The Antifungal Drug Amphotericin B Promotes Inflammatory Cytokine Release by a Toll-like Receptor- and CD14-dependent Mechanism*

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Amphotericin B is the most effective drug for treating many life-threatening fungal infections. Amphotericin B administration is limited by infusion-related toxicity, including fever and chills, an effect postulated to result from proinflammatory cytokine production by innate immune cells. Because amphotericin B is a microbial product, we hypothesized that it stimulates immune cells via Toll-like receptors (TLRs) and CD14. We show here that amphotericin B induces signal transduction and inflammatory cytokine release from cells expressing TLR2 and CD14. Primary murine macrophages and human cell lines expressing TLR2, CD14, and the adapter protein MyD88 responded to amphotericin B with NF-κB-dependent reporter activity and cytokine release, whereas cells deficient in any of these failed to respond. Cells mutated in TLR4 were less responsive to amphotericin B stimulation than cells expressing normal TLR4. These data demonstrate that TLR2 and CD14 are required for amphotericin B-dependent inflammatory stimulation of innate immune cells and that TLR4 may also provide stimulation of these cells. Our results provide a putative molecular basis for inflammatory responses elicited by amphotericin B and suggest strategies to eliminate the acute toxicity of this drug.

Amphotericin B (AmB) is the mainstay of therapy for severe, fatal fungal infections due to the high efficiency and potency of this drug against many fungi. AmB, a polyene antifungal, is an amphipathic fermentation product of the Gram-positive bacterium, Streptomyces nodosus. AmB kills fungi by binding to the fungal membrane sterol, ergosterol, thus forming pores that cause lethal leakiness to the fungal membrane. AmB has the broadest spectrum of all available antifungal agents, and despite extensive use, fungal resistance to AmB is rare (1).

Unfortunately, therapeutic use of AmB is limited by its toxicity to patients, including acute, infusion-related toxicities of fever, chills, anorexia, nausea, and tachypnea, as well as chronic renal toxicity (1). AmB is insoluble in water; thus, conventional drug preparations consist of a parenterally administered suspension of AmB admixed with deoxycholate (1). Infusion-related toxicity occurs in up to 70% of patients (2, 3) receiving AmB. These are generally seen 1–3 h after the start of the infusion and mimic a “sepsis-like syndrome” characterized by inflammatory cytokine release (4).

AmB toxicity has provided the impetus for the development of multiple, alternative antifungal drugs, including lipid formulations of AmB, the azoles, and the echinocandins. Despite these alternate therapies, AmB is still widely administered because it is relatively inexpensive, is rapidly fungicidal, has the broadest spectrum of antifungal activity, and rarely induces resistance.

AmB dosages of 0.3–1.5 mg/kg/day are commonly administered to patients, resulting in peak AmB plasma concentrations of ~2 μg/ml (1). With extended use, AmB accumulates in the liver, lung, and kidneys, and at these sites, as well as the site of infusion, AmB concentrations may exceed 2 μg/ml. However, at concentrations above 5 μg/ml, AmB is cytotoxic, leaving a narrow therapeutic index for this drug. Nonetheless, after one-half century of clinical use, despite considerable toxicity and discomfort of administration, AmB remains the gold standard for the treatment of many life-threatening fungal infections.

AmB stimulates transcription and production of inflammatory cytokines (TNF-α, IL-6, IL-1Ra, IL-1β), chemokines (IL-8, MCP-1, MIP-1β), nitric oxide, prostaglandins, and ICAM-1 (intercellular adhesion molecule-1) from murine and human innate immune cells (IC) in vitro (2–4). Moreover, in vivo induction of TNF-α, IL-6, IL-1Ra, and IL-1β has been demonstrated in persons administered AmB (4). The remarkable similarities between AmB infusion-related toxicities and many of the manifestations of sepsis led us to hypothesize that similar mechanisms could be responsible for both (5, 6).

Toll-like receptors (TLRs) represent a family of conserved, mammalian cellular receptors (TLR1-TLR10 in humans and TLR1-TLR9 in mice) (6, 7). Microbial products are recognized by TLRs on IC and stimulate inflammatory cytokine release.
from these cells via TLR-dependent signal transduction. Ligands for TLR2 and TLR4 have been the best studied. Examples of TLR2 ligands include Gram-positive bacteria, peptidoglycan, lipoteichoic acid, and zymosan, whereas TLR4 ligands include LPS from Gram-negative bacteria, Taxol, and Cryptococcus neoformans capsular polysaccharide (6, 8).

Toll-like receptors bear homology to the IL-1 receptor and share a similar signaling cascade that activates inflammatory cytokine genes, including TNF-α and IL-8 (6, 8–11). Briefly, it is presumed that receptor-ligand interactions trigger polymerization of TLRs and result in recruitment of the IRAK (IL-1 receptor-associated kinase)-bound adapter protein, MyD88 (myeloid differentiation primary response gene 88). In the case of TLR4-dependent LPS signaling, another protein (Mal/TIRAP) may also serve as an adapter molecule with or in place of MyD88 (12). In most cases, however, complexation of TLRs, MyD88, and IRAK(s) promotes autoprophosphorylation of IRAK(s), initiating a signaling cascade resulting in the activation of NF-κB as well as p38 and JNK MAP kinases (6). The coordinate effect of NF-κB and MAPK activation enables cytokine gene expression and results in cytokine production and secretion. For example, the interaction of the bacterial product, LPS, specifically with TLR4 and CD14 receptors on IC, induces inflammatory signaling in IC, resulting in cytokine production (13, 14) and many of the manifestations of Gram-negative bacterial sepsis (13).

Because AmB is a microbial product that mimics these downstream events of TLR activation (inflammatory cytokine and chemokine release), we hypothesized that AmB stimulates inflammatory cytokine release from IC via TLR-mediated signal transduction. In many cases, the glycosylphosphatidylinositol-anchored co-receptor, CD14, is required for optimal responses to TLR2 and TLR4 agonists, particularly the amphiphilic molecule, LPS (14). As AmB is also amphiphilic, we postulated that CD14 could be required for AmB to stimulate inflammatory cytokines via TLRs. To address these hypotheses, we performed experiments to determine the dependence of AmB-induced inflammatory responses on TLRs and CD14. As 1) major side effects of AmB include fever and chills; and 2) as these are in part thought to be mediated by NF-κB secretion; and 3) as parasitoids have elevated plasma TNF-α levels in response to AmB intravenous administration, we chose to investigate the NF-κB response following AmB stimulation in cells with varied TLR expression. We analyzed TNF-α production, and in selected cases IL-1β production, following AmB stimulation of peritoneal macrophages (pMΦs) from mice mutated in the adapter protein MyD88, TLR2, TLR4, or CD14. We also examined AmB-dependent induction of an NF-κB-driven reporter gene in the human monocytic cell line, THP-1, overexpressing CD14, as well as IL-8 production and NF-κB-dependent reporter activity in the HEK293 human embryonic kidney cell line transiently transfected with TLRs and/or CD14. We found that TLR2 and CD14, acting through the adapter protein MyD88, are required for AmB-induced NF-κB signal transduction and proinflammatory cytokine release. We found that TLR4 may also function as a signaling receptor for AmB to induce cytokine release. These data demonstrate a receptor-mediated mechanism for the influx-related toxicity of AmB and specify targets for therapeutic strategies to eliminate these untoward side effects.

**EXPERIMENTAL PROCEDURES**

**R eagents—**Thiglycollate (Remel, Lenexa, KS), RPMI 1640, Genetin (sensitivity to 15 pg/ml), mouse IL-1β, and Genetin (0.5 μg/ml, for stable cells).

**Luciferase Assays—**THP-1 and HEK293 cells were transiently transfected using DEAE-dextran (22) and Polyfect reagent (Qiagen, Valencia, CA), respectively. Plasmids transfected contained genes coding for the NF-κB-dependent pELAM-luciferase reporter (23), TLR2 (24), TLR4 (25), CD14 (26), and MD2 (27). Cells (5 × 10^4 THP-1 or 1 × 10^5 HEK293) were added to 12-well plates, washed after 18 h, and stimulated for 5 h. Cells were then lysed with reporter lysis buffer (Promega, Madison, WI) as directed, and lysates were analyzed for luminescence using Promega luciferase substrate and a Monolight 3010 luminometer (Pheramgen).

**ELISA—** Supernatants from peritoneal macrophages or HEK293 cell lines were harvested and stored at –20 °C until use. Sandwich ELISAs were performed on the same plates using DuPont ELISA kits for mouse TNF-α (sensitivity to 15 pg/ml), mouse IL-1β (sensitivity to 15 pg/ml), and human IL-8 (sensitivity to 10 pg/ml) (R&D Systems) as per the manufacturer’s instructions. ELISA plates were read at 450 nm, and data were collected and analyzed using SOFTMAX PRO software (Molecular Devices Corp., Sunnyvale, CA).
RESULTS

MyD88 Is Required for AmB-induced TNF-α Production from Macrophages—TLR signaling in IC generally results in the activation of NF-κB and MAP kinases via the adapter protein, MyD88, to stimulate production of the inflammatory mediators TNF-α and IL-8 (28). To determine the role of MyD88 in AmB-dependent stimulation of IC, we compared the responses of peritoneal macrophages from normal (C57BL/6 MyD88+/+) mice with those from mice containing a deletion of the MyD88 gene (C57BL/6 MyD88−/−). MyD88−/− macrophages failed to respond to AmB stimulation. In contrast, MyD88+/+ cells secreted TNF-α in a dose-dependent fashion in response to AmB stimulation (Fig. 1). Deoxycholate (DC), at concentrations present in the AmB suspension, stimulated neither the wild-type nor the null cells. Consistent with previous data, LPS (29) stimulated greatly reduced amounts of TNF-α in the null cells, whereas the MyD88-dependent stimulus, peptidoglycan (11), did not stimulate detectable TNF-α production from MyD88−/− cells. These data suggest a role for TLRs, acting through the adapter protein, MyD88, in AmB-stimulated cytokine production.

TLR2 Is Required for AmB-induced TNF-α and IL-1β Production from Macrophages—Amphotericin B is a product of the Gram-positive bacterium, S. nodosus. Therefore, we surmised that IC might recognize AmB as they do other Gram-positive bacterial products, via TLR2 (25). We compared the responses of C57BL/6 TLR2−/− peritoneal macrophages with those of C57BL/6 TLR2+/+ peritoneal macrophages and found that unlike TLR2+/+ macrophages, TLR2−/− macrophages did not produce TNF-α in response to AmB stimulation (Fig. 2). In these experiments, the TLR2-dependent stimulus, B. burgdorferi lysate (30), stimulated responses only in TLR2+/+ cells, whereas the TLR2-independent stimulus, LPS (16), stimulated similar responses in TLR2−/− and TLR2+/+ cells. DC did not stimulate TNF-α production from either cell type.

To determine whether AmB induced IL-1β production from elicited peritoneal mouse macrophages, we compared IL-1β production from C57BL/6 TLR2−/− and C57BL/6 TLR2+/+ peritoneal macrophages in response to AmB. TLR2+/+ macrophages produced IL-1β when stimulated with 1, 2, and 4 μg/ml AmB (data not shown). In contrast, TLR2−/− macrophages secreted undetectable amounts of IL-1β in response to AmB stimulation.

AmB-induced TNF-α Production from Macrophages Is Partially TLR4-dependent—Due to the similarity between physiologic responses to LPS and AmB, we investigated whether TLR4 (the signaling receptor for LPS) could also act as a signaling receptor for AmB. We examined the TNF-α response of C3H/HeJ (TLR4−/−) peritoneal macrophages that express mutant, non-functional TLR4 (31) in comparison with those of the C3H/HeOuJ (TLR4+/+) mice, which are genetically similar to the C3H/HeJ mice but express wild-type TLR4 (31). As such, the TLR4-dependent stimulus, LPS, could only stimulate high levels of TNF-α from the C3H/HeOuJ mice (Fig. 3). In contrast, AmB stimulated similar amounts of TNF-α release from both types of macrophages in response to 2 μg/ml AmB (Fig. 3). The responses of C3H/HeJ macrophages were reduced by 69% (p = 0.021) however, in comparison with those of the C3H/HeOuJ macrophages stimulated with 4 μg/ml AmB. This dose exceeds the peak plasma concentration of AmB generally achieved in patients (~2.5 μg/ml for AmB-deoxycholate (32, 33)) and therefore may not represent a response that is present in most cases in vivo.

CD14 Is Required for AmB-induced TNF-α Production from Macrophages—Many TLR2 and TLR4 ligands are dependent on the CD14 co-receptor for optimal TLR activation. To determine the dependence of the TLR signaling response to AmB on CD14, we compared the TNF-α response of peritoneal macrophages from CD14 knockout mice (C57BL/6 CD14−/−) with those from CD14 wild-type (C57BL/6 CD14+/+) mice. As such, AmB-stimulated similar amounts of TNF-α release from both types of macrophages in response to 2 μg/ml AmB (Fig. 3). The responses of C3H/HeJ macrophages were reduced by 69% (p = 0.021) however, in comparison with those of the C3H/HeOuJ macrophages stimulated with 4 μg/ml AmB. This dose exceeds the peak plasma concentration of AmB generally achieved in patients (~2.5 μg/ml for AmB-deoxycholate (32, 33)) and therefore may not represent a response that is present in most cases in vivo.

CD14 Overexpression Renders Human Monocytic Cells Responsive to AmB—The THP-1 human monocyte cell line naturally expresses TLR2, TLR4, and very low levels of CD14 (21). We found that THP-1 cells stably overexpressing CD14 (THP1-CD14) responded, in a dose-dependent fashion, to AmB stimulation with robust NF-κB-dependent luciferase production, whereas THP-1 cells stably transfected with empty vector (THP1-RSV) did not respond (Fig. 5). Addition of polymyxin B (PB) prior to AmB did not affect AmB-elicted responses, demonstrating that the AmB-dependent NF-κB stimulation of luciferase production was not due to endotoxin contamination (Fig. 5). DC, with or without PB, did not stimulate either cell
The CD14-dependent stimulus, LPS, induced a response only in cells overexpressing CD14, and this response was inhibited by PB. The CD14-independent stimulus, TNF-α/H9251, stimulated similar responses in both cell types, demonstrating that both were able to activate NF-κB-dependent gene expression. Thus, the data with the THP-1 cell lines lend support to our findings with the CD14 knockout mice that CD14 is required for AmB stimulation.

TLR2 expression renders HEK293 cells responsive to AmB—HEK293 human embryonic kidney cell lines do not express messages for TLR2, TLR4 (34), 2 and CD14 (35) and require exogenous CD14 (23, 36) for CD14-dependent responses. We transiently transfected HEK293 cells with the NF-κB-dependent reporter plasmid, pELAM-luc, alone (luc) or in combination with a plasmid containing the gene for human TLR2 (luc+TLR2). Cells were then stimulated with either AmB (2 μg/ml) or the TLR2-dependent stimulus B. burgdorferi (Fig. 6c). Only cells transfected with TLR2 responded to AmB and B. burgdorferi with NF-κB-dependent luciferase production. These data provide further support for a role for TLR2 as a signaling receptor for AmB. These experiments do not exclude a requirement for CD14 in addition to TLR2 as HEK cells are able to utilize soluble CD14 from FBS present in cell culture media (23, 35, 36).

Fig. 2. TLR2 expression is required for amphotericin B-induced TNF-α production. TNF-α production by thioglycollate-elicited peritoneal macrophages from C57BL/6 TLR2+/− and C57BL/6 TLR2−/− mice stimulated for 18 h with AmB, deoxycholate vehicle control (DC), LPS, B. burgdorferi lysate (BB), or left unstimulated is shown. TNF-α measurements were determined by sandwich ELISA and are based on a standard curve for purified murine TNF-α. The results are expressed as the mean plus standard error for n = 8. *, p < 0.05 versus unstimulated cells of the same cell type by one-way ANOVA.

Fig. 3. Amphotericin-B-induced TNF-α production is diminished in TLR4 mutant macrophages. TNF-α production by thioglycollate-elicited peritoneal macrophages from C3H/HeJ TLR4<sup>−/−</sup> and C3H/HeOuJ TLR4<sup>−/−</sup> mice stimulated for 18 h with AmB, deoxycholate vehicle control (DC), LPS, or left unstimulated is shown. TNF-α measurements were determined by sandwich ELISA and are based on a standard curve for purified murine TNF-α. The results are expressed as the mean plus standard error for n = 3. *, p < 0.05 versus unstimulated cells of the same cell type by one-way ANOVA. #, p < 0.05 versus C3H/HeJ cells stimulated with the same stimulus by one-way ANOVA.

TLR2 expression renders HEK293 cells responsive to AmB—HEK293 human embryonic kidney cell lines do not express messages for TLR2, TLR4 (34), 2 and CD14 (35) and require exogenous CD14 (23, 36) for CD14-dependent responses. We transiently transfected HEK293 cells with the NF-κB-dependent reporter plasmid, pELAM-luc, alone (luc) or in combination with a plasmid containing the gene for human TLR2 (luc+TLR2). Cells were then stimulated with either AmB (2 μg/ml) or the TLR2-dependent stimulus B. burgdorferi (Fig. 6c). Only cells transfected with TLR2 responded to AmB and B. burgdorferi with NF-κB-dependent luciferase production. These data provide further support for a role for TLR2 as a signaling receptor for AmB. These experiments do not exclude a requirement for CD14 in addition to TLR2 as HEK cells are able to utilize soluble CD14 from FBS present in cell culture media (23, 35, 36).

<sup>2</sup> D. T. Golenbock, unpublished data.
tions tested. In addition to CD14 and TLR4, MD2 is required for LPS-stimulation of HEK293 cells (27, 35). However, whereas transient transfection of HEK293 cells with CD14, TLR4, and MD2 rendered HEK293 cells responsive to LPS, it did not render them responsive to AmB (Fig. 6b). Moreover, co-transfection with MD2 did not augment AmB-stimulated responses of HEK293 cells transfected with CD14 and TLR2 (data not shown). These data suggest that MD2 does not play a role in TLR-mediated responses to AmB and further exclude LPS contamination of our AmB preparation. For all experiments with transiently transfected HEK293 cells, IL-8 production paralleled NF-κB-dependent luciferase production (data not shown). In all experiments, DC stimulation induced only background levels of NF-κB-dependent reporter activity and cytokine production, similar to unstimulated cells.

**HEK293 Cells and Peritoneal Macrophages Expressing TLR2 and CD14 Fail to Respond to Lipid Formulations of AmB—**

Cellular responses to the conventional deoxycholate AmB preparation were compared with responses to two lipid formulations of AmB, L-AmB and ABLC. HEK293 cells were transiently transfected with the pELAM-luc plasmid (indicated by luc on the figure), along with combinations of plasmids containing genes for TLR2, TLR4, CD14, or MD2. Cells were then stimulated with AmB (1, 2, or 4 μg/ml), L-AmB (2, 4, 10, or 40 μg/ml), ABLC (2 or 4 μg/ml), MALP-2 (10 μg/ml), or LPS (100 ng/ml) (Fig. 6c). As in previous studies, responsiveness of HEK293 cells to AmB was conferred upon transfection of TLR2 and CD14 but not upon transfection of TLR4 with CD14 and MD2. However, the lipid formulations of AmB did not stimulate NF-κB-dependent luciferase production from either of the two transfected HEK293 cell lines. Consistent with these data, C57BL/6 CD14−/− peritoneal macrophages produced very little TNF-α or IL-1β in response to L-AmB or ABLC lipid formulations of AmB (data not shown). These data suggest that
FIG. 6. HEK293 cells expressing TLR2 or TLR2 and CD14 produce luciferase from an NF-κB-dependent reporter construct following amphotericin B stimulation. As shown in a–c, HEK293 cells were transiently transfected with the NF-κB-dependent pELAM-luc luciferase reporter and either TLR2, TLR4, CD14, or MD2. Cells were transfected using Polyfect and placed in culture at 2.5 × 10⁵ cells/well. After 18 h of culture, cells were stimulated with AmB, deoxycholate vehicle control (DC), L-AmB, ABLC, LPS, B. burgdorferi lysate (BB) (a TLR2-dependent stimulus), MALP-2 (a TLR2-dependent stimulus), or were left unstimulated. After 5 h of stimulation, cells were harvested and assayed for luciferase production. Results are expressed as -fold induction over unstimulated cells for each transfection group for a representative experiment of 2–5 experiments.
at concentrations found in serum, the lipid formulations of AmB do not stimulate TLR/CD14-dependent responses to a significant degree.

Cytotoxicity Is Not Responsible for AmB Inductive Effect in Our Studies—Finally, we verified that doses of AmB used in these experiments were not causing cell death or significant membrane permeability. Peritoneal macrophages, THP1-CD14, THP1-RSV, and HEK293 cells treated with the AmB concentrations used in our assays (up to 4 μg/ml) did not show evidence of cellular toxicity by visual inspection and with the LIVE/DEAD viability/cytotoxicity assay (Molecular Probes, Eugene, OR; data not shown).

DISCUSSION

The data presented herein, taken together, indicate a role for TLR2 and CD14 as signaling receptors for AmB in IC. Our data demonstrate that, at therapeutic concentrations found in human serum, AmB stimulates proinflammatory cytokine production from murine peritoneal macrophages and human monocytic and HEK293 cell lines. These data correlate with the results of Arning et al. (4), who found elevated TNF-α, IL-6, and IL-1ra in the plasma of patients given AmB, and with McCarthy et al. (38), who found that human peripheral blood mononuclear cells stimulated ex vivo with AmB produce IL-1β.

In our study, the AmB-dependent release of the proinflammatory cytokines TNF-α and IL-8 is dependent on TLR2 expression and is enhanced by the expression of the CD14 co-receptor. The AmB-dependent release of the proinflammatory cytokine IL-1β is also dependent on TLR2 expression. Thus, macrophages from mice null for TLR2 or CD14 failed to make TNF-α when stimulated with AmB. Likewise, macrophages from mice null for TLR2 failed to make IL-1β when stimulated with AmB. Moreover, transfection of TLR2 and CD14 into HEK293 cells rendered them responsive to AmB stimulation. As activation of TLR2 with CD14 expressed on innate immune cells mediates initiation of inflammatory responses, we have thus ascribed a putative molecular mechanism for the acute, inflammatory toxicity elicited by AmB in patients.

Interestingly, lipid formulations of AmB did not stimulate significant cytokine production from mouse peritoneal macrophages, nor did they induce TLR/CD14-dependent NF-κB activation in HEK293 cells. These data correlate with the significantly reduced toxicity of the lipid formulations relative to conventional AmB for patients (1). We speculate that the lack of response elicited by lipid formulations of AmB is due to the low concentration of free, non-lipid-associated AmB in these preparations. Amphoterin B in the lipid preparations is embedded within lipid micellar/bilayer structures, leaving little of the molecule exposed for interaction with TLR2/CD14 or any other receptor.

Our data comparing macrophages from C3H/HeJ and C3H/HeOuJ mice indicate that, in addition to TLR2, TLR4 may play a role in AmB-dependent signaling. However, this effect was induced only with AmB concentrations above those commonly found in the serum of patients receiving this drug. Moreover, HEK293 cells transfected with TLR4, with or without CD14 and MD2, did not respond to AmB. Nonetheless, AmB stimulated an NF-κB-dependent reporter gene when Chinese hamster ovary cells were stably transfected with TLR4 and CD14,3 despite the fact that Chinese hamster ovary cells do not express functional TLR2 (39). Interestingly, recent reports have noted interspecies variation in TLR expression and activation (40, 41). Thus, under some conditions, TLR4 may act as a signaling receptor for AmB.

TLR family members may cooperate by forming signaling complexes to generate a TLR-mediated signaling event. Reports have demonstrated the cooperative function of TLR1 or TLR6 co-receptors with TLR2 on IC for stimulation by some TLR2-specific ligands (42–44). Other TLR2 ligands presumably also stimulate TLR2 heterodimers to initiate a signaling response, as TLR2 homodimers cannot initiate a signaling event (45). This cooperative function may serve to increase the diversity of the signaling responses a cell has at its disposal. Because HEK293 cells express message for TLR1 and TLR6 (34),2 our data do not preclude the involvement of TLR1 or TLR6 as part of the TLR2/CD14 receptor complex required for AmB stimulation of IC. Moreover, whereas our data demonstrate that TLR2-CD14 form a likely candidate receptor complex for AmB, we have also shown that TLR4 can mediate responses to AmB in some systems. Thus, TLR4 may function as an alternate receptor that, in complex with TLR2 and CD14 (or in lieu of TLR2), enhances AmB responses. This type of cooperation between TLR2 and TLR4 has been reported for Pseudomonas aeruginosa Poly-M (46), HSP60 (47), and Entero- coccus hirae glycolipid (48).

Other investigators have suggested that the infusion-related toxicity experienced by patients given AmB stemmed from the toxicity of AmB for cell membranes (49, 50). Specifically, because AmB interacts and intercalates with cholesterol and phospholipids in mammalian cell membranes (49, 50), it was believed that AmB formed pores in these membranes. However, more recent studies demonstrate that the toxicity of AmB for mammalian cells occurred at AmB concentrations that far exceeded the peak plasma concentrations (2.5 μg/ml) generally attainable in patients (50, 51). We found that the AmB concentrations utilized in our studies had no direct cellular toxicity. Indeed, our data support the concept that the inflammatory effects of AmB are distinct from the membrane-related toxicity of AmB (50). The latter may be responsible for some of the chronic toxicity associated with AmB treatment, as with long term use, AmB may accumulate in specific organs.

AmB binds and intersperses with cholesterol and phospholipids and can transiently concatameterize into multimer formations within cell membranes. Thus, AmB can modify the ordered, cholesterol-, and glycolipid-rich microdomains, termed lipid rafts, causing aggregation of raft matrices (52, 53). Alterations in lipid raft composition and distribution have been associated with signal transduction (53). As CD14 is always present within lipid rafts, and TLR4 (54, 55), and possibly, TLR2 associate with lipid rafts during activation, it is possible that AmB could stimulate multiple receptors through indirect routes such as lipid raft clustering (56), reorganization of rafts (53), or redistribution of lipid raft proteins (56). We believe that the ability of AmB to create more ordered lipid structures in cellular membranes will facilitate the interaction of AmB with multiple TLRs as these receptors associate with the lipid moieties and signaling complexes forming within the plasma membrane. The reduced TLR- and CD14-dependent responses that we observed with the lipid formulations of AmB could be the result of a diminished association of AmB with cellular membrane lipids and cholesterol. We are currently investigating these intriguing hypotheses. Preliminary data demonstrate that AmB has a considerable effect on lipid raft distribution and TLR surface expression.4

The AmB preparation that we used is the conventional preparation given to patients. As AmB is a microbial product, the possibility that the TLR-stimulatory capacity of our AmB is due to a minor contaminant of the pharmaceutical preparation or due to the combinatorial effect of AmB with deoxycholate

3 K. Sau and S. M. Levitz, unpublished data.

4 K. Sau, E. Latz, and S. M. Levitz, unpublished data.
must be considered. We feel that this is unlikely for three reasons. First, the pharmaceutical preparation we used is a highly pure, certified endotoxin-free preparation. In accordance with USP guidelines, the AmB used in our studies was certified by the manufacturer to contain fewer than 0.005 endotoxin units/μg. We confirmed that our preparation contained fewer than 0.005 endotoxin units/μg of AmB (see Experimental Procedures). Also, in our studies, the LPS antagonist polymyxin B did not inhibit responses to AmB (see Fig. 5). Second, by NMR spectroscopic analysis, no contaminants were observed in our AmB preparations. 3 Third, high pressure liquid chromatography-purified AmB (Sigma) reconstituted with Me2SO rather than deoxycholate stimulates CD14- and TLR-dependent signaling. 4 Therefore, we believe that bona fide AmB is causing the observed TLR-dependent stimulation of inflammatory mediators.

Serum constitutes ~50% of blood, and serum factors are known to bind AmB (1). However, in two independent experiments, we found no differences between the responses elicited by AmB (TLR2 and CD14-dependent NF-κB activation) in the presence of 50% serum when compared with stimulation in the presence of 10% serum. 5

In conclusion, we find that AmB stimulates inflammatory cytokine production from innate immune cells via an interaction that requires CD14 and TLRs. These data demonstrate a putative mechanism for the long recognized, inflammation-related toxicity of AmB. Our work demonstrates that the induction of inflammatory cytokines following AmB treatment is a direct effect of AmB on blood and tissue cells possessing signaling molecules of the TLR family. This mechanism also requires the expression of the integral lipid raft protein, CD14. We predict that AmB utilizes CD14 perhaps as LPS does, to allow transfer of AmB to the plasma membrane in the context of TLR signaling complexes (55). Our data provide a rationale for AmB-deprivation complexes (55). Our data provide a rationale for AmB-de-