Introduction

• For at least four decades, amphibian populations have been declining.
• Although the causes are complex, most agree that emerging diseases are one of the most important causes.
• Disease agents linked to amphibian declines are
  – Ranaviruses
  – Batrachochytrium dendrobatidis (Bd)
  – Batrachochytrium salamandrivorans (Bsal)
Introduction

- My lab has focused our research on *Batrachochytrium dendrobatidis* (*Bd*), a chytrid fungal pathogen that causes the skin disease chytridiomycosis.
- Until recently, *Bd* was the only known chytrid pathogenic to vertebrates.
- In 2013 a new species, *Batrachochytrium salmandrivorans* (*Bsal*) was described.
  - Likely originated in Asia
  - Lethal to European salamanders
  - Significant threat to North American salamanders. USA is “hot spot” of salamander diversity.

Yellow-flecked Glassfrog (*Cochranella albomaculata*)

Fire salamander (*Salamandra salamandra*)

Simplified evolutionary tree of fungi

<table>
<thead>
<tr>
<th>Domain: EUKARYA</th>
<th>Kingdom: FUNGI</th>
<th>Phylum: Chytridiomycota</th>
<th>Class: Chytridiomycetes</th>
<th>Order: Rhizophydiales</th>
<th>Genus: <em>Batrachochytrium</em></th>
<th>Species: <em>dendrobatidis</em></th>
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Life Cycle of Batrachochytrium dendrobatidis (Bd)

- Zoospore
- Germlings
- Zoosporangium

[Stages that occur within frog skin cells]

Duration of life cycle is 4-5 days at 22°C

J.E. Longcore et al. 1999. Mycologia 91:219

Bd appears to kill by disturbance of skin functions

- Electrolyte transport across ventral skin is impaired in diseased frogs.
- Plasma sodium and potassium concentrations are significantly reduced.

Ack ! Ack! My ion balance is disturbed!


Hyla cinerea (green treefrog)
Chytridiomycosis is a disease of the skin epidermis

- Unlike other fungal diseases, this pathogen does not migrate to other organs.
- Therefore, to understand immune defenses against chytridiomycosis, we need to understand the skin defenses.

Model of immune defenses in the skin


The dermis of the skin of many amphibians is rich in granular glands which produce and store antimicrobial peptides (AMPs)

AMPs
- 10-50 amino acid residues
- Cationic, hydrophobic and usually adopt an α-helical conformation
- Act by binding to charged residues or through hydrophobic interactions with target cells and disruption of membrane function

Are AMPs present on the skin of resting and active frogs and do they inhibit Bd?
**Peptide collection and MALDI-TOF**

1. Apply stimulus
2. Place frog in collection buffer for 15 mins
3. Add 1% TFA to inactivate proteases
4. Enrich peptides over C18 Sep-paks
5. Determine peptide concentration by microBCA
6. Determine peptide profile by Mass spectrometry

**Direct sampling of skin secretions for MALDI-TOF MS**

1. Carbon embedded film
2. Activate in 100% methanol
3. Apply directly to frog
4. Perform MALDI-TOF
MALDI-TOF profile of skin AMPs by direct sampling

Chased frogs release more skin peptides

Resting frog sampled with carbon embedded film

MALDI-TOF analysis shows AMPs in resting and chased frogs


Peptides collected from resting and chased frogs have antimicrobial activity

Following secretion, how long are the skin peptides detectable?

Use of an external standard allows us to determine relative concentrations of AMPs on the skin by mass spectrometry.
Use of an external standard shows that individual AMPs persist for about 60 min

Can we deplete and exhaust skin peptide stores?


After depletion, peptide renewal is slow

Depletion of skin peptides in Rana pipiens juveniles results in greater susceptibility to Bd

**Summary of AMP studies**

- Frogs constitutively release low amounts of AMPs that inhibit *Bd*, and AMP defenses are elevated following a simulated predator attack.
- AMPs are effective inhibitors of *Bd* at these low constitutive concentrations but degrade within two hours, protecting the integrity of the skin and commensal bacteria.
- Depletion of AMP responses increases susceptibility to *Bd*.

**What is the role of the adaptive immune system in control of *Bd*?**

- Is there a role for B lymphocytes?
- What is the role for T lymphocytes?
- Does *Bd* evade adaptive immune defenses?
Model of immune defenses in the skin


Dermis

Mucus: Antimicrobial peptides, lysozyme, antibodies, and bacterial products

Epidermis

Granular gland

Sublethal X-irradiation reduced resistance to Bd infection

Infection intensity (mean log zoospore equivalents + 1)

- X-irradiated
- X-irradiated & peptide-depleted
- No treatment

Days after first exposure to B. dendrobatidis

**X-irradiation reduced lymphocyte numbers but did not impair function of granular glands**


**Immunization against Bd resulted in development of high-titer antibody responses**

IgY antibody responses

Skin mucus of Bd exposed frogs contains antibodies of three classes that can bind specifically to Bd


- Collectively, these studies suggest that both innate defenses (antimicrobial peptides) and conventional lymphocyte-mediated immune responses help to protect frogs from lethal Bd infections.
- If the immune system is capable of recognizing this pathogen, why are many amphibian species not protected?
How does Bd escape immune surveillance?

- Successful immunity against fungal pathogens begins with recognition by phagocytic cells, which begin to control the infection.
- The phagocytic cells then recruit lymphocyte effectors.
- The lymphocytes amplify the response and recruit more phagocytic cells to clear the infection.

Macrophages and neutrophils can phagocytose Bd

- Frog neutrophil has engulfed Bd cells
- Frog macrophage has engulfed Bd cells
- Developing thallus with rhizoids
- Maturing zoosporangium
**Bd culture supernatants have a minimal effect on the ability of peritoneal leukocytes to engulf zymosan particles**

Zymosan is a protein-carbohydrate complex prepared from yeast cells. It binds to TLR 2 on macrophages to induce proinflammatory responses.

**Overnight treatment of peritoneal leukocytes with Bd Sup does not impair accessory function**

Live or heat killed Bd cells inhibit T lymphocyte proliferation

Live Bd co-cultured with T cells

Killed Bd co-cultured with T cells


Live Bd cells also inhibit B lymphocyte proliferation

Inhibition of lymphocyte proliferation occurs even when Bd cells are separated from lymphocytes by a 0.4 µm membrane or replaced by Bd supernatants.

Bd-induced inhibition of lymphocytes is not limited to frogs.

Therefore, the lymphotoxic factors target a vulnerability shared by lymphocytes of amphibians and mammals.

With Sarah Parker Collier and Tom Aune
Bd factors also inhibit proliferation and cytokine production by human T cells

Proliferation of human T cells stimulated with anti-CD3 and anti-CD28 was impaired by Bd supernatants. Supernatants from Bd cultures inhibited secretion of IL-2 and IFN-γ by purified human CD4+ T cells.

A nonpathogenic chytrid Homolaphlyctis polyrhiza does not inhibit lymphocyte proliferation

JEL197 = nonpathogenic chytrid, Homolaphlyctis polyrhiza

One mechanism of inhibition is the induction of lymphocyte apoptosis by killed Bd

Annexin V stains phosphatidylserine

Lymphocyte apoptosis is also induced by Bd supernatants

Lymphocyte apoptosis induced by Bd supernatant is diminished in the presence of a pan caspase inhibitor

Pathways of Apoptosis

**Bd supernatants appear to induce apoptosis via both the intrinsic and extrinsic pathway**

Caspase Activity (RLUs×10⁻³)

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<td>Caspase 9</td>
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**Zoospores can inhibit only if they mature in culture; zoospore supernatants do not inhibit**

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What is the nature of the immunotoxic factor or factors?

Bd supernatants retained their capacity to inhibit lymphocyte proliferation following treatment with high heat or proteinase K

Production of the Bd factors is impaired by treatment with agents that inhibit cell wall synthesis

Therefore, the inhibitory factors may be components of the cell wall.

Isolated cell walls have significant inhibitory activity against T cells

Therefore, some inhibitory factors are enriched in the cell wall.


Jack Lee
Inhibition of T cells is positively correlated with carbohydrate concentration of supernatants

\[ y = 25.942x + 37.745 \]
\[ R^2 = 0.89929 \]

\[ y = 18.628x + 58.007 \]
\[ R^2 = 0.35691 \]

Therefore, the some inhibitory factors may be soluble carbohydrates associated with the cell wall.

Tim Chappell

HPLC analysis of cell-free Bd supernatants show the presence of tryptophan, kynurenine, and MTA (methylthioadenosine)

Kevin Minbiole

Thomas Umile
Summary of immune evasion by Bd

- *Bd* factors inhibit T and B cells by induction of apoptosis.
- The inhibitory factors are water soluble and can cross a cell-impermeable barrier.
- The inhibitory factors are heat-resistant and protease-resistant, suggesting that they are not proteins or peptides.
- *Bd* factors produced after treatment with the chitin synthase inhibitor, nikkomycin Z, have reduced activity, suggesting that they may be cell-wall components.

Summary of immune evasion by Bd

- Enriched cell-wall preparations alone inhibit T cells.
- Inhibitory activity correlates with carbohydrate content.
- *Bd* also releases small metabolites, MTA and kynurenine, which may also induce T-regulatory cells or otherwise inhibit lymphocytes.
Summary of immune evasion by Bd

• Taken together, these results suggest that Bd has evolved strategies to resist immune surveillance in order to survive in amphibian skin.
• Ongoing studies aim to identify additional fungal immunotoxic factors and define their mechanism of action.

Acknowledgements

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