Liposomal amphotericin B activates antifungal resistance with reduced toxicity by diverting Toll-like receptor signalling from TLR-2 to TLR-4

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Objectives:: Neutrophils play a crucial role in the control of the Aspergillus fumigatus infection and act in concert with antifungal drugs. This study was undertaken to obtain insights into the possible involvement of Toll-like receptors (TLRs) in the interaction of liposomal amphotericin B (L-AmB; AmBisome) with neutrophils in response to A. fumigatus.

Methods: For generation of bone marrow-transplanted mice, irradiated C57BL6 mice were infused with T cell-depleted allogeneic donor cells. For infection, mice were injected intranasally with Aspergillus fumigatus conidia and treated with L-Amb and deoxycholate amphotericin B prophylactically or therapeutically. For TLR-dependent antifungal functions, murine neutrophils were preincubated with antifungals or TLR ligands before the addition of Aspergillus conidia.

Results: The results show that: (a) neutrophil activation by Aspergillus occurs through TLR signalling pathways differently affecting the oxidative and non-oxidative mechanisms of the killing machinery; (b) by diverting signalling from TLR-2 to TLR-4, liposomes of AmBisome activate neutrophils to an antifungal state while attenuating the pro-inflammatory effects of deoxycholate amphotericin B; (c) this translates in vivo to the optimization of the AmBisome therapeutic efficacy in mice with aspergillosis.

Conclusions: These results provide a putative molecular basis for the reduced infusion-related toxicity of AmBisome and suggest that TLR manipulation in vivo is amenable to the induction of optimal microbicidal activity in the absence of inflammatory cytotoxicity to host cells.

Keywords: AmBisome, Aspergillus fumigatus, neutrophils

Introduction

Invasive aspergillosis (IA) is an important cause of morbidity and mortality in patients with cancer, immunodeficiencies and transplant recipients.¹ Despite a recent expansion in the armamentarium of newer antifungals,² there is a need for therapeutic advances against aspergillosis, as the mortality rate associated with IA is nearly 100% in some patient groups.³ Treatment with deoxycholate amphotericin B (D-AmB; Fungizone), one first drug of choice, is limited by the dose-related toxicity of the drug that precludes full-dose therapy in patients who have undergone bone marrow transplantation (BMT).⁴ Several lipid-based formulations of amphotericin B have been developed to permit delivery of higher doses while sparing toxicity.⁵ This reduction in toxicity occurs primarily through avoidance of renal clearance and infusion-related side effects. Liposomal amphotericin B (L-AmB; AmBisome), a small unilamellar formulation of amphotericin B, differs from several other lipid formulations of amphotericin B in that it forms true liposomes with uniform, stable, spherical single membrane vesicles of <0.1 µm in diameter. The intact liposomes accumulate in infected tissues where their disruption allows amphotericin B to bind to ergosterol in the fungal cell membrane,⁶ thus contributing to the potent fungicidal activity with reduced toxicity of L-AmB.⁷

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It has been postulated that the higher infusion-related toxicity of D-AmB versus L-AmB is the result of pro-inflammatory cytokine production, including tumour necrosis factor (TNF)-α, by innate immune cells. A putative molecular basis for the inflammatory response elicited by D-AmB has recently been ascribed to the stimulation of Toll-like receptors (TLRs)-2, CD14 and the adapter protein MyD88 on macrophages. TLRs are a family of conserved, mammalian cellular receptors that mediate cellular responses to structurally conserved pathogen-associated microbial products. It is recognized that the intricacies of how TLRs signal will ultimately provide an explanation for the molecular basis for how cells involved in innate immunity dictate the processes of host defence specific to the provoking pathogen.

There is no doubt that innate defence mechanisms are primarily responsible for the elimination of the fungus from the lungs and that antifungal drugs must act in collaboration with host phagocytes in the microenvironment of infected tissues. A recent study has shown that amphotericin B formulations exert additive antifungal activity in combination with phagocytes against A. fumigatus. At variance with D-AmB, L-AmB displayed additive effects with polymorphonuclear neutrophils (PMNs) more than with macrophages.

As PMNs are rapidly recruited (within 4h) in the lung upon infection and play a crucial role in the control of the infection and in determining the type of pathology associated with IA in different clinical settings cells, this study was undertaken to obtain insights into the possible mechanisms in vitro and functional consequences in vivo underlying the interactions of Ambisome with PMNs in response to A. fumigatus. With this aim, the involvement of different TLR-activation pathways was evaluated in vitro and in vivo in murine and human PMNs exposed to the fungus in the presence of L-AmB or D-AmB. The results provide evidence that Ambisome attenuates the pro-inflammatory promoting activity of amphotericin B by providing signalling through TLR-4. This translates in vivo to the optimization of the Ambisome therapeutic efficacy in BMT-mice with IA.

**Materials and methods**

**Mice**

Female, 8- to 10-week old, BALB/c and C57BL/6 mice were from Charles River (Calco, Italy). Breeding pairs of homozygous TLR-2- and TLR-4-deficient mice, raised on C57BL/6 background, were bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and Perugia University Animal Care Committee guidelines.

**Microorganism, culture conditions, infection and treatments**

Resting conidia and hyphae from A. fumigatus were obtained as described previously. For infection, mice received 2 × 10⁶ conidia in 20 µL saline intranasally for three consecutive days. Quantification of fungal growth in the lungs was done by the chitin assay and results expressed as micrograms of glucosamine per pair of lungs as described previously. For generation of BMT mice, C57BL/6 mice were exposed to a lethal dose of 9 Gy and infused with T-cell-depleted donor cells from BALB/c mice. D-AmB (Fungizone, Bristol-Myers Squibb, Sermoneta, Italy) and L-AmB (Ambisome, GILEAD, Milan, Italy) were prepared fresh daily in accordance with the manufacturers' instructions and diluted in a 5% glucose–water solution. In accordance with the USP guidelines, D-AmB and L-AmB, used in our study were approved for patient use and certified by the manufacturer to contain fewer than 0.005 endotoxin units/µg. Using the limulus amoebocyte lysate kinetic assay (QLC-1000, BioWhittaker, Walkersville, MD, USA), we confirmed that our preparations contained fewer than 0.005 endotoxin units/µg. Liposomes of similar size, charge and composition (hydrogenated soy phosphatidylcholine and distearoyl phosphatidylglycerol) to those of Ambisome were from Sigma (St Louis, MO, USA) and were prepared as per the manufacturer’s instructions. Small uniform-sized liposomes were obtained by extrusion through a series of polycarbonate filters (Nuclepore, Pleasanton, CA, USA) of decreasing pore size (450 → 220 → 100 nm). Using high-pressure liquid chromatography analysis of our D-AmB, L-AmB and liposome preparations, no contaminants were observed (data not shown). Antifungals were administered intraperitoneally in BMT mice 5 days before Aspergillus infection (prophylactic treatment) or 5 days after the last injection of conidia (therapeutic treatment). Controls received the diluent alone.

**Cell isolation**

Human PMNs were separated from blood of normal donors (>95% pure on FACS analysis and cytoospin) by Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation as described previously. Typical recovery was 3 to 5 million PMNs per millilitre of collected blood. Murine CD11b+Gr-1+ PMNs (>98% pure on FACS analysis and cytoospin) were positively selected with magnetic beads (Miltenyi Biotec, Bologna, Italy) from the peritoneal cavity of mice 8 h after the intraperitoneal injection of 1 mL of endotoxin-free 10% thioglycollate solution (Difco). Endotoxin was depleted from all solutions with Detoxi-gel (Pierce, Rockford, IL, USA). PMNs were isolated before each experiment and used immediately.

**Phagocytosis, cultures and antifungal effector activity**

For phagocytosis, PMNs were incubated at 37 °C with resting Aspergillus conidia for 60 min at an effector to fungal cells ratio of 1:3. The percentage of internalization was calculated on Giemsa-stained cytospin preparations, as described previously. For staining of degranulated PMNs, the cytospin preparations were subjected to Methylene Blue (for azurophil granules) or eosin (for non-azurophil granules) staining following standard procedures. For fungicidal activity, PMNs were incubated with unopsonized resting conidia for 120 min, and the percentage of colony forming unit inhibition (mean ± S.E.M.), referred to as conidiocidal activity, was determined as described. PMN production of reactive oxygen intermediates (ROI) was done by quantifying the superoxide anion (O₂-) production by measuring the SOD-inhibitable reduction in cytochrome c. For TLR stimulation, PMNs were pre-exposed for 120 min with Zymosan (10 µg/mL), LPS (10 µg/mL) from Salmonella minnesota Re 595 (Sigma), D-AmB (1.25 µg/mL) or L-AmB (2.5 µg/mL)
before the addition of conidia without any washing procedure. Anti-
fungals were used at the concentrations that achieved <50% activity
against the fungus when they were used alone. Cytokine production
was assessed in 24 h culture supernatants of PMNs stimulated with
conidia heat-inactivated (to prevent germination) in RPMI 1640
medium with no serum but with polymyxin B, in the presence of
D-AmB or L-AmB at 10 μg/mL, as cytokine expression response to
amphotericin B is known to be dose-dependent.23 Zymosan and LPS
(10 μg/mL) or liposomes at 9.6 μg/mL (a concentration equivalent
to that present in 10 μg/mL of AmBisome). No effects on PMN
availability or degranulation were observed at the concentrations of
antifungal used. Cells were pre-exposed to cytochalasin D,
216 g/mL at 37°C for 30 min. Photographs were taken using a high-
resolution microscopy colour camera, AxioCam Color, using the
AxiVision Software Rel. 3.0 (Carl Zeiss S.p.A., Milan, Italy).

Cytokine assays
The levels of cytokines in culture supernatants were determined by
Kit ELISAs (R&D Systems, Inc., Space Import-Export S.r.l, Milan,
Italy). The detection limits (pg/mL) for the assays were <32 for
TNF-α, <3 for IL-10 and IL-12 p70.

Quantification of cytokine transcripts by RT–PCR
For RT–PCR, total RNA was extracted from PMNs pre-exposed to
L-AmB or D-AmB or exposed to unopsonized Aspergillus conidia
or hyphae for 15 min. The synthesis of cDNA was done as described
previously.17 For real-time RT–PCR, total RNA was extracted from
and reverse transcribed as described previously.18 PCR amplification
of the housekeeping eukaryotic 18S rRNA gene was carried out for
each sample to control for sample loading and allow normalization
between samples as per the manufacturer’s instructions (Applied
Biosystems, Foster City, CA, USA). The eukaryotic 18S rRNA-
normalized data were expressed as relative cytokine mRNA (ΔΔCT)
in experimental groups compared with that of naive mice.18

Statistical analysis
The log-rank test was used for paired data analysis of the Kaplan–
Meier survival curves. Student’s t-test or analysis of variance
(ANOVA) and Bonferroni’s test were used to determine the statisti-
cal significance of differences in organ clearance and in vitro assays.
Significance was defined as P<0.05. In vivo groups consisted of
4–6 animals. The data reported were pooled from 3–5 experiments,
unless otherwise specified.

Results
AmBisome activates PMNs for antifungal activity
Although PMNs are considered to exert antifungal activity at the
level of hyphal formation and growth, PMNs still may phagocytose
resting conidia, as revealed by TEM after 15 min (Figure 1a)
or 120 min (Figure 1b) incubation and kill them (between 22%
and 31% killing at 120 min), an activity that may be of benefit
to the host in conditions of functional impairment of conidioci-
dal activity of alveolar macrophages21 or after swelling of coni-
da.22 A recent study has shown that L-AmB displayed additive
effects with human PMNs in damaging Aspergillus hyphae.16
We have shown that AmBisome also additionally augmented the
conidiocidal activity of both murine (from 20% of PMNs alone
and 18% of L-AmB to 46% in combination) and human (from
22% of PMNs alone and 18% of L-AmB to 49% in combi-
nation) PMNs, a finding suggesting that L-AmB exerts an immu-
nomodulatory role on PMN antifungal effector functions or that
L-AmB renders conidia more susceptible to PMN conidiocidal
activity.

AmBisome induces TLR expression on PMNs exposed
To A. fumigatus
Because D-AmB activated TLR-dependent signalling on mono-
nuclear cells,10 we examined whether TLR expression is acti-
vated by D-AmB on PMNs, known to express TLRs and to
exhibit TLR-dependent responses to microbes.25,26 As D-AmB
induces signal transduction through TLR-2 and, partially,
TLR-4,10 and both TLRs are involved in the host response to the
fungus in vitro and in vivo,15,26 the levels of expression of both
TLRs were assessed in murine and human PMNs upon exposure
to resting conidia and hyphae of Aspergillus and/or L-AmB or
D-AmB. We have shown that the expression of both TLR-2 and
TLR-4 was induced upon exposure of human and murine PMNs
to conidia, whereas TLR-4 expression only was induced upon
exposure to hyphae. D-AmB up-regulated the expression of
TLR-2, while decreasing that of TLR-4 in PMNs unexposed or
after the exposure to both fungal morphotypes. In contrast,
L-AmB seemed to favour the expression of TLR-4 over that of
TLR-2 in unstimulated and stimulated PMNs. No TLRs
expression was induced upon incubation of cells with the anti-
fungal diluent alone (Figure 2). The fact that similar results were
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obtained in the presence of the LPS antagonist polymyxin B (data not shown), confirms the absence of endotoxin contamination. Because L-AmB failed to activate TLR-dependent signalling in macrophages, these data confirm that the effects of L-AmB on the different phagocytic cells are divergent.

AmBisome activates the TLR-4-dependent signalling pathway on PMNs exposed to A. fumigatus

To correlate the pattern of TLR expression with the functional activity of TLRs on PMNs, purified murine PMNs were assessed for their ability to phagocyte and kill conidia, oxidant production and degranulation, as both the oxidative and non-oxidative pathways are essential for the efficient killing of fungi, as well as release of pro-inflammatory and anti-inflammatory cytokines upon exposure to Aspergillus conidia and/or D-AmB, L-AmB, the TLR-2 (Zymosan) and the TLR-4 (LPS) ligands. Similar to D-AmB and L-AmB, LPS and Zymosan increased the phagocytosis and conidiocidal activity of PMNs although to a much greater extent for LPS than Zymosan. In terms of quality of antifungal effector mechanisms elicited by the different stimuli, \( \text{O}_2^-/\text{H}_2\text{O}_2 \) generation in response to conidia was potentiated by Zymosan and D-AmB but not by LPS or L-AmB (Table 1). Zymosan and D-AmB but not LPS or L-AmB also

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>% Phagocytosis</th>
<th>% Conidiocidal activity</th>
<th>( \text{O}_2^-/\text{H}_2\text{O}_2 ) (nM/10^6 cells)</th>
<th>TNF-( \alpha ) (pg/mL)</th>
<th>IL-10 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30 ± 3</td>
<td>22 ± 3</td>
<td>1.4 ± 0.6</td>
<td>220 ± 18</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Zymosan</td>
<td>38 ± 2*</td>
<td>33 ± 4*</td>
<td>4.2 ± 1.1*</td>
<td>298 ± 31*</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>LPS</td>
<td>49 ± 4*</td>
<td>48 ± 6*</td>
<td>1.1 ± 0.3</td>
<td>195 ± 25</td>
<td>138 ± 8*</td>
</tr>
<tr>
<td>D-AmB</td>
<td>39 ± 4*</td>
<td>39 ± 4*</td>
<td>5.2 ± 1.6*</td>
<td>361 ± 22*</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>L-AmB</td>
<td>46 ± 3*</td>
<td>51 ± 7*</td>
<td>1.7 ± 0.5</td>
<td>115 ± 20*</td>
<td>155 ± 10*</td>
</tr>
</tbody>
</table>

Murine neutrophils were pre-treated with Zymosan, LPS, D-AmB or L-AmB, exposed to resting conidia and assessed for phagocytosis, fungicidal activity, \( \text{O}_2^-/\text{H}_2\text{O}_2 \) and cytokine production.

*\( P<0.05 \); TLR ligands or antifungals versus stimulation with conidia alone (none).

Figure 2. AmBisome and Fungizone induce TLR expression on neutrophils exposed to Aspergillus conidia or hyphae. Murine (a) and human (b) neutrophils were pre-exposed to Fungizone (D-AmB) or AmBisome (L-AmB) before the addition of resting conidia. After 15 min of incubation, TLR expression was assessed by RT–PCR. cDNA levels were normalized against the HPRT gene. None, cells exposed to the diluent alone. The data shown are representative of three experiments.

Figure 3. AmBisome induces degranulation of neutrophils exposed to A. fumigatus. Degranulation was assessed by eosin (pink colour) and Methylene Blue (blue colour) staining on murine neutrophils pre-exposed to Zymosan (Zym), LPS, D-AmB or L-AmB and then to resting conidia. Arrows indicate fungal elements. In the inset, myeloperoxidase-positive cells are shown.
induced $O_2$ production in the absence of conidia ($4.3 \pm 0.7$ and $3.8 \pm 0.4 \text{ nM/10}^6 \text{ cells}$, for Zymosan and D-AmB, respectively). In contrast, degranulation, as observed upon eosin staining of non-azurophil granules (pink staining; Figure 3) or Methylene Blue staining of azurophil granules (blue staining; Figure 3), was maximally induced upon stimulation with conidia and LPS (particularly the extracellular secretion of azurophil granules containing myeloperoxidase, in the inset to Figure 3) or L-AmB compared with Zymosan or D-AmB (Figure 3). Together, these data indicate that the expression of the antifungal effector functions of PMNs seems to occur through the involvement of distinct members of the TLR family, each likely activating specialized antifungal effector functions on PMNs. The analysis of pattern of cytokine production following stimulation with the different stimuli and conidia further confirms this point by showing that the balance between pro-inflammatory and anti-inflammatory cytokine production is differentially affected by the different stimuli. Zymosan and D-AmB both favoured the production of TNF-$\alpha$ over that of IL-10, whereas the balance was skewed toward the production of IL-10 over that of TNF-$\alpha$ by LPS and L-AmB (Table 1). Therefore, TLR-4 stimulation and L-AmB both favour a mechanism of fungal killing involving degranulation more than ROI production and divert the cytokine production by PMNs from pro- to anti-inflammatory.

AmBisome behaves like D-AmB in the absence of TLR-4

To directly assess the relative contribution of TLR-2 and TLR-4 stimulation to the effects of D-AmB and L-AmB on PMNs, purified PMNs from TLR-2- or TLR-4-deficient mice were assessed for phagocytosis and killing activity, $O_2$ production and release of pro- and anti-inflammatory cytokines upon exposure to conidia in the presence of the relevant TLR ligands or the antifungal agents. We have already shown that both the phagocytosis and the conidiocidal activity are impaired in TLR-4-deficient PMNs but virtually unaffected in TLR-2-deficient PMNs. Here we confirm this finding and show that severe impairment is also the collaborative action of L-AmB on conidiocidal activity of PMNs from TLR-4-deficient mice; such an activity was instead retained with PMNs from TLR-2-deficient mice. For D-AmB, the collaborative activity with PMNs was impaired in the relative absence of TLR-2 more than TLR-4 (Figure 4a). Oxidant production was almost undetectable in TLR-2-deficient but present in TLR-4-deficient PMNs upon stimulation with TLR ligands and, intriguingly, with both antifungals (Figure 4b). Similarly, the production of IL-10 predominates over that of TNF-$\alpha$ in TLR-2-deficient PMNs and the opposite was true for TLR-4-deficient PMNs (Figure 4c). In vivo studies confirmed the ablation of TNF-$\alpha$ production in D-AmB-treated TLR-2-deficient mice and the production of IL-10 in L-AmB-treated TLR-4-deficient mice (data not shown). Therefore, TLR-2 and TLR-4 stimulation oppositely regulate the balance between the pro- and anti-inflammatory responses of PMNs to A. fumigatus. It is confirmed that D-AmB preferentially signals through TLR-2, but interestingly, L-AmB appears to work preferentially via TLR-4. In the relative absence of TLR-4-dependent signalling, however, L-AmB behaves like D-AmB as both agents divert the response of PMNs to pro-inflammatory.

Liposomes divert the PMN response from pro- to anti-inflammatory

As liposomes are known to efficiently target PMNs and to attenuate the inflammatory response in vivo, we assessed whether the small, negatively charged liposomes of L-AmB could be responsible for the preferential TLR-4 stimulation of L-AmB. Negatively charged 100 nm liposomes of composition similar to those of AmBisome were added to PMNs stimulated with Aspergillus conidia or D-AmB and the effects on $O_2$ and cytokine production were then assessed. The adding of liposomes significantly reduced TNF-$\alpha$ and $O_2$ production and increased that of IL-10 also in response to

![Figure 4. AmBisome behaves like D-AmB in the functional absence of TLR-4-signalling. Murine neutrophils from TLR-2- or TLR-4-deficient mice were treated as detailed in the legend to Figure 3. $*P<0.05$, TLR ligands or drugs versus no stimulation (none).](https://academic.oup.com/jac/article-abstract/55/2/214/856848/255x292/14521214886644?download=true)
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**Table 2.** Liposomes divert the neutrophil’s response to *Aspergillus* conidia from pro- to anti-inflammatory

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Neutrophils</th>
<th>Conidia</th>
<th>Liposomes</th>
<th>O$_2$ (nM/10$^6$ cells)</th>
<th>TNF-$\alpha$ (pg/mL)</th>
<th>IL-10 (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>TLR-4$^{++}$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TLR-4$^{++}$</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>1.8 ± 0.7$^a$</td>
<td>240 ± 23$^a$</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>TLR-4$^{-/-}$</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND$^*$</td>
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<tr>
<td>TLR-4$^{++}$</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND$^{**}$</td>
<td>98 ± 15$^{***}$</td>
<td>117 ± 10$^{***}$</td>
</tr>
<tr>
<td>TLR-4$^{-/-}$</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1.5 ± 0.4</td>
<td>208 ± 22</td>
<td>22 ± 2</td>
</tr>
</tbody>
</table>

Murine neutrophils from wild-type (TLR-4$^{++}$) or TLR-4 deficient (TLR-4$^{-/-}$) mice were pre-exposed to liposomes and then to resting conidia before the assessment of O$_2$ and cytokine production. ND, not detectable.

$^a$Cells were pre-exposed to cytochalasin D before the addition of liposomes.

$^*P<0.05$, compared with no stimulation;

$^{**}P<0.05$, cytochalasin-treated or TLR-4-deficient versus wild-type neutrophils;

$^{***}P<0.05$, compared with conidia alone.

similar results were obtained with liposomes and D-AmB (data not shown).

**AmBisome shows an improved therapeutic activity with reduced toxicity in BMT mice with IA.**

All the above data would predict that AmBisome might have therapeutic efficacy with reduced inflammatory pathology *in vivo* in mice with aspergillosis. To test this hypothesis, we resorted to a well-described pre-clinical model of allotgenic BMT that replicates the immunodeficiency observed in BM transplanted patients. Mice were treated with AmBisome or Fungizone either before or after the infection. BMT mice succumbed to IA and susceptibility to infection correlated with a high level of chitin in the lungs (Figure 5a). However, both prophylactic and therapeutic treatment with the highest doses of AmBisome (>60 days survival) cured the mice of IA, as revealed by the increased survival after the infection that paralleled the reduced fungal burden in the lungs. Interestingly, and in line with results obtained by others, the prophylactic administration was superior to therapeutic treatment in terms of reduction in fungal growth in the lungs. In contrast, D-AmB failed to induce full protection at the dosages tested and significantly increased the survival and restricted the fungal growth when given therapeutically at the highest tolerated dose of 4 mg/kg. It is of interest that, at the same dosages, D-AmB cured the mice of the infection in the chemotherapy-induced neutropenic model (data not shown), a finding suggesting that the toxicity of D-AmB is increased in BMT. Because in mice with IA, resistance to infection correlates with the activation of interferon (IFN)-$\gamma$-producing Th1 cells, we assessed cell recovery by FACS analysis in the lung, as blood neutrophil levels do not predict susceptibility to aspergillosis, together with the pattern of local cytokine production and antifungal activity of effector phagocytes. The numbers of CD4$^+$ and Gr-1$^+$ neutrophils were significantly increased in the lungs of mice upon therapeutic treatment with 4 mg/kg D-AmB or 5 mg/kg L-AmB. No differences were observed in the number of lung F4-80$^+$ cells with and without treatment (Figure 5b). Recovered myeloid cells were functionally active, as indicated by their fungicidal activity against conidia (38% and 45% killing by PMNs from D-AmB- and L-AmB-treated mice, respectively). In terms of pattern of cytokine gene expression, Figure 5(c) shows that L-AmB, more than D-AmB, increased the expression of IFN-$\gamma$ and decreased that of IL-4 on CD4$^+$ splenocytes. On lung homogenates, the levels of TNF-$\alpha$ were greatly reduced in L-AmB-treated compared with D-AmB-treated mice. Taken together, these data indicate that L-AmB had a superior efficacy, compared with D-AmB, in BMT mice with IA.

**Discussion**

Innate and acquired immunity are both required for the development of optimal resistance to pathogenic fungi. In the case of IA, although neutropenia has historically been the most important risk factor for infection, the increased incidence of IA observed concomitantly with the occurrence of graft-versus-host disease in transplantation, attests to the importance of specific defects in both the innate and adaptive immune effector mechanisms in the pathogenesis of the disease. Both Fungizone and AmBisome were able to activate antigen-specific Th1 responses to *Aspergillus* in transplanted mice. However, AmBisome, better than Fungizone, fulfills the requirement of inducing effective antifungal resistance with a finely tuned inflammatory response at the site of infection.

This study demonstrates a putative mechanism for the reduced infusion-related toxicity of D-AmB. Consistent with the expression of TLR-2 and TLR-4 on PMNs exposed to *Aspergillus* conidia in the presence of D-AmB and L-AmB, respectively, each agent activates the antifungal state of PMNs through distinct preferential mechanisms. PMN activation by *Aspergillus* may occur through the generation of both oxidants with potent antimicrobial activity and a multitude of mediators with non-oxidative antifungal activity contained in PMNs granules. The quantity and specificity of delivery of these toxic neutrophil products ultimately will determine the relative efficiency of fungicidal activity versus inflammatory cytotoxicity to host cells.
Figure 5. AmBisome shows an improved therapeutic activity in mice with aspergillosis. Bone marrow-transplanted mice were intranasally infected with *Aspergillus* conidia a week after the transplant. Drugs were given for 5 days either before or after the infection. (a) Percentage survival and fungal growth (chitin content) in the lung at the time of death for mice dying earlier or 6 days after the infection. Bars indicate the standard errors. *P < 0.05 (treated versus untreated mice). (b) The numbers refer to the percentages of positive cells, as assessed by FACS analysis in the lung 3 or 6 days after the infection (untreated or mice treated after the infection with 5 or 4 mg/kg L-AmB or D-AmB, respectively). (c) Cytokine gene expression on CD4⁺ splenocytes by real time PCR and (d) the levels of TNF-α in the broncoalveolar lavage fluids were assessed at the time of FACS analysis. *P < 0.05, infected (open bars) versus uninfected (filled bars) mice; **P < 0.05 untreated (open bars) versus D-AmB-treated (dark grey bars) or L-AmB-treated (light grey bars) mice.
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without complimentary non-oxidative mechanisms. Actually, host proteins rather than those of engulfed microbes are found to be the targets of oxidative reactions in PMNs. Therefore, because products of the oxidative stress impede the phagocytic-dependent clearance of inflammatory products, ROI production may adversely affect the host ability to oppose the inflammatory pathology.

As in macrophages, D-AmB activates the oxidative mechanism leading to a pronounced pro-inflammatory state via TLR-2 signalling; in contrast, diverting signalling from TLR-2 to TLR-4, L-AmB elicits degranulation more than ROI production, and IL-10. Thus, the exploitation of TLR-4, more than TLR-2, by AmBisome attenuates the toxicity associated with the pro-inflammatory state induced by conventional amphotericin B. Studies are under way to find out whether the diversion of TLR signalling also associates with reduced renal toxicity.

The effect on TLR diversion is mediated by the small size and negatively charged liposomes of AmBisome, known to be taken up efficiently by macrophages. We found here that liposomes of similar size and charge are taken up by PMNs and divert the response from pro-inflammatory to anti-inflammatory. The internalization through a phagocytic process appears to be required for the liposome-mediated effect, as clearly shown by the results obtained in the presence of cytochalasin B. Liposomes have been shown to traffic to the trans-Golgi area where TLR-4 also localizes in epithelial cells. Although it remains to be demonstrated whether the liposome interaction with TLR-4 may occur intracellularly in PMNs, wherever it occurs, the TLR-4 activation may counteract the ability of free AmB to activate TLR-2, an activity postulated to occur by alterations of cellular lipid raft composition and distribution. Therefore, the ability to activate PMNs to a unique antifungal state together with the tendency to accumulate preferentially at sites of infection may help explain the superior efficacy with reduced toxicity of AmBisome.

These results indicate that TLR manipulation in vivo may be amenable to the induction of optimal fungicidal activity in the absence of inflammatory cytotoxicity to host cells. The role of TLR signalling pathways in mediating the therapeutic effects of different formulations of amphotericin B may suggest a rationale for pre-emptive antifungal therapy in disparate clinical settings.

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