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Immunomodulatory Metabolites Released by the Frog-Killing Fungus *Batrachochytrium dendrobatidis*

Louise A. Rollins-Smith,[a,b] J. Scott Fites,[b]* Laura K. Reinert,[a] Andrea R. Shiakolas,[b] Thomas P. Umile,[c] Kevin P. C. Minbiole[d]

Department of Pathology, Microbiology, and Immunology, and Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA[a]; Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee, USA[b]; Division of Natural and Computational Sciences, Gwynedd Mercy University, Gwynedd Valley, Pennsylvania, USA[c]; Department of Chemistry, Villanova University, Villanova, Pennsylvania, USA[d]

***Batrachochytrium dendrobatidis* is a fungal pathogen in the phylum Chytridiomycota that causes the skin disease chytridiomycosis. Chytridiomycosis is considered an emerging infectious disease linked to worldwide amphibian declines and extinctions. Although amphibians have well-developed immune defenses, clearance of this pathogen from the skin is often impaired. Previously, we showed that the adaptive immune system is involved in the control of the pathogen, but *B. dendrobatidis* releases factors that inhibit *in vitro* and *in vivo* lymphocyte responses and induce lymphocyte apoptosis. Little is known about the nature of the inhibitory factors released by this fungus. Here, we describe the isolation and characterization of three fungal metabolites produced by *B. dendrobatidis* but not by the closely related nonpathogenic chytrid *Homolaphlyctis polyrhiza*. These metabolites are methylthioadenosine (MTA), tryptophan, and an oxidized product of tryptophan, kynurenine (Kyn). Independently, both MTA and Kyn inhibit the survival and proliferation of amphibian lymphocytes and the Jurkat human T cell leukemia cell line. However, working together, they become effective at much lower concentrations. We hypothesize that *B. dendrobatidis* can adapt its metabolism to release products that alter the local environment in the skin to inhibit immunity and enhance the survival of the pathogen.**

In recent years, amphibian populations around the world have declined due, in part, to the emerging fungal disease chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (1–6). Heavily infected frogs show lethargy, loss of appetite, and behavioral changes (1, 3, 7, 8), and they die due to a failure to maintain a balance of essential ions across the damaged skin (9). Although amphibians have robust and complex immune defenses (10), immune responses against this pathogen are impaired (11–13). Our previous studies showed that sublethal X irradiation of South African clawed frogs (*Xenopus laevis*) resulted in decreased lymphocyte numbers in the spleen and increased fungal burdens, demonstrating that lymphocyte-mediated adaptive immune responses are important for control of the infection (14). Further studies demonstrated that *B. dendrobatidis* cells release molecules that inhibit proliferation of lymphocytes and transformed cell lines and induce apoptosis of lymphocytes (11). Enriched fungal supernatants also interfere with the development of a delayed-type hypersensitivity (DTH)-like response to injected phytohemagglutinin (PHA) in *X. laevis* (12). Thus, the inhibitory factors can act locally in the skin environment. The nature of these inhibitory molecules has not previously been determined. Using liquid chromatography coupled with mass spectrometry (LC-MS) and UV-visible (UV-Vis) detection, we isolated three small-molecule metabolites from *B. dendrobatidis* supernatants (*B. dendrobatidis* Sup) with putative immunomodulatory activity. These metabolites are methylthioadenosine (MTA), tryptophan, and an oxidized product of tryptophan, kynurenine (Kyn). The pure molecules were tested for their effects on cultured lymphocytes. Independently, MTA and Kyn inhibited lymphocyte survival and proliferation. Kyn was active only at supraphysiological concentrations, but the addition of a suboptimal concentration of MTA greatly enhanced the activity of Kyn, bringing its range of activity much closer to physiologically relevant concentrations. These results support the hypothesis that *B. dendrobatidis* can adapt its metabolism to release products that alter the local environment of the skin to inhibit immunity and enhance survival of the pathogen.

MATERIALS AND METHODS

Culture of *B. dendrobatidis* and *H. polyrhiza* and preparation of aqueous supernatants. *B. dendrobatidis* isolate JEL197 (2) was cultured and maintained in 1% tryptone broth, as previously described (11). The nonpathogenic chytrid fungus *Homolaphlyctis polyrhiza* (isolate JEL142) (15) was cultured in medium containing 0.1% peptonized milk, 0.1% tryptone, and 0.5% glucose. We chose to use mixed cultures of zoospores and maturing zoosporangia because they produced highly active supernatant factors in previous studies. Pure zoospores lacked the capacity to produce lymphocyte-inhibitory factors (11), and a preliminary high-performance liquid chromatography (HPLC) analysis of supernatants from zoospores showed an absence of peaks for MTA and tryptophan. Cultures were incubated at 21°C and subcultured twice weekly. After 6 to 7 days of culture, *B. dendrobatidis* or *H. polyrhiza* cells were centrifuged, washed with sterile glass-distilled water, resuspended at 10^7 cells/ml in sterile dis-

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Address correspondence to Louise A. Rollins-Smith, louise.rollins-smith@vanderbilt.edu.

* Present address: J. Scott Fites, Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, University of Wisconsin—Madison, Madison, Wisconsin, USA.

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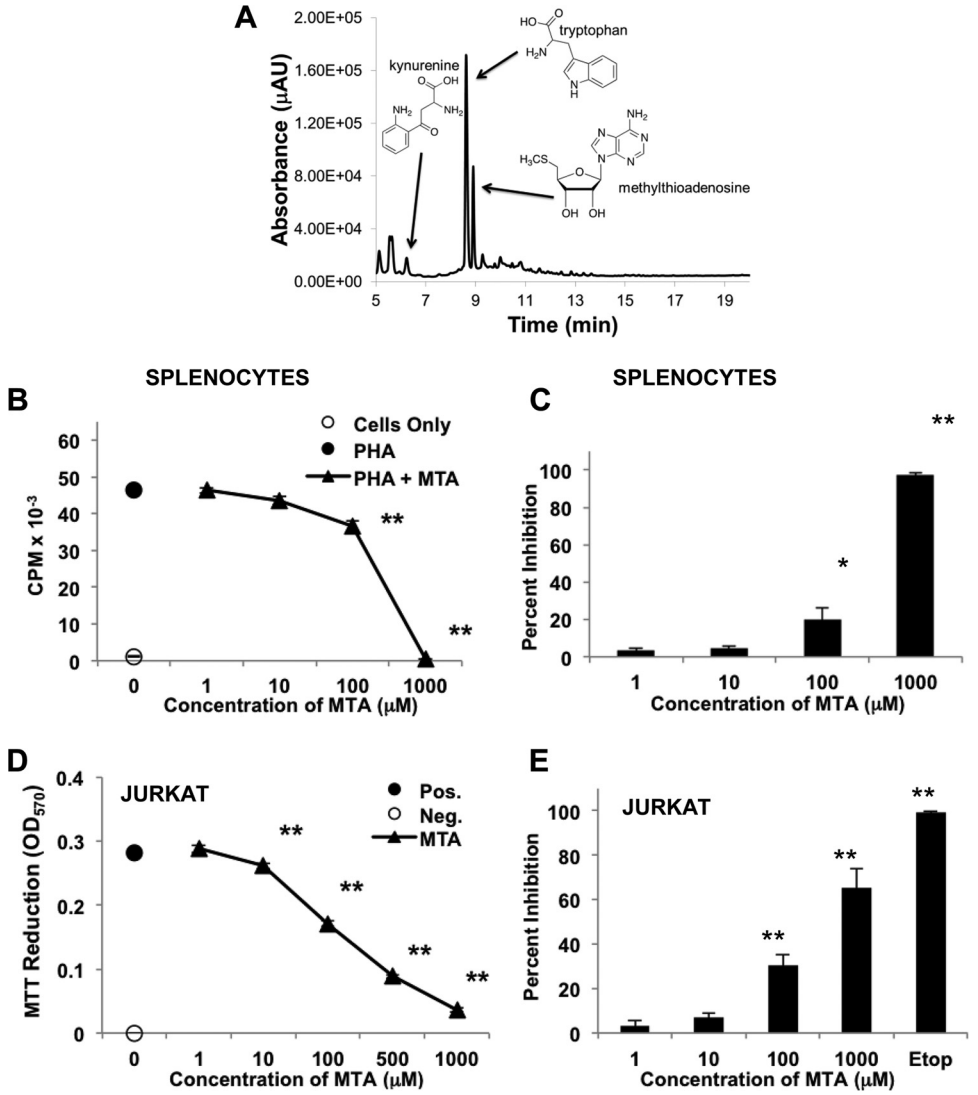

FIG 1 (A) HPLC chromatogram of the lyophilized cell-free supernatant of *B. dendrobatidis* cells (JEL197) showing the presence of tryptophan, Kyn, and MTA. Absorbance is reported as micro-absorbance units (μAU). (B and C) Inhibition of PHA-stimulated *X. laevis* spleen cells by MTA. (B) Data from one representative of five similar experiments. *X. laevis* splenocytes were cultured alone or with PHA. PHA-stimulated cells were incubated with increasing concentrations of MTA, as shown. (C) Percent inhibition of growth at each concentration ($n = 4$ or 5 experiments at each concentration). (D and E) Inhibition of Jurkat T cells by MTA. (D) One representative experiment (seven total) showing inhibition of growth measured as the reduction of MTT at 570 nm. The positive control (Pos.) was medium alone, and the negative control (Neg.) was treated with etoposide (Etop). OD_{570}, optical density at 570 nm. (E) Summary of data from seven experiments reported as percent inhibition of growth. For all panels, error bars show standard errors, and the indicated treatments were significantly different from the positive control (PHA alone for splenocytes and medium alone for Jurkat cells). *, $P < 0.05$; **, $P < 0.01$ (determined by one-way analysis of variance with a Tukey *post hoc* test).

tilled water, and incubated at 21°C for 24 h in large flasks. Cells were centrifuged, and supernatants were passed through 0.2-μm filters (Fisher, Waltham, MA, USA) to remove any cells. Supernatants were then frozen and lyophilized (11).

Extraction of *B. dendrobatidis* supernatants and HPLC isolation of fractions. A lyophilized, cell-free supernatant of *B. dendrobatidis* (JEL197) (*B. dendrobatidis* Sup) (2) was reconstituted in 1.0 ml of HPLC-grade water and analyzed by LC-MS (Shimadzu LC-20 liquid chromatograph equipped with an ACE C_{18} column [3 μm; 150 by 4.6 mm], a Shimadzu SPD-M20A diode array detector, and an Applied Biosystems Sciex API 2000 triple-quadrupole mass spectrometer). After elution (ramping from acidified H_2O to acidified acetonitrile, as described previously by Umile et al. [16]), the chromatogram was compared to that for lyophilized water and the *H. polyrhiza* supernatant. Compounds unique to *B. dendrobatidis* were isolated by semipreparative HPLC (ACE C_{18} column [5 μm; 250 by 10 mm]).

Identification of metabolites by NMR. Tryptophan was identified on the basis of ^{1}H nuclear magnetic resonance (NMR), HRMS-ESI (high-resolution mass spectrometry–electrospray ionization), and UV-Vis spectroscopy. MTA was characterized on the basis of HRMS-ESI and UV-Vis spectroscopy. Although the isolated compound was too dilute for complete ^{1}H NMR characterization, the ^{1}H NMR spectrum showed singlet peaks at 8.345 and 8.232 ppm, corroborating the postulated adenine chromophore. Kyn was identified on the basis of its characteristic UV-Vis chromophore (257 nm; 362 nm). For all compounds, authentic standards were purchased and shown to have identical retention times under the LC-MS conditions used in their initial discovery.

For tryptophan, ^{1}H NMR (500 MHz, CD_3OD, δ) 7.70 (d, $J = 7.9$ Hz,

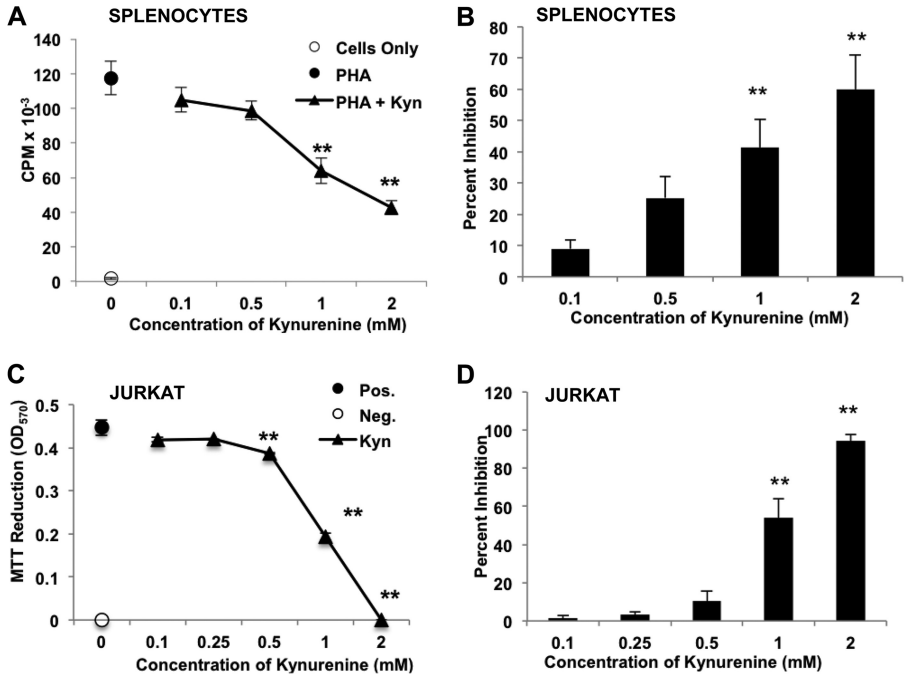

FIG 2 Inhibition of splenocytes and Jurkat cells by Kyn. (A) Inhibition of PHA-stimulated *X. laevis* splenocytes by Kyn (data from one representative of eight similar experiments). Splenocytes were cultured alone or with PHA. PHA-stimulated cells were incubated with increasing concentrations of Kyn, as shown. (B) Percent inhibition of growth at each concentration ($n = 8$ experiments). (C) Inhibition of Jurkat T cells by Kyn. Data from one representative experiment of six show inhibition of growth measured as the reduction of MTT at 570 nm. The positive control (Pos.) was medium alone, and the negative control (Neg.) was treated with etoposide. (D) Summary of data from seven experiments shown as percent inhibition of growth. For all panels, error bars show standard errors, and the indicated treatments were significantly different from the positive control (PHA alone for splenocytes and medium alone for Jurkat cells). **, $P < 0.01$ (determined by one-way analysis of variance with a Tukey *post hoc* test).

1H), 7.36 (d, $J = 7.4$ Hz, 1H), 7.20 (s, 1H), 7.12 (t, $J = 8.0$ Hz, 1H), 7.05 (t, $J = 8.0$ Hz, 1H), 3.86 (dd, $J = 9$ Hz, 4 Hz, 1H), 3.51 (dd, $J = 15$ Hz, 4 Hz, 1 H), 3.16 (m, 1H). HRMS-ESI (*m/z*) $[M + H]^+$ calculated for $C_{11}H_{13}N_2O_2$, 205.0977; found, 205.0969; UV λ_{max}, 278 nm.

For MTA, ^{1}H NMR (500 MHz, CD_3OD, δ) 8.345 (s), 8.232 (s), other peaks obscured. HRMS-ESI (*m/z*) $[M + H]^+$ calculated for $C_{11}H_{16}N_5O_3S$, 298.0974; found, 298.0971; UV λ_{max}, 257 nm.

For kynurenine, UV λ_{max}, 257, 362 nm.

Assays of lymphocyte inhibition. Spleen cells from *X. laevis* were isolated and cultured with PHA and increasing concentrations of purified metabolites, as previously described (11). Splenocytes were incubated at 26°C in an atmosphere of 5% CO_2–95% air for 3 days before harvesting, as previously described (11). All wells were pulsed with 0.5 μCi [^{3}H]thymidine (5 μCi/ml; specific activity, 2 Ci/mmol) (PerkinElmer, Waltham, MA, USA) during the last 24 h prior to harvesting.

Jurkat T cells were cultured in RPMI medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin) at 37°C with 5% CO_2–95% air and passaged twice weekly. Cells were centrifuged at ~200 × *g* for 10 min and resuspended in RPMI medium. Five or more replicates of cells (10^4 cells/well) in 50 μl were cultured for 3 days with or without increasing concentrations of purified MTA or L-kynurenine or with 25 μg/ml etoposide (negative control for growth) in 50 μl of RPMI medium (final concentration in culture, 12.5 μg/ml). After incubation, 100 μl of MTT (thiazolyl blue tetrazolium bromide) (500 μg/ml) was added to each sample, and cells were incubated for another 2 to 4 h at 37°C. The samples were then centrifuged for 30 min at ~2,200 × *g*, culture supernatants were removed, and the contents of each well were resuspended in 100 μl of dimethyl sulfoxide (DMSO). The plate was incubated 37°C for 10 min, and the absorbance was read with a BioTek ELx808 plate reader at 570 nm after 2 min of fast shaking.

Percent inhibition of PHA-induced splenocyte proliferation was determined by subtracting the average reduced counts per minute divided by the average control PHA-induced counts per minute from 1 and multiplying this value by 100 {% inhibition = [1 − (avg. reduced cpm/avg. PHA-only cpm)] × 100%}. Percent inhibition of Jurkat growth was determined by subtracting the average reduced MTT absorbance divided by the average absorbance of untreated Jurkat cells from 1 and multiplying this value by 100 {% inhibition = [1 − (avg. reduced MTT abs./avg. MTT abs. of untreated cells] × 100%}.

RESULTS

***B. dendrobatidis* metabolites and their lymphotoxic activity.** Because preliminary studies suggested that lymphotoxic activity could be detected in low-molecular-weight fractions derived from the *B. dendrobatidis* Sup, we used LC-MS with UV-Vis detection to investigate small molecules produced by the original type strain of *B. dendrobatidis* (isolate JEL197) (2) but not by the closely related nonpathogenic chytrid *H. polyrhiza* (15, 17). The *B. dendrobatidis* Sup from cultures of maturing zoosporangia grown overnight in water (11) consisted of a mixture of two major light-absorbing components as well as a number of less abundant components not present in the *H. polyrhiza* supernatant (Fig. 1A). One major metabolite was identified as MTA, which is a compound known to have immunomodulatory effects in mammalian systems (18, 19). This compound inhibited the proliferation of *X. laevis* splenocytes at concentrations of ≥100 μM (Fig. 1B and C) and inhibited the division and survival of Jurkat T cells at concentrations of ≥10 μM (Fig. 1D and E). Another major component was identified as tryptophan. While tryptophan itself is not inhibitory to lymphocytes, an oxidized tryptophan metabolite, Kyn,

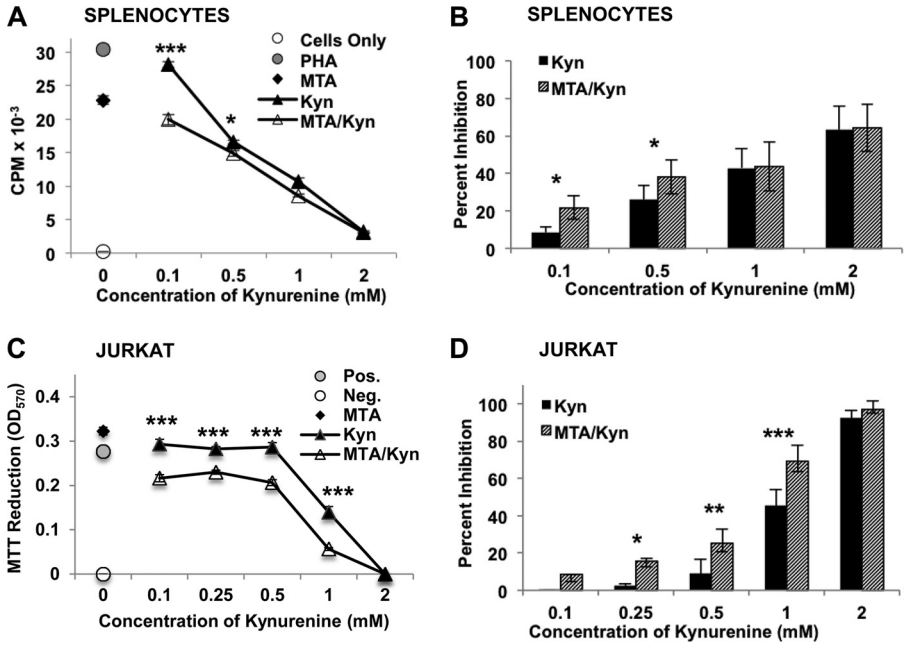

FIG 3 Synergism of MTA and Kyn. (A) Inhibition of PHA-stimulated *X. laevis* spleen cells by Kyn alone at the concentrations shown, MTA alone (10 μM), or Kyn plus 10 μM MTA at the concentrations shown (data from one representative of seven similar experiments). (B) Percent inhibition of PHA-stimulated splenocytes induced by Kyn alone or MTA and Kyn in seven replicate experiments. (C) Inhibition of Jurkat T cells by Kyn alone at the dilutions shown, MTA alone (10 μM), or Kyn plus 10 μM MTA at the concentrations shown (representative of data from five similar experiments). The addition of etoposide (12.5 μg/ml) as a negative control (Neg.) for Jurkat cells completely inhibited growth. (D) Percent inhibition of Jurkat cells by Kyn alone or MTA plus Kyn in five replicate experiments. For all panels, error bars show standard errors, and the indicated MTA/Kyr treatments were significantly different from the Kyr-alone treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (determined by two-way analysis of variance with Bonferroni comparisons). For panels B and D, analysis of variance was done with multiple repeats.

was also detected in the *B. dendrobatidis* Sup and is known to have immunosuppressive effects (20–22). Kynurenine was inhibitory for frog splenocytes (*X. laevis*) at supraphysiological concentrations of 1 to 2 mM (Fig. 2A and B) and for Jurkat cells at concentrations of >0.5 mM (Fig. 2C and D), well above the concentrations found in human or mouse serum (2 to 6 μM) (23, 24). Although Kyn alone was inhibitory only at micromolar concentrations, the addition of 10 μM MTA (a suboptimal concentration) significantly enhanced the inhibitory activity of Kyn at much lower concentrations (0.1 to 0.5 mM) in PHA-stimulated frog splenocytes (Fig. 3A and B) and Jurkat cells (Fig. 3C and D), suggesting a synergistic inhibition of T cell proliferation. Preliminary HPLC analysis of supernatants of purified *B. dendrobatidis* zoospores showed an absence of the UV-visible peaks for MTA and tryptophan.

DISCUSSION

Possible roles of *B. dendrobatidis* supernatant factors in *B. dendrobatidis* metabolism and immune evasion in frogs. MTA is a product of the methionine salvage pathway common to prokaryotes and eukaryotes (25). A search of the *B. dendrobatidis* genome (Broad Institute *Batrachochytrium dendrobatidis* Database [http://www.broadinstitute.org/annotation/genome/batrachochytrium_dendrobatidis/MultiHome.html]) shows the presence of enzymes necessary for the synthesis of MTA as well as enzymes that use MTA as a metabolic precursor to adenine and methionine (25). Thus, MTA is a natural product of *B. dendrobatidis* metabolism. We estimate its concentration in a 10× *B. dendrobatidis* Sup (previously shown to be strongly inhibitory [11]) to be ~2 μM. Its abundance in a cell-free *B. dendrobatidis* Sup may result from the stress of a rapid shift from a rich growth medium (tryptone broth) to water during the preparation of the supernatant shown in Fig. 1A. Alternatively, it may be a natural product of rapidly growing *B. dendrobatidis* cells that accumulates when fungal cells grow at a high cell density, as occurs in bacterial biofilms (26). Its role in *B. dendrobatidis* metabolism awaits further study, but its presence in inhibitory supernatants suggests that it may be one factor that contributes to the immune evasion of *B. dendrobatidis in vitro* and *in vivo* (11, 12). Although the mechanism of action of MTA in the inhibition of lymphocytes is not well understood, this small molecule can inhibit both T lymphocytes and B lymphocytes in mammalian model systems, and it has been proposed for use in the treatment of melanoma and autoimmune disorders (18, 27). Thus, if it is produced abundantly and released by *B. dendrobatidis* within the skin, it could contribute to local immune suppression, allowing zoosporangia to mature and release more propagules onto the skin surface to support further colonization.

The concentrations of tryptophan and Kyn released by *B. dendrobatidis* within the skin compartment are unknown. However, it seems unlikely that Kyn is released at a sufficiently high concentration to directly inhibit lymphocyte viability. Instead, it may have the potential for blunting inflammatory responses *in vivo*. Tryptophan metabolism, particularly the oxidation product Kyn, promotes regulatory T cell development (20). An analysis of the transcriptional response of *X. laevis* following exposure to *B. dendrobatidis* showed that some proinflammatory responses were downregulated (28). Regulatory T cells produce interleukin-10

(IL-10), which suppresses inflammatory responses (29). A recent study showed that IL-10 was upregulated on the skin of *Atelopus zeteki* frogs during *B. dendrobatidis* infection (13). *Candida albicans*, a fungal pathogen of humans, manipulates tryptophan metabolism, promoting the production of 5-hydroxytryptophan to decrease inflammatory type 17 responses, which are typically protective against fungal infections (30). Tryptophan and its oxidation products released by *B. dendrobatidis* might inhibit inflammation, potentially explaining the lack of leukocyte recruitment during chytridiomycosis (1, 3). The *B. dendrobatidis* genome (Broad Institute *Batrachochytrium dendrobatidis* Database [http://www.broadinstitute.org/annotation/genome/batrachochytrium_dendrobatidis/MultiHome.html]) also shows the presence of indoleamine 2,3-dioxygenase, which is involved in the metabolism of tryptophan to Kyn. Cancer cells have been shown to release Kyn, stalling effector lymphocytes (31). Thus, *B. dendrobatidis* may be actively converting tryptophan to Kyn and releasing it in the local skin environment to create a barrier to effective T cell immunity. The synergism of MTA with Kyn was a novel and unexpected finding. There appear to be no previous reports of these agents acting together to inhibit lymphocyte proliferation. The presence of a very small amount of MTA can greatly facilitate the activity of Kyn, bringing it closer to the perceived physiologically important range of activity. Taken together, our results suggest that rapidly growing *B. dendrobatidis* cells have the potential to alter the local microenvironment in the skin and inhibit effective T cell-mediated fungal clearance.

Tryptophan and MTA may be considered natural primary metabolites of *B. dendrobatidis*. Many fungi produce other small molecules, which have been termed secondary metabolites, that are produced at defined and restricted stages of the life cycle and are dispensable for growth (32). In ongoing studies, we plan to look for new secondary metabolites produced by *B. dendrobatidis* that may have other pharmacological properties.

Future studies of possible roles of *Batrachochytrium salamandrivorans* supernatant factors. In addition to *B. dendrobatidis*, a second species in the genus *Batrachochytrium* (*B. salamandrivorans*) was reported in 2013 (33). Unlike *B. dendrobatidis*, which infects and causes pathogenesis in frogs, toads, and salamanders, *B. salamandrivorans* appears to be highly pathogenic to salamanders but spares frogs and toads (34). Thus, these two species appear to have distinct pathogenic profiles, and determining the lymphotoxicity and metabolic profile of *B. salamandrivorans* will be an important area for future research.

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