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## The invasive chytrid fungus of amphibians paralyzes lymphocyte responses

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### Abstract

The chytrid fungus, *Batrachochytrium dendrobatidis*, causes chytridiomycosis and is a major contributor to global amphibian declines. Although amphibians have robust immune defenses, clearance of this pathogen is impaired. Because inhibition of host immunity is a common survival strategy of pathogenic fungi, we hypothesized that *B. dendrobatidis* evades clearance by inhibiting immune functions. We found that *B. dendrobatidis* cells and supernatants impaired lymphocyte proliferation and induced apoptosis; however, fungal recognition and phagocytosis by macrophages and neutrophils was not impaired. Fungal inhibitory factors were resistant to heat, acid, and protease. Their production was absent in zoospores and reduced by nikkomycin Z, suggesting that they may be components of the cell wall. Evasion of host immunity may explain why this pathogen has devastated amphibian populations worldwide.

Although causes of global amphibian declines are complex (1), the chytrid fungus, *B. dendrobatidis* (2, 3), is now recognized as a leading contributor (1, 4). Previous studies in *Xenopus laevis* suggest that both innate immune defenses, such as antimicrobial peptides in the mucus, and adaptive immunity contribute to resistance against *B. dendrobatidis* infection (5). However, lack of extensive lymphocyte infiltration in diseased skin (2, 6) suggests an impaired immune response (7–9). Because evasion of host immunity is a common strategy employed by pathogenic fungi (10, 11), we hypothesized that *B. dendrobatidis* avoids clearance by inhibiting critical immune functions. To test this hypothesis, we examined the effects of *B. dendrobatidis* on peritoneal leukocytes enriched for macrophages, and we cultured *X. laevis* splenocytes stimulated with T-lymphocyte-specific activators (12) or heat-killed bacteria to stimulate B lymphocytes (13) in the presence of *B. dendrobatidis* [See materials and methods in supplementary materials (14)]. Viability and functions of peritoneal phagocytes were not impaired by *B. dendrobatidis* (figs. S1–S2). However, when splenic lymphocytes were cultured with either live or heat-killed *B. dendrobatidis*, T-cell proliferation was reduced (Fig. 1, A and B and fig. S3, C and E). Live *B. dendrobatidis* cells also inhibited B-cell proliferation (fig. S3A). When lymphocytes were separated from *B. dendrobatidis* by a cell-impermeable membrane in a transwell culture system, the fungal cells inhibited lymphocyte proliferation, but less effectively than in co-culture (Fig. 1C). The

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inhibitory effects of *B. dendrobatidis* were replicated using *X. laevis* T- and B-cell populations enriched by magnetic sorting (fig. S4). Inhibition of T- and B-lymphocyte proliferation by *B. dendrobatidis* was also observed when the splenocytes were isolated from another frog, *Rana pipiens* (fig. S5). Induced T- and B-cell proliferation was inhibited in a dose-dependent fashion by 24-hour supernatants derived from *B. dendrobatidis* incubated in water (Figs. 1D and fig. S3, B, D, and F). Proliferation of mouse and human lymphocytes was also inhibited by *B. dendrobatidis* supernatants (fig. S6). Frog splenocytes pretreated with *B. dendrobatidis* supernatants for 48 hours had reduced proliferative capacity in response to phytohemagglutinin (PHA), and delayed addition of supernatants at 24 hours after PHA stimulation still inhibited proliferation (fig. S7). Thus, *B. dendrobatidis* can prevent activation and interfere with proliferation after lymphocyte activation has been induced. Further, *B. dendrobatidis* supernatants derived from killed cells inhibited proliferation (fig. S8). Cells and supernatants from the closely related non-pathogenic chytrid, *Homolaphlyctis polyrhiza* (15), inhibited splenocytes poorly in comparison with those from *B. dendrobatidis* (fig. S9). Proliferation of mammalian epithelial cell lines (HeLa and CHO) was also inhibited by *B. dendrobatidis* supernatant (fig. S10). These data suggest that *B. dendrobatidis* releases a soluble factor that prevents lymphocytes and other cell types from proliferating and renders lymphocytes incapable of normal functions. Thus, immune paralysis is not due to defects in the initial innate immune response of macrophages and neutrophils. Instead, it is due to defects in the lymphocyte-mediated effector arm of the response.

Pathogenic fungi, including *Cryptococcus neoformans* (16), *Aspergillus fumigatus* (17), and *Paracoccidioides brasiliensis* (18) inhibit immune defenses by activating apoptosis signaling pathways. Some fungal products directly induce lymphocyte apoptosis (19). Thus we investigated whether *B. dendrobatidis* induces splenocyte apoptosis. Resting *X. laevis* splenocytes were cultured across a cell-impermeable membrane from live *B. dendrobatidis* cells in transwells and analyzed for apoptosis by flow cytometry using propidium iodide and annexin V staining (20). Splenocytes exposed to *B. dendrobatidis* in transwell cultures showed significantly increased percentages of apoptotic cells at 48 and 72 hours in comparison with controls (Fig. 2A and fig. S11A). When splenocytes were gated to distinguish T and B cells, apoptosis appeared to be preferentially induced in T cells, but some B cells were also apoptotic (fig. S11B). Concentrated *B. dendrobatidis* supernatants also induced splenocyte apoptosis at 48 hours (Fig. 2B and fig. S12), which was significantly decreased by the pan-caspase inhibitor Z-VAD-FMK (Fig. 2C and fig. S13A) but not by necrostatin-1, an inhibitor of programmed necrosis (21) (fig. S13, B and C). The *B. dendrobatidis* supernatants appeared to activate both the intrinsic and extrinsic caspase signaling pathways in splenocytes, as shown by increased activity of caspases-3 and -7 (Fig. 2D), caspase-8 (Fig. 2E), and caspase-9 (Fig. 2F). These results indicate that *B. dendrobatidis* inhibits splenocyte function by activating apoptotic signaling pathways.

*B. dendrobatidis* has two discernible life stages, the zoospore, which lacks a cell wall, and the mature sporangium (3). To identify the life stages that inhibit lymphocytes, zoospores were purified (22) and mixed directly with PHA-stimulated splenocytes (Fig. 3A). During three days of incubation, zoospores matured (Fig. 3B) and inhibited proliferation (Fig. 3A, solid bars, fig. S14). Because zoospores can mature rapidly at 26°C, we tested whether heat-killed zoospores (Fig. 3C) also inhibit splenocytes. In contrast to live zoospores or freshly killed mature cells, heat-killed zoospores were incapable of inhibiting lymphocyte proliferation (Fig. 3A, 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. 3D). Even when zoospores were placed with splenocytes in the top chamber of a transwell, proliferation was not inhibited in the bottom chamber (fig. S14). Furthermore, supernatants from zoospores were not inhibitory (Fig. 3E).

These results show that zoospores do not release splenocyte inhibitory factors until they mature.

To biochemically characterize the lymphocyte inhibitory factors released by *B. dendrobatidis*, concentrated *B. dendrobatidis* supernatants were subjected to heat, proteinase K, or acid. The supernatants retained the capacity to inhibit splenocyte proliferation following incubation at 100°C for 30 minutes (Fig. 4A) or treatment with either proteinase K (Fig. 4B) or trifluoroacetic acid (fig. S15). Proteinase K digested the protein present in the supernatant (fig. S15A), yet the supernatants retained inhibitory activity (Fig. 4B). These results suggest that the inhibitory factors are not proteins.

Some inhibitory factors produced by other fungi are cell-wall components (23); therefore, factors produced by *B. dendrobatidis* may also be located in the cell wall. This idea is consistent with the failure of zoospores, which lack a cell wall, to inhibit. To determine whether inhibitory factors are derived from the cell wall, we interfered with cell-wall synthesis using nikkomycin Z (NZ), a chitin synthase inhibitor (24). Pre-culturing *B. dendrobatidis* with NZ significantly decreased inhibition by both *B. dendrobatidis* cells and supernatants (Figs. 4, C and D). These experiments, along with the observation that non-inhibitory zoospores lack cell walls, suggest that the inhibitory factors produced by *B. dendrobatidis* are cell wall components. Chitin and  $\beta$ -1,3-glucan are the main structural cell-wall components of many fungi (25). Therefore we conducted experiments to determine whether they might be inhibitory. Treatment of *B. dendrobatidis* supernatants with  $\beta$ -glucanases and chitinases did not affect the inhibitory activity (fig. S16). Furthermore, treatment of proliferating lymphocytes with a soluble  $\beta$ -glucan (laminarin) did not inhibit function (fig. S16). Thus, the inhibitory factor does not appear to be a  $\beta$ -glucan or chitin.

We conclude that *B. dendrobatidis*, like other pathogenic fungi, produces toxic factors that inhibit potentially protective host immune responses and likely impair the function of other cells in close proximity. Soluble molecules released by *B. dendrobatidis* inhibited proliferation of amphibian and mammalian lymphocytes and induced apoptosis of target cells by activating both intrinsic and extrinsic pathways. The role of phagocytic cells (macrophages and neutrophils) in controlling chytridiomycosis is not yet well understood. These cells can engulf *B. dendrobatidis*, and accessory functions do not appear to be impaired by *B. dendrobatidis* supernatants. Because these soluble mycotoxins inhibited proliferation and caused death of non-lymphoid cell lines, they are more broadly cytotoxic and could be responsible for other symptoms of chytridiomycosis including disruption of the skin (2, 7, 26) and behavioral changes, such as lethargy and loss of righting reflex (6, 7). One or more of the factors produced by *B. dendrobatidis* may be derived from the cell wall. The capacity of *B. dendrobatidis* to evade protective immune responses helps to explain how this fungus can be so lethal to amphibians lacking effective innate defenses (27) and why some amphibian species with more robust innate responses persist with mild infections as *B. dendrobatidis* reservoirs (28–30).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

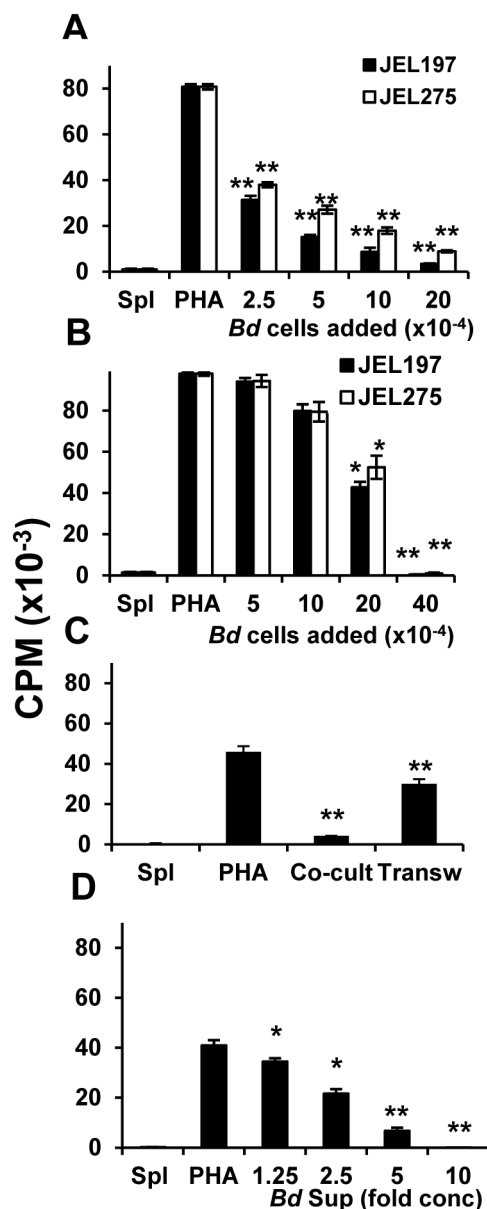
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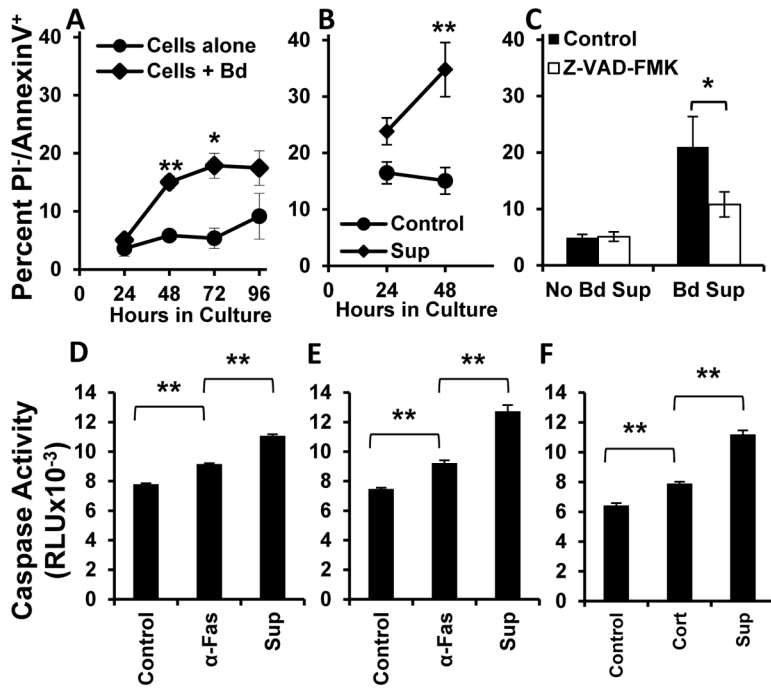
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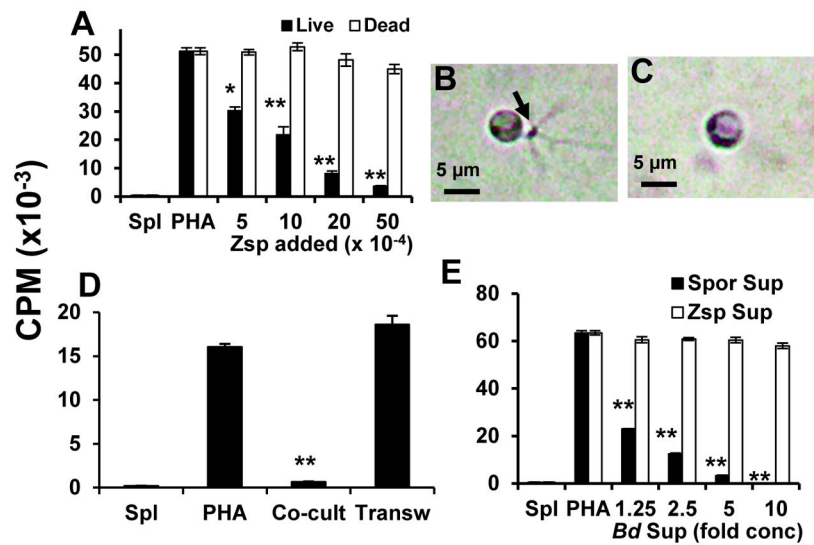
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**Fig. 1.** Inhibition of lymphocyte proliferation by *B. dendrobatidis* (*Bd*). Splenocytes (Spl) from *X. laevis* were cultured alone or with phytohemagglutinin (PHA). PHA-stimulated Spl were cultured alone or with increasing numbers of live (**A**) 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  $\mu$ m 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 <sup>3</sup>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  $\pm$  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.

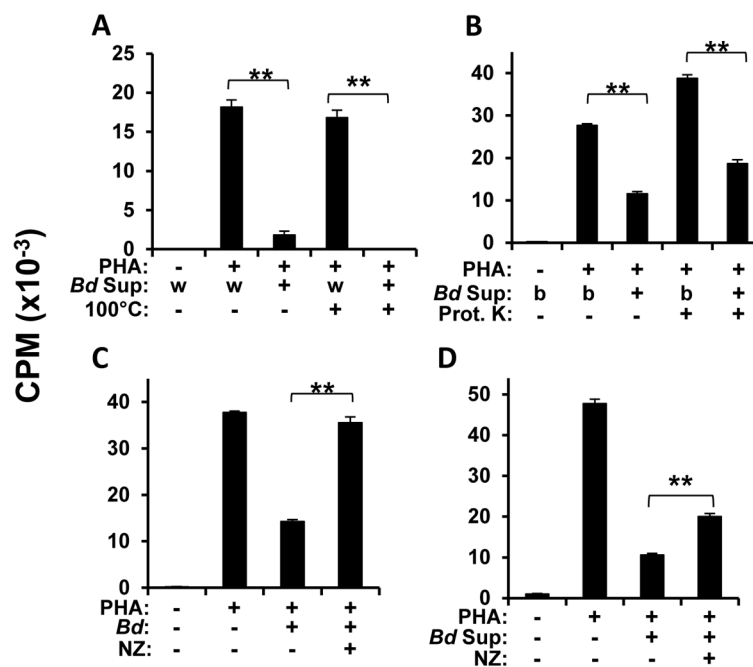


**Fig. 2.** Lymphocyte apoptosis induced by *B. dendrobatidis* (*Bd*). Average percent apoptosis  $\pm$  SEM of splenocytes (A) cultured with or without *Bd* cells separated by a 0.4  $\mu$ m filter (20:1 *Bd* to splenocytes) in transwell (three experiments); (B) cultured with or without *Bd* supernatant (Sup) (four experiments); (C) cultured for 48 hours with or without *Bd* Sup and with or without Z-VAD-FMK (five experiments) and quantified by flow cytometry. (D–F) Caspase activity assays for caspase-3/7 (D), caspase-8 (E), and caspase-9 (F) induced by *Bd* Sup, anti-Fas ( $\alpha$ -Fas) mAb, or corticosterone (Cort) (representative of four experiments). For panels A and B, percent apoptosis in the presence of *Bd* cells or Sup was significantly greater than that observed for splenocytes alone by a paired Student's *t*-test; \* $p < 0.05$ , \*\* $p < 0.01$ . For panel C, percent apoptosis induced by *Bd* Sup alone was significantly greater than that for splenocytes with no *Bd* Sup or splenocytes treated with both *Bd* Sup and Z-VAD-FMK by a paired Student's *t*-test, \* $p < 0.05$ . For panels D–F, Sup treatments induced significantly greater caspase activity than that of splenocytes alone and of positive controls, \*\* $p < 0.01$  (ANOVA with post hoc test).

**Fig. 3.**

Effects of *B. dendrobatidis* (*Bd*) zoospores (*Zsp*) or zoospore supernatants on lymphocyte proliferation. (A) Splenocytes (*Spl*) were cultured alone or with PHA. PHA-stimulated *Spl* were incubated with increasing numbers of live or heat-killed *Bd* *Zsp*. Live *Zsp* (B) developed into germlings during the 3-day culture as shown by the formation of rhizoids (arrow); dead *Zsp* (C) failed to develop (bar represents 5  $\mu$ m). (D) *Spl* were cultured as in A, and PHA-stimulated *Spl* were co-cultured (Co-cult) with or separated from live *Zsp* by a 0.4  $\mu$ m filter in transwell (Transw). (E) Supernatant from whole *Bd* cultures containing both zoospores and sporangia (Spor Sup) or from zoospores alone (*Zsp* Sup) were concentrated and incubated with PHA-stimulated *Spl*. Significantly reduced <sup>3</sup>H-thymidine uptake compared to the control treatment, \**p*<0.05, \*\**p*<0.01 (ANOVA with post hoc test). CPM in each panel are averages  $\pm$  SEM of five or more replicate wells and represent three similar experiments.





**Fig. 4.** Partial characterization of *B. dendrobatidis* (*Bd*) inhibitory factors. **(A)** Splenocytes were cultured alone or stimulated with PHA. PHA-stimulated splenocytes were exposed to water only (w) or concentrated *Bd* supernatants (Sup) (5X) that had (+) or had not (-) been incubated at 100°C. **(B)** Splenocytes were cultured as in A, and PHA-stimulated splenocytes were incubated with control buffer (b) or with *Bd* Sup (5X) previously treated with (+) or without (-) proteinase K-conjugated agarose beads (Prot. K). **(C and D)** Splenocytes were treated as in A, and PHA-stimulated splenocytes were mixed with (+) or without (-)  $10^5$  *Bd* cells (C) or 5X *Bd* Sup (D) that had (+) or had not (-) been pre-treated with 10  $\mu$ g/ml (C) or 5  $\mu$ g/ml (D) nikkomycin Z (NZ). For panels A–D, proliferation was monitored by  $^3$ H-thymidine uptake and CPM were averages  $\pm$  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.