Toward Immunogenetic Studies of Amphibian Chytridiomycosis: Linking Innate and Acquired Immunity

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Recent declines in amphibian diversity and abundance have contributed significantly to the global loss of biodiversity. The fungal disease chytridiomycosis is widely considered to be a primary cause of these declines, yet the critical question of why amphibian species differ in susceptibility remains unanswered. Considerable evidence links environmental conditions and interspecific variability of the innate immune system to differential infection responses, but other sources of individual, population, or species-typical variation may also be important. In this article we review the preliminary evidence supporting a role for acquired immune defenses against chytridiomycosis, and advocate for targeted investigation of genes controlling acquired responses, as well as those that functionally bridge the innate and acquired immune systems. Immunogenetic data promise to answer key questions about chytridiomycosis susceptibility and host-pathogen coevolution, and will draw much needed attention to the importance of considering evolutionary processes in amphibian conservation management and practice.

Keywords: chytridiomycosis, amphibian decline, acquired immunity, toll-like receptors, MHC

**Recent global amphibian declines are now unequivocally linked to chytridiomycosis, an emergent infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998, Longcore JE et al. 1999, Daszak et al. 2003, Lips et al. 2006). This pathogen shows low genetic diversity across wide geographic areas (Morehouse et al. 2003), little correlation between fungal genotype and geography (Morehouse et al. 2003, Morgan et al. 2007), a wavelike spread across landscapes (Lips et al. 2008), and often causes acute die-offs in naïve populations (Berger et al. 1998, Daszak et al. 2003, Lips et al. 2006). Molecular phylogenetic studies of Fungi also show that *Bd* evolved from within an ancient group of species that feed exclusively on dead or decaying organic matter, providing evidence that its ability to infect vertebrate hosts is an evolutionarily derived feature (Harris et al. 2006). These findings are consistent with the hypothesis that *Bd* is undergoing rapid population expansion around the world; however, the reasons for its sudden emergence as an amphibian pathogen remain the subject of intensive study, and numerous interacting factors are most likely responsible (Carey et al. 1999, Collins and Storfer 2003).

Approximately 200 frog species are known to be affected by chytridiomycosis (Skerratt et al. 2007), although the number is likely to be much higher because most species have not been studied. Outbreaks have been recorded on nearly every continent but are most pervasive in tropical habitats of Australia and Central and South America. In Central America, nearly 70% of mid- and high-elevation frog species are susceptible to *Bd*, with more than 50% of these now extirpated from certain areas (Lips et al. 2003, 2006). The disease has also been documented in temperate amphibian populations, where die-offs are more temporally sporadic than in the tropics (Kriger and Hero 2007, Longcore JR et al. 2007). This difference in epidemiology may be linked to the tendency for fungal pathogens to thrive in humid tropical habitats and to seasonal temperature flux at more northern latitudes, which can exceed the thermal optima for fungal growth and replication (Piotrowski et al. 2004). The temperature dependence of amphibian immunity is also likely to be important to the dynamics of this disease (Carey et al. 1999, Woodhams et al. 2003, Andre et al. 2008), because individuals may be more susceptible to infection during times of the year when *Bd* is most active (Berger et al. 2004, Kriger and Hero 2007). Recent studies further suggest that global climate change acts synergistically with disease outbreaks to accelerate amphibian declines in areas that were outside the environmental envelope tolerated by the fungus just a few years ago (Pounds et al. 2006, Bosch et al. 2007). However, the role of climate change in the spread of this disease remains controversial (Lips et al. 2008).

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Frog declines documented to date show that species differ in their susceptibility to chytridiomycosis, even in cases where potential hosts are sympatric (Alford and Richards 1999, Lips 1999, Stuart et al. 2004). Some species have high susceptibility and quickly become extirpated once *Bd* is introduced, others have moderate susceptibility resulting in severe declines without the complete loss of all individuals, and a few are apparently able to tolerate infections without developing clinical signs of disease (Berger et al. 1998, Daszak et al. 2003, Lips et al. 2006). Similar variation exists at the population level; individuals of some populations recover more readily from infections than others (Davidson et al. 2003, Briggs et al. 2005). Repeated examples of taxon-specific differences in susceptibility among geographic regions have led researchers to investigate the sources of this variation. In this article, we review the ecological and immunological factors currently identified as contributing to host response, and argue that an overlooked but potentially critical source of chytridiomycosis resistance—molecular variability of genes that participate in acquired immunity—should be rigorously pursued in future research.

Environmental correlates of disease susceptibility and immune response

The earliest and most widely accepted finding of *Bd* epidemiological studies was that the environment is a principal driver of the chytridiomycosis epidemic (Lips et al. 2003, Berger et al. 2004, Pounds et al. 2006, Bosch et al. 2007). The greater incidence of outbreaks in cool, moist habitats, particularly at higher elevations in the tropics, is related in part to *Bd*'s dependence on temperature, humidity, and photoperiod for survival and reproduction. The fungus has a broad thermal and acidic tolerance (4 to 25 degrees Celsius [°C], pH 4 to 8), with preferred growth conditions occurring between 17°C and 25°C and pH 6 to 7 (Johnson and Speare 2003, Piotrowski et al. 2004). Above 30°C, infection loads can become markedly reduced or eliminated altogether in host individuals (Woodhams et al. 2003, Berger et al. 2004, Andre et al. 2008), probably because of the thermal dependency of amphibian immune function and the lethal effects of high temperatures on the pathogen.

Differences in the spatiotemporal dynamics of the disease are evident between temperate and tropical regions. In the tropics, chytridiomycosis outbreaks have occurred as epidemic waves, causing naïve populations to become infected in succession (Williams and Hero 1998, Lips et al. 2008). Once a tropical site is infected, fungal colonies can remain viable in moist soils or water and may impose continued selection on susceptible resident hosts or migrants. In contrast, at temperate sites, high seasonal variability in temperature might lead to episodic *Bd* outbreaks, with pulses of strong selection intermixed with periods of relaxed selection; elevated temperatures can reduce or eliminate the pathogen altogether during the warmest months of the year (Woodhams et al. 2003, Andre et al. 2008), whereas lower temperatures, higher moisture, and compromised immune defenses may increase mortality during other parts of the year (Carey et al. 1999). This idea has yet to be tested, but empirical studies of wildlife populations have provided evidence of temporal variability in selection pressure as a result of the prevalence of particular pathogens (see reviews in Bernatchez and Landry 2003). This mode of fluctuating selection can have important consequences for the maintenance of polymorphism in immunity genes (Hedrick 2002, Charbonnel and Pemberton 2005). Thus, the differences in *Bd* epidemiology between regions may have significant implications for the mode and strength of selection in temperate and tropical environments, despite infection by the same selective agent.

Although environmental conditions largely dictate where *Bd* can persist, they fail to explain the differences in infection response among sympatric species. A growing body of empirical data indicate that amphibians have innate immune defenses against chytrid infections, and that differences in innate immunity among species may contribute to different infection outcomes (Woodhams et al. 2006, 2007). In fact, most studies of chytridiomycosis immunity to date have emphasized innate over acquired mechanisms of pathogen defense (box 1). This bias results mainly from prior knowledge that amphibian skin produces large quantities of host defensive peptides, many of which have antimicrobial activity and inhibit *Bd* growth (Rollins-Smith and Conlon 2005, Woodhams et al. 2006). Indeed, specific innate mechanisms have been linked to different levels of resistance among species. Synergistic mixtures of these skin-secreted peptides and circulating granulocytes (see box 2) can combine to form defenses against *Bd* infections, with low-susceptibility species showing more potent antifungal activity (Woodhams et al. 2007). Combined, these findings contribute to the general consensus that species ecology and innate immunity are key factors in determining why some frogs succumb to chytridiomycosis while others do not.

The current bias toward studies of innate immune defenses to chytridiomycosis is also based on the efficacy of innate immunity as a rapid first line of defense and on the assumption that acquired immunity may be too slow to protect against *Bd* infection. Acquired defenses to novel pathogens take time to develop because they involve the recruitment, activation, and proliferation of a highly specific subset of memory cells and effector T and B cells (boxes 2 and 3). If a pathogen such as *Bd* kills its host before an acquired immune response can be mounted, such a response would indeed be ineffective. However, evidence of innate defenses against *Bd* is in fact a good reason to presume that acquired immunity plays an important role in chytridiomycosis resistance. Separation of vertebrate immunity into innate and acquired components is an artificial distinction that overlooks the considerable crosstalk among innate and acquired immune modulators, cells, and signaling pathways (Flajnik and Du Pasquier 2004, Degli-Esposti and Smyth 2005). New insights into the function of dendritic cells and the characterization of Toll-like receptors (TLRs) and other costimulatory molecules show that these immune components are particularly

Box 1. Innate versus acquired immunity.

Historical perspectives in immunology separate the immune system into the innate and acquired (also called "adaptive") pathways. Although some distinctions can be made, recent findings in comparative immunology demonstrate that the two systems are functionally integrated, and that both should be considered when investigating possible mechanisms of disease resistance (Flajnik and Du Pasquier 2004).

Innate immunity: First-line host defense that limits infection in the minutes or hours following pathogen exposure. These responses are nonspecific, do not confer long-lasting protection (i.e., no memory), and consist of a limited repertoire of molecules. Innate immunity is phylogenetically conserved from insects to mammals, suggesting that the two systems arose from a common ancestor.

Major functions: The recruitment of specialized cells to the infection site; inflammation; tagging and destroying of infected cells; removal of foreign substances detected in organs, tissues, blood, and lymph; and stimulating the acquired immune system.

Acquired immunity: Specialized host defense that becomes activated when a pathogen breaches the innate immune system and generates a threshold level of antigen. The acquired immune system is traditionally considered to be a derived feature of jawed vertebrates, but recent studies have uncovered immune defenses that functionally resemble those of an acquired immune system in jawless fish (Flajnik 2004) and in primitive chordates (DeTomasa et al. 2005).

Major functions: Recognition of self from nonself antigens, the generation of responses that are tailored to specific pathogens or infected cells, and the development of immunological memory, where individual pathogens are "remembered" by a signature antigen so that pathogens can be eliminated quickly in subsequent infections.

important at the innate-acquired interface (box 2), especially for fungal and cutaneous pathogens (Netea et al. 2002, Kaisho and Akira 2003, Romani 2004). These discoveries have led to a more integrated view of the vertebrate immune response; innate immunity acts to rapidly control pathogen growth while simultaneously signaling the acquired immune system to mount a response in the event that innate defenses are breached (Janeway and Medzhitov 2002). Because the magnitude and specificity of innate defenses are not as robust as acquired defenses, the swift and generalized response of the innate system can be viewed as a stopgap mechanism to buy time for the development of antigen-specific acquired responses. Investigation of innate amphibian defenses is therefore important and justified, but clearly insufficient if we are to understand the ultimate mechanisms of chytridiomycosis defense.

New experimental evidence for acquired immunity

Experimental assays on acquired immunity to *Bd* are in their infancy, but several exciting studies show that frogs with previous *Bd* exposure are able to survive secondary infections better than can immunologically naïve frogs. A recent investigation of two *Bd*-susceptible New Zealand frogs, *Litoria ewingii* and *Litoria raniformis*, showed that a majority of infected individuals treated with identical doses of chloramphenicol, a bacteriostatic antimicrobial, were completely free of *Bd* within 18 days following experimental infection (others were cleared shortly thereafter). Differences in the response time to treatment were also

Box 2. Glossary of immunological terms.

Cross-dressing: The ability of certain antigen-presenting cells to acquire activating receptors of another cell type, dampening or enhancing their normal physiological response to foreign antigens.

Cross-presentation: The ability of certain antigen-presenting cells to switch the processing pathway by which extracellular foreign antigens are presented to T cells.

Dendritic cells: A type of immune cell that incorporates, degrades, and displays small peptides derived from pathogens on their cell surface using major histocompatibility complex molecules, resulting in T-cell activation and differentiation.

Granulocyte: Type of white blood cell filled with microscopic corpuscles that contain enzymes for digesting pathogens.

Hyperkeratosis: Overgrowth of the upper layer of the skin.

Langerhans cell: A type of dendritic cell that is unique to the interstitial layers of the epidermis.

Macrophage: Immunity cells that engulf and digest (i.e., phagocytose) pathogens to stimulate lymphocytes and other immune cells to respond to pathogenic antigens.

Memory cells: A subset of antigen-specific T and B cells that retain an ability to "remember" previously encountered pathogens, thereby stimulating faster antibody production in future infections.

Ortholog: A gene in two or more species that has evolved from a common ancestor.

Skin hyperplasia: Irregular increase in the number of normal skin cells without tumor formation.

Toll-like receptors: Cell-surface receptors produced by macrophages and dendritic cells that initiate the acquired immune response by recognizing specific chemical signatures of pathogens.

Box 3. Differences between T cells and B cells.

T cells are a type of white blood cell (i.e., lymphocyte) that develop in the thymus and have specialized receptors, or TCRs, for recognizing "processed" antigens bound to major histocompatibility complex (MHC) molecules. Several examples of different T-cell types are described below, each with a distinct function.

$CD4^+$ cells (also known as helper T cells, T_h cells, or effector T cells) secrete substances called cytokines that activate other T cells to initiate inflammatory responses or stimulate B cells to produce antibodies. They also possess $CD4^+$ TCRs that recognize antigens bound to MHC class II molecules.

$CD8^+$ cells (also known as killer T cells, T_c cells, or cytotoxic T cells) induce death in virally infected cells and tumor cells. They possess $CD8^+$ TCRs that recognize antigens bound to MHC class I molecules.

Natural killer T cells assist in bridging the innate and adapative immune systems. They recognize lipid antigens presented by CD1d molecules in mammals rather than MHC molecules, produce signaling peptides (i.e., cytokines), and release cytolytic molecules, enabling them to perform functions ascribed to helper and cytotoxic T cells.

B cells are a type of lymphocyte that develops in the bone marrow and matures in the spleen and lymph nodes in mammals. In amphibians, B cells develop in the liver, spleen, or kidney. Their general function is to make two types of antibodies: one that circulates in the blood and lymph and another that binds to the cell surface (called a B-cell receptor, or BCR). Unlike T cells, which can recognize only processed antigens bound to an MHC molecule, B cells use BCRs to recognize antigens in their native form. Activated B cells differentiate into one of the following cell types:

Plasma cells secrete different types of antibodies that destroy microbes by binding to them and making them easier targets for macrophages.

Memory B cells develop from activated B cells that recognize specific antigens generated during the primary immune response, allowing the immune system to "remember" the same pathogen for rapid antibody production in future infections.

noted; *L. raniformis* took longer to eliminate the fungus than did *L. ewingii,* despite having similar initial infection loads (Poulter et al. 2007; Russell Poulter, Department of Biochemistry, University of Otago, Dunedin, New Zealand, 24 October 2008, personal communication). These same frogs were reinfected, but they cleared the pathogen within 35 days even without further chloramphenicol treatment. In a second study, North American boreal toads (*Anaxyrus [Bufo] boreas*) were experimentally infected and were allowed to choose either a dry environment in an enclosure on an incline or were forced to remain flat in a wet environment. The majority of the dry-choice toads survived the first infection whereas all of the wet-choice toads died rapidly. Later, the survivors were reinfected and maintained in wet conditions. Secondary infections ultimately resulted in the death of all test subjects; however, survival after secondary infection was significantly longer in the dry-choice toads than in controls exposed for the first time under wet conditions (Cynthia Carey, Department of Integrative Physiology, University of Colorado at Boulder, 12 November 2008, personal communication). In a third study, sublethal X-irradiation was used to suppress acquired immunity in the model frog *Xenopus laevis,* a species that is relatively resistant to chytridiomycosis (Louise A. Rollins-Smith, Vanderbilt University Medical Center, 22 October 2008, personal communication). Irradiated frogs had greater numbers of zoospores on the skin relative to nonirradiated controls, suggesting that dampened acquired immunity weakened the capacity to clear infection. Although these studies are not conclusive, they all suggest some induction of acquired immune function in response to *Bd* exposure.

New studies evaluating gene expression in *Bd*-exposed frogs provide genetic evidence for the recruitment of acquired immune function in combating *Bd* infections. Using controlled inoculation experiments and whole-genome assays in the model frog *Xenopus tropicalis,* Erica Bree Rosenblum (one of the authors of this article) and colleagues evaluated gene expression profiles in immune function tissues at two time intervals following infection. They discovered differential expression in sick frogs for a number of genes involved in the acquired immune response (e.g., major histocompatibility genes, immunoglobulin, and interleukin). The most compelling patterns of up-regulated immune function genes were found for the spleen just three days after *Bd* exposure. This finding suggests that not only is there a response to *Bd* from various components of the acquired immune system, but genetic regulation of these components occurs at a time scale that is relevant for frogs to develop an acquired immune response under natural conditions. At the same time, several immune function genes also showed evidence of down-regulation, a pattern that suggests that *Bd* virulence factors may act to suppress acquired immunity in infected hosts. However, the genetic and biochemical underpinnings of *Bd* virulence are unknown, and much remains to be discovered about the immunogenetic regulation of responses to *Bd.* These findings underscore the need not only for more genetic studies on amphibian acquired immunity but also for more studies on the mechanisms by which this novel pathogen might evade the immune defenses posed against it.

Recommendations for future research on acquired immunity

Evidence now clearly indicates that activation of the innate immune system is a prerequisite for the induction of acquired immunity (Akira and Takeda 2004), and that acquired responses involve cascading pathways with many genes controlling various stages of the process. Thus, the decision of which genes to investigate first is not straightforward; however, the location of the primary infection site offers

some valuable guidance. *Batrachochytrium dendrobatidis* attacks an individual's first line of disease defense, the skin. Infections are localized to keratinized epidermal tissue and cause skin hyperplasia, hyperkeratosis (box 2), lethargy, poor righting reflex, convulsions with extended limbs, and mortality (Berger et al. 1998). Some researchers have suggested that mucosal antibodies within the epidermis may offer some protection against infection (Kurtz and Scharsack 2007). Antibodies occur in the cutaneous mucus of fish and retard infection by aquatic protozoan pathogens (Maki and Dickerson 2003, Hamuro et al. 2007). Genomic studies of the *X. laevis* immune system have identified immunoglobulin X (IgX) as the frog ortholog (box 2) of the mammalian secreted antibody class, IgA, and expression assays suggest functional as well as structural homology (Mussmann et al. 1996). Determining the presence and function of secreted IgX antibodies on amphibian skin is an avenue of research worth pursuing in future chytridiomycosis studies.

The mobilization of a highly specialized type of dendritic cell known as Langerhans cells (box 2) may also constitute a primary line of defense against *Bd* infection. Langerhans cells are ubiquitous in the epidermis and are well equipped to isolate, engulf, and degrade pathogens into small peptides. Once activated, these cells develop into potent immunostimulators that prime T cells for antigen-specific responses (box 3) or tolerize them when the response is no longer appropriate. Use of cell type–specific antibodies has confirmed the presence of Langerhans cells in *X. laevis* epidermis, where they appear in midlarval stages and significantly increase in number at metamorphosis (Du Pasquier et al. 1989, Du Pasquier and Flajnik 1990, Mescher et al. 2007). Langerhans cells are also present in adult *Lithobates [Rana] catesbeianus* epidermis (Carillo-Farga et al. 1990), suggesting similar cutaneous immune anatomy across all frogs. Thus, amphibians apparently do not suffer from a generalized inability to detect epidermal pathogens.

Because amphibians possess the requisite cellular architecture to carry out acquired defenses, it is important to learn which components of the acquired immune system might explain why certain individuals, populations, or species are able to survive *Bd* infections. The key may lie in the genetics of receptors and signaling molecules that interface the innate and acquired immune pathways, or are directly involved in antigen presentation. Given the known role of innate defenses in combating *Bd* infections, one might initially look to genes encoding molecules that not only sense the invasion of fungal pathogens in the skin but also instruct the development of acquired immunity.

Toll-like receptors and antifungal immunity

One family of evolutionarily conserved receptors that functionally bridges the innate and acquired immune systems in vertebrates is the TLRs. These receptors play an important role in early host defense by recognizing the chemical signatures of a wide variety of microorganisms, including fungi, and are expressed by a number of cells involved in both innate and acquired immunity (e.g., T and B cells, macrophages, and dendritic cells). Upon pathogen recognition, TLRs initiate signaling cascades that result in the production of cytokines, inflammatory mediators, and other effector molecules and cells (figure 1), while simultaneously stimulating dendritic cells to capture foreign peptides and transport them to immunostimulatory centers such as the lymph nodes (mammals) or spleen (amphibians). During this migration, the dendritic cells develop from a surveillance stage to an antigen-presenting stage that ultimately triggers T- and B-cell production. Thus, TLRs play a critical role in linking early innate responses to downstream acquired immunity.

Toll-like receptors may have particular relevance to *Bd* infections because of their ability to recognize a variety of fungal pathogens in other vertebrates (e.g., *Candida albicans, Aspergillus fumigatus, Cryptococcus neoformans,* and *Pneumocystis carinii*) (Roeder et al. 2004, Luther and Ebel 2006). In fact, homologues of the TLR family are an essential component of the innate immune response to fungal infections in insects, and mutations affecting the Toll pathway are known to lower resistance to fungal pathogens (Lemaitre et al. 1996). This provides evidence that TLR function in antifungal immunity is evolutionarily ancient, thus making it possible that regulatory mutations affecting their expression might influence *Bd* resistance in frogs as well. Interestingly, pathogen-associated molecular patterns (PAMPs) in the cell wall of many fungal species have been identified as TLR ligands, but ligands have not yet been described for all receptors. Thus, it may prove useful to search for associations between *Bd* PAMPs and candidate TLRs in future chytridiomycosis studies. At the very least, the ability of both the innate and acquired immune systems to utilize a variety of TLRs in combating fungal pathogens suggests that their activity may be important for understanding *Bd*-related mortality.

Major histocompatibility complex

Another key group of molecules expressed on antigen-presenting cells include those encoded by genes of the major histocompatibility complex (MHC). These genes are present in all gnathostomes (i.e., jawed vertebrates), and are known to confer resistance to a broad range of pathogens, including some that cause cutaneous and fungal diseases (Restrepo et al. 1983, Ashman 1987, McClelland et al. 2003, Monteiro de Almeida et al. 2005). MHC orthologs are easily detectable in animals as distantly related as *Xenopus* and mammals, which diverged from a common ancestor 300 million to 350 million years ago (Ohta et al. 2003). Their major function is also phylogenetically conserved to discriminate self from nonself peptides and to regulate the acquired immune response. The pathway by which a pathogen enters a cell, either through infection or by phagocytosis (box 2) into antigen-presenting cells (macrophages and Langerhans cells; figure 1), determines the type of MHC molecule used to combat that infection (box 4). In both cases, the pathogen is degraded into small peptides intracellularly before being loaded onto specific MHC molecules. Infected cells use MHC class I molecules to usher

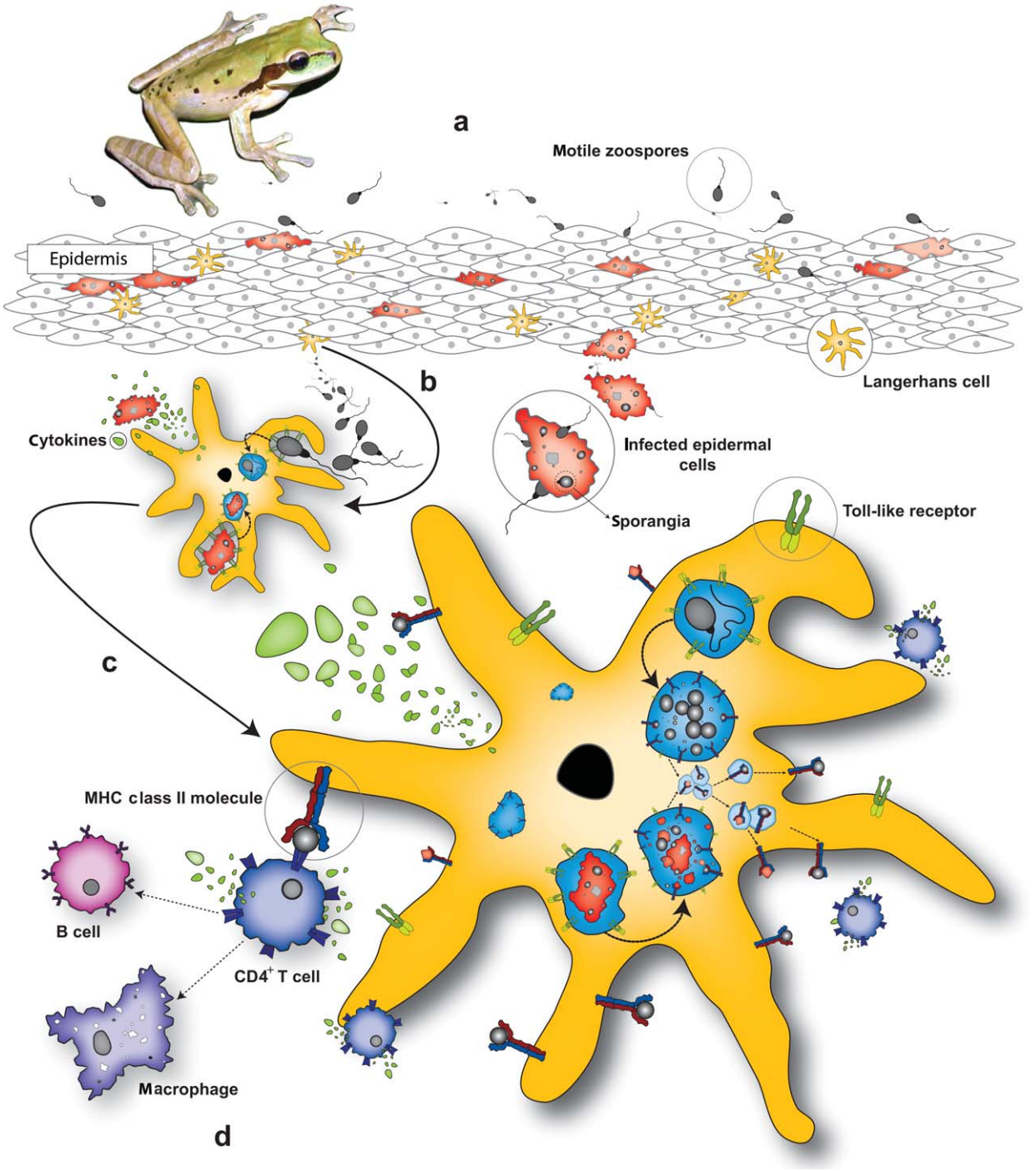

Figure 1. Proposed activation of acquired immunity against* Batrachochytrium dendrobatidis *infection in amphibians. *(a) Infection occurs by skin contact in moist soils or water, where fungal zoospores attack keratinized epidermal tissue on the toepads, the belly, and the underside of the legs. (b) The immunosurveillance stage: Zoospores enter cells either through infection of epidermal cells (shown in red), with zoospores maturing into sporangia and reproducing within the cytoplasm, or by phagocytosis of zoospores and infected cells by antigen-presenting cells (i.e., macrophages and Langerhans cells). Membrane-bound Toll-like receptors recognize the chemical signatures of pathogens and facilitate the release of cytokines, which signal macrophages and lymphocytes to attack the foreign invader. (c) Maturation and migration of Langerhans cells: Following pathogen capture, proteasomes within the cytoplasm (shown as blue vesicles) degrade the pathogen into small peptides that are loaded onto major histocompatibility complex (MHC) class II molecules and transported to the cell surface. (d) MHC class II molecules display antigens derived from pathogens to $CD4^+$ T cells; T-cell receptor binding causes the release of more cytokines as the T cells migrate to the spleen, where they activate plasma B cells, macrophages, and killer T cells.***

Box 4. Gene clusters making up the major histocompatibility complex.

Gene clusters within the vertebrate major histocompatibility complex (MHC) are hypothesized to have evolved from a common ancestral region through large-scale block or chromosomal duplications (Flajnik and Kasahara 2001). The MHC gene family includes two main groups of immunologically active molecules, referred to as MHC class I and class II, that have common structural and functional features. A third, gene-rich class III subfamily also exists, but class III molecules are not involved in antigen presentation (although some have important innate and acquired immune function). Amphibians, as well as mammals, have MHC class I, II, and III genes.

Class I molecules primarily monitor the intracellular environment and present endogenous peptides derived from intracellular pathogens (e.g., viruses) to $CD8^+$ cytotoxic T cells. They are expressed on the surface of nearly all nucleated somatic cells, except sperm cells and some neurons.

Class II molecules primarily monitor the extracellular environment and present peptides derived from bacteria, fungi, and parasites to $CD4^+$ T cells. Expression is restricted to epithelial cells in the thymus and antigen-presenting cells in the periphery (B cells, macrophages, and dendritic cells).

these peptides to the cell surface for presentation to $CD8^+$ T cells, whereas antigen-presenting cells rely on MHC class II molecules to present peptides to $CD4^+$ T cells (box 3). The binding of a T-cell receptor to an MHC-peptide complex is the key interaction that triggers an acquired response; the range of peptides presented to T cells depends on an individual's MHC repertoire, thus MHC variability is often associated with immunocompetence (Hughes and Yeager 1998).

The intra- and extracellular location of different *Bd* life history stages invokes a role for both MHC class I and class II molecules. Traditional views in immunology dichotomize class I molecules as presenters of intracellular peptides and class II as presenters of extracellular peptides. *Batrachochytrium dendrobatidis* is primarily but not exclusively an intracellular pathogen (figure 1). Motile zoospores enter frog epidermal cells, mature into zoosporangia within the cytoplasm, reproduce asexually, and release nascent zoospores through discharge tubes into the extracellular space (Berger et al. 1998). Pathogenic responses could therefore be elicited through both extracellular (e.g., antibodies, TLRs, phagocytosis) and intracellular (e.g., cell-to-cell contact, cytotoxic responses, apoptosis) signaling pathways. Particular class I and class II molecules may also be important in *Bd* infections because of nontraditional antigen-presentation pathways such as cross-presentation and cross-dressing (box 2) of peptide-MHC complexes from necrotic cells (Cresswell et al. 2005, Donlan et al. 2006). Given the known specificity of disease resistance to single class I or class II alleles in many taxa (Piertney and Oliver 2005), it is likely that both MHC gene classes will offer insight on chytridiomycosis susceptibility and resistance, and that several MHC-dependent resistance mechanisms combined are important in combating *Bd* infections (box 5).

Box 5. Overdominance and frequency-dependent selection.

Two major histocompatibility complex (MHC)-dependent mechanisms may be important for determining differential survivorshop to *Batrachochytrium dendrobatidis* (*Bd*) infection: overdominance and frequency-dependent selection (Takahata and Nei 1990, Slade and McCallum 1992).

Overdominance

Prediction: Heterozygous individuals are expected to have a fitness advantage over homozygotes when exposed to a diversity of pathogens and parasites.

Theory: Compared with homozygotes, heterozygotes have a larger MHC repertoire for antigen presentation, increasing their resistance to a wider variety of pathogens (Doherty and Zinkernagel 1975).

This "numbers game" may also have advantages in defending against emergent diseases because heterozygotes are more likely to possess genetic variation that offers some ability to biochemically interact with novel antigens.

Frequency-dependent selection

Predition: Individuals with a rare MHC genotype are expected to have a fitness advantage over individuals with common genotypes.

Theory: Pathogens are more likely to be adapted to common host MHC genotypes, thus providing a fitness advantage to individuals that possess the rare genotype (Lively and Dybdahl 2000).

Balanced polymorphisms can evolve over time if particular alleles are selected against when they are common but favored when they are rare (e.g., rare-allele advantage; Meyer and Thomson 2001). Different rare alleles might also confer similar fitness advantages in separate populations, particularly if strains diverge locally, in which case directional selection could drive the fixation of alternative MHC genotypes in different parts of a species' range. Indeed, numerous *Bd* strains are known from around the world (Morehouse et al. 2003, Longcore et al. 2007, Retallick and Miera 2007) and in some areas, sexual reproduction and recombination may be contributing to newly resistant sporangia (Morgan et al. 2007).

A variety of fungal pathogens activate the acquired immune system in vertebrates, if not the MHC specifically (Gil-Lamaignere et al. 2005, Lin et al. 2005). For example, several human studies have demonstrated both positive and negative associations between developing paracoccidioidomycosis, a chronic disease caused by the fungus *Paracoccidioides brasiliensis,* and the expression of particular MHC class II alleles (Restrepo et al. 1983, Goldani et al. 1991, Monteiro de Almeida et al. 2005). Similarly, experimental assays in mice show that the magnitude and duration of pulmonary infections caused by common yeast genera (*Candida* and *Cryptococcus*) are

affected by whether an individual expresses a homo- or heterozygous MHC genotype (Ashman 1987, McClelland et al. 2003). In lung infections caused by soilborne spores of *Histoplasma capsulatum,* dendritic cells in class II-deficient mice acquire and present extracellular antigens on MHC class I molecules (i.e., cross-presentation [box 2] or cross-priming) to generate a protective $CD8^+$ T-cell response (Lin et al. 2005). Taken together, these examples demonstrate a role for both types of MHC molecules in combating fungal infections, and that variability in MHC genotypes can determine differential disease susceptibility or resistance.

A rising incidence of human fungal infections caused by pathogenic zygomycetes (i.e., zygomycosis) may have particular relevance to amphibian chytridiomycosis, as several of these species arose early in the evolution of Fungi along with *Bd* (Harris et al. 2006). In fact, zygomycetes and *Bd* are more closely related to each other than to any other known vertebrate pathogens. Although zygomycosis resistance has yet to be associated specifically with MHC variability, studies have demonstrated a role for acquired immune responses in protection against this disease (Gil-Lamaignere et al. 2005). Like chytridiomycosis, zygomycosis can also manifest itself as a cutaneous disease, most often in immunocompromised individuals (Roden et al. 2005). Infections occur predominantly in the tropics, with transmission occurring by implantation of spores through minor skin trauma or by contact with mucocutaneous sites. Ongoing advances in understanding amphibian chytridiomycosis may therefore provide insight into human zygomycosis outbreaks (and vice versa), emphasizing a need for exploration of the similarities between global epidemics in natural wildlife populations and those in humans.

Other candidate receptor molecules involved in fungal recognition

Toll-like receptors and MHC molecules are almost certainly not the only signal transducers involved in defending against *Bd* infection. A number of different receptors collaborate in the phagocytotic uptake of fungal-derived antigens, including complement, mannose receptor, and opsonic Fcχ receptor, to name a few, and thus many candidate genes are worth consideration in future chytridiomycosis studies. We emphasize TLRs and MHC molecules in this article because they are both critical upstream regulators of immune response, making it easier to confirm their activity in a particular response pathway. Both receptors also have an ability to respond to fungal microbes, and each provides a unique advantage for different types of immunological analyses. For example, TLRs are good targets for biochemical assays because pathogen-exposed individuals may vary in their ability to recruit specific TLRs that respond to *Bd* PAMPs. Alternatively, MHC molecules are better suited for immunogenetic studies because the genes encoding these receptors may contain sequence variation that directly affects the ability to bind specific antigenic peptides. The high polymorphism at these loci resulting from the accumulation of point mutations over many millions of years, the conversion of existing MHC alleles into new alleles through recombination, and gene duplication provide ample opportunities for species to diverge in their capacity to fight infectious disease (Parham and Ohta 1996).

Conclusions

Various genes are now known to control different aspects of innate and acquired immune responses. However, as stated previously, existing studies of the amphibian immune response to chytridiomycosis are limited primarily to studies of innate defenses. Given the essential role of TLRs and MHC molecules in bridging innate and acquired systems, as well as the potential for MHC allele-specific resistance to fungal infections, these gene families are logical starting points for investigating how host immunogenetics might contribute to different species-typical responses to *Bd* infection. Other components of the acquired immune response, such as mucosal antibodies, complement factors, and cytokine receptors, may also be important, and we urge investigators to consider these candidate gene groups in future biochemical and genetic studies of chytridiomycosis. Finally, the role of *Bd* virulence factors in suppressing amphibian immune defenses must also be considered. Recent whole-genome analyses have revealed a large expansion of the fungalysin metallopeptidases in the *Bd* genome that are highly expressed in intracellular zoosporangia (Rosenblum et al. 2008). This gene family may also contribute to pathogenicity of dermatophytic fungi (Jousson et al. 2004), a group that parasitizes vertebrate skin in much the same way as *Bd* does (Hainer 2003, Odom 1993). There are also genes with differential expression between *Bd* life-history stages that show sequence similarity to genes that might be involved in viral or bacterial evasion of the vertebrate immune system. Clearly, specific interactions between *Bd* gene products and the amphibian immune system are possible.

Host genetics of acquired immunity may have other important implications for the conservation of declining populations. That this epidemic is rapidly changing the genetic composition of amphibian populations lends new significance to the role of evolutionary processes in conservation biology. At the same time, host populations exert selection pressure on the pathogen, perhaps driving the evolution of newly resistant strains. Understanding how the genetics of resistance, the strength and mode of pathogen-mediated selection, the evolutionary rates of immunity genes, and the degree to which demographic processes affect disease spread is critical for predicting the population response to novel pathogens. Immunogenetic data could be used in predictive models that incorporate environmental, demographic, and population genetic parameters to assess extinction vulnerability in populations that have yet to be exposed to the pathogen. This would improve the ability to focus conservation efforts on populations that are predisposed to disease outbreaks, allowing managers to take preventive action to minimize future extinction risk. Thus, studies on the immunogenetic

correlates of *Bd* susceptibility can bridge a collaborative research endeavor among conservation biologists, ecologists, immunologists, and evolutionary biologists: understanding how to preserve amphibian biodiversity in the face of emerging diseases and other environmental threats.

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