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Inhibition of Local Immune Responses by the Frog-Killing Fungus *Batrachochytrium dendrobatidis*

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Amphibians are suffering unprecedented global declines. A leading cause is the infectious disease chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis*. Chytridiomycosis is a skin disease which disrupts transport of essential ions leading to death. Soluble factors produced by *B. dendrobatidis* impair amphibian and mammalian lymphocytes *in vitro*, but previous studies have not shown the effects of these inhibitory factors *in vivo*. To demonstrate *in vivo* inhibition of immunity by *B. dendrobatidis*, a modified delayed-type-hypersensitivity (DTH) protocol was developed to induce innate and adaptive inflammatory swelling in the feet of *Xenopus laevis* by injection of killed bacteria or phytohemagglutinin (PHA). Compared to previous protocols for PHA injection in amphibians, this method induced up to 20-fold greater inflammatory swelling. Using this new protocol, we measured DTH responses induced by killed bacteria or PHA in the presence of *B. dendrobatidis* supernatants. Swelling induced by single injection of PHA or killed bacteria was not significantly affected by *B. dendrobatidis* supernatants. However, swelling caused by a secondary injection of PHA, was significantly reduced by *B. dendrobatidis* supernatants. As previously described *in vitro*, factors from *B. dendrobatidis* appear to inhibit lymphocyte-mediated inflammatory swelling but not swelling caused by an inducer of innate leukocytes. This suggests that *B. dendrobatidis* is capable of inhibiting lymphocytes in a localized response to prevent adaptive immune responses in the skin. The modified protocol used to induce inflammatory swelling in the present study may be more effective than previous methods to investigate amphibian immune competence, particularly in nonmodel species.

Amphibians are declining at a rate faster than any other vertebrate taxon (1, 2). Infectious diseases, particularly chytridiomycosis, are major contributors to amphibian declines (3–5; reviewed in references 6, 7, and 8). Chytridiomycosis is a lethal skin infection of amphibians caused by the chytrid fungus *Batrachochytrium dendrobatidis* (9–11) and has been responsible for over 200 mass mortality events and an increasing threat of extinctions in many parts of the world (7, 12). The presence or absence of skin defenses predicts whether an amphibian species or population will persist with *B. dendrobatidis* infection (13–15; reviewed in reference 16). Despite the fact that immune responses may play a major role in survival of amphibians susceptible to chytridiomycosis, very little is known about the interactions between the amphibian immune system and *B. dendrobatidis* within the skin.

Adaptive immune responses, particularly those mediated by T lymphocytes, are essential for the clearance of fungal pathogens (reviewed in reference 17). Immunity to the potentially lethal amphibian-infecting chytrid fungus, *B. dendrobatidis*, also appears to require lymphocyte responses (15, 18). *B. dendrobatidis* probably resists host immunity in the skin by producing inhibitory factors that impair infiltrating lymphocytes. These factors inhibit *in vitro* responses of both amphibian and mammalian lymphocytes but do not appear to impair the viability or defenses of amphibian phagocytes (19). This immune evasion strategy allows *B. dendrobatidis* to infect immunocompetent hosts and helps to explain why species lacking innate mucosal immune defenses are so susceptible to chytridiomycosis (reviewed in reference 16).

Due to the current limitations in molecular tools for studies of 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 models for studies of amphibian immunology, and yet many of the reagents necessary to determine which cell populations and cytokines are important in immunity to chytridiomycosis (20) are not available. Therefore, investigations of amphibian immunity must rely on more classical immunological techniques. Amphibian leukocytes respond to mitogens (21) or bacterial products (19, 22) similar to other vertebrates. Phytohemagglutinin (PHA), phorbol-12-myristate 13-acetate, concanavalin A, and killed bacteria are mitogenic for amphibian B and T lymphocytes (19, 21). Also, the intraperitoneal injection of killed *Escherichia coli* into *X. laevis* induces infiltration of macrophages and neutrophils into the peritoneum (19, 23).

PHA injection is a common method used to investigate immunocompetence in nonmodel vertebrates. In birds, PHA is injected subcutaneously in avian patagia (wing-webs), and inflammation is measured by swelling at the site of injection (24). PHA induces robust T cell proliferation *in vitro* in amphibians (19, 21) and can also induce *in vivo* inflammatory swelling in adults, metamorphs, and tadpoles (25–28). *In vivo*, PHA induces swelling through the recruitment of granulocytes, phagocytes, thrombocytes, and lymphocytes (24, 27). Leukocyte recruitment typically begins with

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infiltration of neutrophils/heterophils and macrophages that are later followed by lymphocytes. Significant lymphocyte (primarily T cell) recruitment and activation induced by PHA typically requires a second injection of PHA (27, 29). Evaluation of T cell responses using PHA injection in amphibians probably requires two PHA injections because the inflammation of the primary response is overwhelmingly comprised of innate phagocytes and granulocytes.

In adult frogs, investigators have injected PHA into several different sites, including the toe (25), the thigh (26), and toe webbing (27). In these studies, subcutaneous injection of PHA induced less than a millimeter of swelling compared to buffer controls, with most recorded differences being about 0.1 mm. Such small differences require very precise measuring tools and handling, and they may not represent a biologically significant response. In pilot experiments with a large (>100 g) *X. laevis*, we observed no noticeable or measurable difference in swelling after subcutaneous PHA injection in different locations of the foot. Therefore, the technique was modified for the present study by the injection of PHA deeper into the foot tissue.

To determine the effects of *B. dendrobatidis* inhibitory factors on amphibian innate or adaptive immune responses *in vivo*, this modified method of foot injection was used. A primarily innate immune response was induced with killed *E. coli*, known to stimulate phagocyte infiltration into the peritoneum (19, 23). Injection of PHA was chosen as a way to induce mixed innate and adaptive immune responses. Here we show that immune inhibitory factors produced by *B. dendrobatidis* inhibited adaptive immune responses induced locally in the foot after an intraperitoneal priming injection and a second PHA injection in the foot but did not impair swelling caused by a single injection of killed *E. coli* or PHA into the foot. These data support our previous *in vitro* observations that *B. dendrobatidis* produces soluble factors that inhibit lymphocyte responses but do not appear to inhibit innate phagocyte responses (19).

MATERIALS AND METHODS

Animals. Outbred *X. laevis* from Xenopus I (Dexter, MI) or Nasco (Fort Atkinson, WI) were kept in polystyrene containers at a density of six to eight frogs per 16 liters of dechlorinated tap water. Females ranging in size between 110 and 225 g were used for foot injection experiments. During foot injection experiments, frogs were housed individually in polystyrene containers so that the experiments could be measured in a blinded fashion. Before foot injection and measurements, frogs were anesthetized in ethyl-*m*-aminobenzoate methanesulfonate salt (MS-222; MP Biomedicals LLC, Solon, OH) at 5 g/liter until all movement ceased. Frogs were then immediately placed in dechlorinated water to wash off the anesthetic. After measurements were taken, frogs were carefully observed in fresh dechlorinated water until they were able to make voluntary motions. Due to possible harmful effects of multiple exposures to MS 222, individuals were anesthetized three or fewer times. All animal procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University School of Medicine.

Induction of inflammatory swelling in the foot region. The site and depth of injection were determined based on pilot injections of PHA-P (Sigma, St. Louis, MO) in amphibian phosphate-buffered saline (APBS) (18) at 2 mg/ml or killed bacteria (*E. coli*, 10^{10} killed CFU/ml) into several sites in the foot from the ankle to the toes. Subcutaneous injection yielded insignificant swelling. Deeper, intramuscular injections in the foot region caused more swelling than injections in the ankle or toes. After this optimization, injections were made intramuscularly into the feet of *X. laevis* in the middle of the plantar side of the foot (Fig. 1A) using 1-ml syringes with

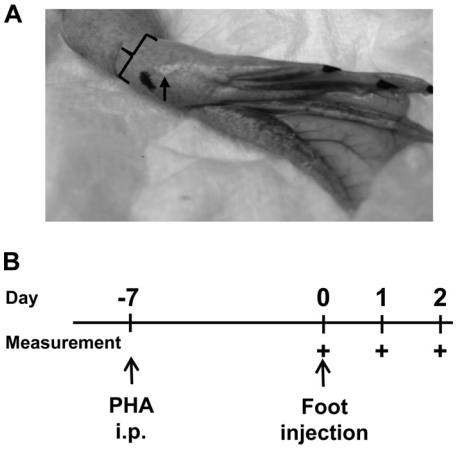

FIG 1 Experimental design for injection of feet with inducers of inflammatory swelling. (A) *X. laevis* feet were injected intramuscularly in the middle of the foot on the plantar side (arrow). Measurements of the foot thickness (bracket) and width (perpendicular to the thickness) were recorded with a caliper. (B) Schematic of foot injections. Foot injections occurred on day 0 with either APBS, PHA alone, killed *E. coli* alone, PHA and *B. dendrobatidis* supernatant (Bd Sup), or killed *E. coli* and *B. dendrobatidis* supernatant. Intraperitoneal (i.p.) injection with PHA only occurred for defined experiments in which frogs were primed with PHA. The time points at which feet were measured are indicated (+).

25 or 30 gauge needles. Dimensions of the foot were measured to the nearest 0.1 mm using a Mitutoyo plastic digital caliper (model 4LB11; Mitutoyo USA, Morgan Precision Tools, Aurora, IL). The foot width (in the plane of the foot) and foot thickness (perpendicular to the plane of the foot) were measured. The dimensions of each foot were measured at least three times at each time point, and these measurements were averaged to determine a more accurate measurement of foot dimension.

Foot perimeter (P) was calculated using the foot width and thickness measurements to calculate the perimeter of an ellipse using one-half of the foot width (a) and one-half of the foot thickness (b) as follows:

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

The peak of inflammatory swelling induced by PHA in previous amphibian studies occurs at about 24 h (Fig. 1B) (25–27). Therefore, foot dimensions were measured before injection and 24 h after injection in all experiments. Foot dimensions were also measured at 48 h after injection to follow the kinetics of PHA-induced swelling. The percent increase in foot size was determined by dividing the foot dimension at 24 or 48 h postinjection by the foot dimension from the same foot before injection. Because the natural foot width and thickness had a range of ~3.5 mm between largest and smallest frogs, the percent increase was corrected for the variation in size of individual frogs.

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 h postinjection, frogs were randomly reassigned new identification so that the person measuring the foot dimensions was blinded to the injection treatment. The identity of frogs was revealed after measurements were recorded.

Coinjection with *B. dendrobatidis* supernatants. *B. dendrobatidis* supernatants were prepared from isolate JEL197 as previously described (19). Briefly, *B. dendrobatidis* cells were centrifuged, washed with sterile glass distilled water, resuspended at 10^7 matured cells (all cells beyond zoospore stage) per ml in sterile distilled water, and incubated at 21°C for 24 h in large flasks. Cells were centrifuged, and supernatants were passed

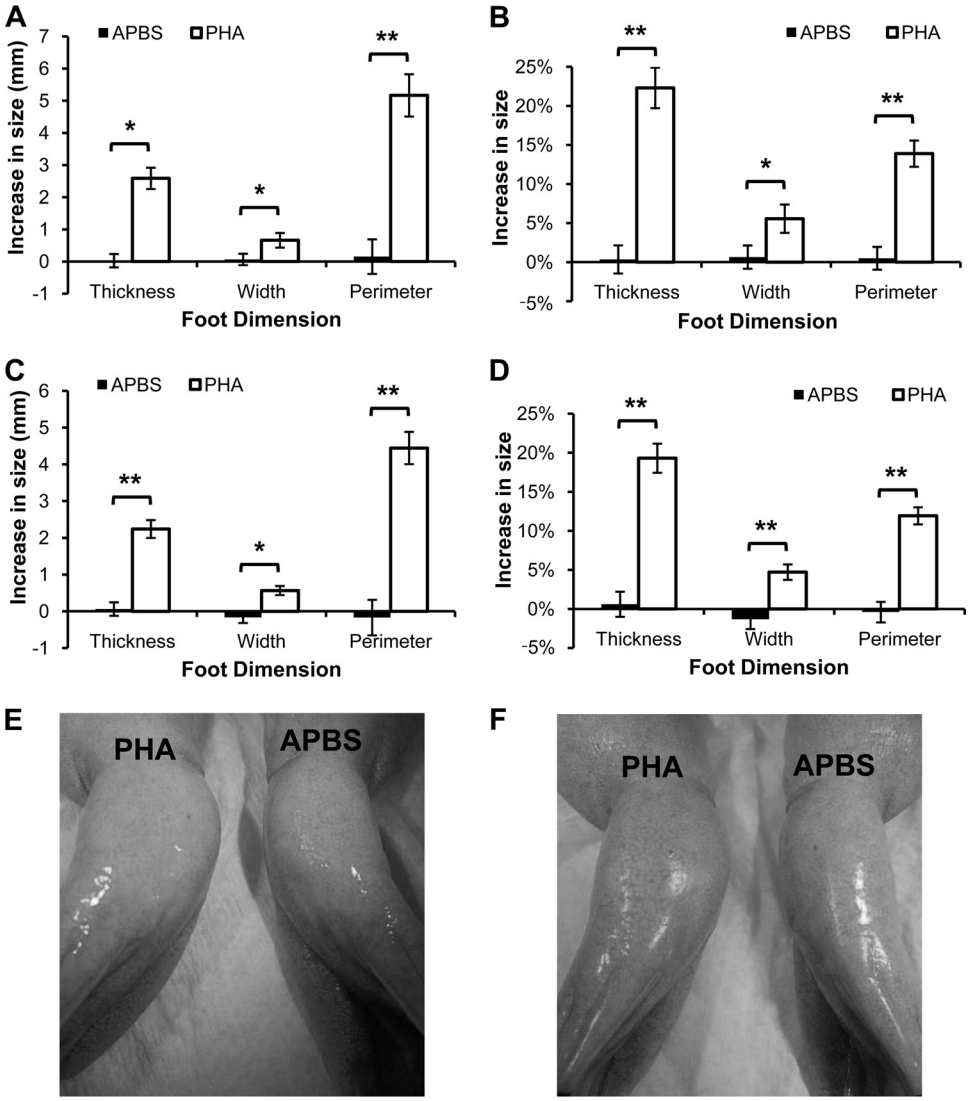

FIG 2 Intramuscular injection of phytohemagglutinin (PHA) into the foot of *X. laevis* induces inflammatory swelling after 24 h (A, B, and E) and 48 h (C, D, and F). PHA injections induced significantly greater swelling than buffer (APBS) controls (*, $P < 0.01$; **, $P < 0.001$ [paired Student's *t* test; alpha set to 0.017 for multiple tests]). The data show the mean (± the SEM) increase in actual size (A and C) or percent increase (B and D) in foot size compared to each foot's measurement before injection from both feet of six frogs. Representative photographs of individuals at 24 h (E) or 48 h (F) after injection of APBS in the left foot and PHA into the right foot (pictures show ventral side of frogs).

through 0.2-μm-pore-size filters (Fisher, Waltham, MA) to remove any cells. Supernatants were lyophilized and resuspended in APBS to be injected with inducers of inflammatory swelling. Supernatants were injected into the feet at a concentration of 10× of the original supernatant concentration before lyophilization.

To quantify PHA-induced inflammatory swelling, PHA-P in APBS at 2 mg/ml or APBS buffer alone was injected in a volume of 100 μl into the right or left foot of six individual *X. laevis*. At 7 days before foot injection with PHA and *B. dendrobatidis* supernatant, *X. laevis* were or were not primed with an intraperitoneal injection of 100 μl of 1 mg/ml PHA in APBS (Fig. 1B). When PHA (1 mg/ml) was injected with *B. dendrobatidis* supernatants (10×), 200 μl of either PHA alone or PHA with *B. dendrobatidis* supernatant (10×) were injected into the feet of 6 (without PHA priming) or 12 (with PHA priming) individual *X. laevis*.

A culture of heat-killed *E. coli* was prepared as previously described (19). Briefly, *E. coli* (strain DH 5α) was grown to a concentration of approximately 5.7×10^8 CFU/ml. The cells were boiled for 1 h in a water bath, washed twice, and resuspended in sterile APBS to 10^{10} killed CFU/ml. 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* were injected with *B. dendrobatidis* supernatants, 200 μl of either killed *E. coli* alone or killed *E. coli* with *B. dendrobatidis* supernatant (10×) were injected into the feet of 12 individual *X. laevis*.

Correlation of *B. dendrobatidis* infection with spleen size. Spleens were previously harvested from *X. laevis* to obtain a lymphocyte population to investigate the effects of *B. dendrobatidis* on amphibian lymphocytes (19). The individuals used in this experiment were males and females ranging in mass between 50 and 250 g. The number of splenocytes from each individual spleen was counted after splenocytes were enriched by centrifugation over a Ficoll-Hypaque cushion (Sigma). Individuals not screened for *B. dendrobatidis* infection ($n = 75$) were used to confirm that spleen size does significantly correlate with total mass in *X. laevis*. Because larger individuals tend to have larger spleens, the number of splenocytes

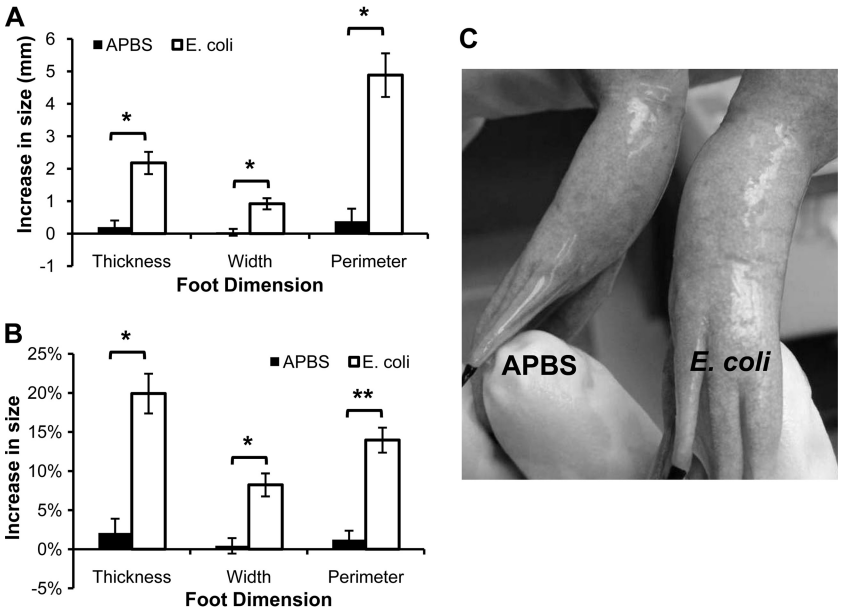

FIG 3 Intramuscular injection of killed *E. coli* into the foot of *X. laevis* induces inflammatory swelling after 24 h. *E. coli* injections induced significantly greater swelling than buffer (APBS) controls (*, $P < 0.01$; **, $P < 0.001$ [paired Student's *t* test; alpha set to 0.017 for multiple tests]). The data show the mean (± the 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 h after injection of APBS in the right foot and of killed *E. coli* into the left foot (the picture shows the ventral side of the frog).

was normalized by dividing the total number of splenocytes 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 ($n = 61$) were swabbed before euthanasia and spleen harvesting. DNA was extracted from these swabs to determine the number of zoospore equivalents on the skin according to the methods of Boyle et al. (30) and Hyatt et al. (31) by quantitative PCR. Swabbing procedure, PCR conditions, and standard curve calculation were as described by Ramsey et al. (18). Zoospore equivalents (z) determined by quantitative PCR were log transformed to calculate *B. dendrobatidis* infection load (*B. dendrobatidis* load) using the following equation *B. dendrobatidis* load $= \log_{10}(z + 1)$.

Statistics. The increase in size due to inflammatory swelling from the two compared treatments in foot injection experiments was analyzed by using 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-foot dimension measurements, so the alpha for statistical significance was set to 0.017. To determine whether there was a correlation between infection load and spleen size (relative number of splenocytes) a regression analysis was used. A *P* value of <0.05 was considered statistically significant.

RESULTS

Injection of PHA and killed *E. coli* to induce inflammatory swelling. Before injection with PHA or killed *E. coli*, the average foot width among all *X. laevis* individuals in all experiments (42 frogs, 84 feet) was 11.65 ± 0.08 mm (mean ± the standard error of the mean [SEM]), and the average foot thickness was 11.21 ± 0.08 mm (mean ± the SEM). Comparisons were made between left and right feet irrespective of treatment, and there were no significant differences between the left feet and right feet for any experiment at any time point (data not shown).

Intramuscular PHA injection into the foot area caused significantly greater swelling in all dimensions compared to the injection of APBS vehicle control (Fig. 2A and C). Swelling induced by PHA increased the width of the foot by about 5% and the thickness by about 20% (Fig. 2B and D). PHA induced a large increase in the foot size 24 h after injection (Fig. 2A and B), which was not significantly changed after 48 h (Fig. 2C and D); however, the difference in swelling between PHA and APBS treatments was more significant at 48 h than at 24 h. Feet injected with PHA were visibly larger than feet injected with APBS for all individuals (Fig. 2E and F).

X. laevis feet injected with heat-killed *E. coli* showed profound swelling 24 h after injection, and control feet injected with APBS buffer did not show a significant change in foot size in comparison with the size determined before injection (Fig. 3). Injection of killed *E. coli* caused a significant increase in the size of the feet (Fig. 3A). This increase represented an increase in the foot width by about 8% and foot thickness by 20% after *E. coli* injection (Fig. 3B). As with injection with PHA, killed *E. coli* induced a visible increase in foot size compared to the APBS control (Fig. 3C).

Redness is a classical symptom of acute inflammation (32). However, no reddening of the foot near the site of injection was visible for either the PHA or the killed *E. coli* injections. The only visible effect of injection with inflammatory inducers was an increase in the size of the foot (Fig. 2E and F; Fig. 3C). The foot swelling did not appear to cause discomfort and did not obstruct movement of the *X. laevis*.

***In vivo* inhibition of a local immune response by *B. dendrobatidis*.** In our previous study demonstrating paralysis of lymphocytes by *B. dendrobatidis* factors, the *B. dendrobatidis* infection burdens of many *X. laevis* used to obtain splenocytes were assessed (19). Other frogs not screened for *B. dendrobatidis* infection were used to confirm that spleen size was significantly correlated with total mass in *X. laevis* (Fig. 4A). The data obtained from frogs with detectable *B. dendrobatidis* infections was used here to determine whether *B. dendrobatidis* infection in the skin would affect lym-

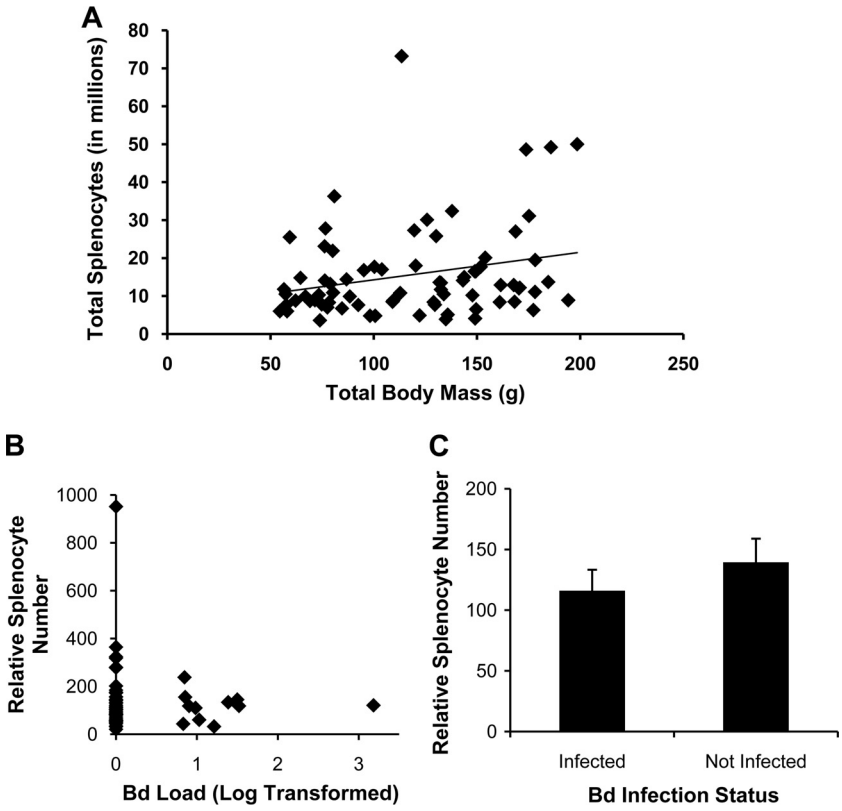

FIG 4 *B. dendrobatidis* infection does not affect the number of leukocytes present in the spleen (splenocytes) of *X. laevis*. (A) Splenocyte number positively correlates with size in *X. laevis*. A significant correlation exists ($P = 0.03$) between the total body mass of individuals, and the total number of splenic leukocytes. $n = 75$, $R = 0.25$; the best fit line is shown. (B) No significant relationship exists between the infection load and relative number of splenocytes (relative to body mass) by regression analysis (all: $P = 0.64$; infected: $P = 0.99$). Bd Load, *B. dendrobatidis* load. (C) There is also no significant difference in the mean number of splenocytes (± the SEM) between individuals that were or were not infected with *B. dendrobatidis* by two-tailed Student's *t* test ($P = 0.37$). The number of splenocytes was normalized (B and C) to the size of each individual by dividing the total splenocytes by the mass in mg of the frog. The Bd load was determined by quantitative PCR using zoospore equivalent standards and log transformed. $n = 50$ for individuals that were not infected; $n = 11$ for infected individuals. The mean infection load (± the SEM) for infected individuals was 17.8 ± 0.9 zoospore equivalents.

phocyte populations in the spleen (the only secondary lymphoid organ in frogs). There was no effect of intensity of infection (Fig. 4B) or infection status (Fig. 4C) on the number of splenocytes obtained from *X. laevis*. Because the *X. laevis* harbored very low infection burdens in these studies, *B. dendrobatidis* did not appear to significantly affect splenocyte populations. Therefore, the foot injection approach described here was taken to measure the effects of *B. dendrobatidis* soluble factors on a local induced immune response in the foot.

B. dendrobatidis supernatants were injected along with PHA to determine whether inhibitory factors decreased the swelling caused by innate and adaptive leukocytes activated by PHA. *B. dendrobatidis* supernatants had no significant impact on the increase of foot size following a single PHA injection (Fig. 5). Previous studies suggested that a single PHA injection typically promotes a more robust innate leukocyte response, and a second PHA injection promotes recruitment of phagocytes again but with greater activation and immigration of lymphocytes (27, 29). To investigate whether *B. dendrobatidis* factors impaired adaptive immune responses *in vivo*, *X. laevis* frogs were primed with a single intraperitoneal PHA injection before receiving foot injections of PHA alone or PHA with *B. dendrobatidis* supernatants. After PHA priming, *B. dendrobatidis* supernatants caused a significant reduction in foot swelling induced by PHA (Fig. 6). At 24 h after injection, the difference in swelling was substantial but not significant (Fig. 6A and B). However, by 48 h, the swelling of feet receiving *B. dendrobatidis* supernatants with PHA was significantly diminished compared to the feet receiving PHA alone (Fig. 6C and D). In some individuals, the decrease in swelling caused by *B. dendrobatidis* supernatants was visible without the need for measuring devices at both 24 and 48 h (Fig. 6E and F).

B. dendrobatidis supernatants were also injected along with heat-killed *E. coli* to determine whether factors present in the supernatants 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 dimensions were not different between the feet receiving *E. coli* alone and those receiving both *E. coli* and *B. dendrobatidis* supernatants (Fig. 7). The increase in foot size of this experiment was comparable to what was observed when only one foot received the killed *E. coli* injection.

DISCUSSION

Inhibition of local adaptive immune responses by *B. dendrobatidis*. Early studies of chytridiomycosis noted minimal leukocyte

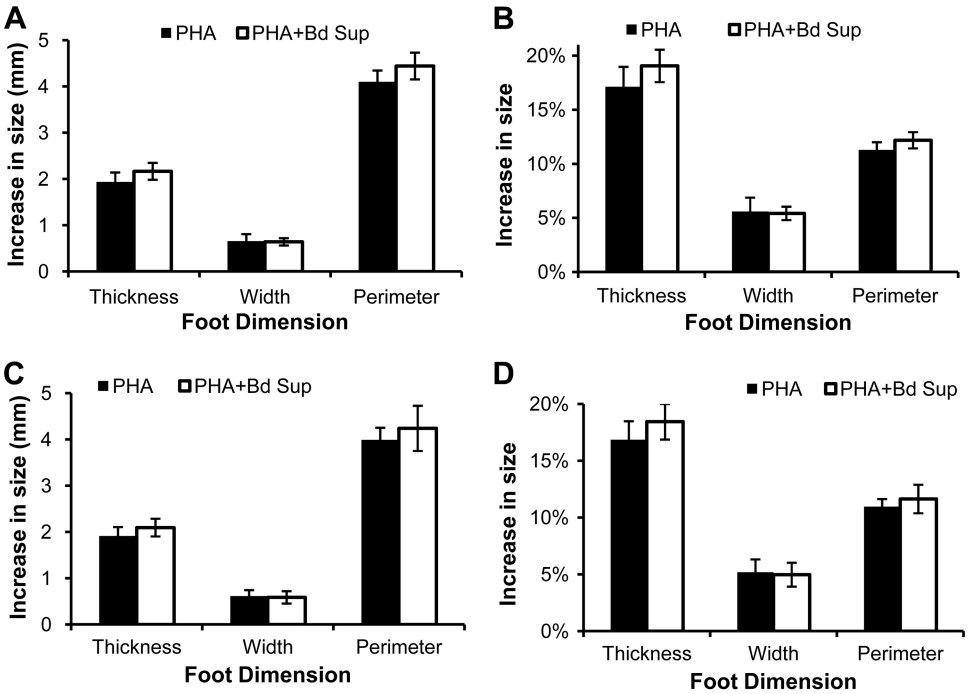

FIG 5 *B. dendrobatidis* supernatant (Bd Sup) does not impair inflammatory swelling induced by a single PHA injection (frogs were not primed with PHA). *X. laevis* feet were injected with PHA alone or with PHA and Bd Sup. Feet were measured before injection and at 24 h (A and B) and 48 h (C and D) after injection. The data show the mean (± the 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).

infiltration into *B. dendrobatidis*-infected skin (9, 11, 33). Transcriptional studies of early infections also found little activation of immune gene expression during chytridiomycosis (34, 35). The absence of robust immune responses to *B. dendrobatidis* is not likely due to the incapacity of amphibian immunity. Adaptive immune defenses can be activated and promote resistance to chytridiomycosis in some species (15, 18, 36, 37). The ineffective clearance of *B. dendrobatidis* is most likely the result of immune evasion due to suppressive factors produced by the fungus (19, 37). *B. dendrobatidis* impairs lymphocytes *in vitro* (19), and very recent transcriptional expression studies in the highly susceptible frog, *Atelopus zeteki*, also seem to show a robust immune activation which is subsequently suppressed (37). Both observations may explain the lack of robust immunity in the most susceptible species.

The factors that impair and kill amphibian lymphocytes *in vitro* do not appear to inhibit amphibian phagocytes, suggesting that the targets of evasion are effector lymphocytes (19). The present 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* induces a primarily innate immune response mainly causing an infiltration of phagocytic leukocytes (19, 23, 27, 29). The inflammatory swelling induced by these injections was not significantly affected when *B. dendrobatidis* supernatants were simultaneously injected, suggesting no impairment of innate immune responses. Amphibian macrophages and neutrophils were not impaired *in vitro* by *B. dendrobatidis* supernatants in our previous study (19), and soluble *B. dendrobatidis* factors did not appear to kill or impair recruitment of innate leukocytes into the foot in the present study.

In a previous study, a single injection of PHA did induce a small amount of lymphocyte recruitment in an amphibian host, but this is likely to be a minor component of the inflammatory response causing swelling (27). A second PHA injection promoted a more robust lymphocyte response in birds (29) and induced greater swelling in amphibians (27). 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 with *B. dendrobatidis* supernatant. The priming injection of PHA allowed for a greater lymphocyte response during the second injection. Unlike the response induced with a single PHA injection, the inflammatory swelling induced by the second injection was significantly reduced by factors present in *B. dendrobatidis* supernatants, suggesting that lymphocytes were impaired by *B. dendrobatidis* factors *in vivo* as previously characterized *in vitro*. The full effect of *B. dendrobatidis* supernatants did not become significant until 48 h after injection. This correlates well with the observed peak of *B. dendrobatidis*-induced amphibian lymphocyte apoptosis (19). The delayed effect of *B. dendrobatidis* supernatant also may be explained by the later recruitment of lymphocytes during PHA injection compared to the infiltration of innate leukocytes (27). Because *B. dendrobatidis* factors inhibit activation and induce apoptosis in lymphocytes (19), the mechanism of decreased swelling induced by a secondary PHA injection probably is mediated by the inhibition of lymphocyte proliferation and induction of lymphocyte apoptosis after recruitment. However, *B. dendrobatidis* supernatants may also inhibit lymphocyte infiltration by disrupting or damping chemokine signaling (37).

We assumed that injection of *E. coli* induces a rapid immigration of neutrophils and macrophages rather than a lymphocyte-

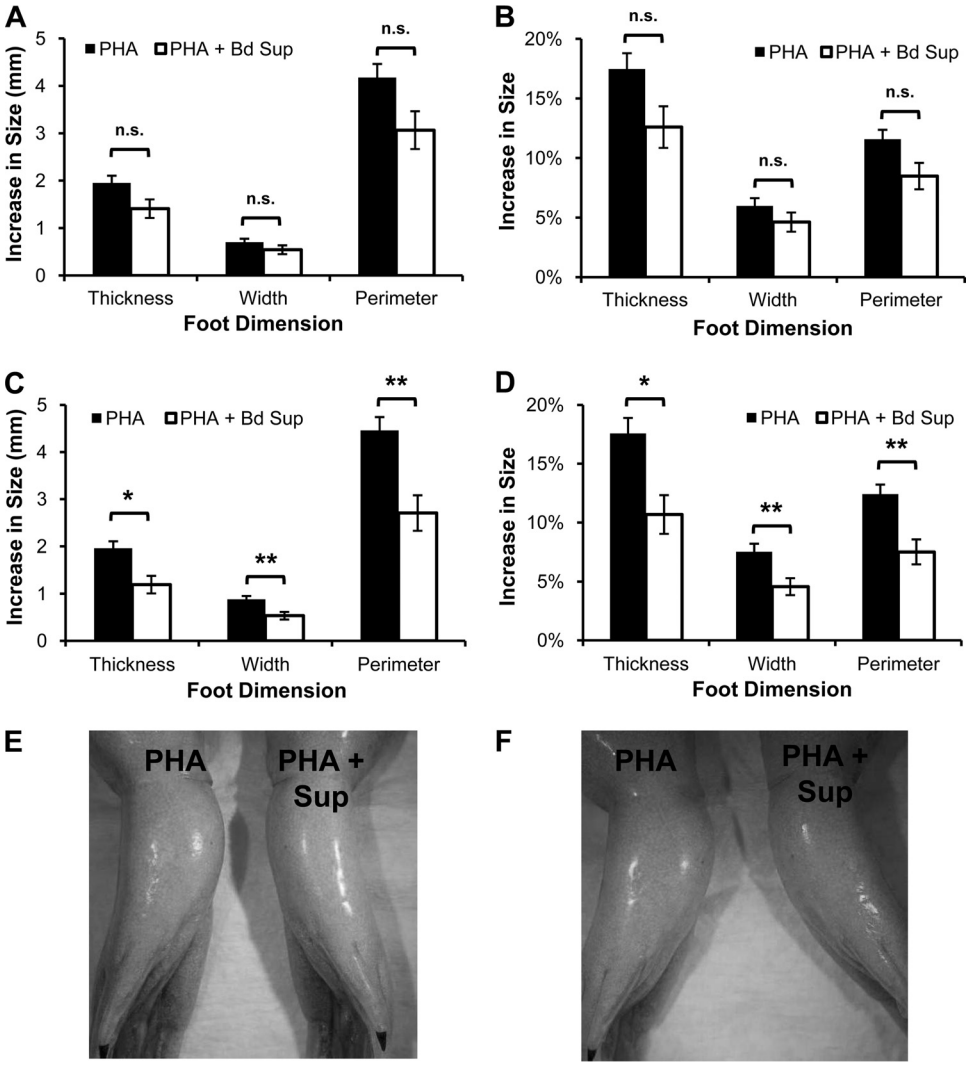

FIG 6 *B. dendrobatidis* supernatant (Bd Sup) reduces inflammatory swelling induced by a second PHA injection. *X. laevis* feet were injected with PHA alone or with PHA and Bd Sup 7 days after priming with intraperitoneal injection of PHA. Feet were measured before injection and at 24 h (A, B, and E) and 48 h (C, D, and F) after injection. The data show the mean (± the SEM) increase in actual size (A and C) or percent increase (B and D) in foot size compared to each foot's measurement before injection from both feet of 12 frogs. Swelling was only significantly different between treatments in feet 48 h after injection. n.s., $P > 0.017$; *, $P < 0.01$; **, $P < 0.001$ (paired Student's *t* test; alpha set to 0.017 for multiple tests). Representative photographs of an individual at 24 h (E) and 48 h (F) after the injection of PHA and Bd Sup in the left foot and PHA alone into the right foot (the pictures show the ventral side of the frog).

specific response because these cells are recruited during a single intraperitoneal injection of *E. coli* into *X. laevis* (19, 23). However, we did not test whether intraperitoneal priming with *E. coli* would result in enhanced swelling of the foot, as was observed with priming and injection of PHA. If priming with *E. coli* does induce an *E. coli*-specific lymphocyte response, we would expect that the *B. dendrobatidis* supernatants would inhibit that response as well.

The *in vivo* injection of *B. dendrobatidis* demonstrated that soluble factors from *B. dendrobatidis* inhibited local adaptive immune responses at the sites of lymphocyte recruitment and activation in *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 alone resembled the amount of inflammation caused by PHA when no *B. dendrobatidis* supernatant was injected into *X. laevis*. However, a systemic inhibition of immunity may be possible during an infection that lasts longer than the duration of the foot injection experiment. The lymphotoxic factors released by *B. dendrobatidis* in the skin may be released into the bloodstream at a high enough concentration to have an impact on lymphocytes further away from the sites of infection. When we investigated the effect of low level *B. dendrobatidis* infection on splenocytes, we saw no effect of low-intensity infection on *X. laevis* splenocyte number. However, studies of gene transcription in *A. zeteki* late in infection and with heavy lethal pathogen burdens support the hypothesis that fungal products from the skin infection can alter splenic lymphocyte numbers and functions (37). These results suggest that the factors released by *B. dendrobatidis* may, indeed, interact with the immune system outside the skin.

An improved method for eliciting a DTH response in amphibians. PHA injection is a common method used to measure a delayed-type-hypersensitivity (DTH) immune response in non-

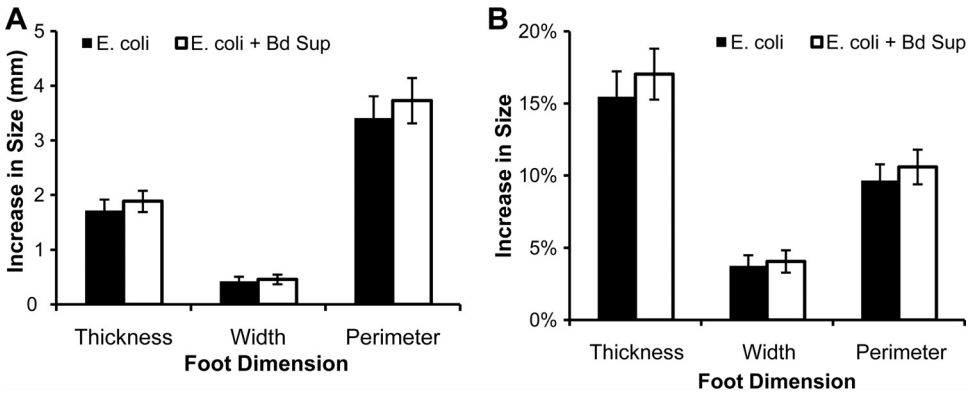

FIG 7 *B. dendrobatidis* supernatant (Bd Sup) does not impair inflammatory swelling induced by killed *E. coli*. *X. laevis* feet were injected with either killed *E. coli* alone or *E. coli* with Bd Sup. Feet were measured before injection and 24 h after injection. The data show the mean (± the SEM) 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).

model vertebrates. In birds a consensus protocol of injecting patagia has been developed (24), but no such site has previously been determined for anuran amphibians. In previous studies, many of the sites chosen for injecting adult frogs showed a very small amount of swelling induced by PHA (26, 27). Such small differences in size require very precise tools and a large number of replicates to obtain statistical significance. For our experimental design, in which factors from *B. dendrobatidis* were examined to determine whether they would decrease swelling, greater inflammatory swelling was necessary to determine significant inhibition of inflammatory processes. 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 and 20 times greater than previously recorded with subcutaneous injection in frogs (25–28). The amount of inflammatory swelling induced by intramuscular injection was sufficient so that a noticeable difference was visible between PHA and buffer injections. This method was also suitable for measuring swelling induced by killed bacteria.

Priming with a prior injection of PHA appears to be necessary to induce a robust lymphocyte response in our studies. In birds, a second injection of PHA into the patagium induces a greater infiltration of lymphocytes than a single injection (29). In amphibians, a single injection of PHA does induce some lymphocyte recruitment, and a second injection of PHA induces significantly greater swelling (27). In our experimental design, we injected PHA intraperitoneally 7 days before the second injection of PHA into the feet. The peritoneum was chosen for PHA priming so both feet did not have to be injected and because the peritoneum has previously been used as the site to induce immune responses in *X. laevis* (18, 19, 23).

Taken together, our studies suggest that intramuscular foot injection of PHA or other inducers of inflammation may be a better method for investigating DTH immune responses in amphibians than previously published sites of injection. Deeper injection into the foot and the increased difference in swelling requires less precision in injection and measuring, thus decreasing experimental error and decreasing the need for many experimental replicates. The simplicity of the method may also make it preferable for future studies.

In summary, we used an improved immunological method in amphibians to provide *in vivo* evidence that *B. dendrobatidis* is capable of inhibiting local lymphocyte responses in its amphibian host to promote infection. This adaptation by *B. dendrobatidis* to evade adaptive immune responses may help to explain how it is such a successful pathogen known to infect more than 500 species of amphibians (38) and why amphibians lacking robust innate immune responses are so susceptible to chytridiomycosis (reviewed in reference 16).

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We have no conflicts of interest to declare.

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