al., 1997), and serum concentration of L-kynurenine are elevated in patients with ovarian cancer (de Jong et al., 2011). In mice, typical serum concentrations of L-kynurenine range between 2 and 3 μM but increase to 6 μM when mice are injected with LPS (Wang et al., 2010). *Xenopus laevis* splenocytes were treated with the same physiological range of L-kynurenine without an effect on PHA-induced proliferation (Fig. 4-17 D). L-kynurenine is present at low concentrations in *B. dendrobatidis* supernatants and does not impair lymphocyte proliferation at greater than 20-fold normal human and murine serum concentrations; therefore, L-kynurenine in *B. dendrobatidis* supernatants is probably not a lymphotoxic factor. L-kynurenine may play a role in promoting Treg responses over inflammatory Th17 responses in chytridiomycosis, but it does not impair lymphocyte proliferation.

Analysis of *B. dendrobatidis* supernatants with solid phase extraction chromatography

The components of *B. dendrobatidis* supernatants appear to be polar. The manner in which supernatants are prepared tends to enrich for soluble factors present in the water removed from *B. dendrobatidis* cells. Lyophilized supernatants re-suspend easily in water or media also suggesting that they are composed mostly of polar molecules. Culture supernatants were also mixed with organic solvents to obtain components of different polarity from *B. dendrobatidis*, and the organic phase of this extraction did not contain molecules that inhibited lymphocyte proliferation (data not shown). To further analyze the polarity of lymphotoxic factors, *B. dendrobatidis* supernatants were fractionated by C18 chromatography.

C18 chromatography is reverse-chromatography meaning that components are eluted off of C18 columns by polarity where the least polar compounds are retained the longest. C18 columns are often eluted with increasing concentrations of acetonitrile (ACN) to elute fractions by polarity. Fractionation of *B. dendrobatidis* supernatant revealed that much of the inhibitory components of *B. dendrobatidis* supernatants eluted without any ACN suggesting that most of the inhibitory components are very polar (Fig. 4-18 A). All of the inhibitory components in the supernatant eluded with up to 60% ACN suggesting that there are some moderately polar inhibitory components of the *B. dendrobatidis* supernatants (Fig. 4-18 A, B). Most of the activity still eluted from the C18 column with 50% ACN, but some activity was still eluted with 60% ACN after 50% ACN elution (Fig. 4-18 C). Together these data indicate that the majority of inhibitory factors were very hydrophilic and some were moderately hydrophilic, but no hydrophobic molecules in *B. dendrobatidis* supernatants were inhibitory to lymphocyte proliferation (Fig. 4-19).

Based on the polarity of the *B. dendrobatidis* inhibitory factors along with the observations that lymphocyte inhibition is not reduced by acid, heat, or proteases, the larger molecular weight lymphotoxic factors are probably carbohydrates. Fungal polysaccharides, especially those associated with the cell wall, have been shown to modulate immunity (De Jesus et al., 2010; Gravelat et al., 2013). Analysis of supernatants by the phenol-sulfuric acid (P-S) method indicates that there is approximately 60 μg of hexose sugar per mL of 1X *B. dendrobatidis* supernatant. Therefore, the search for the identity of the lymphotoxic factors will focus on carbohydrates, along with small molecules as described in the previous section. *Batrachochytrium dendrobatidis* supernatants were also fractionated by solid phase extraction (SPE) using strong anion exchange (SAX) and cyano (CN) chromatography. Fractions

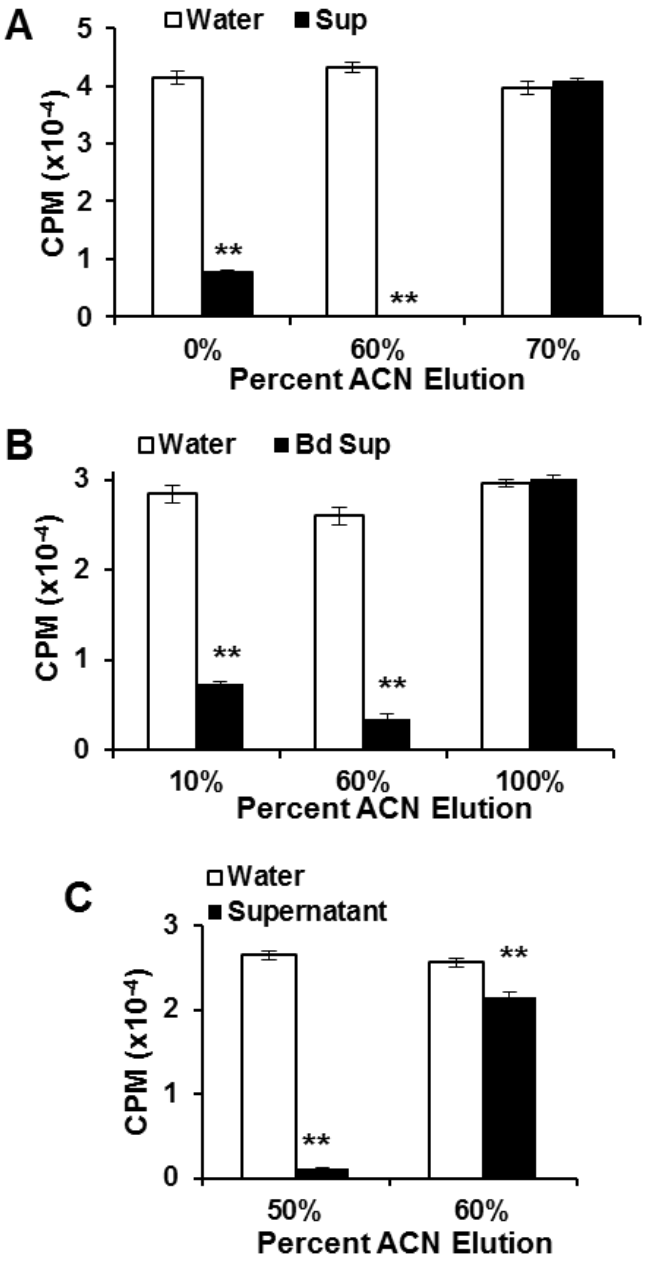

Fig. 4-18. *B. dendrobatidis* (*Bd*) lymphotoxic factors are mostly polar. *Bd* supernatants (Sup) or water controls were added into C18 columns. C18 columns were eluted with different dilutions of acetonitrile (ACN) mixed in water in an increasing gradient. (**A**) *Bd* Sup or water control was eluted with 0% (water), 60%, and 70% ACN in that order. (**B**) *Bd* Sup or water control was eluted with 0%, 10%, 60%, and 100% ACN in that order. (**C**) *Bd* Sup or water control was eluted with 0%, 50%, and 60% ACN in that order. Samples were dried after elution and re-suspended to be concentrated 5-fold from the original supernatant in *X. laevis* splenocyte culture with PHA stimulation. All fractionations were eluted with 0% ACN because of the nature of the column setup, but the effect of this fraction on lymphocyte proliferation is only shown in (A). *Bd* supernatant fractions that significantly inhibited lymphocyte proliferation compared to the water control are indicated: **$p<0.01$, ANOVA with Tukey post-hoc test. Data show mean CPM (±SEM) of at least five replicate wells and represent at least two experimental repeats.

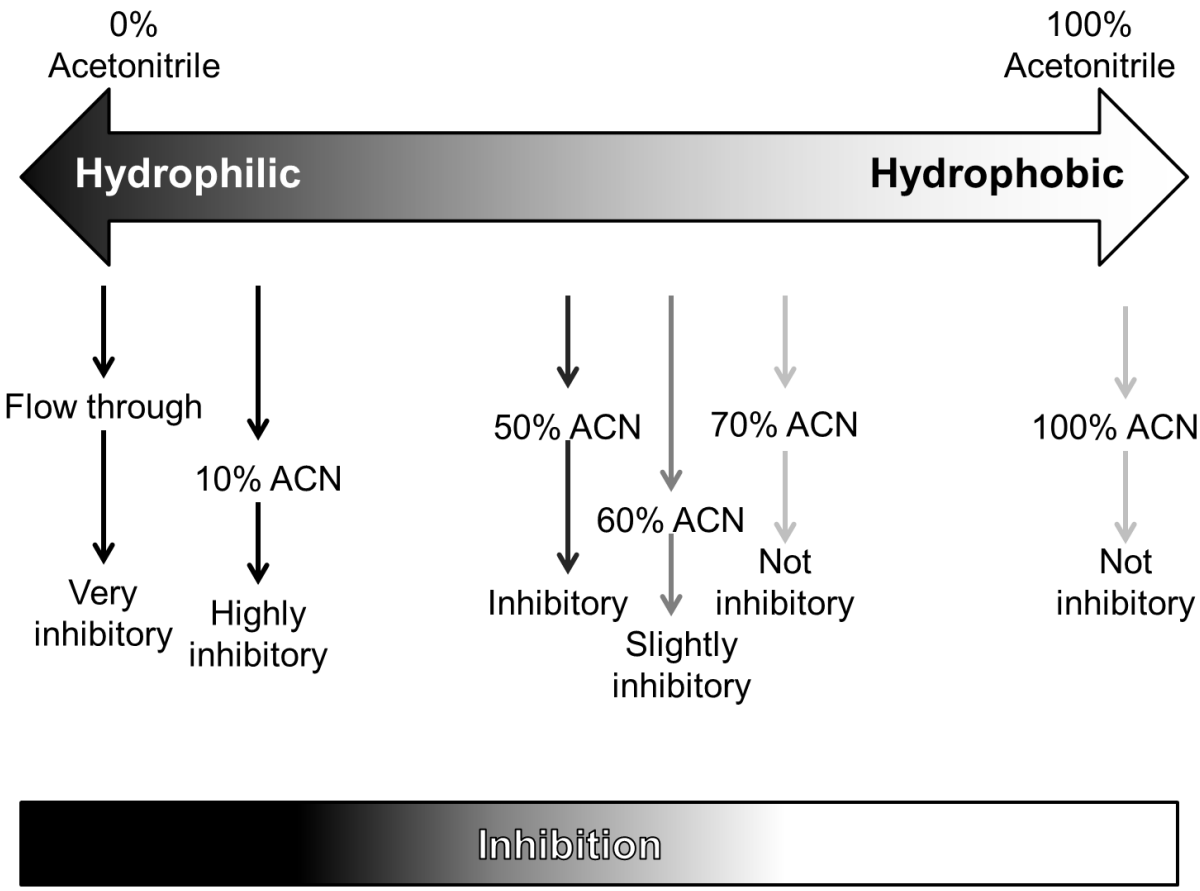

Fig. 4-19. Summary of C18 chromatography separation of *B. dendrobatidis* (*Bd*) supernatant. The relative inhibition of *X. laevis* lymphocyte proliferation induced by PHA is indicated for the different fractions of *Bd* supernatants. Data from representative experiments are shown in Fig 4-18.

were assayed with the P-S method and then tested for inhibition of Jurkat cells. SAX chromatography separates samples by charge, eluting positively charged components first. Only the first fraction of *B. dendrobatidis* supernatant eluted from SAX was significantly more inhibitory than its water control (Fig. 4-20 A). Although the inhibitory factors may be spread among the SAX fractions, they were most concentrated in the first fraction suggesting that the lymphotoxic factors are positively charged. CN chromatography separates fractions by polarity in a similar manner as C18. Unlike C18 fractionation, CN fractionation concentrated all inhibitory activity into one fraction (Fig. 4-20 B). The fraction that impaired Jurkat cells was the most polar fraction, corroborating other observations of the polar nature of factors. Because CN fractionation successfully retained activity into a single fraction, this SPE method will likely be used for further investigation of the lymphotoxic factors.

Certain fractions of *B. dendrobatidis* supernatant obtained by SPE with SAX and CN were enriched for carbohydrates as indicated by the P-S method. Importantly, the fractions with the most carbohydrate from each SPE method were also the most inhibitory to Jurkat cells (Table 4-3). CN chromatography enriched the carbohydrate content of the supernatant almost entirely into one fraction, and this fraction likely contained all of the lymphotoxic factors. These data highly suggest that carbohydrates are responsible for the impairment of lymphocytes by *B. dendrobatidis*.

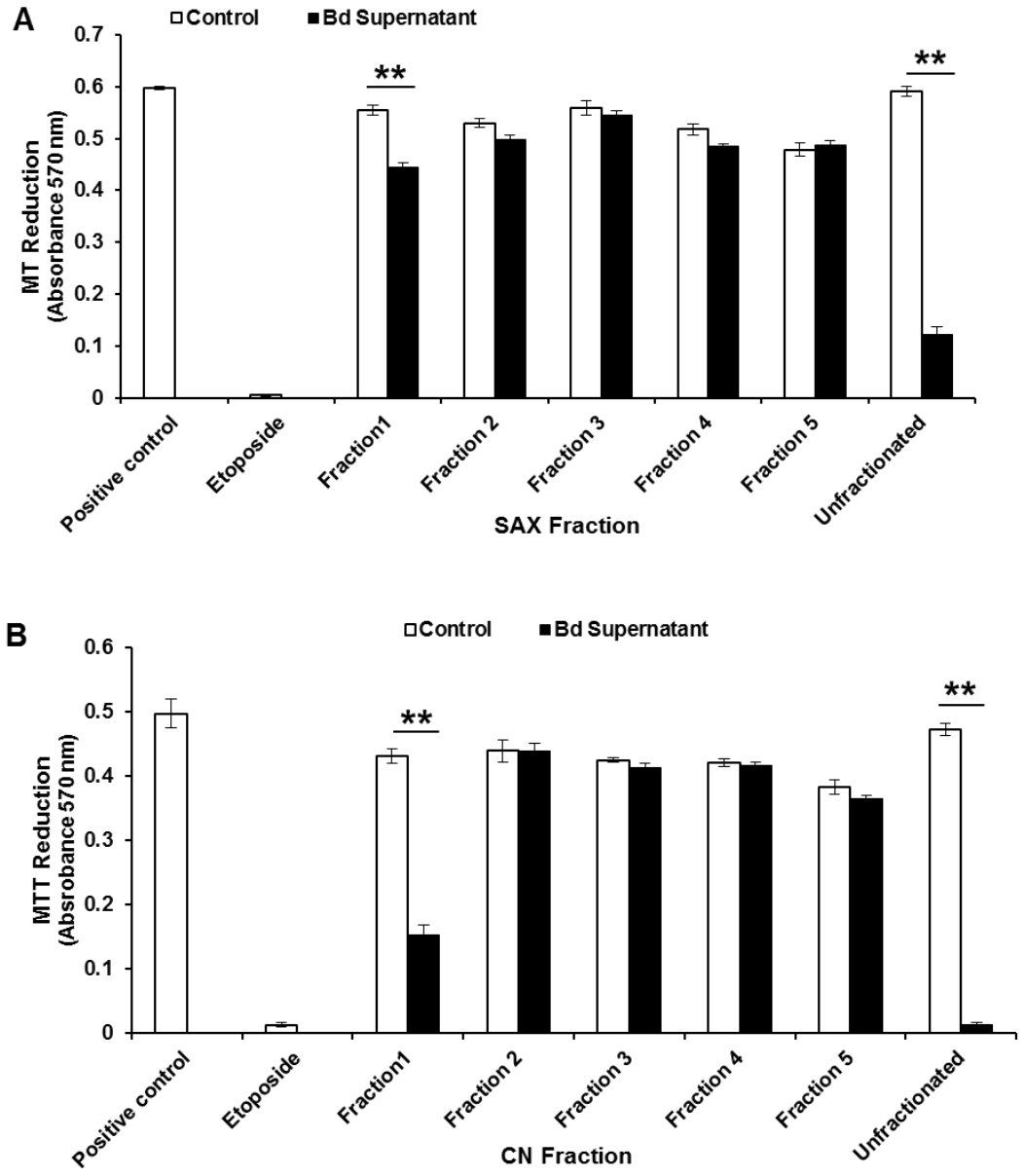

Fig. 4-19. SAX and CN chromatography separation of *B. dendrobatidis* (*Bd*) supernatants. *Bd* supernatants or water (Control) were added into SAX (**A**) or CN (**B**) columns. Columns were eluted by decreasing polarity (A) or decreasing charge (B). Samples were dried after elution and re-suspended to be concentrated 5-fold from the original supernatant in Jurkat cell culture. Jurkat cell proliferation and viability was quantified by the MTT assay. Assay controls were Jurkat cells alone in RPMI (Positive control) and a negative control receiving 12.5 μg/mL etoposide. An unfractionated *Bd* supernatant (different from the supernatant used for SPE) was also mixed with Jurkat cells as an added control. Fractions that significantly inhibited Jurkat cells are indicated: **$p<0.01$, ANOVA with Tukey post-hoc test. (Experiments completed with Alex Roberts.)

Table 4-3. The fractions from SAX and CN SPE enriched for carbohydrates had the greatest inhibition of Jurkat cells. The percent inhibition of Jurkat cell proliferation was calculated from the MTT assay relative to the Jurkat cells alone control. The carbohydrate content was determined by the P-S method displaying the absorbance (Abs) values of each fraction at 490 nm. (Experiments completed with AlexRoberts.)

Fraction	Percent Inhibition (mean±SEM)	Carbohydrate Content (P-S Abs at 490 nm)
SAX1	*25.7±1.3%	0.344
SAX2	16.6±1.4%	0
SAX3	9.2±1.6%	0
SAX4	19.2±0.9%	0.056
SAX5	17.7±1.7%	0.14
CN1	*69.3±3.3%	1.174
CN2	11.6±2.4%	0
CN3	12.4±1.6%	0.328
CN4	11.6±1.3%	0
CN5	22.9±1.3%	0

*$p<0.01$ compared to the no *B. dendrobatidis* supernatant water control.

Discussion

Batrachochytrium dendrobatidis zoospores do not produce lymphotoxic factors

Batrachochytrium dendrobatidis zoospores do not to inhibit lymphocyte proliferation unless they mature in co-culture with lymphocytes. Zoospores that were heat-killed to prevent maturation were not inhibitory to *X. laevis* splenocytes, but zoospores that were not heat-killed matured to germlings and inhibited lymphocyte proliferation. Living zoospores did not appear to release the inhibitory factors because zoospore supernatants were not inhibitory. When living zoospores were place in culture separate from lymphocytes in a transwell, these cells did not inhibit lymphocyte proliferation. The results from zoospore transwell assays suggested that during the early stages of maturation *B. dendrobatidis* can inhibit proliferation but only in close proximity to lymphocytes. When zoospores were not highly purified (containing many encysted zoospores and some germlings) or when a highly enriched zoospore population was given time to mature, *B. dendrobatidis* cells did produce some inhibitory factors, but not as much as a culture containing more mature cells.

The fact that *B. dendrobatidis* zoospores did not inhibit lymphocytes but did soon after maturing is important for two reasons: zoospores are not present inside of host tissue and zoospores do not have cell walls. Zoospores are free from the host and never come in contact with immune cells (Berger et al., 2005a). The purpose of a zoospore is to move away from the parent zoosporangium to infect a new host or to infect the same host in a new location. Production of lymphotoxic factors would only be beneficial for the mature *B. dendrobatidis* cells present in the skin. One of the major differences between *B. dendrobatidis* zoospores and mature *B. dendrobatidis* cells is that zoospores do not form a cell wall until time at which they invade the host (Longcore et al., 1999; Van Rooij et al., 2012; Greenspan et al., 2012).

The lymphotoxic factors are associated with the *B. dendrobatidis* cell wall

Based on the results showing that *B. dendrobatidis* zoospores did not produce inhibitory factors, I hypothesized that the *B. dendrobatidis* lymphotoxic factors are components the *B. dendrobatidis* cell wall. This hypothesis was supported by several observations. 1) *Batrachochytrium dendrobatidis* cells are much more inhibitory when cultured in close proximity to lymphocytes than when separated in a transwell culture. If the lymphotoxic factors exist on the surface of *B. dendrobatidis* cells in or on the cell wall, they may be shed as soluble factors but would be more inhibitory to lymphocytes adjacent to *B. dendrobatidis* cells. This is probably why *B. dendrobatidis* supernatants have to be concentrated to impair lymphocytes. 2) Dead *B. dendrobatidis* cells with cell walls still inhibit lymphocyte proliferation. *Batrachochytrium dendrobatidis* cells even release lymphotoxic factors after being killed and washed. Although *B. dendrobatidis* could release cytosolic components after death, the cell walls of *B. dendrobatidis* are present after killing and would be a logical source of the lymphotoxic factors. 3) Heat appears to promote the release of lymphotoxic factors from *B. dendrobatidis* even when *B. dendrobatidis* cells have been killed. The rate of which components of the cell wall are shed probably increases with temperature because components of the cell wall are more likely to dissociate or denature at higher temperatures. Particularly interesting was the observation that more inhibitory factors were released at 26° C than near the optimal *B. dendrobatidis* growth temperature of 21° because 26° is closer to the optimal temperature for the amphibian immune response in *X. laevis*.

To test the hypothesis that the lymphotoxic factors are associated with the *B. dendrobatidis* cell wall, *B. dendrobatidis* cells were treated with a chitin synthesis inhibitor, nikkomycin Z (NZ). The effects of NZ have been characterized on many fungi, but never before

in *B. dendrobatidis* (Cohen 1987; Hector 1993, Gaughran et al., 1994). NZ was found to inhibit maturation and proliferation, cause large increases in cell diameter, and induce cell lysis in *B. dendrobatidis* cells (now published in Holden et al., 2014). The inhibitory effects of NZ on *B. dendrobatidis* greatly suggest that NZ weakens the *B. dendrobatidis* cell wall. NZ also decreases the capacity of *B. dendrobatidis* to impair lymphocytes, giving more support to the hypothesis that the lymphotoxic factors are present in the cell wall.

The data supporting the cell wall hypothesis so far are only correlative. To confirm whether or not *B. dendrobatidis* cell walls actually have lymphotoxic components, current work is focusing on isolating the *B. dendrobatidis* cell wall. Chytrid cell walls are likely very different from the cell walls of higher fungi (Ruiz-Herrera & Ortiz-Castellanos, 2010), so protocols used to isolate fungal cell walls may not be applicable for chytrid cell wall isolation. Also, because the components of the chytrid cell wall are almost entirely unknown, confirming isolation of the *B. dendrobatidis* cell wall will be difficult.

Batrachochytrium dendrobatidis lymphotoxic factors may be small molecule metabolites

Crude size separation of *B. dendrobatidis* supernatants indicated that a small molecule of less than 10 kDa inhibits lymphocyte proliferation. The inhibitory molecule or molecules in this fraction may be a small macromolecule or could be a metabolite. Spectrometric analyses of *B. dendrobatidis* supernatants indicated that only a few dozen metabolites are present in *B. dendrobatidis* supernatants. Few of these metabolites have been identified, but the identified molecules did not inhibit lymphocyte proliferation.

Tryptophan was found to be present in high abundance in *B. dendrobatidis* supernatants. Although tryptophan is not an immunomodulatory molecule, tryptophan metabolism is important

in down-regulating inflammatory responses. *Candida albicans* can modify tryptophan metabolism to decrease inflammatory responses (Cheng et al., 2010). Tryptophan metabolism can cue tolerance to fungi which is important along epithelia where fungi are present but not necessarily pathogenic (Romani, 2011). Kynurenine is a metabolite product of tryptophan metabolism and is very important in down-regulating inflammation (Mellor, 2004; Belladonna et al. 2007). Kynurenine is a component of *B. dendrobatidis* supernatants but does not seem to play a role in impairing or killing lymphocytes *in vitro*. The kynurenine produced by *B. dendrobatidis* may play an important role *in vivo* to inhibit inflammatory responses that might clear the infection.

The production of tryptophan by *B. dendrobatidis* may be important in the protection against chytridiomycosis. Tryptophan is necessary for the synthesis of the antifungal metabolite violacein (Durán et al, 2010; Hoshino, 2011). Violacein production by symbiotic skin bacteria, particularly by the bacterium *Janthinobacterium lividum*, has been correlated with protection from chytridiomycosis in amphibians (Brucker et al., 2008; Becker et al., 2009; Lam et al., 2010). Tryptophan produced by *B. dendrobatidis* may be used subsequently by symbiotic bacteria to produce molecules that inhibit *B. dendrobatidis*. However, current understanding of *B. dendrobatidis* pathogenesis is that *B. dendrobatidis* grows inside of host tissue and only exists on the surface of the skin as a zoospore or an encysted cell (Berger et al., 2005a; Van Rooij et al., 2012; Greenspan et al., 2012). Zoospores do not release tryptophan into supernatants. If only zoospores are present at the skin surface where symbiotic bacteria are, then producing tryptophan is not likely to promote antifungal metabolite production by skin symbionts.

Batrachochytrium dendrobatidis lymphotoxic factors are likely to be carbohydrate in nature

Along with a small molecular component, *B. dendrobatidis* supernatants have a larger molecular component of at least 50 kDa that inhibited lymphocytes. Various treatments of *B. dendrobatidis* supernatants before mixing into lymphocyte culture limited most molecules in this size range. The lymphotoxic factors were resistant to high heat, acid, RNase, and proteases. The factors were polar in nature, and analysis of *B. dendrobatidis* supernatants by a lipidomics group found no evidence that the factors might be lipids. Although exceptions may exist where the lymphotoxic factors could be highly resistant proteins or unique lipids, the most likely explanation is that the large molecular weight component is a carbohydrate.

Fungal cell walls are primarily composed of carbohydrates (Klis et al., 2002; Nimrichter et al., 2005). Because the lymphotoxic factors appear to be associated with the *B. dendrobatidis* cell wall, they are likely to be one of the components present in the *B. dendrobatidis* cell wall. The lymphotoxic factors are not likely to be the common fungal cell wall carbohydrates β-1,3-glucan and chitin. These carbohydrates are PAMPs and typically induce immune responses (Brown, 2011). *B. dendrobatidis* also lacks the genes to synthesize β-1,3 and β-1,6-glucans (Ruiz-Herrera & Ortiz-Castellanos, 2010), and analysis of *B. dendrobatidis* cell walls has not found these β-glucans (personal communication with Jason Stajich). *B. dendrobatidis* does have chitin in its cell wall, but chitin is probably not present in *B. dendrobatidis* supernatants. Chitin is insoluble in water (Pillai et al., 2009), and digestion of *B. dendrobatidis* supernatants with Glucanex^{TM} which contains chitinases (Lorito et al., 1993) did not greatly increase the amount of digested sugar. The *B. dendrobatidis* cell wall is mainly uncharacterized, and carbohydrates components of the *B. dendrobatidis* cell wall may have immunomodulatory effects.

Batrachochytrium dendrobatidis supernatants have substantial amounts of carbohydrates. Solid phase extraction (SPE) of supernatants indicated that fractions enriched for carbohydrates inhibited lymphocytes to a greater extent than fractions with little to no carbohydrates. SPE fractionation also indicated other features of the lymphotoxic factors. Polar fractions of *B. dendrobatidis* supernatant were the most inhibitory. Anion exchange chromatography also suggested that the lymphotoxic factors were positively charged.

Fractionation of *B. dendrobatidis* supernatants, especially by size, suggested that multiple molecules present in *B. dendrobatidis* supernatants inhibit lymphocyte proliferation. However, a single factor may be present in the different fractions. If the effects on lymphocytes are only caused by a single molecule, this molecule is likely to be a carbohydrate. Lymphocyte impairment is associated with the *B. dendrobatidis* cell wall which is a large molecular complex. It is possible that a single lymphotoxic factor can be inhibitory to lymphocytes both in a large complex and as a monomer. Carbohydrates are present in the fungal cell wall complex, and large soluble complexes are likely shed from fungal cell walls during growth and normal remodeling. Also, a large polysaccharide would still likely have the same effect if broken into smaller carbohydrates because of the repetitive nature of most polysaccharides. An oligosaccharide containing up to 60 glucose residues is less than 10 kDa, and even larger carbohydrates may pass through a 10kDa centrifugal column depending on their structures. The wide array of polarities at which lymphotoxic factors were eluted from C18 may also be explained by a single carbohydrate factor as well. Large polysaccharides can be insoluble in water. Galactosaminogalactan, a polysaccharide present on the surface of *Aspergillus fumigatus* that induces apoptosis in neutrophils and dendritic cells, is very insoluble in water (Fontaine et al., 2011; Lee et al., 2014). If lymphocyte inhibition were to be caused by a single carbohydrate, then

the carbohydrate may be less water-soluble in a complex or large polysaccharide and more water-soluble as a free carbohydrate or oligosaccharide.

Great progress has been made to better isolate and understand the lymphotoxic factors. Much evidence suggests that a carbohydrate is responsible for inhibition of lymphocytes. Future work will work to isolate inhibitory carbohydrates so that they may be analyzed and potentially identified. Also attempts have begun to study the relationship between carbohydrates present in supernatants from various *B. dendrobatidis* isolates and the relative inhibition these supernatant have on lymphocytes.

CHAPTER V

CONCLUSIONS AND FUTURE PROGRESS

Much research is still needed to identify the evasion tactics of *B. dendrobatidis* used to prevent immune clearance of infection. The research in this thesis characterized one evasion strategy employed by *B. dendrobatidis* to impair adaptive immune responses. The factors responsible for immune impairment have yet to be identified, and the exact mechanism of their effect is still unclear. Most of the interactions between *B. dendrobatidis* and host cells are completely unknown, especially the important interaction between *B. dendrobatidis* and the immune system. Knowledge obtained from investigations of immunity to fungi in humans and mice (Chapter I) can give insight into some of the host-pathogen interaction in chytridiomycosis. The future of treating and mitigating chytridiomycosis to protect threatened amphibian populations will require a more complete understanding of immunity to *B. dendrobatidis*.

Future Progress Understanding the Lymphotoxic Factors

The most important step in understanding the role the *B. dendrobatidis* lymphotoxic factors play in chytridiomycosis is to identify these factors. Once identified, the mechanism of lymphocyte impairment and induction of apoptosis can be determined. At this point, it is possible that there are multiple factors present in *B. dendrobatidis* supernatant causing different effects on host cells. Only by isolating and testing each factor responsible will it be possible to determine the specificity of the effects on lymphocytes and other host cells.

Identification of the lymphotoxic factors will allow for analysis of how these factors induce effects in host cells. Investigations can answer which receptors or host proteins are interacting partners, which signaling pathways are induced by factors, and what expression changes the factors induce. Determining the mechanism of cellular impairment will be important in understanding how adaptive immune responses are impaired. Identification of the inhibitory mechanism will also give insight to how these factors may be developed into drug therapies.

The relative presence of the lymphotoxic factors in non-pathogenic, hypovirulent, and hypervirulent chytrids will be essential to understand how *B. dendrobatidis* has acquired these virulence factors. Acquiring virulence factors to evade host immunity was probably a critical step for *B. dendrobatidis* to become a vertebrate pathogen. By identifying *B. dendrobatidis* lacking or with excess lymphotoxic factors, genomic or expressional analysis could determine the genes responsible for the synthesis and excretion of the factors. Such analysis could proceed before identification of the factors. For example, gene expression analysis of *B. dendrobatidis* has compared zoospores and mature cells (Rosenblum et al., 2008). Genes highly expressed in mature cells and weakly expressed in zoospores are good candidates for genes important in the production of lymphotoxic factors. Confirmation of each gene's importance would still likely required identification of the lymphotoxic factors. Once the genetic components necessary for production of the *B. dendrobatidis* lymphotoxins have been identified, attenuated *B. dendrobatidis* strains could be created or found. An attenuated *B. dendrobatidis* strain would be very useful in understanding the pathogenesis of chytridiomycosis. Attenuated *B. dendrobatidis* could also be used to promote natural immunity in amphibian populations in the form of a vaccine.

A Protective Immune Response against Chytridiomycosis

Hypothetical protective immune responses against chytridiomycosis can be predicted based on the understanding of fungal diseases in humans and murine models. Immune receptors and signaling pathways are conserved among vertebrates (Roach et al., 2005; Ishii et al., 2007; Robert & Ohta, 2009). Adaptive immune responses in amphibians have not been as well described as those in humans and mice, but amphibians have B and T lymphocytes that share common receptors, origin, and function with mammalian lymphocytes (Robert & Ohta, 2009; Robert & Cohen, 2011). Immune responses that have been found to be protective against fungal diseases in mice and humans might be expected to be protective in amphibians against chytridiomycosis. Here we outline what would be expected in a protective immune response against chytridiomycosis in amphibians.

Detection of *B. dendrobatidis* in the skin

Batrachochytrium dendrobatidis is an intracellular pathogen of amphibian keratinocytes (Loncore et al., 1999; Berger et al., 2005a). By entering keratinocytes via a germination tube (Van Rooij et al., 2012; Greenspan et al., 2012), *B. dendrobatidis* evades phagocytic detection. However, PAMPs from *B. dendrobatidis* may be exposed to resident DCs or LCs if rhizoids exit host cells or if PAMPs leak out of dead cells. *Batrachochytrium dendrobatidis* should be primarily recognized by host epithelial cells. Amphibian epithelial cells should express PRRs and have the capacity to alert the immune system when infected or damaged.

Unless *B. dendrobatidis* masks all of its PAMPs, PRRs on epithelial cells or resident skin DCs are likely to recognize *B. dendrobatidis* infection. Twenty TLRs have been identified in *Xenopus* and share close homology with TLRs in mice and humans (Ishii et al., 2007). TLR2 and

TLR4 would likely play a role in detection of mannans in the *B. dendrobatidis* cell wall, although the exact composition of the *B. dendrobatidis* cell wall has not yet been determined. *X. laevis* has two TLR2 genes with very close homology with mammalian TLR2, but TLR4 is much less conserved potentially explaining the minimal response of amphibian cells to LPS (Ishii et al., 2007, Bleicher et al., 1983). TLRs 3, 7, and 9 have been shown to be important in recognizing fungal nucleic acids (Garcia-Vidal et al., 2013), and this is probably true for these TLRs in amphibians as well. TLR7 is very well conserved among vertebrates, but TLRs 3 and 9 are more divergent in amphibians. *Xenopus* TLR9 is even predicted to have an extra transmembrane domain near the N terminus (Ishii et al., 2007).

Unlike TLRs, CLRs have not been characterized in amphibians, yet they would be expected to play an important role in recognizing *B. dendrobatidis*. Dectin-1 has been determined to be an important PRR activating antifungal inflammatory responses in mammals (Haridson & Brown, 2012). Because the primary ligand for Dectin-1, β-glucan, is not predicted to be present on *B. dendrobatidis* (Ruiz-Herrera & Ortiz-Castellanos, 2010), a β-glucan CLR is not likely to play any role in chytridiomycosis. However, other CLRs recognize other carbohydrate components of the fungal cell wall which probably are in chytrid cell walls as well. The outer mannoprotein layer of fungi is thought to be present in the chytrid cell wall (Ruiz-Herrera & Ortiz-Castellanos, 2010) which is recognized by a variety of CLRs including Dectin-2, Dectin-3, Minkle, and the MR (Haridson & Brown, 2012; Zhu et al., 2013).

Chitin is an important component of the *B. dendrobatidis* cell wall and would be an important PAMP for the recognition of *B. dendrobatidis* (Ruiz-Herrera & Ortiz-Castellanos, 2010; Holden et al., 2013). Although no definitive PRR has been identified for chitin recognition, chitin does activate immune responses in mice (Da Silva et al., 2008; Da Silva et al.,

2009; Roy et al., 2012; Roy et al., 2013). Compared to other fungi, the *B. dendrobatidis* cell wall is probably composed of more chitin, so chitin should be an important target for recognition and destruction by the amphibian immune system.

The presence of *B. dendrobatidis* within host cells and the damage caused by infection can also activate immune responses. The inflammasome is a cytosolic protein complex that is often activated by intracellular pathogens, often via recognition of NLRs (Lupfer & Kanneganti, 2013). The inflammasome cleaves IL-1 and IL-18 into the active forms which activate inflammatory immune responses. The inflammasome appears to be important in immunity to several fungal pathogens (Roy & Klein, 2012; Garcia-Vidal et al., 2013; Mao et al., 2013; Tavares et al., 2013). Evidence that IL-1β expression is increased in *B. dendrobatidis*-infected skin (Rosenblum et al., 2012a) suggests that inflammasome activation may also be important in immunity to chytridiomycosis.

Along with activation of the inflammasome, the damage *B. dendrobatidis* inflicts upon its host cell can promote immune activation by DAMPs (Bianchi, 2007). This is especially true if *B. dendrobatidis* disrupts the host plasma membrane. Rhizoid structures on *B. dendrobatidis* can protrude out of host cells (Pessier et al., 1999). The damage done by rhizoids and especially the leaking of the host cytosolic components are important DAMPs that may activate inflammatory responses. If *B. dendrobatidis* induces necrotic death in host cells, these necrotic cells can activate inflammation as well, by revealing or releasing DAMPs (Zitvogel et al., 2010). My studies suggest that *B. dendrobatidis* may induce apoptosis in non-lymphoid host cells which is a much less inflammatory process (Chapter II); however, apoptosis of host cells may lead to cross-presentation of *B. dendrobatidis* antigens as noted for *H. capsulatum* (Hsieh et al., 2011).

Although, *B. dendrobatidis* is likely to have evasion strategies to avoid detection by the immune system, the vertebrate immune system has many safe-guards to promote pathogen recognition and clearance. The damage induced by *B. dendrobatidis* to host skin that eventually causes many hosts to die (Voyles et al., 2009) should be enough to cue recognition of DAMPs if *B. dendrobatidis* is not already recognized by its PAMPs.

Activation of immune responses against *B. dendrobatidis*

Once a pathogen has been detected in tissue that has been breached by a pathogen, inflammatory cytokines are released to promote recruitment of phagocytic cells into the infected tissue. IL-1, IL-18, IL-8, and TNFα are important cytokines produced after pathogen recognition in mammalian hosts. These cytokines promote recruitment and activation of phagocytes and also can have an important role in priming Th1 and Th17 responses, all leading to sterilizing immunity of the fungal pathogen (Wüthrich et al., 2012a). Infiltration of inflammatory monocytes also is important in immunity to several fungal pathogens; these monocytes are recruited by CCL2 and CCL7 chemokines via the CCR2 receptor (Blease et al., 2000; Szymczak & Deepe, 2009; Hohl et al., 2009; Ersland et al., 2010). IL-1, IL-18, IL-8 and TNFα homologues have been identified in amphibians (Huising et al., 2004; Morales et al., 2010; Cui et al., 2011); and CCL2 and CCL7 and their shared receptor CCR2 have been described in mammals yet not defined for amphibians (Laing & Secombes, 2004). The production, release, and effect of these early cytokines on the immune system during chytridiomycosis are not well understood. Some expressional analysis of *B. dendrobatidis*-infected skin has suggested an increase in expression of IL-1β but not any of these other early cytokines (Rosenblum et al., 2012a). Unfortunately, no commercially-available antibodies to amphibian cytokines exist, so it would be difficult to

determine if, where, and when these inflammatory mediators are produced to promote immunity to *B. dendrobatidis*. If leukocytes could be isolated from *B. dendrobatidis*-infected skin, expression of cytokines could be determined by PCR or RNAseq methods. Imaging mass spectrometry analysis (Schone et al., 2013) of infected tissue would also provide a method to follow all of the proteins, including cytokines, produced in *B. dendrobatidis*-infected skin.

Typically, innate immune responses alone are not sufficient for pathogen clearance, and robust inflammatory T-cell responses are essential for resistance to fungal pathogens (Romani, 2011; Wüthrich et al., 2012a; Garcia-Vidal et al., 2013). T cells must first be activated and primed through the production of cytokines to promote immunity. In the skin of mice, fungal cells and antigens are typically carried to the nearest lymph nodes where they are presented to lymphocytes (Ersland et al., 2010; Igyártó et al., 2011). Amphibians do not have organized lymph nodes but appear to have lymphatic components associated with the skin described as SALT (skin-associated lymphatic tissue) (Robert & Ohta, 2009; Egawa & Kabashima, 2011; Xu et al., 2013). Once DCs, LCs, or macrophages engulf *B. dendrobatidis* cells, *B. dendrobatidis* antigens, or infected keratinocytes, antigens can be presented to lymphocytes most likely in the SALT. Amphibians do have a spleen that acts as a secondary lymphoid organ (Robert & Ohta, 2009), but it is not known whether antigens can or need to be transported to the spleen to prime lymphocytes to respond in amphibian skin. Antigen presentation influences T-cell polarization through secondary signals outside of the TCR. With the correct co-stimulatory signals, T cells can be activated to produce the cytokines necessary to promote resistance to *B. dendrobatidis*, but the protective responses in chytridiomycosis remain unknown.

Which lymphocyte responses are protective in chytridiomycosis?

Some evidence suggests that lymphocyte-mediated responses are important for resistance to *B. dendrobatidis* (Table 1-1). The presence of virulence factors that target lymphocytes suggests that *B. dendrobatidis* can only persist in host skin when adaptive immune responses are impaired (Chapter II). Evidence is lacking to show which types of immune response are important for immunity to *B. dendrobatidis.* *Xenopus laevis* exposed to or immunized against *B. dendrobatidis* produce antibodies against *B. dendrobatidis* antigens (Ramsey et al., 2010). However, *B. dendrobatidis*-specific antibodies have not been shown to be protective, and similar immunization protocols using *Rana muscosa* and *Bufo boreas* did not led to resistance to chytridiomycosis (Rollins-Smith et al., 2009; Stice & Briggs, 2010). T cell responses are thought to be more important than antibody responses in resistance to fungal infections. Major histocompatibility complex (MHC) class II alleles, important for presentation of antigens to $CD4^+$ T cells, have been linked with survival in one amphibian population (Savage & Zamudio, 2011). Also, lymphotoxic factors produced by *B. dendrobatidis* appear to be more effective against T cells than B cells (Chapter II) suggesting that *B. dendrobatidis* has adapted to evade T cell responses over B cell responses. Further investigation of the immune responses elicited or suppressed by *B. dendrobatidis* is needed to determine how an amphibian may resist chytridiomycosis.

Based on what is known about mammalian immunity to fungi, Th1 and Th17 responses are likely to be very important in immunity to chytridiomycosis. During a Th1 response, the pathogen-killing capacity of phagocytes, particularly macrophages, is augmented by IFNγ, TNFα, and GM-CSF. Th1 responses have generally been shown to be important in immunity to fungi particularly with fungi that can reside inside of macrophages (Romani, 2011; Wüthrich et

al., 2012a; Garcia-Vidal et al., 2013). In *Xenopus tropicalis*, IFNγ exists in a locus with IL-22 and IL-26, and all three cytokines are up-regulated when frogs are stimulated with PAMPs (Qi & Nie, 2008). Th1-type immune responses are likely to influence immunity to *B. dendrobatidis* if phagocytes actually play a prominent role. However, if *B. dendrobatidis* exists mostly or entirely as an intracellular pathogen of keratinocytes, then Th1 responses would not necessarily promote resistance.

During a Th17 response, recruitment of phagocytes, primarily neutrophils, and the activation of AMP production is promoted by IL-17A, IL-17F, and IL-22. Protection by IL-17 has been established in multiple mouse models of fungal infection (Rudner et al., 2007; Conti et al., 2009; Wozniak et al., 2011; Wüthrich et al., 2011). IL-17 genes are well conserved among most metazoans and even appear to mediate inflammation in invertebrates as well (Roberts et al., 2008). The function of IL17A/F is conserved throughout vertebrates, promoting inflammation and AMP production in fish (Monte et al., 2013). Very little is known about the role of IL-17 in amphibians, but homologues of IL-17 genes are expressed in *X. laevis* γδ T cells (Jackson et al., 2012). The γδ subset of T cells has been identified in the skin of amphibians (Mescher et al., 2007) and may be capable of producing IL-17 to activate inflammation during chytridiomycosis. Also, the presence of IL-1 promotes Th17 polarization and activity (Wüthrich et al., 2013), and amphibian skin is known to increase the expression of IL-1β during *B. dendrobatidis* infection (Rosenblum et al., 2012a). Thus, Th17 immunity may be activated during chytridiomycosis. Due to the gap in knowledge about $CD4^+$ T cells in amphibians, the presence of Th17 cells and their role immunity is unknown. However, due to the overall conservation of IL-17 and adaptive immunity, Th17 cells probably are present in amphibians and would play a protective role in chytridiomycosis if activated and recruited to the skin.

In many studies, Th2 responses have been shown to be ineffective in promoting resistance to fungal pathogens in mice and humans (Romani, 2011; Wüthrich et al., 2012a). However, resistance to *P. murina* and potentially other fungi may be mediated by Th2 responses (Nelson et al., 2011). Th2 cytokines promote alternative activation of macrophages which promotes fungal persistence inside of macrophages (Szymczak & Deepe, 2009; Wüthrich et al., 2012a). The role of Th2 responses in chytridiomycosis may be protective if production of class-switched antibodies promotes resistance and if the role of phagocytes is not important for resistance. The investigation of antibodies against *B. dendrobatidis* have not shown that these antibodies protect against *B. dendrobatidis*, and it is possible that IgM and IgX antibodies that are thymus-independent may provide enough protection without T cell help (Ramsey et al., 2010). Phagocytosis may be important in resistance to *B. dendrobatidis*. *Xenopus laevis* macrophages and neutrophils engulf *B. dendrobatidis in vitro* (Chapter III) When *X. laevis* peritoneal leukocytes were activated *in vivo* with killed bacteria to obtain these phagocytes, they behaved like "activated" cells suggesting that classical activation may be essential for phagocytic killing of *B. dendrobatidis*. Therefore, Th2-type responses are most likely not protective in chytridiomycosis.

$CD8^+$ T cells and other cytotoxic lymphocytes may play a much larger role in chytridiomycosis than in other fungal diseases because *B. dendrobatidis* is an intracellular pathogen of epithelial cells. Cytotoxic lymphocytes have been shown to promote resistance to fungi through the production of IFNγ and IL-17, but very little evidence suggests that the cytotoxic capacity of $CD8^+$ T cells or other lymphocytes is important in fungal immunity (Wüthrich et al., 2003; Lin et al., 2005; Cohen et al., 2011; Nanjappa et al., 2012). MHC class I expression is increased in the skin during chytridiomycosis (Rosenblum et al., 2012a) which

could aid in presentation of *B. dendrobatidis* peptide antigens to $CD8^+$ T cells. If *B. dendrobatidis* is an obligate intracellular pathogen requiring the niche of a keratinocyte to develop and reproduce, killing host cells soon after infection would stop the life cycle and diminish *B. dendrobatidis* infection. However, if the keratinocyte is just a location for *B. dendrobatidis* cells to obtain nutrients and hide from phagocytes, killing host cells would not kill *B. dendrobatidis* but may make *B. dendrobatidis* more susceptible to phagocytes. In *X. laevis* skin allografts, $CD8^+$ cells infiltrate into the skin to promote rejection (Ramanayake et al., 2007), so $CD8^+$ T cells can theoretically infiltrate into sites of *B. dendrobatidis* infection where they may actually promote resistance to chytridiomycosis.

B cells may also play a protective role in chytridiomycosis. Although B cells have not been identified in amphibian skin, the presence of skin mucosal antibodies suggests their presence in amphibian SALT (Ramsey et al., 2010). Antibodies directed against surface structures and virulence features can be protective in mouse models of fungal disease (Cassone & Casadevall, 2012). Antibodies produced in amphibian skin against *B. dendrobatidis* have not been shown to protect against chytridiomycosis, but these antibodies could inhibit *B. dendrobatidis* adhesion, invasion, or replication and could activate killing by complement-mediated mechanisms.

Amphibian resistance to chytridiomycosis

Unrestricted *B. dendrobatidis* infection of amphibian skin leads to skin disruption which causes electrolyte dysregulation and eventual death (Voyles et al., 2009). To survive chytridiomycosis, amphibians must slow *B. dendrobatidis* replication or kill *B. dendrobatidis* cells. Prevention of pathogen invasion in the mucus, inhibition of *B. dendrobatidis* growth in

host cells, and clearance of *B. dendrobatidis* by immune cells are all mechanisms to promote resistance to chytridiomycosis.

The mucus covering the skin of amphibians may be the most important defense against chytridiomycosis. Certain AMPs or antifungal bacterial metabolites present in amphibian mucus have been shown to kill or at least inhibit the growth of *B. dendrobatidis in vitro* (Rollins-Smith & Conlon, 2005; Harris et al., 2006; Woodhams et al, 2007b; Lauer et al., 2008; Ramsey et al., 2010). Bacterial and AMP defenses have also been correlated with survival from chytridiomycosis (Woodhams et al., 2005; Woodhams et al., 2007a; Becker & Harris, 2010; Lam et al., 2010). Antibodies in the mucus may also play an important role in limiting infection (Ramsey et al., 2010). Defenses in the mucus are likely to impair or kill invading zoospores preventing invasion of host epidermis. Amphibian AMPs can disrupt the cell membrane of zoospores (Daum et al., 2012), and because zoospores lack a cell wall (Longcore et al. 1999, Pessier et al. 1999, Berger et al. 2005a), AMPs in the mucus are probably more effective at impairing *B. dendrobatidis* than AMPs in the skin. Several antifungal metabolites are known to be produced by skin symbionts on amphibian skin (Brucker et al., 2008a; Brucker et al., 2008b). Although the mechanism of *B. dendrobatidis* inhibition by these metabolites is unknown, growth of *B. dendrobatidis* is greatly impaired in the presence of these metabolites. Mucosal antibodies against *B. dendrobatidis* have not been shown to confer protection but theoretically could convey protection in various ways. Antibodies could target *B. dendrobatidis* for complement-mediated killing, prevent adhesion by zoospores, disrupt germination tube formation, or impair cell wall formation of encysted zoospores. Antibodies are known to affect the expression of metabolism of *C. neoformans* (McClelland et al., 2010); amphibian antibodies may have similar effects on *B. dendrobatidis*.

Once *B. dendrobatidis* enters the skin, the immune mechanisms that slow or stop the infection are completely unknown. Adaptive immune responses appear to be important because when lymphocytes are killed by sub-lethal X-irradiation, *X. laevis* attains higher *B. dendrobatidis* infection loads (Ramsey et al., 2010). T cell-mediated responses are likely to be the most important in promoting resistance to *B. dendrobatidis*, particularly the more inflammatory immune responses.

IL-17 plays an important role to recruit phagocytes especially neutrophils. Neutrophils can mediate killing by phagocytosis, secretion of an arsenal of antimicrobials, or autolysis to produce extracellular chromatin traps (Gunzer, 2013). Infiltration of neutrophils would cause the localized destruction of tissue which might promote killing of *B. dendrobatidis* and would promote survival if immunopathology to the skin is limited. In amphibians IL-17 and IL-22 should induce production of AMPs that may promote the killing of *B. dendrobatidis* both in and on the surface of the skin.

IFNγ, GM-CSF, and TNFα are important in resistance to most pathogenic fungi (Wüthrich et al., 2012a). These Th1 cytokines may play an important role in activating phagocytic killing and antigen presentation during chytridiomycosis if *B. dendrobatidis* cells exit host keratinocytes. If *B. dendrobatidis* cells are engulfed by phagocytes *in vivo*, Th1 cytokines would be important in promoting killing of *B. dendrobatidis* cells in phagosomes and would limit any evasion mechanism *B. dendrobatidis* has to evade destruction.

Antibodies, and potentially Th2-mediated activation of antibody production, may have protective roles in *B. dendrobatidis*-infected skin. Antibodies could opsonize *B. dendrobatidis* cells to allow for phagocytosis or complement activation and killing. NK-cell killing of infected host epithelial cells may also be mediated by antibody-dependent cell-mediated cytotoxicity.

Xenopus laevis splenocytes express Fc receptors for IgY and IgM (Coosmans & Hadji-Azimi, 1988), so it is possible that antibodies may promote lymphocyte-mediated immunity to *B. dendrobatidis*.

Killing of epithelial cells by cytotoxic lymphocytes might be protective against *B. dendrobatidis*. Host cell death at an appropriate time would prevent the replication of the resident *B. dendrobatidis* cell inside of the host cell. Even if *B. dendrobatidis* were resistant to killing of host cells, the induction of cell death would target host cells for removal by phagocytes which could promote immune activation after *B. dendrobatidis* is phagocytized with the apoptotic host cell. Because *B. dendrobatidis* can grow free of its host in nutrient media (Longcore et al., 1999), lymphocyte-mediated killing of host cells would probably release *B. dendrobatidis* into the extracellular space. Freed from host cells, *B. dendrobatidis* may be susceptible to phagocytosis and other killing mechanisms. *Batrachochytrium dendrobatidis* appears to be capable of moving from one epithelial cell to another (Van Rooij et al., 2012), but this appears to be a rare event (Berger et al., 2005a). If host cells were targeted for destruction by cytotoxic lymphocytes, *B. dendrobatidis* might be able to use germination tubes to escape into other epithelial cells, although this has not been observed.

The immune system has a variety of mechanisms to kill pathogens. *Batrachochytrium dendrobatidis* is likely susceptible to many of these mechanisms but appears to have several evasion strategies to either dampen or avoid immune defenses. The differential survival of amphibians when infected with *B. dendrobatidis* may entirely or partially be explained by the ability of the immune system to clear or deter infection.

Evasion of immunity by *B. dendrobatidis*

Batrachochytrium dendrobatidis is mostly, if not entirely, an obligate pathogen of amphibians. Some very fragmentary evidence suggests that *B. dendrobatidis* can infect arthropods (McMahon et al., 2013) and nematodes (Shepard et al., 2012). However, because *B. dendrobatidis* is known to infect over 500 species of amphibians (Olson et al., 2013) and the closest known relative to *B. dendrobatidis, B. salamandrivorans,* also infects salamanders (Martel et al., 2013), the likely niche of choice for *B. dendrobatidis* is the skin of amphibians. In order to complete its life cycle in an amphibian host, *B. dendrobatidis* must have adapted multiple strategies for inhibiting immune responses. Fungi have acquired many evasion tactics that *B. dendrobatidis* could also employ (Table 1-2). Several studies suggested that *B. dendrobatidis* impairs immune responses, but only recently has my work provided definitive evidence of evasion showing that *B. dendrobatidis* inhibits lymphocyte responses (Chapter II). My study only identifies one of the multitude of immune evasion strategies *B. dendrobatidis* may be able to use to avoid clearance by the normally effective vertebrate immune system.

Due to the low infiltration of leukocytes into *B. dendrobatidis* infected skin (Pessier et al., 1999; Berger et al., 2005b), *B. dendrobatidis* likely impairs cytokine signaling. The lack of significant leukocyte recruitment could be the result of impaired T cell responses which recruit neutrophils and macrophages (Chapter III). However, activation of immune responses in the absence of lymphocytes should still initiate some leukocyte infiltration and activation. If *B. dendrobatidis* were to interfere with cytokine and chemokine signaling during early immune activation, the observed modest leukocyte response would result. Any number of the proteases produced by *B. dendrobatidis* could cleave and inactivate cytokines that activate and recruit leukocytes. Fungal proteases and host proteases activated by funi have been shown to diminish

immune responses by inactivating cytokines or complement (Speth et al., 2008; Rambach et al., 2010; Wüthrich et al., 2012b). Proteases have also been shown to activate allergy responses in allergic bronchopulmonary aspergillosis (Denning et al., 2006) which may be a mechanism to promote non-protective Th2 responses. The *B. dendrobatidis* genome has extensive gene expansions in proteases compared to its closest non-pathogenic relative suggesting that these proteases have an important role in virulence (Joneson et al., 2011). Although most of these proteases probably play important roles in invasion, tissue disruption, and digestion of host cell components (Piotrowski et al., 2004; Moss et al., 2010; Brutyn et al., 2012), several may be essential for evasion of host immunity. Proteases from *B. dendrobatidis* have been shown to cleave amphibian AMPs (Thekkiniath et al., 2013). Some of the proteases produced by *B. dendrobatidis* should activate immune responses by producing DAMPs (Romani, 2011), but *B. dendrobatidis* may also have proteases that inactivate immune activation or down-stream cytokines. Future studies should further investigate whether *B. dendrobatidis* proteases inactivate cytokines, chemokines, complement, or immune receptors.

No direct evidence shows impairment of phagocytes by *B. dendrobatidis*, but production of cytokines and pathogen killing has not been investigated. *B. dendrobatidis* may completely evade phagocyte detection and phagocytosis inside of host keratinocytes. However, since *B. dendrobatidis* does appear to send rhizoids out of host cells and can live free of host cells in culture medium, phagocytes in the skin do have the chance to interact with *B. dendrobatidis* cells during infection. *B. dendrobatidis* does not appear to evade phagocytosis (Chapter III), but could inhibit killing mechanisms inside of the phagocyte much like other pathogenic fungi. *Histoplasma capsulatum*, *C. albicans*, and *C. neoformans* prevent maturation of the phagosome compartment (Woods, 2003; Fernandez-Arenas, 2009; Tucker & Casadevall, 2009). *Aspergillus*

fumigatus, *C. albicans*, and *C. neoformans* can escape out of phagosomes (Brown, 2011; Coelho et al., 2013), but *B. dendrobatidis* does not appear to be capable of similar phagosomal escaper (Chapter III). Most fungi can resist reactive oxygen and nitrogen with anti-oxidants, catalase, superoxide dismutase, or inhibitors of nitric oxide synthesis (Brown, 2011). Genes with putative catalase, peroxidase, and superoxide dismutase activities have been identified in the *B. dendrobatidis* genome (Joint Genome Institute, http://genome.jgi-psf.org/cgi-bin/ToGo?species=Batde5). If *B. dendrobatidis* can inhibit robust inflammatory responses to prevent leukocyte infiltration, defensive strategies to prevent killing by phagocytes may not be necessary. The immune system is safe-guarded against pathogen evasion, so *B. dendrobatidis* is likely to encounter phagocytes during infection.

All living organisms require metal nutrients for the proper function of various proteins. The immune system utilizes this requirement of bacterial and eukaryotic pathogens by limiting the presence of metals such as iron, zinc, calcium, and manganese in the extracellular environment and inside of phagosomes. The sequestration of nutrients to limit the growth of pathogens is called "nutritional immunity" and is essential for limiting the growth of most microorganisms (Hood & Skaar, 2012). In order for a pathogen to survive in a nutrient depleted environment, it must obtain nutrients from the host. Fungal pathogens can scavenge metals with siderophores or by directly obtaining metals from host metal-binding proteins (Kornitzer, 2009; Gauthier et al., 2010). The metal nutrient requirements of *B. dendrobatidis* or chytrids are unknown, but *B. dendrobatidis* is likely to require metals and obtain them from the host. Like other fungal superoxide dismutases, the *B. dendrobatidis* superoxide dismutase is predicted to require metal cofactors to resist ROS (Subramanian Vignesh et al., 2013; Joint Genome Institute, http://genome.jgi-psf.org/cgi-bin/ToGo?species=Batde5). *Batrachochytrium dendrobatidis* may

use the intracellular environment of host epithelial cells to obtain metal nutrients along with the organic nutrients necessary for growth and replication. However, inside of phagocytes, *B. dendrobatidis* would be in metal-depleted conditions where, in order to survive, *B. dendrobatidis* would need to scavenge metals. Nutritional immunity may not play a role in chytridiomycosis, but further studies are needed to understand the nutritional needs of *B. dendrobatidis* and to determine the possible importance of metal sequestration in the skin.

The host epithelial cells infected by *B. dendrobatidis* should be the primary cells to detect the presence of *B. dendrobatidis* and activate the production of inflammatory cytokines and chemokines. Some transcriptional evidence suggests that a few cytokines are up-regulated in amphibian skin (Rosenblum et al., 2012a), but whether or not these cytokines are being released and subsequently are activating innate immune processes is unknown. The lack of any significant leukocyte infiltration (Pessier et al., 1999; Berger et al., 2005b) suggests that even the early, innate responses are being impaired. Unfortunately, transcriptional analysis, one of the few tools currently available to investigate the effect on host keratinocytes during chytridiomycosis, is not sufficient to answer these questions. More molecular tools in amphibians and *in vitro* systems that mimic *B. dendrobatidis* pathogenesis are still needed. Future studies will need to determine whether *B. dendrobatidis* activates the inflammasome or PRRs in host keratinocytes, if keratinocytes secrete active cytokines, and if these cytokines are degraded by *B. dendrobatidis* or host proteases.

Amphibians have a robust immune system that is present and active in the skin (Ramanayake et al., 2007; Robert & Ohta, 2009; Rollins-Smith et al., 2009). The only way *B. dendrobatidis* can persist and complete its replication in host skin is to inhibit inflammatory processes and immune defenses in the skin. *Batrachochytrium dendrobatidis* produces factors

that impair lymphocytes which would also diminish inflammatory processes mediated by Th1 and Th17 cells. Pathogenic fungi are known to possess an array of evasion tactics (Table 1-2). As a deadly pathogen across an entire class of vertebrates, *B. dendrobatidis* is likely to employ a multitude of evasion strategies yet to be identified.

Future Progress in Chytridiomycosis Research

No successful mitigation strategy has yet been developed to aid populations of amphibians susceptible to chytridiomycosis (Woodhams et al., 2012). If declining populations are to be saved or if expatriated populations are to be reintroduced, these populations must be resistant to *B. dendrobatidis* now ubiquitous across most amphibian ecosystems. Various mitigation strategies have been suggested including providing probiotic bacteria (Bletz et al., 2013), decreasing host density (Woodhams et al., 2011), directly treating infected amphibians to remove infection (Woodhams et al., 2012), modifying the ecosystem by diminishing canopy cover (Becker et al., 2012), and introducing zooplankton to prey on *B. dendrobatidis* (Searle et al., 2013). Strategies that have attempted to promote an amphibian's natural immunity have been attempted as well but have not yet been successful. Immunizing *X. laevis* systemically against *B. dendrobatidis* did promote antibody production (Ramsey et al., 2010), but similar immunizations have not protected susceptible species (Rollins-Smith et al., 2009; Stice & Briggs, 2010). This method of immunization may not induce antibodies in the skin mucus where they would be the most effective against *B. dendrobatidis*. Also, exposing and clearing amphibians of *B. dendrobatidis* with antifungal drugs has not yet been shown to confer protection against chytridiomycosis (Cashins et al., 2013). The ability of *B. dendrobatidis* to inhibit lymphocytes

may prevent the development of long-term immunity to chytridiomycosis in these studies (Chapter II). *Batrachochytrium dendrobatidis* strains could be identified that lack this virulence feature, or genetic modification could attenuate *B. dendrobatidis*. Immunization with an attenuated *B. dendrobatidis* could be much more successful, as noted in several fungal vaccination studies (Cassone & Casadevall, 2012; Wüthrich et al., 2013). Before natural immunity can be induced to mitigate chytridiomycosis in natural populations, many additional questions about amphibian immunity and *B. dendrobatidis* pathogenesis must be answered so as to develop a method to promote appropriate antifungal responses with immunological memory.

Outstanding questions in amphibian immunology

No studies have demonstrated how an amphibian can acquire and retain resistance to chytridiomycosis. Most of the evidence demonstrating resistance is correlated with AMPs or bacterial symbionts in the skin mucus (Woodhams et al., 2005; Woodhams et al., 2007a; Becker & Harris, 2010; Lam et al., 2010). The role of adaptive and innate immune defenses against *B. dendrobatidis* in the skin is still very poorly understood with some studies suggesting an absence of immune responses while others suggest a capacity of the immune system to respond and clear infection (Table 1-2). The differences in results likely originate from differences in choice of host species and the absence of an understanding of the kinetics of a protective immune response to chytridiomycosis. Future immunological studies must recognize that amphibian immune responses are much slower to develop than those of mammals, especially at cooler temperatures (Robert & Ohta, 2009), and a robust adaptive immune response in an amphibian may take multiple exposures to develop (Table 5-1; McMahon et al., submitted).

Table 5-1. Kinetics of immune responses of *X. laevis* to pathogens and allo-antigens.

Response to:	**Peak of infiltration**	**Days until clearance**	**Type of response**	**Reference**
MHC-class-I disparate skin transplant	7 days	20 days at optimal temperature	CTL	Ramanayake et al., 2007
Minor-H disparate skin transplant	15 days	30-35 days, temperature dependent	CTL	Ramanayake et al., 2007
Ranavirus	Primary: 6 days; Secondary: 3 days	About 30 days (can be up to 60 days)	CTL, Antibody, likely Th1 and Th2 responses	Morales et al., 2010; Chen & Robert, 2011
Bd infection	Unknown*	Infection peak at 30 days; no complete clearance	Unknown*	Ramsey et al., 2010

*Immunized *X. laevis* could produce antibodies against *B. dendrobatidis* but only after 49-56 days and three immunizations.

The defenses against chytridiomycosis present in the skin mucus have been the best defined. However, much is still unknown about the environment of the mucus and how it may prevent *B. dendrobatidis* invasion. Potentially the most important contributors to the mucus are the mucosal antibodies. Ramsey et al. (2010) characterized antibodies of all three antibody classes in the mucus of *X. laevis* which also recognized *B. dendrobatidis*. No study has yet investigated what protective effect anti-*B. dendrobatidis* antibodies may have. *In vivo* studies investigating the role of amphibian antibodies may be difficult, but *in vitro* studies could investigate whether antibodies may neutralize adhesion, prevent germination tube formation, or impair cell wall development of zoospores. Antibodies may also activate complement-mediated killing of zoospores in the mucus. In the skin mucus, there is potential for interaction among AMPs, bacteria, and antibodies to either promote or impair resistance. AMPs and a single bacterial metabolite have been shown to act synergistically to inhibit *B. dendrobatidis* (Myers et al., 2012). AMPs and antibodies may also influence the skin flora, potentially to promote the growth of beneficial bacteria. AMPs inhibit the growth of bacteria on the skin, which may aid or hinder antifungal resistance. Human IgA appears to promote the formation of bacterial biofilms in the gut (Bollinger et al., 2003), and the amphibian analogue IgX may play a similar role on the skin. A core set of bacteria have been described for *Pletodon cinereus* (Loudon et al., 2013), and it is possible that these are maintained by the immune system.

One important unknown that will aid in understanding immunity to chytridiomycosis is how the amphibian immune system recognizes *B. dendrobatidis*. Several amphibian PRRs have been identified (Ishii et al., 2007; Robert & Ohta, 2009), but very little is known about the PAMPs on *B. dendrobatidis* that may activate immune responses. Also yet to be determined are the cells that recognize *B. dendrobatidis*. Are PRRs or the inflammasome activated in host

keratinocytes? Does *B. dendrobatidis* infection release DAMPs to activate immune responses? Do innate immune cells, particularly LCs resident to the epidermis, have a chance to recognize *B. dendrobatidis* PAMPs?

How the amphibian immune system may promote inflammatory responses in the skin is unknown. The cytokines involved in activation and inflammation are likely to be the same between amphibians and mammals, but certain cytokines may be more important or have slightly different effects in amphibians. Transcriptional evidence suggests that IL-1β is an important cytokine that is activated during chytridiomycosis (Rosenblum et al., 2012a), but the absence of clear immune gene expression changes, possibly caused by immune evasions mechanisms, prevents determining which immune responses may be beneficial for resistance. Transcriptional analysis probably overlooks the differential gene expression in leukocytes, but *in vitro* studies exposing phagocytes to *B. dendrobatidis* or *B. dendrobatidis* antigens could determine how leukocytes respond to the presence of *B. dendrobatidis*.

In the absence of lymph nodes, the migration of antigen presenting cells (APCs) and lymphocytes is very much unknown in amphibians. Amphibians have spleens to process antigen and activate lymphocytes, but the spleen may not process antigens from the skin. APCs in the skin may simply present antigen in the skin to promote adaptive immune responses. It will be important to determine where *B. dendrobatidis* antigens are transported by APCs and if *B. dendrobatidis* antigens do arrive in the spleen. In chytridiomycosis, antigen presentation is important because MHC class II alleles have been correlated with survival suggesting that effective antigen presentation can promote $CD4^+$ T cell immunity (Savage & Zamudio, 2011). Also, amphibian phagocytes exposed to *B. dendrobatidis* soluble antigens can promote lymphocyte activation *in vitro* (Chapter III). If certain alleles can promote survival, resistance to

chytridiomycosis may be dependent on the kinetics and repertoire of T cell priming to limit infection before *B. dendrobatidis* can inhibit lymphocyte responses.

The role of lymphocytes has been established during chytridiomycosis (Ramsey et al., 2010), and it appears that impairing lymphocyte responses promotes *B. dendrobatidis* infection (Chapter II). However, whether B cell, T cells, or ILCs all contribute to resistance or only certain lymphocyte populations are important in immunity to *B. dendrobatidis* is not known. The role of T cells can be investigated in thymectomized *X. laevis* which lack T cells (Horton & Manning, 1974; Du Pasquier & Horton, 1976), but more specific lymphocyte responses still need to be investigated in chytridiomycosis.

Specific helper T cell responses have not been characterized in amphibians, but due to the conservation of the adaptive immune system and of T cell cytokines, these responses are likely to be very similar among vertebrates (Qi & Nie, 2008; Jackson et al., 2012; Monte et al., 2013). It would be important to characterize activities of $CD4^+$ T cells in amphibians, so that the role helper T cells play in resistance to chytridiomycosis can be studied. The cytokines most important for Th1 and Th17 responses, IFNγ and IL-17, can be studied in amphibians by following the expression of these important cytokines by leukocytes. Th1 and Th17 are likely to be important in immunity to *B. dendrobatidis*, but until this can be demonstrated, it is possible that other cells or other responses may be more vital to resistance. Characterizing memory in amphibian lymphocytes would also allow for development of long term immunity. Determining which immune responses are important to activate sterilizing immunity in chytridiomycosis will be essential for immunization of susceptible amphibian populations.

Cytotoxic lymphocytes likely play an important role in immunity to chytridiomycosis. $CD8^+$ T cells have been followed in rejection of skin transplants in *X. laevis* (Ramanayake et al.,

2007), and it may be possible to investigate $CD8^+$ T cells in a similar way during chytridiomycosis. Cytotoxic lymphocytes can produce cytokines to promote helper T cell responses during infection, so expression of IFNγ, IL-17, and other cytokines by $CD8^+$ T cells and NK cells may be activated during chytridiomycosis.

Outstanding questions in *B. dendrobatidis* pathogenesis

Despite over a decade of research, *B. dendrobatidis* is still a very mysterious pathogen. Less knowledge is generally available about chytrid fungi compared to higher fungi. For example, the chytrid cell wall, despite being predicted to be much different from higher fungi (Ruiz-Herrera & Ortiz-Castellanos, 2010), has not been characterized. The genome of *B. dendrobatidis* provides an added layer of complexity. Despite appearing to mostly, if not entirely, replicate asexually, the genome of *B. dendrobatidis* is highly unstable with much recombination and great variation in chromosomal copy number (Farrer et al., 2013; Rosenblum et al., 2013). Some of the more important questions about *B. dendrobatidis* pathogenesis have been addressed including how *B. dendrobatidis* develops in the skin (Berger et al., 2005a), how chytridiomycosis leads to death in amphibians (Voyles et al., 2009) and how *B. dendrobatidis* enters host cells from the skin surface (Van Rooij et al., 2012; Greenspan et al., 2012), but many questions still remain.

In vitro growth assays have attempted to reproduce components the environment of the skin mucus in culture with *B. dendrobatidis* cells (Rollins-Smith et al., 2002; Harris et al., 2006), but how *B. dendrobatidis* enters and exists in the mucus is still unknown. *B. dendrobatidis* is predicted to quickly pass through the mucus and may take only a few hours to pass through the mucus, encyst, and invade a host cell (Van Rooij et al., 2012; Greenspan et al., 2012). *B.*

dendrobatidis may grow as it does in culture media on the surface of the skin and also send germination tubes into the skin to infect more cells. Alternatively, *B. dendrobatidis* may spend so little time in the mucus that AMPs and bacterial metabolites may only have a short opportunity to paralyze or kill the invading zoospore. *B. dendrobatidis* zoospores may evade bacterial and host defenses in the mucus (Lam et al., 2011; Thekkiniath et al., 2013) suggesting that a very limited amount of time is spent by *B. dendrobatidis* in the mucus.

As a successful pathogen, *B. dendrobatidis* is likely to have many immune evasion strategies. Soluble factors from *B. dendrobatidis* impair lymphocytes by inducing apoptosis (Chapter II), but other evasion mechanisms to inhibit immune activation and pathogen clearance in the skin are still unknown. Several genomic studies have proposed various *B. dendrobatidis* virulence factors (Sun et al., 2011; Joneson et al., 2011; Abramyan & Stajich, 2012), but no direct evidence suggests which of these actually contributes to pathogenesis.

Proteases are probably responsible for some of the pathogenesis and immune inactivation during chytridiomycosis. *Batrachochytrium dendrobatidis* has gene duplications in serine peptidases that likely arose from horizontal gene transfer from bacteria (Sun et al., 2011). Along with serine-type proteases, *B. dendrobatidis* also has genomic expansions in metalloproteases and aspartyl proteases (Joneson et al., 2011) that are highly expressed when *B. dendrobatidis* is cultured on amphibian skin (Rosenblum et al., 2012b). Also *B. dendrobatidis* has an elastase which likely plays a role in invasion and tissue destruction (Moss et al., 2010). *Batrachochytrium dendrobatidis* proteases are probably responsible for digestion of host cells, dissociation of epithelium, degradation of AMPs, inactivation of cytokines, and cleavage of host receptors. Serine proteases from *Streptococcus* can inactivate chemokines to dampen immune responses (Hildago-Grass et al., 2006; Bryan et al., 2009). Dipeptidyl peptidase 4, a host serine protease,

also has been shown to cleave chemokines (Zhong et al., 2013). Host metalloprotease can be activated by *B. dermatitidis* to inactivate chemokines to decrease inflammation (Wüthrich et al., 2012b). Due to the noticeable lack of leukocyte infiltration, the *B. dendrobatidis* serine and metalloproteases probably inactivate cytokines and chemokines, as well, to prevent inflammatory responses. A subtilisin-like serine protease from *B. dendrobatidis* has been shown to cleave amphibian AMPs (Thekkiniath et al., 2013), but the majority of proteases and their effects still have not been characterized.

Batrachochytrium dendrobatidis evades adaptive immunity by impairing lymphocytes. The soluble factors inhibit proliferation and induce apoptosis in amphibian lymphocytes and inhibit the production of IL-2 and IFNγ in human $CD4^+$ T cells (Chapter II). The factors responsible for this inhibition have not been identified but appear to be carbohydrates associated with the *B. dendrobatidis* cell wall (Chapter IV). *B. dendrobatidis* induces more apoptosis in amphibian T cells than amphibian B cells (Chapter II), but the effect on other lymphocytes and subsets of T cells has not yet been investigated. The *B. dendrobatidis* factors responsible for lymphocyte impairment do not appear to negatively affect phagocytosis, viability, or accessory functions of amphibian phagocytes (Chapter III). However, *B. dendrobatidis* may still be able to endure phagocytosis by inhibiting phagosome maturation, resisting ROS and RNS, scavenging metals, or exiting the phagosome. The effect of *B. dendrobatidis* recognition and phagocytosis on the cytokine production of phagocytes has also not been investigated. The investigation of this evasion strategy has been *in vitro*, but *in vivo* experiments in *X. laevis* corroborate the *in vitro* observations (Chapter III). The inhibition of adaptive immune responses by *B. dendrobatidis* is an explanation of why amphibians are so susceptible to *B. dendrobatidis* when innate immune defenses are lacking (Rollins-Smith et al. 2011); why immunizations have not

promoted resistance to chytridiomycosis (Rollins-Smith et al., 2009; Stice & Briggs, 2010); and why highly resistant species continuously harbor infection (Mazzoni et al., 2003; Ramsey et al., 2010).

Further investigation of chytridiomycosis will require adapting *B. dendrobatidis* as a better organismal model. No genetic manipulation has been successful in *B. dendrobatidis*. Genomic and expressional analysis of *B. dendrobatidis* can pinpoint important virulence genes but only provide hypotheses. Finding and investigating the effects of virulence factors will be difficult unless genes for these can be knocked-down or knocked-out. Due to the absence of a single transfected or transformed *B. dendrobatidis* in the literature, simple transfection or transformation methods probably would not be successful with *B. dendrobatidis*. Transformation with *Agrobacterium tumefaciens* has been shown to transform dimorphic and filamentous fungi (Sullivan et al., 2002; Utermark & Karlovsky, 2008). *Batrachochytrium dendrobatidis* may be transformable with *A. tumefaciens* as well. Until *B. dendrobatidis* can be genetically manipulated, the various strains and isolates of *B. dendrobatidis* may be used to follow pathogenesis. Virulence of *B. dendrobatidis* varies greatly among isolates (Retallick & Miera, 2007; Fisher et al., 2009). Outside of the global panzootic lineage of *B. dendrobatidis*, there are strains that have been identified which may be less pathogenic (Farrer et al. 2011; Farrer et al., 2013; Rosenblum et al., 2013). Analysis of the virulence of these non-panzooitic *B. dendrobatidis* strains along with genomic investigation may lead to better understanding of the virulence features in *B. dendrobatidis*. The newly identified *B. salamandrivorans* may lack some of the virulence factors present in *B. dendrobatidis* especially because it does not cause chytridiomycosis in *Alytes obstetricans*, the midwife toad that is very susceptible to *B. dendrobatidis* (Martel et al., 2013). Investigation of *B. salamandrivorans* may provide great

insight into the transition of *B. dendrobatidis* to become an amphibian pathogen. Identification or generation of a *B. dendrobatidis* strain that lacks one or more virulence features will not only aid in understanding the pathology of chytridiomycosis but could be used as a live-attenuated inducer of natural immunity in susceptible amphibians.

Future progress in treating and preventing chytridiomycosis

Treating individual amphibians to remove *B. dendrobatidis* infection is still a valuable way to limit chytridiomycosis. Although difficult and time consuming, treating individuals to protect wild populations may be effective at maintaining populations of susceptible species outside of captivity. Treatment of captive population is essential for limiting the movement of *B. dendrobatidis* around the globe and to maintain good health of zoo and lab amphibians. If restriction are made to prevent the movement of *B. dendrobatidis* in the trade of amphibians for food, pets, and scientific model organism (Kriger & Hero, 2009), then simple, effective, and inexpensive treatments need to be available to clear *B. dendrobatidis* from amphibians.

Attempts to limit chytridiomycosis in wild populations with antifungal treatment have not been very successful, but most antifungal treatments consist of itraconazole which can be toxic to amphibians and may limit amphibians' natural immune defenses (Jones et al., 2012; Woodhams et al., 2012). Improved antifungal therapies especially those that promote natural immunity are needed. Anti-*B. dendrobatidis* therapies cannot impair natural mucosal defenses and must leave the skin biota and mucosal AMPs intact. Many drugs also cause toxic side effects that impair adaptive immunity, so it is important to find drugs with limited effects on amphibians. Finding drugs that target proteins present in *B. dendrobatidis* that are not shared among fungi and animals is essential. Many antifungal drugs target cell wall or ergosterol

synthesis absent from animal metabolic pathways. These, including itraconazole, are effective at treating fungal pathogens in general, but targeting pathways and proteins specific to *B. dendrobatidis* pathogenesis may be more beneficial. For example, many serine proteases in the *B. dendrobatidis* genome share homology with similar proteases from bacteria (Sun et al., 2011); these proteases are probably virulence factors and may be different enough from host protease to be good drug targets.

Nikkomycin Z (NZ) may be an effective drug to treat chytridiomycosis. NZ is a chitin synthase inhibitor (Gaughran et al., 1994) that greatly impairs the *B. dendrobatidis* cell wall (Holden et al., 2013). NZ also decreases the ability of *B. dendrobatidis* to impair lymphocytes (Chapter IV) and likely makes PAMPs available in the *B. dendrobatidis* cell wall to activate immunity (Goodridge et al., 2009). Treating chytridiomycosis with NZ should decrease fungal burden while promoting natural immunity. Clearing *B. dendrobatidis* with itraconazole does not appear to give protection to amphibians that are exposed to *B. dendrobatidis* again (Cashins et al., 2013). However, treating with NZ may actually support natural clearing of *B. dendrobatidis* by the immune system promoting immunological memory and long-term protection.

Instead of clearing an infection with drugs, long-term immunity may also be activated through immunization. Attempts to vaccinate amphibians against chytridiomycosis have failed to protect susceptible frogs (Rollins-Smith et al., 2009; Stice & Briggs, 2010), but this is not surprising because commercially-available antifungal vaccines are not available for humans (Cassone & Casadevall, 2012). Most vaccines have been discovered serendipitously without complete understanding of what immune responses are activated and how long-term immunity is actually achieved. Many vaccines that have succeeded, particularly for fungal pathogens in mouse models, have been live-attenuated pathogens that infect without causing disease usually

due to a lack of virulence factors (Cassone & Casadevall, 2012; Wüthrich et al., 2013). The vaccination attempts for chytridiomycosis have used dead *B. dendrobatidis* mixed with an adjuvant such as Freund's complete adjuvant (Ramsey et al., 2010; Stice & Briggs, 2010). Dead *B. dendrobatidis* still possesses immunomodulatory factors (Chapter II) and may prevent a robust immune response. An appropriate adjuvant might increase the inflammatory response to killed *B. dendrobatidis*, but the adjuvant must activate the right immune responses to result in resistance. In a recent study, Wüthrich et al. (2013) investigated vaccination using killed *B. dermatitidis* with various adjuvants and found that Freund's complete adjuvant and LPS were not adequate adjuvants to promote resistance to this fungal pathogen; however, IL-1 was a successful adjuvant and promoted Th17 immunity. Once more is known about which immune responses are protective in chytridiomycosis, a vaccine that actually promotes resistance can be developed.

Along with an appropriate vaccine unit and adjuvant, the location of vaccination may also be important to induce immunity in the skin. If vaccines are given at a mucosal surface, mucosal immunity may be more likely to respond to pathogen challenge. Oral immunization in *X. laevis* promotes the production of antigen-specific mucosal IgX production (Du et al., 2012). However, *B. dendrobatidis* may possess evasion mechanisms to inhibit immune response in the skin but not elsewhere. Attenuated *B. dermatitidis* can promote vaccine immunity when given subcutaneously but not through the normal route of infection in the lungs because infiltration of inflammatory monocytes is impaired by the yeast (Ersland et al., 2010; Wüthrich et al., 2012b). *X. laevis* can be immunized with killed *B. dendrobatidis* intraperitoneally to produce serum antibodies, but infection with *B. dendrobatidis* allows for *B. dendrobatidis*-specific mucosal antibody production (Ramsey et al., 2010). Immunization with killed *B. dendrobatidis* into the dorsal lymph sac in highly susceptible *Rana muscosa* has not been successful at conveying

resistance (Stice & Briggs, 2010), but this may be due more to the vaccine than mode of delivery. In order to promote long-term immunity to chytridiomycosis, the amphibian SALT will probably need to be exposed to *B. dendrobatidis* antigens with a live-attenuated *B. dendrobatidis* strain.

Vaccination protection of amphibians against chytridiomycosis would be an important mitigation strategy to maintain susceptible populations. Unfortunately, vaccines would only give resistance to vaccinated individuals, so vaccinations would have to be continuous to ascertain resistance in progeny. Vaccination of the majority of a population would promote herd immunity, so not all individuals would necessarily need to be immunized. Continuous immunization of individuals in a population would be very time-consuming but, compared to other mitigation strategies, would be less drastic and as easily administered. Also, once a vaccinated population has been established, these resistant individuals may be able to reproduce enough to maintain the population without continual mitigation. Vaccination could essentially allow for natural selection of resistant individuals without the threat of losing breeding individuals.

Chytridiomycosis as a disease model

Aside from being important for the conservation of amphibians which are the most threatened group of vertebrates, chytridiomycosis provides an opportunity to study themes infectious disease. Chytridiomycosis offers a chance to investigate a panzootic outbreak, a lethal fungal disease, a chytrid fungus, and mucosal interactions between host and microbiota. The disease progression of chytridiomycosis in susceptible populations has allowed for modeling of disease outbreaks and population declines due to infectious disease (Briggs et al., 2010). The

white-nose syndrome of bats is a cutaneous fungal disease of bats that, like *B. dendrobatidis*, has caused population declines in multiple species. Due to the similarities between the zootic diseases, lessons from chytridiomycosis can be applied to white-nose syndrome (Blehert, 2012). Another important aspect of chytridiomycosis is that, unlike fungal pathogens in humans, *B. dendrobatidis* is suited to infect a host to propagate itself. *B. dendrobatidis* provides a model of a fungal pathogen that is very transmissible and highly virulent that other fungal disease models do not offer.

Concluding Remarks

Fungal pathogens are an increasingly important threat to human health and populations of plants and animals (Fisher et al., 2012). Many aspects of fungal pathogenesis and immunity to fungi are completely unknown making a shift to focusing on fungal pathogenesis necessary for medical and conservation research. Fungi are often devastating to hosts causing great damage in both tissue-specific and systemic infections. The tenacity of fungi to adapt to various environments and the shared biology of fungi and animals makes treating fungal diseases especially difficult. Typically robust host immunity and presence of protective microbiota hold fungi in check, so impairment of antifungal defenses often leads to resilient fungal infections.

Of fungal pathogens, maybe of pathogens in general, *B. dendrobatidis* has been the worst to infect vertebrates threatening species across an entire taxonomic class. Chytridiomycosis has caused hundreds of population declines in amphibians contributing to extinctions (Wake & Vredenburg, 2008). If species in the path of chytridiomycosis are to be preserved and if species

only saved from *B. dendrobatidis* in captivity are to be reintroduced, mitigation strategies inducing natural immunity to *B. dendrobatidis* are essential to save these amphibians.

APPENDIX A

ANALYSIS OF ZOOSPORE ENRICHMENT

Several recent studies have investigated *B. dendrobatidis* virulence features such as the production of proteases or inhibition of immune responses (Fisher et al. 2009, McMahon et al. 2013, Thekkiniath et al. 2013, Chapter II). Some of these studies have been careful to examine the presence of these features in the different life stages of *B. dendrobatidis*, especially between the zoospore present on the host's exterior and the more matured cells (germlings, thalli, and zoosporangia) present in the host epithelium (Berger et al., 2005). Expression across the genome greatly differs between substrate-independent (zoospores) and substrate-dependent (germlings, thalli, and sporangia) (Rosenblum et al. 2008). The typical procedure for obtaining zoospores is to flood an agar-plate culture with water or medium to allow the release of zoospores from zoosporangia (Longcore et al. 1999). Several studies have added an enrichment step where this volume is passed through membrane filters which collect clumps and larger cells (Rollins-Smith et al. 2002a, Rosenblum et al., 2008; Gammill et al., 2012; Rosenblum et al., 2012b). It was previously stated that enrichment over a 20 μm pore filter enriches the zoospore purity to a greater than 99% (Rollins-Smith et al. 2002a), but it is likely that this study identified encysted cells that may have germ-tubes or newly sprouting rhizoids as zoospores. The substrate independent fraction obtained by flooding liquid over an agar culture likely contains mostly zoospores but should also contain encysted cells that have not adhered to the agar substrate. Also germlings, thalli, sporangia, and clumps of *B. dendrobatidis* can be dislodged when liquid is added or removed from agar plates. Filter-enrichment over 20 μm pore filter paper should

remove clumps of *B. dendrobatidis* cells and large sporangia but not most individual *B. dendrobatidis* cells that typically are less than 20 μm in diameter (Berger et al. 1999, 2005, Longcore et al. 1999). Because *B. dendrobatidis* zoospores are typically 3-5 μm in diameter (Berger et al., 2005), an optimal zoospore purification method would use a filter with pores between 3 and 5 μm.

The data in this appendix shows that enrichment of zoospores is necessary to obtain a population with greater purity. The type of enrichment will depend on the need for pure cells in the study. For studies concerned about which life stage is present, filtering to enrich zoospores is essential for obtain a greater purity of zoospores. Certain studies although declaring they had a population of zoospores, did not to purify the zoospores (Brutyn et al., 2012; McMahon et al., 2013). The results of these studies are greatly put into question when this method does not appear to produce a highly pure population of zoospores.

Zoospores were enriched as described in Chapter III Materials and Methods. Unenriched fraction, that was not filter, was counted to determine purity without enrichment. Three different enrichment filters were used with pore sizes of 20, 5, and 3 μm. The purity of zoospores were determined by counting all cells and defining the cells which were zoospores, encysted zoospores, intermediate cells (cells with visible rhizoids termed germlings and thalli). To determine if the presence of matured cells, zoospore preparations were stained with calcofluor white (as in Chapter IV). Also, the zoospore yield for the 3 μm pore-filter enrichment was noted to be very low, so cells were counted before and after enrichment to determine yield loss.

Zoospore purity significantly increased when filter-enriching the population (Fig A-1 A). Without enrichment, the zoospore purity was 70.4±2.0% (±SEM). The purity was significantly enriched when zoospore populations were filtered through filter of 20 μm pore-sizes to

83.3±1.4% purity. Zoospores were significantly more enriched with 5 μm pore-sizes filters to 92.9±0.5% purity. Almost complete purity was achieved enriching the 3 μm pore-sizes filters, 98.7±0.5%. Not only did filtering zoospores with smaller pore-sized filters increase zoospore purity; filter-enrichment greatly decreased the variability in populations obtained from different agar cultures on different days. Such population variability in the unenriched fractions would likely contribute to greater experimental variability.

The high zoospore purity using 3 μm pore-sizes filters came at a high cost of yield. The mean percent of zoospore remaining after passing through the 3 μm filters was 12.4±3.7%, and the median percent yield was 9.3%. When higher yields at or near 100% were achieved, the zoospore yield was very low at 4.2±1.2%, but when purities were less than 99%, the zoospore yields were much greater at 20.7±5.1% (Fig. A-1 B). The decrease in purity and increase in yield are correlated; imperfections in the filter may have contributed to greater passage of zoospores and encysted cells through the filter.

The majority of cells in zoospore populations which were not zoospore could best be defined as encysted cells. In other studies, these cells were likely classified as zoospore, but these should not be classified as zoospores. Encysted cells have a cell wall, as indicated by chitin cell wall staining (Fig. A-1 C, D). These cells often appear to have sprouting structures which may be either germinal tubes (Van Rooij et al., 2012; Greenspan et al., 2012) or very early rhizoids (Longcore et al., 1999; Berger et al., 2005a). A few cells in the unenriched population and 20 μm pore filter-enriched population had visible rhizoids (Fig. A-1 C, E). The cells possessing rhizoids were mostly germlings, but there were some which may be classified as thalli in the unenriched population. No germlings or thalli were present in the 3 and 5 μm pore filter-enriched populations. There were very few germlings present in the 20 μm pore filter-enriched population,

and the number of cells with rhizoids was significantly decreased by filtering with the 20 μm pore filter (Fig. A-1 E).

The results presented in this appendix section indicate the importance of enriching *B. dendrobatidis* zoospore populations before experimental analysis. Enrichment method was important for preparation of zoospores in this study, because cells with cell walls inhibited lymphocyte proliferation (Chapter IV). Future studies investigating *B. dendrobatidis* need to be more careful about defining the population of cells they are using in studies, and better zoospore enrichment would better help obtaining a more homogenous *B. dendrobatidis* cell population.

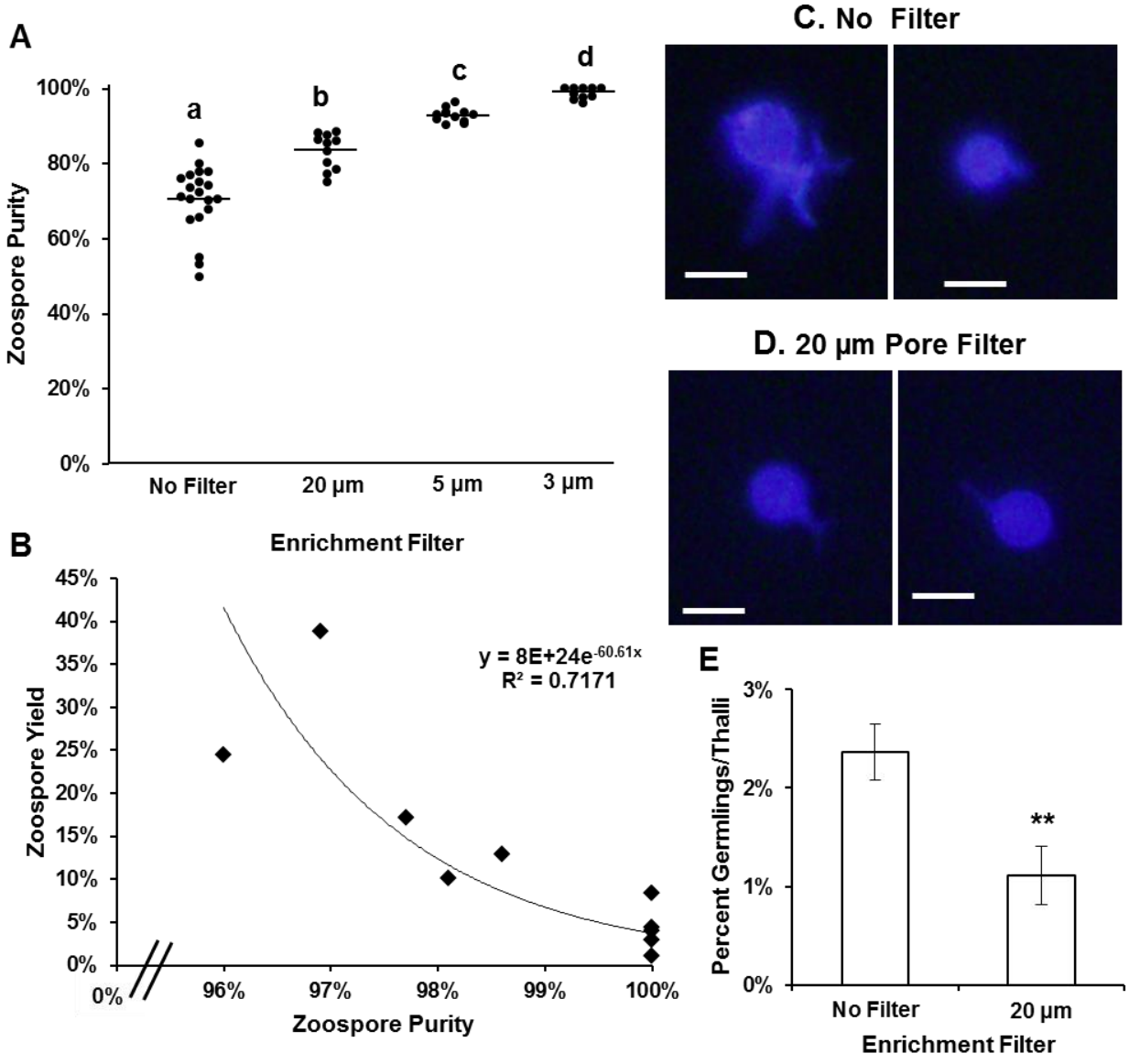

Fig. A-1. Analysis of *B.dendrobatidis* zoospores after filter enrichment. (**A**) Zoospore purity was determined by counting cells of the enrichment *Bd* populations. Each symbol represents a single enriched population; lines indicate the mean zoospore purity. Zoospore purity was analyzed with an ANOVA with Tukey post-hoc, and different letters indicate groups with significantly different zoospore purities. More information on the following page.

Fig. A-1. Analysis of *B.dendrobatidis* zoospores after filter enrichment (Continued). (**B**) Analysis of the 3 μm pore filter enrichment zoospore yield. Each symbol indicates an experiment where the total number of zoospores was determined before and after enrichment. The equation for the best fit curve is shown with the R-square value. (**C**) Representative images of cells from an unenriched zoospore population stained with calcofluor white (a chitin [cell wall] fluorescent stain). (**D**) Representative images of cells from a 20 μm pore filter enrichment zoospore population stained with calcofluor white. (**E**) The relative number of rhizoid-bearing cells (germlings or thalli) present in enriched or unenriched zoospore populations. No germlings and thalli were seen after enrichment with 3 or 5 μm pore filters.

APPENDIX B

INVESTIGATION OF THE INHIBITION OF LYMPHOCYTES BY INOCULA FROM *BATRACHOCHYTRIUM DENDROBATIDIS*

A recent study investigated whether or not *B. dendrobatidis* infects arthropod hosts (McMahon et al., 2013). This study proposed that crayfish (*Procambarus* species) found in the same environment as amphibians could be reservoirs for *B. dendrobatidis* and showed some evidence that *B. dendrobatidis* could infect the gastrointestinal tract of crayfish. An interesting experiment in this paper was that the *B. dendrobatidis* zoospore inocula was filtered to remove cells and treated on crayfish. *Procambarus alleni* crayfish exposed to these cell-free inocula had significantly higher mortality and gill recession than a mock control without *B. dendrobatidis* cells. This experiment suggested that a soluble factor produced by *B. dendrobatidis* was causing pathology in crayfish. The inocula prepared in this study were made by washing *B. dendrobatidis* cultures growing on agar plates with sterile water similar to how zoospores were obtained as described in Chapter III and Appendix A. The inocula contain anything washed off the agar plate including both cells and any molecules secreted or released by *B. dendrobatidis* and any free components of the agar or medium. McMahon et al. (2013) removed the cells by filtering, so the components of the cell-free inocula would probably include all the soluble molecules present on a *B. dendrobatidis* agar culture.

The cell-free inocula probably contain the lymphotoxic factors, so inocula were prepared as described by McMahon et al. (2013) to test on Jurkat cells. A mock control was also prepared in which 1% tryptone agar plates were seeded with 1% tryptone broth instead of 1 ml of liquid *B.*

dendrobatidis culture. Plates were washed with a smaller volume of water (6 mL) than described by McMahon et al. (7 mL) to have a higher concentration of soluble factors and cells. The inocula washed from plates were mostly zoospores, but contained a large number of matured *B. dendrobatidis* cells (see Appendix A). The inocula were diluted so that the final cellular concentration was 2 x 10^6 cells/mL (cells included both zoospores and matured cells). The inocula were centrifuged and the supernatants were filtered through 0.2 μm pore filters and saved. Also to determine if any inhibitory factors were coming from the agar plate or cells in suspension, the cellular pellet was re-suspended in an equal amount of water and incubated for 20 minutes at room temperature. These "washed" inocula were not prepared by McMahon et al. After the 20 minute incubation to allow for release of any soluble factors, the cells were centrifuged and the supernatants were filtered through 0.2 μm pore filters to remove any cells which may have been still in suspension. "Washed" and "unwashed" inocula from *B. dendrobatidis* and mock plates were lyophilized and re-suspended in RPMI medium to test on Jurkat cells. Inocula were re-suspended to final concentrations between 0.5X and 10X the original concentration before lyophilization. These concentrations were equivalent to inocula containing 10^6 *B. dendrobatidis* cells/mL for the 0.5X treatment and 2 x 10^7 *B. dendrobatidis* cells/mL for the 10X treatment. The effects of inocula were tested on Jurkat cells in a ^{3}H-thymidine assay as described in Chapter II. McMahon et al. (2013) tested cell-free inocula on *P. alleni* that originally contained between 10^2 to 10^6 *B. dendrobatidis* cells/mL seeing an effect at cellular concentrations greater than 10^5 cells/mL. Three different inocula and mock controls were tested in three separate Jurkat assays.

Unexpectedly, the inocula had no negative effect on Jurkat cells compared to mock controls. Oddly, the mock controls were more inhibitory to Jurkat cell proliferation than inocula

that had contained *B. dendrobatidis* (Fig. B-1 A). Mock controls were prepared alongside *B. dendrobatidis* inocula and did not have any cells suggesting that components of the tryptone broth are inhibitory to Jurkat cells. The inhibitory components of tryptone broth must be digested by *B. dendrobatidis* because the inocula from *B. dendrobatidis* culture were less inhibitory to Jurkat cell proliferation. Inocula prepared from "washed" *B. dendrobatidis* were not very inhibitory (Fig. B-1 B). "Washed" inocula had to be concentrated to 5X (equivalent of 10^7 *B. dendrobatidis* cells/mL) to see a significant effect only minimally inhibiting Jurkat cells (Fig. B-1 C). The concentration of "washed" inocula inhibitory to Jurkat cells was about 100 fold higher than the concentration shown to be pathogenic to crayfish (McMahon et al., 2013).

The data from this experiment suggest that at the very least the soluble factors described to cause pathology in crayfish by McMahon et al. (2013) have no effect on lymphocytes. These data also call the results of the McMahon et al. study into question. McMahon et al. used inocula that did not contain *B. dendrobatidis*, but did not specially state that they used mock controls with tryptone broth. It is possible that the components of the *B. dendrobatidis* growth medium actually were responsible for mortality and gill recession in crayfish and not soluble factors released by *B. dendrobatidis*.

The study by McMahon et al., (2013) showed that *B. dendrobatidis* could use infect an arthropod and that *B. dendrobatidis* is present at low prevalence in some crayfish populations. The presence of *B. dendrobatidis* inside of crayfish by PCR may not mean that *B. dendrobatidis*. *Daphnia* species, also crustaceans, have been shown to prey on *B. dendrobatidis* (Searle et al., 2013). The presence of *B. dendrobatidis* DNA in crayfish may be due to crayfish predation on *B. dendrobatidis* instead of infection by *B. dendrobatidis*. McMahon et al. (2013) did show pathology by *B. dendrobatidis* on crayfish, but this may be caused by overloading the crayfish

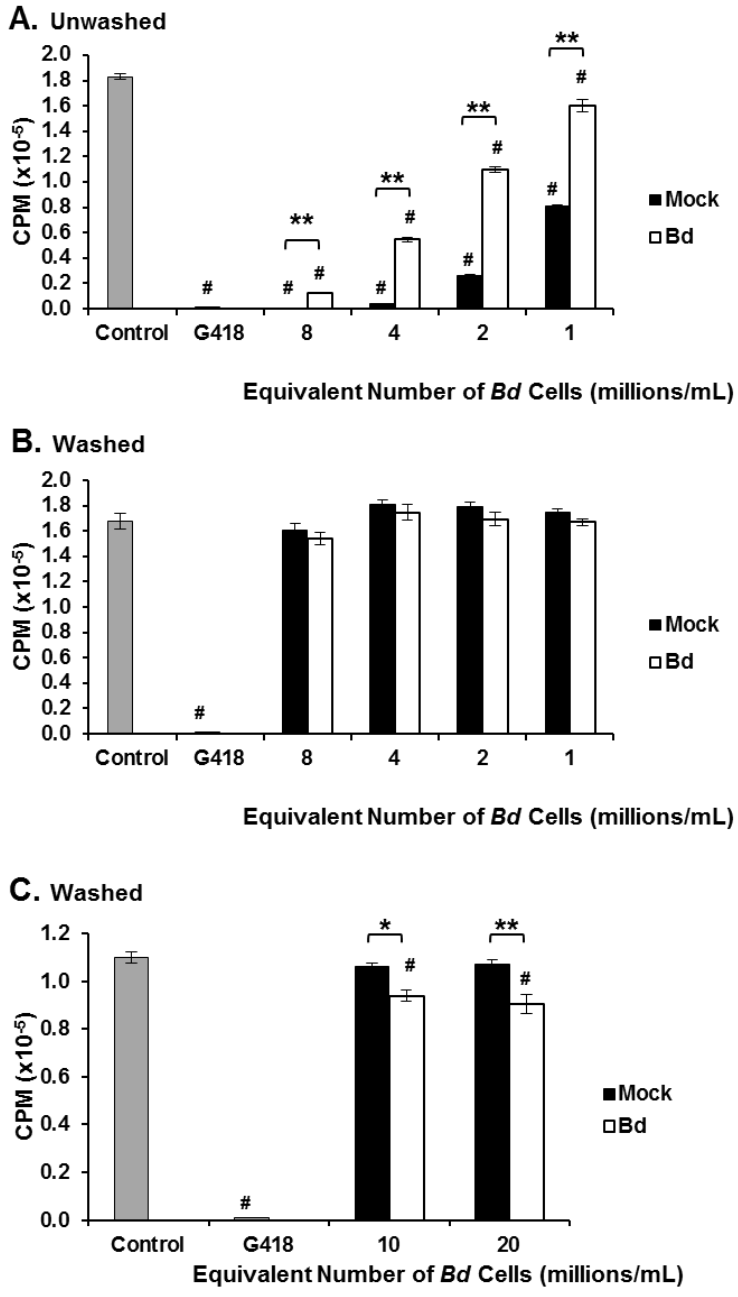

Fig. B-1. Treatment of *B. dendrobatidis* (*Bd*) inocula on Jurkat cells. (**A**) Inocula from unwashed *Bd* cells or *Bd*-free mock controls were re-suspended into Jurkat cell culture. (**B-C**) Inocula from washed *Bd* cells or *Bd*-free mock controls were re-suspended into Jurkat cell culture at lower (B) and higher (C) concentrations. Jurkat proliferation was assayed by ^{3}H-thymidine uptake. Control treatments (gray bars) containing no inoculum were treated with RPMI alone (Control) or G418 at 2 mg/ml. Treatments with significantly decreased inhibition compared to Jurkat cells alone are indicated: $^{\#}p<0.01$. Significant differences in proliferation between *Bd* and mock inocula are indicated under bars: $^{*}p<0.05$, $^{**}p<0.01$. Statistical comparisons were made with a two-tailed, single-factor ANOVA with Tukey tests for individual comparisons. Data are representative of three similar experiments.

with zoospores. An earlier study showed that *B. dendrobatidis* could "infect" *Caenorhabditis elegans* (Shapard et al., 2012), so it may be possible for *B. dendrobatidis* to grow on substrates other than amphibian keratinized tissue, but it still remains to be shown whether non-amphibian species actually are biologically significant reservoirs of *B. dendrobatidis*.

APPENDIX C

CURRICULUM VITAE

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Education

- Doctorate of Philosophy, expected graduation May 2014, GPA 3.88/4.0
  VANDERBILT UNIVERSITY, Nashville, Tennessee
  Dissertation: *Evasion of adaptive immune defenses by the lethal chytrid fungus Batrachochytrium dendrobatidis*. Defense date: February 14, 2014.
- Bachelor of Science in Biology, with Honors, May 2009, GPA 3.83/4.0
  UNIVERSITY OF EVANSVILLE, Evansville, Indiana
  Biology Concentration with a Minor in Spanish, Major GPA 3.93/4.0

Research Experience

Vanderbilt University, Nashville, TN

- Graduate student in the lab of Louise Rollins-Smith in the Department of Biological Sciences (August 2009-present); investigating amphibian adaptive immunity to the pathogenic fungus *Batrachochytrium dendrobatidis*
- Rotation projects from June-May 2009 in the labs of Timothy Cover (*Helicobacter pylori* pathogenesis), Andrew Link (*Saccharomyces cerevisiae* proteomics), David Wright (malaria pathogenesis and immune evasion), Seth Bordenstein (host-symbiont interactions in wasps).

Medical College of Georgia**,** Augusta, GA

- *STAR Program Undergraduate Research Participant* (May-July 2008). Conducted research in the lab of Dr. Wen Chen Xiong studying the role of neogenin, a developmental signal receptor, on spermatogenesis

University of Evansville, Evansville, IN

- Undergraduate Research Assistant (May 2007-December 2007). Sequenced the mitochondrial genome of a water mite *Unionicola foili* using molecular strategies and techniques. The novel sequence of another mite was discovered aiding the description of a new mite species: *Unionicola ernstingi.*

Honors and Awards

- Hickory Stick Award in Teaching Assisting, Department of Biological Sciences, Vanderbilt University, 2013 (and 2012 runner-up)
- Dissertation Enhancement Grant, Vanderbilt University, 2013

- Charlotte Magnum Student Support Award, Society for Integrative and Comparative Biology, 2013
- Travel Award for the 12th Congress of the International Society of Developmental and Comparative Immunology, 2012
- Travel Award for the North American Comparative Immunology Workshop, 2012
- Trustee Award from the University of Evansville, 2005-2009
- Cardinal Ritter High School Valedictorian, 2005
- IU Honors Program in Foreign Language, San Luis Potosí, Mexico, 2003

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1. Holden WM, **Fites JS**, Reinert LK, and Rollins-Smith LA. 2014. Nikkomycin Z is an effective inhibitor of the chytrid fungus linked to global amphibian declines. Fungal Biol. 118:48.
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3. Brown CM, Samir P, **Fites JS**, Villarreal SA, Link AJ. 2013. The yeast eIF2B translation initiation complex interacts with the fatty acid synthesis enzyme YBR159W and ER membranes. Mol. Cell Biol. 33:1041
4. Gammill WM, **Fites JS**, Rollins-Smith LA. 2012. Norepinephrine depletion of antimicrobial peptides from the skin glands of *Xenopus laevis*. Dev. Comp. Immunol. 37:19
5. Ernsting BR, Edwards DE, Aldred KJ, **Fites JS**, Neff CR. 2009. Mitochondrial genome sequence of *Unionicola foili* (Acari: Unionicolidae): a unique gene order with implications for phylogenetic inference. Exp. & Appl. Acarology 49:305

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1. McMahon TM, Sears BF, Venesky M, Brown JM, Deutsch K, Halstead NT, **Fites JS**, Reinert LK, Rollins-Smith LA, Raffel TR, and Rohr JR. Acquired immunological and behavioral resistance to a fungal pathogen causing global amphibian declines. Submitted.
2. **Fites JS**, Reinert LK, Chappell TM, Rollins-Smith LA. Soluble factors from *Batrachochytrium dendrobatidis* locally paralyze amphibian lymphocyte responses *in vivo*. In preparation.
3. **Fites JS**, Rollins-Smith LA. The amphibian immune response to *Batrachochytrium dendrobatidis*: Using insights from fungal disease to predict host-pathogen interactions in chytridiomycosis. In preparation.
4. Holden, WM*, **Fites JS***, Rollins-Smith LA. Differential filtration enriches individual *Batrachochytrium dendrobatidis* life stages from heterogeneous cultures. *Authors contributed equally to work. In preparation.

Presentations

1. Amphibian Disease Meeting; Arizona State University, Tempe, AZ
    - November 9-10, 2013, "Fighting with one arm tied behind the back: soluble molecules from *Batrachochytrium dendrobatidis* inhibit lymphocytes yet do not impair phagocytes"

- November 12-13, 2011, "Further characterization of factors released by *Batrachochytrium dendrobatidis* that inhibit amphibian immunity"
- November 6-7, 2010, "Characterization of factors secreted by *Batrachochytrium dendrobatidis* that inhibit amphibian lymphocytes"

2. 2013 Society for Integrative and Comparative Biology Annual Meeting; January 3-7, 2013; "Batrachochytrium dendrobatidis, an emergent pathogen linked to amphibian declines, produces factors that inhibit adaptive immunity in amphibians and mammals"
3. 12th Congress of the International Society of Developmental and Comparative Immunology; July 9-13, 2012; Fukuoka, Japan "Deadly chytrid fungus can paralyze amphibian lymphocyte responses"
4. Third North American Comparative Immunology Workshop; June 5-8, 2012; Rochester, NY "*Batrachochytrium dendrobatidis*, an emergent pathogen linked to amphibian declines, produces factors that inhibit adaptive immunity in amphibians and mammals"
5. Vanderbilt Department of Biological Sciences Retreat; Nashville, TN
    - October 10-11, 2013, "Arm for the invasion! The war between the killer chytrid and the amphibian immune system"
    - October 4-5, 2012, "Batrachochytrium dendrobatidis, an emergent pathogen linked to amphibian declines, produces factors that inhibit adaptive immunity in amphibians and mammals"
    - October 6-7, 2011, "*Batrachochytrium dendrobatidis*, an emerging pathogen linked to global amphibian declines, produces factors that inhibit immune responses in amphibian hosts"
    - October 14-15, 2010, "Characterization of factors secreted by *Batrachochytrium dendrobatidis* that inhibit amphibian lymphocytes"
6. Vanderbilt Department of Pathology, Microbiology and Immunology Retreat; Nashville, TN; November 10, 2012"Paralysis of Lymphocyte Functions by a Novel Fungal Factor Released by a Primitive Amphibian Pathogen"
7. Vanderbilt Pediatrics Research Retreat; Nashville, TN; May 19, 2012"Paralysis of Lymphocyte Functions by a Novel Fungal Factor Released by a Primitive Amphibian Pathogen"
8. Vanderbilt Department of Microbiology and Immunology Retreat; Fall Creek Falls State Park, TN; May 20-21, 2011 "Characterization of factors from *Batrachochytrium dendrobatidis* that inhibit amphibian lymphocytes"

Societies

- International Society of Developmental and Comparative Immunology
- American Association for the Advancement of Science
- Microbes and Defense Academic Society, Vanderbilt University
- Society for Integrative and Comparative Biology
- Honor Societies: National Society of Collegiate Scholars and Order of Omega

Teaching Experience

Vanderbilt University, Nashville, TN

- Teaching assistant (June 2013) for the Vanderbilt Summer Academy assisting instruction of a course in Microbiology and Immunology for talented students in 9th and 10th grade.
- Teaching assistant (January-May 2013) for cell biology laboratory: BSCI 202.

- Teaching assistant (August 2010-May 2012) for introductory biology laboratories: BSCI 111A and BSCI 111C.

University of Evansville, Evansville, IN

- Lab Assistant for Biology Labs (August 2006-December 2008); prepared labs for different biology classes and was an assistant lab instructor.
- Genetics tutor (January-May 2008); assisted student in a genetics course.

Graduate Coursework

- Bioregulation (biochemistry, molecular biology, genetics, cancer biology, cell signaling, microbiology and immunology, neurobiology)
- Immunology
- Microbial Population Biology
- Responsible Conduct in Research
- Seminars in Biological Sciences

University Service

- Immunology Journal Club
- Biological Sciences Graduate Student Association, Social Media Chair (Designed Biological Sciences GSA Website and managed Facebook and Twitter for the Graduate Student Association)
- Microbes and Defense Academic Society

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