CHARACTERIZATION OF THE γδ T CELL RESPONSE TO AMPHOTERICIN B

by

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ABSTRACT

γδ T cells mediate a wide variety of functions within the host. Putative roles for these cells include tumor infiltration and clearance, pathogen detection and response, tissue repair and homeostasis, and augmentation or regulation of the inflammatory response. However, the precise roles γδ T cells play in the immune response to diseases and other stress-inducing states within the host are still unclear. Therefore, it is of interest to determine what types of molecules and signals are involved in the activation of these cells, altering their functional responses in disease states. As such, we screened numerous natural and synthetic compounds for their ability to activate bovine γδ T cells. Interestingly, a clinically approved antifungal drug, Amphotericin B, was found to prime the γδ T cells for activation. We demonstrate that Amphotericin B can utilize different receptors in a tissue-specific manner, making it a rather unique agonist for use as a therapeutic adjuvant. Amphotericin B was also able to act synergistically with other human and bovine γδ T cell agonists for enhanced activation of these cells. Furthermore, alternate formulations of the drug displayed different capabilities to stimulate γδ T cells. Interestingly, Amphotericin B was found to enhance the clearance of bacterial infections in mice, and the drug demonstrated potential as a potent vaccine adjuvant. Overall, Amphotericin B affects γδ T cell responses, and these cells are involved in a wide variety of immune responses. Therefore, Amphotericin B has the potential to be administered so as to manipulate the γδ T cell response for the benefit of the host during infectious disease.

INTRODUCTION

Adaptive and Innate Immunity

Higher vertebrates possess protective mechanisms that detect and respond to various assaults on the immune system, including bacterial, viral, and fungal infections. Continuous surveillance by the immune system allows for rapid responses via the innate system, which has the ability to alert the adaptive system and allow it to respond accordingly. Innate responses mainly involve natural killer (NK) cells, other innate lymphocytes, such as γδ T cells, and myeloid cells and are driven by activation due to engagement of pattern recognition receptors (PRRs) with molecules expressed by microorganisms or upregulated by host cells in response to cellular stress (Gallucci 99; Janeway 02). Following the interaction, an inflammatory response is typically elicited, which consists of the recruitment of other immune cells and leads to microbicidal and cytotoxic responses for clearance of the pathogen. After the invading agent has been cleared, a tissue repair response occurs, and the immune system is then downmodulated so as to return the host immune system to a state of equilibrium (Medzhitov 08). Because of the memory response it can maintain, the adaptive response is well suited to respond quickly to repeat exposure to pathogens (Danilova 12). The expansion of B and T cells allows for immunological memory to occur, and innate cells play a large role in the induction of these adaptive responses. Innate cells internalize pathogens and break them down into peptide antigens which are then presented via the major histocompatibility complex (MHC) molecules to conventional alpha beta (αβ) T cells. In addition, they send

signals via cytokines, which alert αβ T and B cells to the immunological assault and allow them to respond accordingly (Gallucci 99; Janeway 02; Danilova 12). Typically, innate responses occur before the adaptive system is involved, but there is also reverse cross-talk occurring, in which the adaptive system causes recruitment and activation of innate cells (Bonneville 10). Cells which are involved in this process include specific subsets of αβ T cells and T cells expressing gamma delta (γδ) T cell receptors (TCRs).

γδ T Cells

γδ T cells are evolutionarily well-conserved and are thought to be the precursors of modern B and αβ T cells (Ferrick 00; Richards 00). Originally discovered by two independent groups in 1986, γδ T cells were found to be distinct from αβ T cells due to the genes which encode their TCRs, their ability to recognize antigens without the aid of MHC presentation, and their localization to epithelial tissue (Bank 86; Brenner 86; Hayday 00). γδ T cells participate in innate responses, can recognize a wide variety of antigens, locate to specific tissues, and take part in the inflammatory response. Therefore, several of their functional properties imply that they may be closely related to cells of the myeloid lineage. Global gene expression analysis of sorted bovine γδ T cells revealed several similarities between these cells and those of the myeloid family (Fahrer 01; Meissner 03A). Surface antigens (cluster of differentiation 11b [CD11b] and CD14), cytokines (granulocyte colony-stimulating factor [G-CSF], granulocyte macrophage colony-stimulating factor [GM-CSF], nicotinamide adenine dinucleotide phosphate [NADPH] oxidase, superoxide dismutase [SOD], and nitric oxide [NO]), receptors (Toll-

like receptors [TLRs] and scavenger receptor 1), and transcription factors (B-lymphocyte-induced maturation protein 1 [BLIMP-1]), all of which are often associated with cells of the myeloid lineage, were found to be expressed by γδ T cells (Hedges 03; Meissner 03A).

γδ T cells have been termed non-conventional, innate-like, and/or transitional T cells, due to the fact that their precise function within the immune system appears to be somewhat ambiguous (Hayday 09; Bonneville 10). γδ cells have potent cytolytic activities, produce several different cytokines, can act as antigen presenting cells, and both induce and suppress inflammation (Ferrick 95; Collins 98; Mak 98; Zuany-Amorim 98; Born 99; Egan 00; O'Brien 00; Brandes 05). γδ T cells populate portals of entry into the body, and they are one of the first cell types to respond to invading pathogens which enter via these sites (Komano 95; Boismenu 98; Ferrick 00; Chen 02). These cells can also be effectively recruited to sites of infection and are a major source of interferon-γ (IFN-γ) and interleukin-17 (IL-17) in response to pathogen presence, both of which are activators of myeloid cells (Wilson 99; Lockhart 06; Shibata 07).

γδ T cells have the ability to recognize conserved patterns on the surface of pathogens, known as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), as other innate cells do. It has been shown that γδ cells express PAMP receptor transcripts and will respond to several different PAMPs (Meissner 03B; Hedges 05; Lahmers 06; Lubick 06; Kerns 09). However, γδ T cells can also have a memory aspect, which allows for longer-term specific activation of the cells and induction of their effector functions. The activity of these cells has been linked to

clearance of intracellular and extracellular bacteria, modulation of innate and adaptive immune responses, wound healing, and tumor surveillance (Hayday 09; Bonneville 10).

Tissue Localization and Distribution Among Species

Although all jawed vertebrates have a population of γδ T cells, the number and distribution vary from one species to the next (Hayday 00). Waves of γδ T cells are released from the thymus after development and then populate specific areas throughout the body, depending upon the expression of their TCRs. In mice, the first wave of clonal γδ cells trafficks to the epidermis, and additional waves populate the gut and then the reproductive tissues (Allison 91). Controlling the release of these subsets is suggestive of a pre-programmed selection for various γδ T cells. However, in cattle and chickens, extensive V(D)J recombination occurs in the thymus to produce a population of γδ T cells with unique TCRs (explained in greater detail below), and the γδ T cells which are released and traffic to designated areas throughout the body are not necessarily clonal (Hein 91; Six 96). Therefore, it appears that murine γδ T cells are primed for responses specific to the areas where they traffic, while bovine and chicken γδ T cells have a larger range of epitopes which they have the potential to recognize. In humans, the first γδ T cells to emerge from the fetal thymus (designated Vδ1) typically traffick to epithelial tissues and do not preferentially end up in circulation within the blood (Hayday 01). A different subset, Vγ9Vδ2, is released later and makes up the majority of the circulating γδ T cells in the blood and lymphatic system (Kabelitz 03; Girardi 06).

Based on the idea that γδ T cells are involved in responding to common pathogen-linked molecules, it has been suggested that it may not be necessary for these cells to traffic through lymph nodes or into the T cell zones within the spleen, because these are typically sites involved with sampling for antigens via a specific interaction with the immune cell receptors (Hayday 03). One study which supported this concept found that γδ T cells within the spleen are located throughout the organ rather than being only associated with the white pulp (Bucy 88). These cells are often associated with specific tissues, especially in the case of the large population of γδ T cells found within the epithelia (Goodman 88; Kyes 89; Itohara 90). Because γδ T cells belong to this population of cells, it is predicted that they play a large role in identifying and alerting the immune system of tissue-specific "stress-antigens" that signal tissue distress (Janeway 88; Hayday 00; Hayday 03). γδ T cells also populate other IEL compartments, including the genitourinary tract, the skin, and mucosal surfaces (Wilson 99; Chen 02; Girardi 06). The circulating lymphocytes of bovine neonatal calves can be comprised of up to 70% γδ T cells, and the lymphoid systems of adult ruminants contain a large subset of these cells as well (Goodman 88; Hein 91; Hayday 00). Therefore, the tissue distribution of the various γδ T cell subsets varies among species, and the function of each of the subsets appears to be related to the location of the cells as well as which antigen the TCR recognizes (Girardi 06).

The γδ T Cell Receptor (TCR)

The γδ TCR is a glycoprotein comprised of two heterodimeric components, the gamma and the delta chains, which are structurally similar to the alpha and beta chains of the αβ T cell, respectively. These chains are composed of several different combinations of gene segments, and TCRs are formed in the thymus via a process known as V(D)J recombination. During the recombination event, variable (V), diverse (D), and joining (J) gene segments are combined in a randomized manner and paired with the constant (C) region so as to produce several unique TCRs. The ability of vertebrates to undergo this phenomenon allows for the immune system to develop a wide array of TCRs which have the potential to recognize countless antigens, including proteins associated with viruses, bacteria, and other pathogens. The complementary determining region 3 (CDR3) loops of the TCR are responsible for antigen recognition, and V(D)J recombination accounts for their enormous diversity (Rock 94; Chien 06). Among all antigen receptor chains, the δ chains of the TCR have the highest potential diversity in the CDR3 loop, but the TCR γ and δ loci have fewer commonly used V genes than most of the immunoglobulin (Ig), as well as the α and β TCR, loci (Davis 03; Chien 06).

The CDR3 regions of Ig light chains are short and have narrow length distributions, while the Ig heavy chains are longer and have broad length distributions (Chien 06). In the case of α and β CDR3 regions, the length distributions are similar to one another, but in γδ TCRs, the γ chain CDR3 loops are similar to Ig light chains (short and constrained), while the δ chain CDR3 loops are long and variable like Ig heavy chains (Rock 94; Chien 06). Therefore, because of the similarities between Igs and γδ

TCRs, it was thought that the γδ ligand recognition would be quite similar to that of the Igs (Rock 94; Schild 94). However, although the interaction between the TCR and ligands may be Ig-like, it appears that this idea itself cannot predict what molecule will be a γδ TCR ligand (Born 11).

γδ T cells develop in the thymus, and some evidence exists which supports the idea that thymic selection affects their functional development, as TCR-ligand interactions in the thymus were required for the distinction between IFN-γ- and IL-17A-producing cells (Lewis 06; Boyden 08; Jensen 08). Between the TCR-ligand interaction being similar to the Ig receptor-ligand relationship and the idea of thymic selection, it has been suggested that γδ TCRs are similar to natural Igs with respect to having a specificity for auto-antigen, but this concept has not been demonstrated (Born 11). Peripheral TCR selection has been demonstrated as well, but how the selected TCRs might differ functionally has not been elucidated, because ligands have not been determined, and the binding properties of the TCRs remain largely unknown (Sim 91; Pluschke 92; Shimonkevitz 93; Roark 07; Born 11).

The ligands which interact with the γδ TCR are often small and non-repetitive, meaning they cannot successfully cause TCR cross-linking for activation, which leads to the idea of the ligands being anchored in some way (Born 11). Antigen presentation is typically associated with αβ T cell responses, but the same idea has been suggested for γδ T cell activation as well (Holoshitz 92). Presentation of phospho-antigens might occur, but whether actual presenting molecules are utilized is unknown (Morita 95; Wei 08). In addition, there is evidence that when CD1d is present, γδ T cells can recognize

phospholipids, meaning that CD1d may be a potential presenting molecule for γδ T cells (Agea 05; Russano 06). Many of the ligands which interact with γδ TCRs are MHC molecules or are MHC-related (Chien 07; Champagne 11), such as CD1 (Faure 90; Agea 05; Russano 06), MHC class-I-related chain A/B (MICA/B) (Groh 98), human leukocyte antigen-E (HLA-E) (Barakonyi 02), and MHC-class II molecules (Holoshitz 89). However, there are several molecules not associated with MHC which are recognized by the γδ TCR as well. Such molecules include viral glycoproteins (Sciammas 94), heat shock proteins (Holoshitz 89; O'Brien 89), and ATPase complexes (Scotet 05).

Other γδ T Cell Pathogen Recognition Receptors

Unlike MHC-restricted αβ T cells, which differentiate and expand after the interaction between the TCR and co-stimulatory receptors, γδ T cells can also utilize other receptors, such as TLRs and natural killer receptors (NKRs), in addition to the TCR for activation of effector functions (Bonneville 10). TLRs are PRRs which can recognize a broad range of structurally-conserved molecules from various microorganisms. As recently reviewed (Wesch 11), direct and indirect effects of TLRs on the activation of γδ T cells have been demonstrated, and it is known that TLR ligands can affect the effector functions of these cells. It has been shown that some γδ T cells express surface NKG2D, a stimulating receptor which is also found on $CD8^+$ αβ T cells and NK cells, and interaction of NKG2D with ligands such as MICA or MICB leads to cellular destruction (Bauer 99; Nedellec 10). These ligands are often upregulated during cellular stress and

are found on tumor cells (Groh 99), and it has been suggested that destruction of these cells may occur via infiltrating γδ T cells or other $NKG2D^+$ lymphoid cells (Girardi 06).

Roles in Host Defense

Researchers originally believed that γδ T cells played similar roles to those which αβ T cells do in adaptive immunity, due to the cells having TCRs which resembled one another. However, γδ T cells are considered unconventional T cells due to the shared properties they have with other cells grouped into the category, such as limited TCR diversity, possible autoreactivity, and specific tissue associations (Hayday 03). In addition, they do not typically express CD4 or CD8 and do not require MHC presentation of antigens. Their capacity to respond quickly without the requirement of expansion and production of antigen-specific clones suggests that these cells contribute more to the innate rather than the adaptive response. In support of this theory, it is known that the γδ TCR can recognize the conserved epitopes of a plethora of self and non-self antigens. This phenomenon allows these cells to respond quickly in cases of pathogen presence or cellular distress. The concept that γδ T cells are involved in the first line of defense has been considered because of their localization to portals of entry throughout the body and the idea that they may respond to self-antigens which are expressed by infected or transformed cells, a response that is likely to be critical in the early stages of life before the adaptive response is fully formed (Groh 98). The precise roles of γδ T cells in host defense are still quite unclear; however, it has been suggested that these cells may aid in wound healing, could be a potential therapeutic modality in cancer, and may increase innate resistance to infection (Jameson 02; Kabelitz 07; Bonneville 10).

Studies involving γδ-deficient mice have shown that, depending on the infection, protective γδ T cell function can be compensated for by αβ T cells or they can be absolutely necessary for protection (Hiromatsu 92; Pao 96; King 99; Steele 02). However, the largest defect in γδ-deficient mice appears to be involved with the immunoregulatory response of the animals (Born 99), and exaggerated or expedited immunopathology was observed in these mice after infection with various bacterial pathogens (Mombaerts 93; Fu 94; D'Souza 97; Moore 00). These studies support the idea that γδ T cells are typically involved with downregulating the immune response during infection, but there is a plethora of evidence that γδ T cells play a role in pro-inflammatory responses as well. Additionally, the regulatory functions of γδ T cells in mice are not necessarily consistent with their role in other species.

Circulating γδ T cells can respond to invading pathogens such as bacteria, viruses, and parasites via chemokine receptors on their surface, allowing for the trafficking of these cells to sites of infection for aid in pathogen clearance (Bonneville 10). In addition, γδ T cells have the ability to kill infected or transformed cells via pathways involving death-inducing receptors such as FAS and tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAILR) or by the release of cytotoxic effector molecules (e.g. perforin, granzymes) (Dieli 01; Qin 09). Upon bacterial infection, an expansion of a subset of pro-inflammatory γδ T cells occurs which greatly amplifies the percentage of these cells within the peripheral blood (Kroca 00), and this increase in population subsequently allows for the production of various antibacterial products and a recruitment of other Th1 cells to occur in order for destruction of the invading pathogen to take place

(Agerberth 00; Martino 07). γδ T cells can produce a variety of cytokines after infection, including IFN-γ, tumor necrosis factor (TNF), macrophage inflammatory protein 1α (MIP1α), MIP1β, and IL-17 (Bonneville 10). IFN-γ production by γδ T cells plays an important role in the clearance of a wide range of viral infections, although other pro-inflammatory cytokines are also often involved in the response (Franco 97; Wang 03; Poccia 05). The γδ T cell response to parasitic infections is similar to that of viral assaults in that it appears IFN-γ plays a major role in the pro-inflammatory response to several species of protozoan parasites (Su 00; Sardinha 06; D'Ombrain 07). Overall, the functions of γδ T cells are quite broad and include the killing of infected, activated, or transformed cells, pathogen clearance, production of cytokines involved in protective immunity versus a wide array of infectious agents, and immunomodulation of the innate and adaptive responses.

Because γδ T cells appear to take part in several different aspects of the immune system, it has been of interest to determine what triggers a rapid and potent response from these cells. It has been suggested that the function which a particular γδ T cell has may be dependent upon the class of the receptor which is engaged, the nature of the stimulus, and the signal strength produced by the TCR (Bonneville 10). In order identify possible agonists for γδ T cells, high throughput cell-based screening assays were performed, and it was discovered that a diverse group of substances were potential candidates, including several natural compounds (Holderness 07; Holderness 08; Holderness 11; Graff 07; Graff 09). One peculiar γδ T cell agonist which was discovered is a clinically approved anti-fungal drug, amphotericin B (AmB). AmB belongs to a group of compounds known

as polyene macrolides, and it is the most widely used of the polyenes for the treatment of systemic fungal infections.

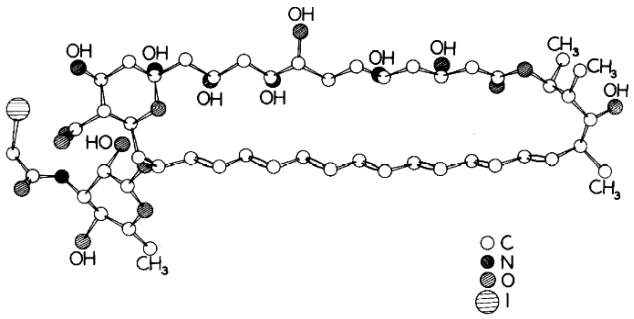
Polyene Macrolides

Polyene macrolides were first discovered as antifungal agents in the 1950s (Hazen 50; Vandeputte 55/56) and are characterized by large lactone rings consisting of 20-44 members with three to eight conjugated double bonds, often joined with a sugar moiety. The double bonds absorb light from the ultraviolet-visible end of the electromagnetic spectrum, giving the compounds a yellow coloring. Derived from *Streptomyces* species, polyene macrolides bind to ergosterol in fungal cell membranes, causing leakage of ions and small molecules. There are over 200 polyene macrolides which have been discovered, but only a select few are utilized in therapy (Omura 84). Such drugs include nystatin, candicidin, pimaricin, methyl partricin, trichomysin, and amphotericin B, all of which have antifungal activity but have little influence on bacterial species (Mandell 96). In general, the polyene macrolides have toxic side effects for humans, but are widely utilized due to the ability of these drugs to act as broad spectrum antifungal agents with limited resistant pathogen emergence (Mandell 96). The ability of these compounds to target cholesterol in mammalian cell membranes is likely one of the contributing factors to the toxicity often seen with therapeutic doses of the drugs, such as renal complications and blood clots (Fisher 78; Mandell 96). However, new formulations of several of the compounds have been aimed at reducing the toxic side effects of these potent antimicrobials.

Amphotericin B

The most widely used polyene antimicrobial which is utilized clinically to treat systemic fungal infections is amphotericin b (AmB). First isolated from *Streptomyces nodosus* in 1955 at the Squibb Institute for Medical Research, AmB is an amphoteric molecule, meaning the compound can react as either an acid or a base (Gold 55; Vandeputte 55/56; Donovick 56; Dutcher 56; Steinberg 56). Another product of *S. nodosus*, amphotericin A, has a very similar structure to AmB; however, it was discovered that AmB was a more potent antifungal compound (Jambor 56; Gold 55; Vandeputte 55/56; Dutcher 56; Aszalos 85).

Figure 1. The Molecular Structure of N-iodoacetylamphotericin B (Mechlinski 70)

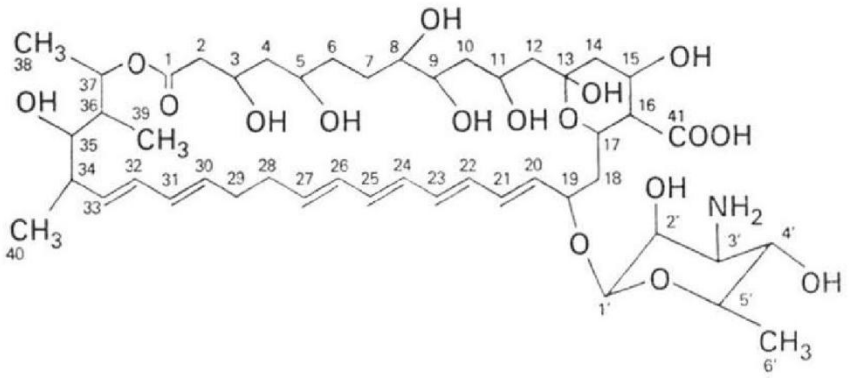


Structure

The structure of AmB was elucidated in the 1970's, and the molecule was described as having a heptaenic macrolactone ring which was connected by a glycosidic bond to a mycosamine amino-sugar (Mechlinski 70). The AmB molecule is comprised of seven conjugated double bonds and possesses several hydroxyl radicals, giving it both a

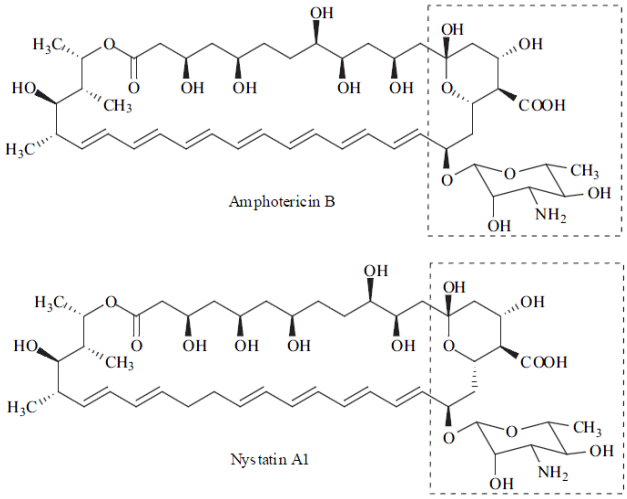
hydrophobic and a hydrophilic portion, respectively. The molecule also contains a polar head group comprised of the carboxylic acid (COOH) and ammonia (NH_2) radicals of the mycosamine sugar (Figure 1).

Figure 2. The Chemical Structure of Amphotericin A (Aszalos 85)



Amphotericin A has an almost identical structure (Figure 2), with the exception of it having a single bond between carbons 28 and 29 rather than the double bond found in AmB (Aszalos 85). In addition, another related molecule, nystatin, has a very similar structure to both AmB and amphotericin A (Figure 3), and this molecule is utilized as a clinical drug to treat fungal infections as well (Hazen 51; Brown 57; Borowski 71; Aszalos 85). The difference between AmB and nystatin structurally is that the latter contains a saturated conjugated double bond.

Figure 3. The Chemical Structures of Amphotericin B and Nystatin A_1



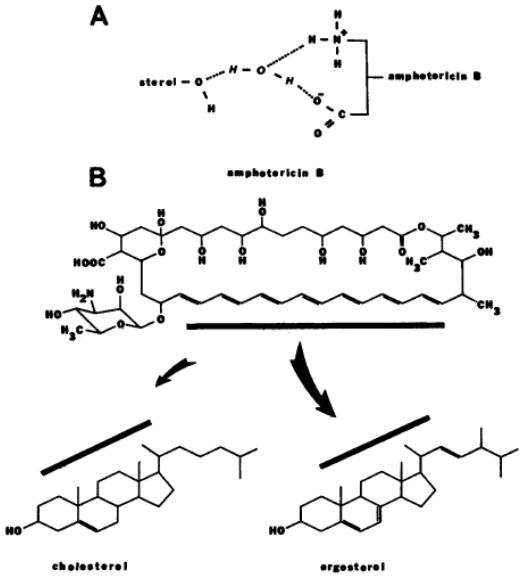
Conserved C8 regions linked to the polyene moiety and comprising the hemiketalic ring with ionizable groups are marked with dashed rectangles. (Adapted from Zotchev 03)

Mechanism of Action

The biological activity of the polyene macrolides was originally believed to be solely due to their ability to interact with the sterols within the cellular membranes of fungi and protozoa (Norman 76; Pfaller 81), but further analyses concluded that other mechanisms were contributing to the antimicrobial properties (de Kruijff 74). Rather than simply interacting or interfering with the sterols present in the membranes, the polyenes were found to form complexes with the sterols (de Kruijff 74), creating hydrophilic pores from which cellular components and ions, such as K^+ and Na^+, would leak and cause cellular death (Hammond 77). Although slightly interactive with the cholesterol contained within mammalian membranes, these compounds generally have higher affinity for ergosterol in fungal membranes, due to the alkyl side chain of ergosterol being more sensitive to the drugs (Kitajima 76; Gruda 80; Teerlink 80; Vertut-Croquin 83; Szponarski 88; Herve 89; Milhaud 89).

AmB is the most studied of the polyene macrolides, and it is believed that eight AmB molecules combine with the fungal sterols to form the hydrophilic channel via an interaction between the sterols and the hydrophobic chromophore of the drug (de Kruijff 74). Two types of binding are believed to occur between the AmB molecule and ergosterol—specific forces and van der Waals interactions (Rinnert 81; Brajtburg 90; Mazerski 95; Khutorsky 96) (Figure 4).

Figure 4. A Schematic Representation of Hydrogen Bond Formation and Nonspecific Interactions between AmB and Sterols



A) Hydrogen Bond Formation. B) Nonspecific Interactions. (Brajtburg 90)

Antifungal Properties

AmB is utilized for the treatment of a wide variety of fungal diseases, and optimal drug concentrations and formulations have been determined for each type of infection. Treatment of fungal infections of the central nervous system (Salaki 84), peritoneum (Eisenberg 86), genitourinary tract (Fisher 82; Nix 85; Frangos 86), and of the eye (Bell

73; Miller 78; Perraut 81; Fisher 83; O'Day 85; Squibb 88) caused by a variety of organisms have been successful with the utilization of AmB (Gallis 90). In addition, neutropenic and AIDS patients have been treated effectively for numerous fungal infections with AmB, and successful prophylactic usage of AmB has been reported in these patients as well (Degregorio 82; Pizzo 82; Stein 82; Holleran 85; Stein 86; Karp 91). Topical AmpB creams are available for dermatological fungal infections (Squibb 88), and pulmonary fungal infections are treated via intravenous administration of AmB as well (Sarosi 83; Stamm 83). However, the most common usage of AmB in the United States is for Candidemia, and AmB is quite successful at eliminating the infection (Edwards 78; Myerowitz 77; Fraser 79).

Susceptibility testing for fungi has seen major advances in recent years, but it still has reliability issues for detecting resistance (Ellis 02). The NCCLS-M27-A method used for the testing of yeasts was the first standardized susceptibility test (Standards 97). It involved broth microdilutions which could be utilized to test for susceptibility of *Candida* spp. and *Cryptococcus neoformans* to antifungals such as AmB. A newer method, termed NCCLS M38-P was developed for the testing of mold susceptibility to antifungal agents (Standards 99). Currently, fungi are considered susceptible at a minimum inhibitory concentration (MIC) of <1 mg/L, intermediate at an MIC of 2 mg/L, and resistant at an MIC of ≥4 mg/L; however, it appears there can be species which fall into several of the categories (Ellis 02). In addition, problems can arise with susceptibility testing due to the fact that some of the causative organisms have yet to be isolated (Ellis 02).

Despite some discrepancies with susceptibility testing, the antifungal spectrum of AmB and all of its formulations (explained in greater detail below) has been well-studied (Bannatyne 77; Hopfer 84; Mullen 97; Johnson 98; Ramage 02; Barrett 03; Clemons 04; Moen 09). Numerous *Candida* species are susceptible to AmB, although a small minority of isolates have been shown as resistant (Pfaller 95; Davey 98; Pfaller 98; Arthington-Skaggs 00), and extensive testing has not been performed with *Malassezia* species, due to the fact that the fungus is difficult to culture (Ellis 02). *Cryptococcus neoformans* and *Saccharomyces cerevisiae* are considered susceptible (Pfaller 95; Davey 98; Arthington-Skaggs 00), while *Trichosporon beigelii* is listed as intermediate (Espinel-Ingroff 98). Most of the dimorphic molds are susceptible to AmB, with a few resistant strains being documented (Ellis 02). In general, *Aspergillus* spp. tend to be susceptible, but a small number have been found to have intermediate susceptibility (Espinel-Ingroff 97; Arikan 99; Rex 00). Many species have been found to have intermediate susceptibility, but treatment failures can still be seen with some susceptible and intermediate species (Ellis 02).

Resistance to amphotericin B is quite rare, but one organism which is highly resistant to antifungal treatment, *Scedosporium prolificans*, has been found in Australia (Cuenca-Estrella 99; Johnson 99). Other species known to be resistant to AmB therapy include some *C. tropicalis* strains, *C. lusiantiae*, and *Sporothrix schenckii* (Ellis 02). Resistance appears to be due to a mutation in the ergosterol biosynthesis pathway of the fungus so that an ergosterol-like compound is produced which AmB cannot bind to as effectively (Ellis 02). Despite some cases of resistance, these species are rarely seen

clinically, and the resistance to AmB has not been profound enough to affect clinical treatment of a wide variety of fungal infections with the drug (Lozano-Chiu 98). Furthermore, it has been demonstrated that resistance in fungal species does not emerge during therapy with AmB (Warnock 91; Casadevall 93; Moosa 02). Overall, AmB covers a wide range of fungal species that are routinely seen in clinical settings, and reports of resistance are not common.

Pharmacokinetics

Oral administration of AmB is not recommended, as the drug is poorly absorbed via this route, but lozenges have been developed in an attempt to overcome the decrease in bioavailability (Louria 58; Hofstra 82; Ching 83). One such preparation of AmB is called Fungilin, and it is marketed by E.R. Squibb and Sons Limited as a treatment for oral candidiasis (thrush). According to the manufacturers, the lozenges are "round, pale yellow" tablets with 10 mg of AmB in each unit, and it is recommended that adults take 4-8 lozenges throughout each day. Although lozenges are effective for treating oral fungal infections, that route is not typically effective for the treatment of systemic infections.

As therapy for invasive fungal infections, AmB is administered intravenously (IV) and is given slowly over several hours. The length of treatment with AmB varies depending upon the type of infection being treated, but it is usually administered at dosages between 0.25-1.0 mg/kg body weight per day. The levels of the drug found within the serum depend greatly upon the dose, frequency, and rate of infusion (Bindschadler 69; Fields 70; Fields 71; Powderly 87). Detection of the drug has been

reported as late as 12 days in the bile and 35 days in the urine (Craven 79). AmB typically has an initial serum half-life of 24-48 hours, but it was discovered that it also has a terminal half-life of about two weeks (Atkinson 78). One pivotal study which was performed on several species of animals suggested that plasma concentrations of AmB drop off more rapidly in small animals than in larger ones, mostly due to the fact that the smaller animals have larger elimination organs with respect to body size (Hutchaleelaha 97). The drug can cross the placental barrier, and concentrations of the drug have been measured in the placental tissue, cord serum, and infant blood even when treatment had been stopped weeks before delivery (Ismail 82; Dean 94). However, the studies noted that the concentrations within these tissues were lower than that of the mother's tissues and blood at the time of measurement.

Distribution of AmB after administration occurs throughout the body, including to the liver, spleen, lungs, kidneys, muscles, skin, and adrenal glands (Atkinson 78; Craven 79; Craven 80; Lawrence 80). Concentrations of the drug tend to be highest in the lung and spleen, but the kidneys often have high concentrations as well (Block 74; Christiansen 85; Collette 89). After AmB has been introduced into the bloodstream, it is readily bound by proteins within the serum (most likely lipoproteins) and has been shown to interact with serum albumin (Fields 70; Brajtburg 84; Wasan 94A; Hartsel 01). Liposomal formulations (described in more detail below), as opposed to the deoxycholate version typically utilized, can allow for the drug to be sequestered more easily in the plasma and tissues, but they also cause the drug to be released so that unbound, protein-bound, and liposomal drug pools are created (Bekersky 01). Therefore, measurement of

AmB in different tissues or in the plasma may be inaccurate, and reports indicating the pharmacokinetics and pharmaceutic potential of the different formulations of AmB are potentially misleading (Fielding 91A). One study found that a liposomal form of the drug increased the total concentration of AmB in plasma but decreased the concentration of unbound AmB (Bekersky 02A). Evidence also exists which supports the idea that liposomal AmB remains in circulation for very long periods of time and causes the sequestration of the drug in the plasma, allowing for the slow release of AmB into tissues via uptake of intact liposomes (Adler-Moore 98; Bekersky 01; Bekersky 02A; Bekersky 02B). In addition, it was discovered that renal and fecal excretion of a liposomal formulation of AmB was much lower than that of the deoxycholate version (8.5% versus 63%), and the authors noted that greater than 95% of the drug was bound in plasma by proteins, such as serum albumin and alpha-1-acid glycoprotein (Bekersky 02A). It has been reported that heat treatment of AmB was found to alter the pharmacokinetics and tissue distribution of the drug in rabbits, such as eliminating the increase in serum creatine concentrations in the body typically seen after treatment, increasing the total body clearance of AmB, and trending toward a decrease in levels of the drug within the kidneys, lung, and spleen versus non heat-treated AmB (Kwong 01).

Adverse Reactions

Several studies have been performed which illustrate the toxicity profile of AmB, and it is widely known that the drug is not well tolerated in many people (Andreoli 73; Hsuchen 73; Maddux 80). Although the drug targets ergosterol in fungal cell membranes, it is thought to also interact with cholesterol in mammalian cell membranes, which may

account for a large portion of the toxicities caused by the administration of AmB (Kinsky 62; Cass 70; Andreoli 73; Hsuchen 73; Maddux 80).

The most concerning complication after administration of the drug is nephrotoxicity. It is believed that AmB causes a decrease in renal blood flow, the glomerular filtration rate, and electrolyte reabsorption from the proximal and distal tubules (Burgess 72; Maddux 80; Branch 88). Signs indicating that renal damage has occurred include increases in magnesium and potassium levels, urinary casts, azotemia (increased levels of urea, creatine, and other nitrogen-rich compounds in the blood), oliguria (low output of urine), and renal tubular acidosis (Butler 64; Douglas 69; Patterson 71; Burgess 72; Maddux 80). Some of these symptoms have been shown to be reversed, even if the patients are still being given AmB (Butler 64; Branch 88); however, reports of irreversible damage have also been reported (Takacs 63; Winn 63).

In addition to renal damage, symptoms such as fever and chills are reported quite often. The term infusion-related reaction (IRR) has been used to describe these symptoms, which are often caused by cytokine and chemokine release. Numerous studies have taken place in order to evaluate the effects of dosage, formulation, and infusion times on the occurrence of IRRs (Burks 80; Gross 86; Arning 95; Wingard 00; Eriksson 01). Some studies point to cytokines such as TNF-α, IL-6, and IL-1 receptor agonist (IL-1ra) as the inducers of toxicity (Chia 90; Cleary 92; Arning 95; Rogers 98). However, it appears that encapsulation of AmB into liposomes (described in more detail below) can attenuate the release of these cytokines from monocytes and macrophages (Arning 95; Walsh 99; Wingard 00; Barrett 03). Continuous infusion of the drug has been shown to

cause fewer side effects versus administering it rapidly, but this method is not always feasible if the patient needs a higher concentration of the drug to overcome the infection (Chabot 89; Oldfield 90; Cruz 92; Ellis 92; Eriksson 01).

Other side effects include thrombophlebitis (swelling of a blood vein caused by a clot) from continuous injections, nausea, vomiting, anorexia, headache, myalgias, muscle/joint pain, rapid heartbeat, sleeplessness, weight loss, and allergic reactions (Maddux 80; Gallis 90). Additionally, drug interactions have been noted, including reports that AmB may enhance the rental toxicity of cyclosporine and aminoglycosides (Kennedy 83). In addition, other drugs might increase the renal toxicity of AmB as well, including the cisplatin and nitrogen mustard compounds (Gallis 90). Despite some adverse drug interactions, enhanced antifungal effectiveness has been reported when AmB has been combined with other drugs, such as flucytosine for the treatment of cryptococcal meningitis and miconazole and ketoconazole versus *Candida* species (Bennett 79; Odds 82; Dismukes 87; Hopfer 87; Francis 92).

Alternate Formulations

Liposomes. Although traditional AmB (AmB deoxycholate, Fungizone) has been the drug of choice for invasive fungal infections (Groll 03; Ostrosky-Zeichner 03), the adverse effects have been overshadowing its efficacy (Tiphine 99; Walsh 99; Hann 01; Cagnoni 02; Dupont 02). Typically, these side effects are treatable to the point that therapy with AmB can continue; however, some of the reactions are severe enough that the therapy may need to be discontinued or a smaller dosage must be given. Often seen

with immunocompromised patients, these effects are intense enough that the therapy can fail, and the mortality rate for these patients can be fairly high (Walsh 88; Denning 90).

One method of circumventing these life-threatening problems without having to decrease the efficacy of AmB is to utilize the properties of liposomes, vesicles which are comprised of water surrounded by bilayered phospholipid membranes. The first person to describe and utilize phospholipids for the purpose of creating enclosed vesicles, termed "smectic mesophases", was Alec D. Bangham (Bangham 63). These molecules were later given the name "liposomes" by a fellow colleague of Bangham's (Ostro 89). The structure of the phospholipids in liposomes consists of hydrophilic heads attached to hydrophobic tails, so that when in contact with water, the molecules spontaneously rearrange into vesicles with the heads facing outward and the tails inward. Water-soluble substances (drugs, enzymes, genes) are often encased within an aqueous phase of a bilayer, whereas fat-soluble molecules can be interspersed throughout the lipid bilayer.

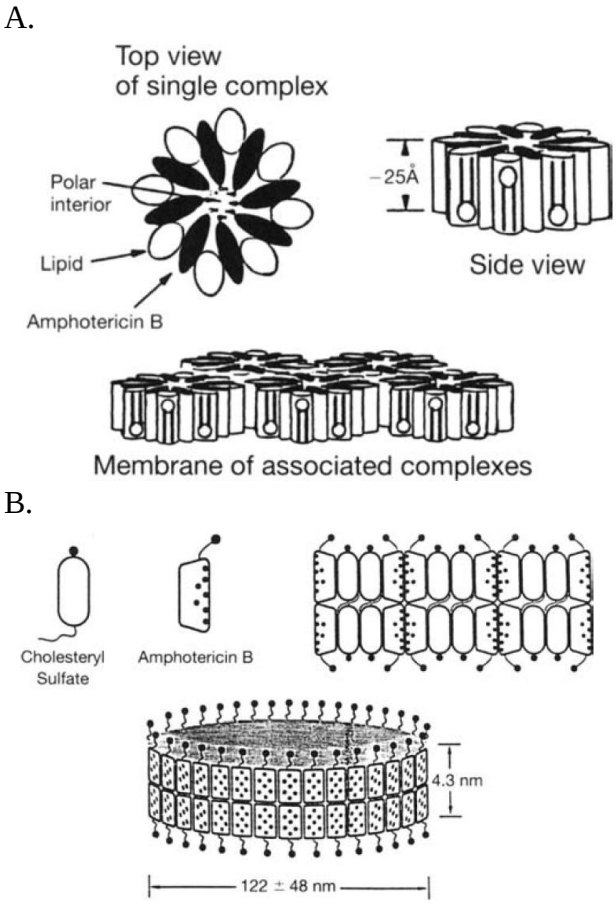
Studies have been performed to determine whether AmB would be suitable for antifungal therapy as a liposomal formulation, and the literature suggests that these forms may be less toxic than, but still retain the potent antifungal properties of, traditional AmB (New 81; Ostro 89; Wong-Beringer 98; Robinson 99). Studies also show that the liposomal versions of AmB do not become concentrated within the kidneys; rather, this alteration to the drug causes it to reside within other organs, such as the liver, spleen, and lungs, allowing for the possibility of less severe side effects (Hiemenz 96). In addition, phagocytic cells have been shown to ingest these formulations of AmB, perhaps causing the drug to be trafficked or targeted to inflammatory sites (Ostro 89; Bangham 92). It has

been suggested that liposomal AmB allows for a higher concentration of the drug in the plasma and tissues while reducing the amount excreted from the body via urine and feces (Bekersky 99A; Bekersky 99B; Bekersky 99C; Bekersky 02B). Studies involving the pharmacokinetics, tissue distribution, and toxicities of these formulations in the presence and absence of other antifungals have been performed, and a great deal of research is devoted to determining the similarities and differences among the different formulations of AmB.

The first studies performed to determine whether liposomal formulations of the drug were less toxic were done in a murine model of leishmaniasis (New 81), followed by experiments to determine whether the therapeutic index of the drug was improved versus the deoxycholate formula in animals infected with cryptococcus (Graybill 82; Taylor 82). The studies suggested that the liposomal formulations of AmB were associated with lower toxicity in the animal models tested while keeping the antifungal properties of conventional AmB intact. More in vivo studies were performed utilizing several species of animals and a wide variety of infectious fungal species, all supporting the idea that liposomal AmB was similarly effective as and less toxic than the deoxycholate form (Lopez-Berestein 84; Lopez-Berestein 85; Lopez-Berestein 87; Clark 91; Janoff 93; Groll 00). An interesting study looked at the effect of an immunomodulator, tuftsin, on the toxicity and pharmacodynamics of a formulation of AmB loaded with the molecule (Khan 06). The results suggested that a reduction in toxicity in the mice and an increase in stability of the drug occurs when liposomal AmB is combined with the immunomodulator. Additionally, the study demonstrated the ability

of the AmB/tuftsin formulation to have greater antifungal properties versus drug sensitive and drug resistant Candida infections in mice. The results support that another modification to liposomal formulations could potentially be an even more tolerable and effective formulation. Three formulations of AmB have been developed and marketed (Figure 5): Liposomal Amphotericin B (L-AmB, AmBisome), Amphotericin B Lipid Complex (ABLC, Abelcet®), and Amphotericin B Colloidal Dispersion (ABCD, Amphotec).

Figure 5. The Putative Structures of Amphotericin B Lipid Complex and Amphotericin B Colloidal Dispersion



A) Amphotericin B Lipid Complex. B) Amphotericin B Colloidal Dispersion. (Adapted from Janoff 93).

Liposomal Amphotericin B (L-AmB, AmBisome). L-AmB (marketed as AmBisome) consists of small unilamellar lipid vesicles which range in size from 60-70 nm, on average, and is the only commercialized formulation which contains liposomal structures. Lipophilic amphotericin B is combined with a hydrogenated soy phosphatidylcholine and distearoyylphosphatidylglycerol bilayer (Adler-Moore 93). In several studies performed on various animal models, AmBisome was found to be as effective as the deoxycholate version of AmB (Anaissie 91; Proffitt 91; Lee 94), but evidence also exists that this formulation is actually less active than AmB deoxycholate (Pahls 94; Ritmeijer 11). Similar to other liposomal formulations of AmB, AmBisome is found most highly concentrated in the liver and spleen after administration, and the reticuloendothelial uptake of the drug appears to be slower than of other liposomal AmB drugs (Proffitt 91; Lee 94). Those studies also discovered that the peak plasma level and circulation time for AmBisome were greater than that of other liposomal AmB complexes. Clinical trials showed that this formulation of AmB was associated with stabilization or reversal of serum creatine levels (indicative of renal toxicity) in patients who switched from the deoxycholate form to AmBisome.

AmBisome has been licensed for use as an intravenous drug which is typically administered over a period of 2 hours, according to the manufacturer. Twenty-four hours after administration of AmBisome, the half-life was found to be 7-10 hours; however, up to 49 days after administration, the mean half-life was 4-6 days (AmBisome.com). Typically, 3-5 mg/kg body weight of AmBisome is administered per day for treatment of

systemic infections from species such as *Aspergillus*, *Candida*, and *Cryptococcus*, with more being administered for immunocompromised patients.

Overall, AmBisome is the most studied lipid-based formulation of AmB and has proven to have the lowest rate of infusion-related adverse events (IRAEs). The most common side effects appear to be elevated levels of transaminase, alkaline phosphatase, and bilirubin, but all of these are reversible, and the drug is typically well tolerated (Wong-Beringer 98).

Amphotericin B Lipid Complex (ABLC, Abelcet®). Another liposomal form of amphotericin B has been developed which consists of a complex of AmB and two lipids, dimyristoyl phosphatidylcholine and dimyristoylphosphatidylglycerol. The discovery of this form came about when researchers noticed "ribbons" within the original formulations of liposomal AmB which were found to be due to the non-liposomal components within the structure (Janoff 88; Janoff 93). When researchers explored these ribbons further, they discovered that, at specific concentrations, AmB and the lipid structures were no longer readily associated with one another, allowing the ribbons to form. This new formulation was termed amphotericin B lipid complex (ABLC). A reduction in nephrotoxicity has been observed, and it is believed to be due to phospholipases being released by vascular smooth muscle, as well as those originating from macrophages and the infectious fungi, breaking down the lipids complexed to the AmB (Wasan 94B; Wingard 97). This action is thought to promote the release of the drug at the specific site of infection. ABLC has a higher molar ratio of AmB in the product, and due to its large size, is rapidly cleared from circulation by the reticuloendothelial system (Madden 90).

Although the levels of AmB found in the liver, spleen, and lungs of rats and mice given ABLC were higher than in animals given AmB deoxycholate, plasma levels of the drug were lower in ABLC-treated animals (Clark 91; Olsen 91; Janoff 93). A reduction in toxicity was seen even when the dosage was increased 10 fold compared to the conventional AmB in mice and four fold in rabbits (Clark 91; Lee 92).

This formulation was initially marketed only as a treatment for Aspergillosis in patients for whom conventional AmB therapy had failed. However, the efficacy of ABLC versus several species of fungi has been evaluated, and its current usage has been expanded to include treatment for all systemic fungal infections. Clinical trials suggest that this formulation of AmB is comparative to AmB deoxycholate for treatment of several types of invasive fungal infections, and it appears to induce less toxicity when compared to conventional AmB (Whitney 89; Clark 91; Clemons 91). A novel study looked at the effects another antifungal drug, Capsofungin, had on the ability of ABLC and L-AmB to combat an *Aspergillus fumigatus* infection in rats (Wasan 07). The study found that the presence of the additional antifungal, while decreasing the fungal load, did not significantly change the plasma concentration over time, the pharmacokinetics, or the tissue distribution of AmB.

ABLC is manufactured by Enzon and is marketed under the name Abelcet®. It is available as a 100 mg/20 ml suspension and is typically administered intravenously at doses of 2.5 mg/kg body weight for 1-2 hours. According to Enzon, the terminal half-life of Abelcet® is about 173 hours after a dosage of 5 mg/kg/day for 5-7 days. Typically, the drug is utilized as a last resort for fungal infections which have failed to be curbed by

conventional AmB. Although the IRAEs appear to be similar to the deoxycholate form, ABLC does have the potential for lower toxic side effects. The most common side effects appear to be increased levels of serum bilirubin and alkaline phosphatase (Wong-Beringer 98).

Amphotericin B Colloidal Dispersion (ABCD, Amphotec). ABCD is a complex consisting of AmB and cholesteryl sulfate, which forms an amphotericin B colloidal suspension in aqueous solutions. The metabolism of this version is different from other formulations, because it is readily ingested by liver macrophages, meaning the kidneys are spared from receiving high levels of the drug. One study suggested that a three- to sevenfold reduction in delivery to the kidneys occurred with ABCD than with traditional AmB, and the concentrations of ABCD found in the liver were two- to threefold higher than regular AmB, with almost 100 percent recovery of ABCD from the liver after 30 minutes of delivery (Fielding 91B). Although ABCD appears to accumulate in the liver, the lower level in the kidneys circumvents many of the serious complications due to nephrotoxicity during treatment with deoxycholate AmB.

In several animal species, ABCD was less toxic than traditional AmB, and increased duration of survival was observed in animals with pulmonary *Aspergillus* infections which were given ABCD versus those given AmB deoxycholate (Fielding 91C; Allende 94). However, deoxycholate AmB was more effective at tissue clearance of fungi when compared to an equal dosage of ABCD (Patterson 89; Fielding 91C). Clinical trials demonstrated that the elimination half-life of ABCD is longer than for traditional

AmB, around 27 hours, but rat studies indicate that plasma levels one hour after injection are lower with ABCD than with the deoxycholate version (Fielding 91C).

ABCD is marketed as Amphocil and is given as an intravenous therapy at concentrations of 3-4 mg/kg body weight per day for treatment of invasive Aspergillosis. The effectiveness of this formulation versus the traditional version of AmB has not been fully supported, and its usage as a salvage therapy after traditional AmB treatment is not as promising as other formulations. The adverse effects of this AmB version have been reported as slightly higher than ABLC, but it has been shown to be less toxic than deoxycholate AmB.

Overall, AmB deoxycholate is often not well-tolerated, but is still the first line of defense for systemic fungal infections. If patients are not responding to or are experiencing adverse reactions and side effects from this therapy, a liposomal formulation of the drug is typically prescribed, especially after levels of creatine are found to be high. Lipid formulations are recommended as first-line therapy if the patient has renal insufficiency or is currently taking nephrotoxic drugs (Dupont 02). In general, the liposomal formulations of AmB have proven to be a significant advance in antifungal therapy, have similar antifungal potencies, and are less nephrotoxic than AmB deoxycholate (Hopfer 84; Mullen 97; Johnson 98; Barrett 03; Clemons 04; Moen 09).

Immunomodulatory Properties

Early studies indicated that amphotericin B was not simply affecting the fungal cells, but it had immunomodulatory effects as well (Hammarstrom 76; Lin 77; Little 78; Lohr 82; Hauser 83; Little 83). Although AmB is effective against *Cryptococcal*

neoformans in healthy subjects (Pfaller 95; Davey 98; Arthington 00; Ellis 02), AIDS patients are rarely cured of cryptococcal meningitis after therapy with the drug, which suggests that the host immune system might be playing a role in the antifungal potency of AmB (Zuger 86; Spitzer 93). It was discovered that the drug alters B cell responses (Shirley 79A) and phagocytosis performed by macrophages (Lin 77). However, there is some evidence that AmB also acts as a T- and B-cell immunosuppressant (Stewart 81; Walls 82; Hauser 83). Interestingly, it has been shown that encapsulation of AmB in liposomes could potentially act as a safeguard for macrophages and T lymphocytes against toxic or immunosuppressive effects which occur with deoxycholate AmB (Mehta 85). There is an abundance of literature devoted to the immunomodulatory effects of AmB, as well as the signalling events which take place after administration, which will be summarized in the following sections.

Effects on Phagocytes. It has been demonstrated that AmB affects macrophages in several different ways, which allows for these cells to combat fungal infections more efficiently by increasing their phagocytic and antibacterial capabilities (Lin 77; Aslanzadeh 91; Tohyama 96). For example, the drug primes macrophages for the release of higher amounts of TNF-α, reactive oxygen metabolites, and NO (Stein 87; Chia 89; Wolf 90; Tokuda 93; Yamaguchi 93; Hermann 94; Tohyama 96; Mozaffarian 97). In addition, one of the studies found that AmB-induced increases in nitrite production by the murine macrophage cell line J774.16 were enhanced in the presence of IFN-γ (Mozaffarian 97). The results suggested that a pathogen-specific antibody administered in conjunction with AmB might allow for the increased macrophage-produced NO in vivo.

Another study demonstrated the ability of AmB in therapeutic doses to induce TNF production in higher amounts when several different macrophage cell lines were first primed with IFN-γ (Chia 89).

Pulmonary macrophages play an essential role in combating *Aspergillus* infections of the lung, and these cells have proven to be toxic to conidia in vitro (Schaffner 83; Schaffner 92; Schaffner 94). The first study which determined the effect cell-associated AmB had on the conidia of *Aspergillus fumigatus* addressed whether AmB could augment the alveolar macrophage response to infection in vitro (Jahn 98). The study found that AmB enhances the ability of human alveolar macrophages to kill the fungus, and the authors suggested that cell-associated AmB may play an important role in the antifungal properties against *Aspergillus* infections in vivo. Another study suggested that stimulation of calcium ion (Ca^{2+}) influx might be a possible mechanism for AmB activation of macrophages (Rogers 99). In addition to alveolar macrophages, polymorphonuclear neutrophils (PMNs) play a role in combating *Aspergillus* infections of the lung (Roilides 98A). Production of cytokines which aid in the host defense against such infections, including macrophage colony-stimulating factor (M-CSF), TNF-α, and IFN-γ, was increased with AmB treatment (Wolf 90; Roilides 98A; Roilides 98B; Gonzalez 01). One study found that several different formulations of AmB enhanced the effect of PMNs on *A. fumigatus* hyphae and conidia in vitro, and the authors suggested that AmB may increase the permeability of the fungal membrane to PMN microbial products as well as induce secretion of such metabolites (Roilides 02).

Neutrophils and monocytes play active roles in the ability to combat invasive fungal infections, such as those caused by *Fusarium* spp. (Antachopoulos 05; Shoham 05). However, studies looking at the relationship between AmB and phagocytic cell oxidative bursts have been contradictory (Yasui 88; Roilides 90; Wilson 91; Sullivan 92). One study determined whether different AmB formulations had an effect on the antifungal activities of PMNs and the ability of these cells to induce a respiratory burst and cause damage to the hyphae of *F. solani* (Dotis 08). The authors found that both deoxycholate and liposomal formulations of AmB did enhance the antifungal activities of these cells versus *F. solani* (as well as against *A. fumigatus*), but that the response was not associated with production of superoxide radicals (O^{2-}). A separate study, looking at the effects of various formulations of AmB on the antifungal activities of monocytes versus *A. fumigatus* hyphae, found that production of H_2O_2 and H_2O_2—dependent intracellular intermediates was augmented by AmB, but that O^{2-} production was not (Dotis 06). Therefore, some studies suggest that certain oxidative mechanisms are responsible for the augmentation of antifungal responses of phagocytes by amphotericin B.

Effects on Lymphocytes. Amphotericin B, as well as other polyene antibiotics, have been shown to act as mitogens for B lymphocytes and can induce polyclonal antibody production from these cells (Hammarstrom 76; Blanke 77; Shirley 79A; Shirley 79B; Seay 82; Mehta 85; Sarthou 86; Henry-Toulme 89; Schindler 93). However, evidence also exists of a suppressive effect AmB has on B cells (Ferrante 79; Walls 82; Schindler 93). One study pointed to the fact that AmB in the deoxycholate form is highly

toxic to mammalian cells, which may account for the discrepancies often observed in studies of this nature (Sarthou 86). Therefore, the authors decided to perform stimulation assays on B cells by a different formulation of AmB which would be less toxic to the cells. The study found that the alternate preparation of AmB was, in fact, a stronger mitogen for B cells than AmB in its native form and that it enhanced B cell proliferation and maturation. In addition, the study found that lipopolysaccharide (LPS) acted in synergy with AmB for B cell proliferation.

AmB has also been described as a stimulator of T cells, but there exists some controversy as to whether it might act as an immunosuppressant for T cell function (Hauser 83; Mehta 85; Henry-Toulme 89; Boggs 91; Schindler 93; Kumar 00; Kretschmar 01). One study determined that AmB has both stimulatory and inhibitory effects on Concanavalin A (ConA)-induced T cell proliferation and that the enhancing effect was only seen after blocking the AmB inhibitory effect (Kumar 00). The authors noted that AmB blocked T cell proliferation after ConA stimulation without affecting cell viability, but it could not affect the proliferation in the presence of ibuprofen, which is an inhibitor of cyclooxygenase. The authors hypothesized that AmB blocks an early step in TCR signalling through oxidative inactivation of proteins which are proximal to phosphoinositide hydrolysis, leading to protein kinase C (PKC) activation and to increased $[Ca^{2+}]_i$. The authors suggested that AmB inhibited T cell proliferation through H_2O_2 production. However, it was noted that AmB only affected the production of H_2O_2 by macrophages, not by the lymphocytes. This finding indicated that the purified T cell preparation may have been contaminated with macrophages, since inhibited proliferation

in the “pure” T cell preparation was observed. The authors concluded that AmB has both stimulatory and inhibitory effects on T cell activation, and the events are mutually exclusive, as suppressing the inhibitory effect of AmB revealed its immunostimulatory effect. Overall, the study indicated that AmB causes macrophages to produce H_2O_2, which then inhibits T cell proliferation. A separate study determined that both standard and liposomal AmB were equally inhibitory of cytotoxic T-cell function in vitro and in vivo after infection with *Listeria monocytogenes*, but the authors found that the effect was dependent upon the dose (Kretschmar 01).

Studies which looked at the relationship between AmB and cells that were stimulated with either LPS or ConA found that lymphocytes do not appear to be stimulated by AmB even in the presence of these mitogens, but results have been contradictory in regard to this subject (Seay 82; Walls 82; Mehta 85; Kumar 00). One study found that the effect was AmB concentration-dependent (Seay 82).

Signalling. There have been several studies which have looked at the effects AmB has on signalling pathways in the immune system (Cleary 88; Chia 89; Chia 90; Cleary 92; Lyman 92; Raponi 93; Tokuda 93; Ghezzi 94; Louie 94; Martin 94; Arning 95). In vivo toxicity is believed to be due to an increase in the levels of IL-1β, TNF-α, and IL-1 receptor agonist (IL-1ra) (Arning 95; Shadkchan 04). These cytokines have been expressed in human peripheral mononuclear cells (PBMCs) (Vonk 98), as well as a leukemic monocytic cell line (Rogers 98; Rogers 99; Rogers 00; Rogers 02), treated with AmB.

One pivotal study determined whether AmB played a role in the balance between pro- and anti-inflammatory responses by looking at its effects on IL-1 and IL-1ra, respectively (Vonk 98). The results indicate that AmB slightly increases the production of pro-inflammatory cytokines by human mononuclear cells and inhibits the anti-inflammatory cytokine IL-1ra. However, some studies support the idea that AmB reduces pro-inflammatory cytokine production only (Raponi 93; Ghezzi 94; Martin 94; Baltch 12). One study determined that alternate formulations of AmB caused different signalling events to occur in monocytes, which may explain some of the discrepancies in previous studies (Turtinen 04).

It has been demonstrated that AmB is recognized by TLR2 and CD14 on the surface of innate immune cells, and through the myeloid differentiation protein 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathways, it allows for the expression of several cytokine genes involved in immunomodulation (Sau 03). This study showed that MyD88, CD14, and TLR2 are all required for AmB-induced TNF-α production from macrophages and that TLR2 is also required for IL-1β production by macrophages. Additionally, the results showed that liposomal formulations of AmB did not cause cytokine production from mouse peritoneal macrophages, and the authors suggested that the absence of signalling induction could account for why liposomal formulations are less toxic. They speculated that the liposomal forms are not free and unbound, as conventional AmB is, so that the interaction between AmB and CD14/TLR2 occurs less often than between conventional AmB and such receptors. One more point the authors noted was that their data support the idea that

TLR4 may also play a role in signalling induced by AmB, but they found that this effect was only significant at AmB concentrations higher than those found in the serum of patients receiving the drug. This study was novel in that it suggested that toxicity related to AmB therapy was not simply due to the fact that the drug may also interact with cholesterol on mammalian cell membranes. It supported the idea that the inflammatory responses induced by the drug were causing toxicity and were separate from membrane-related toxicity.

Another study looked at the relationship between AmB and TLR signalling, and it supported the idea that the various formulations of AmB utilize different signalling pathways (Bellocchio 05). Their results indicate that AmB deoxycholate causes activation of the oxidative mechanism which leads to a pro-inflammatory response via TLR2 and TNF-α. In addition, their data support that liposomal AmB (AmBisome) diverts signalling from TLR2 to TLR4 and elicits degranulation rather than reactive oxygen intermediate (ROI) production due to favored production of IL-10 over TNF-α. They suggest that the utilization of TLR4 instead of TLR2 and the induction of anti-inflammatory responses via IL-10 production are the reasons for the decrease in toxicity associated with AmBisome versus deoxycholate AmB.

Other studies support the idea that the alternate formulations of AmB have significantly different effects on the host immune response in the presence and absence of disease. For example, one study found that there was a decline in untreated monocyte-enriched mononuclear leukocytes of IL-1β, IL-1ra, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1beta (MIP-1β), and TNF-α (Simitsopoulou

05). In addition, deoxycholate AmB and ABCD increased early IL-1β, TNF-α, MCP-1, MIP-1β, and late IL-1ra. The study also found that L-AmB and ABLC decreased or did not affect IL-1β and TNF-α, while ABLC decreased MIP-1β. The authors concluded that deoxycholate AmB and ABCD upregulate the gene expression and production of proinflammatory chemokines and cytokines, whereas L-AmB and ABLC down-regulate or do not affect the same chemokines and cytokines. Their study is also supportive of the idea that the regulation of the host inflammatory response plays a role in causing or dampening the toxicity of the drug.

One study looked at whether the inflammatory response to AmB could be overcome by other drugs, so as to limit the toxicity of AmB. The authors explained that AmB has been found to produce similar but less potent IL-1β dose response curves in mononuclear cells as LPS, and they discovered that this response could be inhibited by the addition of certain premedications, such as hydrocortisone, pentoxifylline, and A81-3138 (Cleary 92). The authors suggest that utilization of such premedications might reduce the some of the febrile reactions which are often associated with AmB therapy.

Objectives of Thesis Project

The long-term objective of this Thesis work was to characterize the relationship between amphotericin B and γδ T cells so as to better understand the roles these cells play in the immune system. The hypothesis that was tested was: amphotericin B is a potent stimulator of bovine and human γδ T cells and has the capability to prime these cells for further activation by other agonists. Using a combination of flow cytometry and

cytokine analyses, we found that amphotericin B treatment of PBMCs leads to a potent and unique stimulatory effect on γδ T cells, which likely is important in the effects of this drug on the immune system.

MATERIALS AND METHODS

Preparation of Bovine and Human PBMCs

Whole blood was drawn from Holstein bull calves, aged 1-3 months, and collected into sodium heparin tubes (Becton Dickinson; Franklin Lakes, NJ). To isolate leukocytes, Histopaque 1077 (Sigma-Aldrich; St. Louis, MO) was used, as previously described (Hedges 03). In addition, red blood cells were removed by hypotonic lysis. Whole blood was collected into ACT tubes (Becton Dickinson) from adult human volunteer donors in healthy condition. Histopaque 1077 (Sigma-Aldrich) was utilized to separate the leukocytes from whole blood, as per the manufacturer's instructions.

Cell preparations were maintained in X-VIVO 15 medium (Cambrex; East Rutherford, NJ) at $1x10^6$ cells/ml for bovine cells or $2x10^6$ cells/ml for human preparations. Cells were cultured at 37ºC, 10% CO_2 in the presence or absence of known or test agonists for 24 hours for bovine cells and 48 hours for human cells, unless otherwise indicated. Samples from a minimum of 3 subjects (human or bovine) were obtained and analyzed per experiment.

All experiments were performed in accordance with the National Institute of Health guidelines and were approved by the Institutional Animal Care and Use Committee and Institutional Review Board of Montana State University.

FACS-Based Analyses of Human and Bovine PBMCs

A FACSCalibur equipped with a High-Throughput Screening (HTS) loader (Becton Dickinson) or a FACSCanto (Becton Dickinson) was used to analyze cells. Bovine cells were stained with monoclonal antibodies (mAbs) against IL-2Rα (LCTB2A) (VMRD; Pullman, WA) and/or γδTCR (GD3.8). Human cells were stained with γδTCR (11F2) (Becton Dickinson) and anti-human CD69 (Biolegend; San Diego, CA).

The mAbs were directly labeled (FITC, PE, PE-Cy5, PE-Cy7, APC, or APC-Cy7) or indirectly labeled with goat-anti-mouse PE, FITC or APC (Jackson ImmunoResearch; West Grove, PA). In order to determine the level of background staining in the FACS analyses, second stage reagents were used alone.

IL-2Rα expression on bovine PBMCs was determined by multi-color FACS after cells were cultured in the presence or absence of AmB for 24 hours. In some experiments, an additional agonist was added for another 24 hours after the initial 24 hour incubation with AmB, and no washing of the cells was performed before addition of the second agonist. In order to determine cell proliferation after incubation with AmB, bovine PBMCs were labeled with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for five minutes in Hank's Balanced Salt Solution (HBSS) (Mediatech; Herndon, VA), washed, and then cultured at 37ºC, 10% CO_2 in X-VIVO medium for 5 days in the presence or absence of AmB. Quantification of cell division was done with multi-color FACS analyses to determine the percentage of cells which had divided at least once, based on a 50% or greater reduction in the intensity of CFSE. Positive control agonists included Concanavalin A (Con A) (Sigma-Aldrich; 1 μg/ml) for bovine cultures and 1-

hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HD-MAPP) (Echelon Bioscience; Salt Lake City, UT; 1 μg/ml) and human rIL-15 (Peprotech; Rocky Hill, NJ; 10 ng/ml) together for human cells. Appropriate negative controls were also employed, including buffers and assay mediums.

Intracellular staining for IFN-γ production by human γδ T cells after being cultured with phorbol 12-myristate 13-acetate (PMA) and ionomycin, AmB, or all three agonists was achieved by utilizing a two-color FACS assay. The cells were incubated in X-VIVO medium at 37ºC in 10% CO_2 in the presence or absence of the agonists for 18 hours. Brefeldin A (eBioscience; San Diego, CA) was added to the cells for 4 hours, and extracellular staining with γδTCR (11F2) (Becton Dickinson) was performed. The cells were fixed using a 4% solution of paraformaldehyde in PBS and then perforated via addition of a 0.2% solution of saponin (Sigma-Aldrich) in PBS/horse serum buffer overnight at 4ºC. Intracellular staining with anti-human IFN-γ (Biolegend) was performed, and the cells were analyzed by flow cytometry for detection of intracellular IFN-γ.

Preparation of Amphotericin B Formulations

Deoxycholate Amphotericin B (Sigma-Aldrich), AmBisome (Gilead Sciences, Inc.; Foster City, CA), and Fungizone (CalBiochem/EMD Millipore; Billerica, MA) were solubilized in deionized water and diluted further in X-VIVO medium at room temperature. Aliquots were stored at -80ºC until use.

Endotoxin Screening

To analyze the concentration of endotoxin which may have been present in the preparations of AmB, a kinetic turbidimetric Limulus Amebocyte Lysate (LAL) (Associates of Cape Cod; East Falmouth, MA) assay was performed as per the manufacturer's instructions. The three formulations of AmB were diluted to concentrations ranging from 56.25 μg/ml to 7.03 μg/ml in LAL Reagent water and analyzed for endotoxin using LAL Reagent resuspended in Glucashield buffer. A ThermoMax Microplate Reader (Molecular Devices; Sunnyvale, CA) was utilized to measure LAL agglutination, and each of the samples was analyzed in triplicate.

RESULTS

Previous Work Performed in the Jutila Laboratory

Amphotericin B in Murine Models of Infection

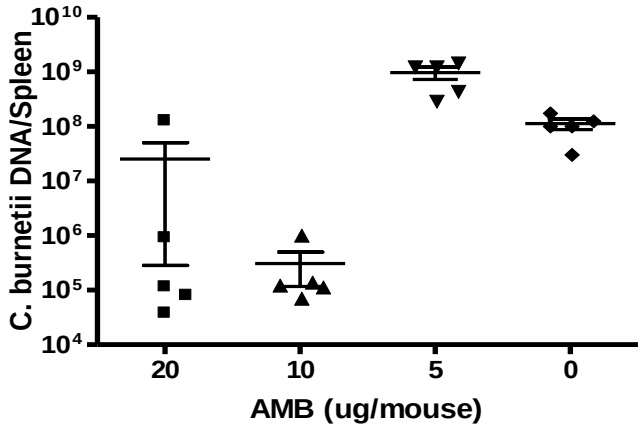
Administration of AmB enhanced the clearance of *Coxiella burnetii*, an intracellular pathogen which typically infects the lungs (Figure 6A). In addition, it was found that AmB administered to the lung induced dendritic cell activation (determined by CD11b and CD11c expression) and neutrophil recruitment (data not shown). IFN-γ is an important cytokine for clearance of *C. burnetii*. Therefore, the ability of AmB to induce production of IFN-γ in cells exposed to *Coxiella* was determined. Lung macrophages and dendritic cells from AmB-primed lungs were found to produce more IFN-γ after ex vivo *C. burnetii* infection than both uninfected AmB-primed cells and infected unprimed cells (Figure 6B).

The pathway AmB utilizes for potent adjuvant activity has been debated, and both TLR2 and TLR4 have been suggested as important to the AmB responses seen in vivo (Bellocchio 05). Thus, the adjuvant activity of AmB in vivo in TLR2- and MyD88-deficient mice was tested using the *C. burnetii* infection model. Adjuvant activity of AmB in the lungs of TLR2-deficient animals was not affected, but the response was diminished in MyD88-deficient mice, suggesting another TLR is playing a role in the AmB response (Figure 7). In the peritoneum, however, there was a clear role for TLR2 (Figure 7). Peritoneal responses in TLR4-deficient mice have not been studied, but nearly all responses were absent from the lungs of TLR4-deficient mice (data not shown).

Therefore, it appears that TLR4 may have a greater role in the lung, while TLR2 might be more important for the AmB adjuvant activity in the periphery during *Coxiella* infection.

Figure 6. Amphotericin B Augments Production of IFN-γ by Mouse Lung Cells in Response to *C. burnetii* Infection In Vivo

A.



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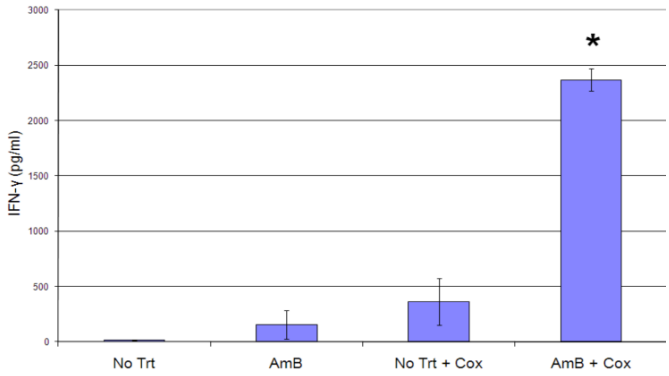
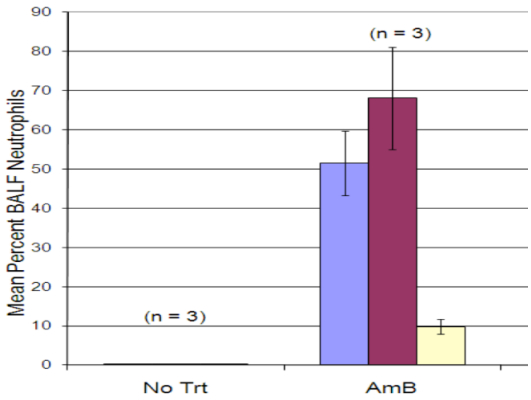


A) Five BALB/C mice were infected i.p. with Phase I *C. burnetii* (1.0 X 10^3). After 24 hours, mice were treated intraperitoneally (i.p.) with AmB (20, 10, or 5 μg/mouse) or the carrier/buffer control and then sacrificed 9 days later. *C. burnetii* from spleens were purified, DNA was extracted, and qPCR quantification of *C. burnetii* genomes/spleen (axis) was performed. B) BALB/c mice (6 animals/group) received no treatment or were treated intratracheally with 10 μg of amphotericin B. Twenty four hours later, lung cells were isolated from all 12 animals and plated in serum-free X-VIVO 15 medium in 48 well plates. *C. burnetii* (1x10^8 phase II) was added to some of the wells containing control or amphotericin B-treated cells. The plates were then incubated for 48 hours, and IFN-γ levels in the supernatant fluids were measured by ELISA. Error bars represent means +/- standard deviation. * Difference between control, plus *C. burnetii* and amphotericin B treated, plus *C. burnetii* was significant at a P value <0.05. Note: Experiments done by Brett Freeman.

Figure 7. Amphotericin B-induced Neutrophil Recruitment is Reduced in the Peritoneum, but not the Lungs, of TLR-2 Deficient Mice.

A.



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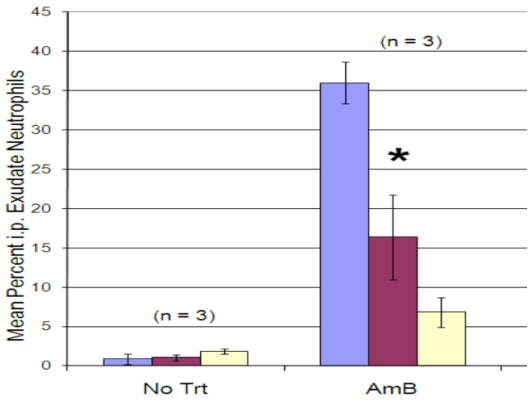
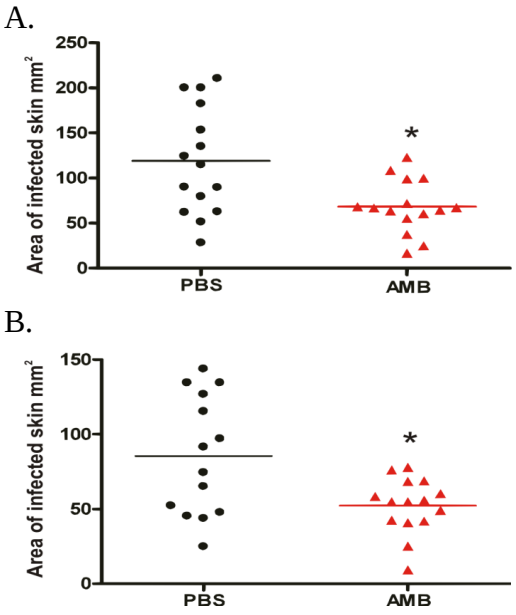


Neutrophil recruitment into the A) lungs and B) peritoneum of C57BL/6 (left bar, blue), TLR2 -/- (middle bar, purple), MyD88-/- (right bar, white), and control mice were compared following injection of amphotericin B (AmB) or no treatment (No trt). Neutrophil recruitment was quantified by FACS. * Denotes a P-value <0.05. Note: Experiments done by Brett Freeman.

The adjuvant potential of AmB was studied in another infectious disease setting, a murine model of skin and soft-tissue infection with *Staphylococcus aureus*. One and four days post infection with *S. aureus*, mice were injected with PBS or PBS plus AmB (5 μg) intraperitoneally, and abscess volume and dermonecrosis area were determined. AmB-

treated mice had a decreased abscess volume and dermonecrosis layer as compared to animals which received PBS alone (Figure 8). Therefore, it appears that the adjuvant activity of AmB spans beyond the lung and peripheral settings, and the antifungal drug could potentially be utilized as an adjuvant versus several systemic, skin, and soft-tissue bacterial diseases.

Figure 8. AmB Reduces MRSA-induced Pathology in the Mouse Skin



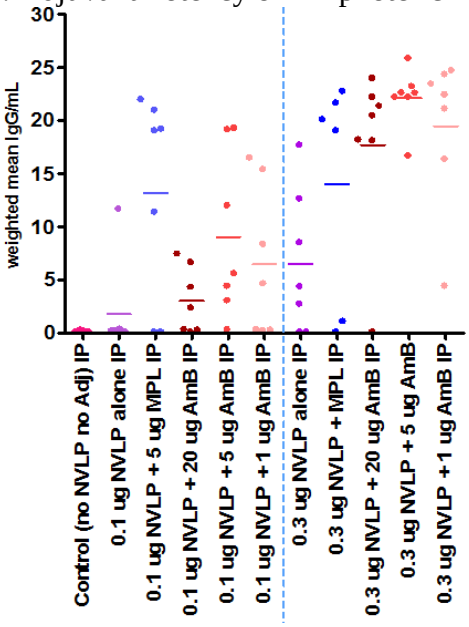
Mice were infected subcutaneously with 1 x10^7 *S. aureus*. At day 1 and day 5 post infection, mice were given an i.p. inoculation of PBS or PBS containing 5 μg of AMB (n=15 mice per treatment). A) Day 3 post infection. B) Day 6 post infection. $P<0.05$ by paired T-test analysis. Note: Experiments done by Brett Freeman, Kirk Lubick, and Jovanka Voyich-Kane.

Adjuvant Potency of Amphotericin B

Activity of AmB in an antigen/vaccine setting was also previously determined in the Jutila laboratory by measuring the antibody titers to a norovirus vaccine construct with and without AmB added to the antigen. It was found that as little as 1 μg of AmB

added to the vaccine was able to induce robust IgG production in immunized mice (Figure 9). Monophosphoryl lipid A (MPL), a known TLR4 agonist widely used as an adjuvant in human vaccines, was utilized as a positive control. The levels of IgG induced by addition of AmB to the norovirus vaccine construct were slightly higher than those induced by addition of MPL, indicating AmB as a potential adjuvant alternative to MPL in a norovirus vaccine (Figure 9).

Figure 9. Adjuvant Potency of Amphotericin B



Mice (n=7) were immunized with a single injection of Norovirus-like particle (NVLP) as a vaccine antigen at 0.1 (left panel) or 0.3 (right panel) µg/mouse, with or without Amphotericin B (AmB) at 1, 5, or 20 µg/mouse, or MPL (5 µg/mouse) as a positive control. Serum was collected on day 14, and anti-NVLP IgG was measured using a direct ELISA. The absorbance mean of duplicate wells performed at 1:50, 1:100, 1:200 serum dilutions was used to calculate individual weighted mean IgG µg/ml based on a murine IgG standard curve in each assay plate. Individual means are shown as dots, and the group mean values are graphed as lines in each group. Experimental controls were non-immunized animals (No NVLP no Adjuvant), NVLP alone, and NVLP plus 5 µg MPL. Note: Experiments done in collaboration with Ligocyte Pharmaceuticals.

Amphotericin B Induces Cell Division and Activation of Bovine γδ T Cells

High throughput screening assays identified both Amphotericin B and nystatin as potent inducers of IL-2R expression on bovine γδ T cells. However, it was discovered that AmB had higher specific activity and was utilized in all further studies concerning the effects of bacterial polyenes on bovine γδ T cells. Cell activation and proliferation induced by AmB was determined by evaluating IL-2Rα upregulation and CFSE staining, respectively, via flow cytometry-based assays. AmB induced upregulation of IL-2Rα on bovine γδ T cells, similar to the response induced by another T cell agonist, ConA (Figure 10). The cells responded strongly after 48 hours of incubation with the antifungal drug, but 24 hours was sufficient to induce the upregulation of IL-2Rα.

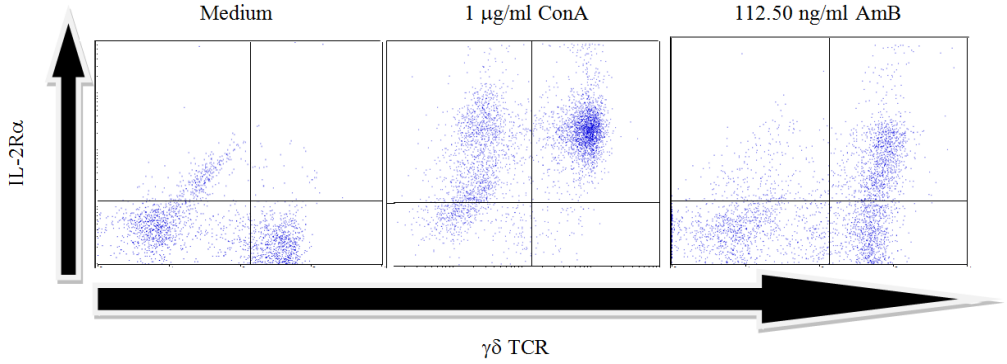
Three formulations of AmB caused different activation profiles in bovine γδ T cells, with the Sigma deoxycholate AmB preparation having the most potent activity, and AmBisome inducing the least amount of activity at the same concentration (Figure 11). Fungizone, another formulation of deoxycholate AmB, was found to have similar, but slightly more potent, effects on the bovine γδ T cells at equal concentrations to AmBisome (Figure 11).

The effect of the antifungal drug on IL-2 receptor upregulation was specific to the γδ T cells within the PBMC population (Figure 10), but not all γδ T cells were activated after exposure to even the highest (non-cytotoxic) concentrations of AmB, suggesting that the drug may only activate specific subsets of γδ T cells. Furthermore, it was observed that removal of monocytes from the PBMC preparation caused a decrease in the

upregulation of IL-2Rα on γδ T cells (data not shown), indicating a potentially crucial role for these cells in the response of γδ T cells to AmB.

Figure 10. AmB Induces the Upregulation of IL-2R on Bovine γδ T Cells

A.



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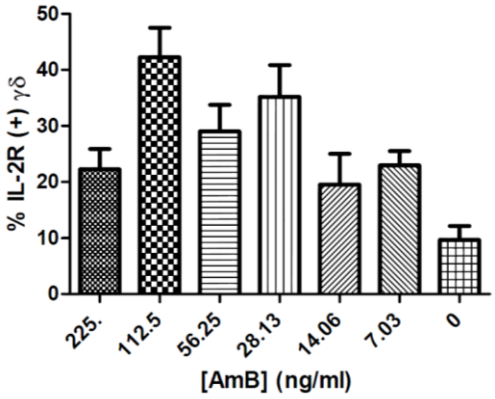
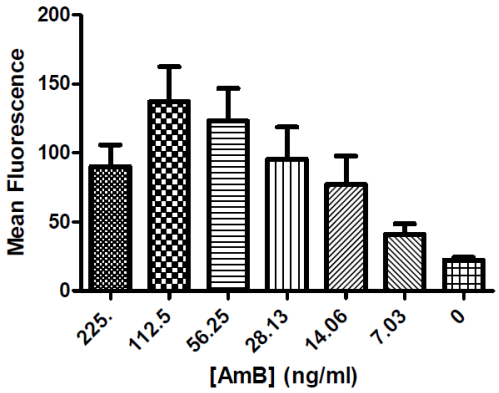


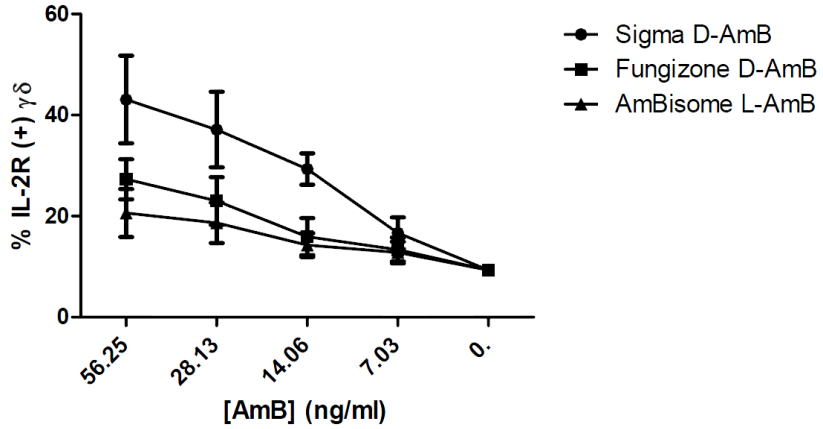
C.



Freshly collected bovine PBMCs were cultured for 24 hours at 37ºC in 10% CO_2 with several concentrations of Amphotericin B, medium alone, or ConA as a positive control. The cells were analyzed by flow cytometry in two-color FACS assays to determine the percentage of γδ T cells expressing IL-2Rα, and the fluorescence intensity of the stain was measured. A) FACS plots depicting upregulation of IL-2Rα on bovine γδ T cells after treatment with X-VIVO medium, ConA (1 µg/ml), or AmB (112.5 ng/ml). B) The percentage of γδ T cells expressing IL-2Rα after treatment with AmB as compared to X-VIVO medium alone. C. The mean fluorescence intensity of staining for IL-2Rα on γδ T cells after treatment with AmB or X-VIVO medium. Error bars represent the standard error from a minimum of 3 independent experiments.

Figure 11. Alternate Formulations of AmB are Less Potent Stimulators of Bovine γδ T Cells

A.



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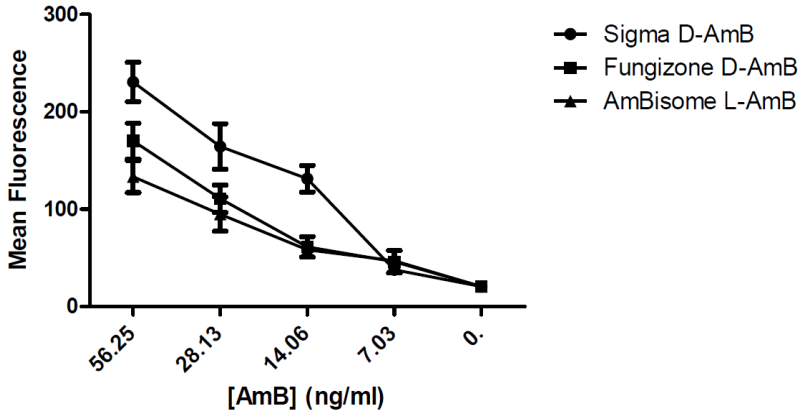
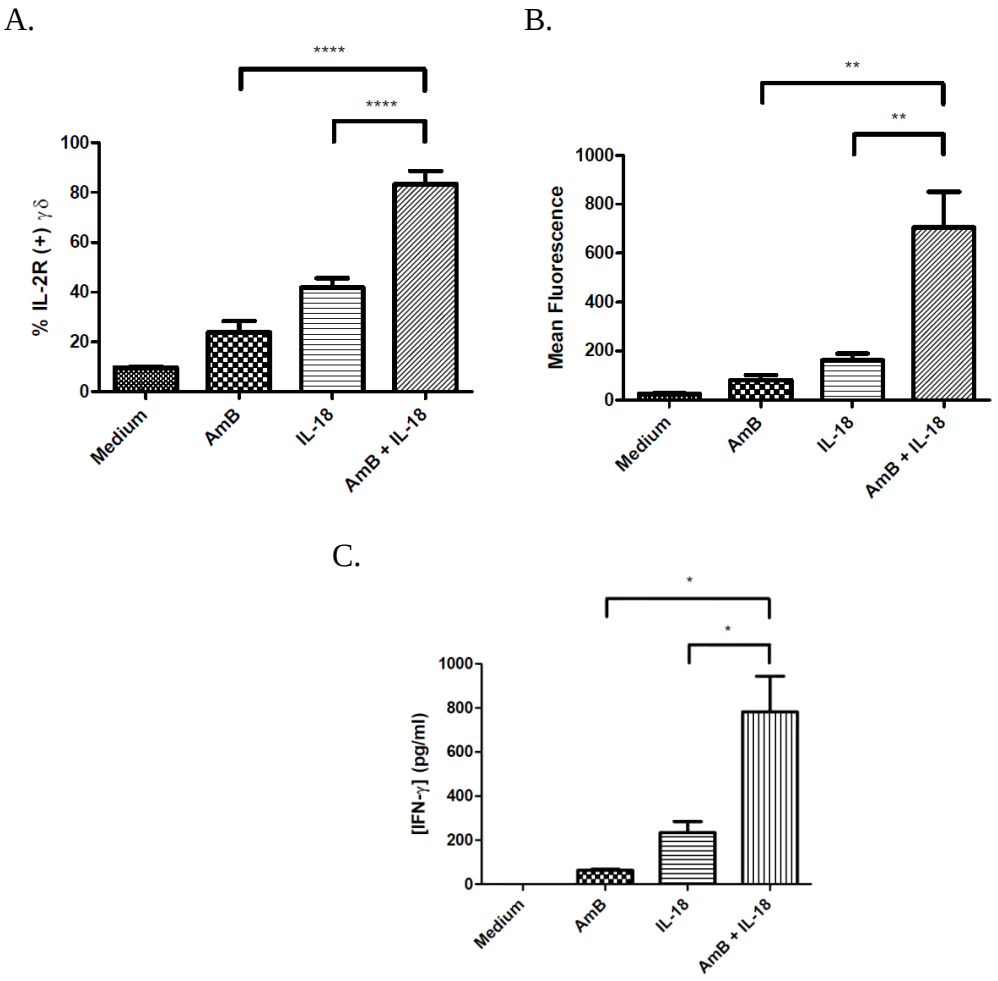


Freshly collected bovine PBMCs were cultured with several concentrations of two formulations of deoxycholate Amphotericin B (Sigma and Fungizone), as well as one liposomal formulation (AmBisome) for 24 hours at 37ºC in 10% CO_2 and analyzed by flow cytometry in IL-2Rα two-color FACS assays to determine the percentage of γδ T cells expressing IL-2Rα, and the fluorescence intensity of the stain was measured. A. The percentage of γδ T cells expressing IL-2Rα after treatment with three different formulations of AmB. B. The mean fluorescence intensity of staining for IL-2Rα on γδ T cells after treatment with three formulations of AmB. Error bars represent the standard error from a minimum of 3 independent experiments.

IL-18 was originally termed “IFN-γ inducing factor”, and it is produced by macrophages and other cells in response to bacterial products such as LPS (Okamura 95). After IL-18 stimulation, cells (such as T cells and NK cells) produce IFN-γ, which can activate additional cells and induce a pro-inflammatory response. Bovine γδ T cells primed with a low dose of AmB in vitro and then stimulated with IL-18 showed an increased response versus treatment with AmB or IL-18 alone (Figure 12), indicating an innate priming response. An increase in IL-2Rα on γδ T cells was observed, and a higher production of IFN-γ from bovine PBMCs was induced after treatment with AmB in combination with IL-18 (Figure 12). Thus, AmB in low doses could act as an agonist which primes cells for activation by other stimulants in vitro. AmB also primed cells for an enhanced response to ConA, a TCR cross-linker, as well as LPS, a TLR4 agonist (Figure 13). Both ConA- and LPS-induced IL-2Rα upregulation on γδ T cells and IFN-γ production by PBMCs were increased after priming with AmB (Figure 13).

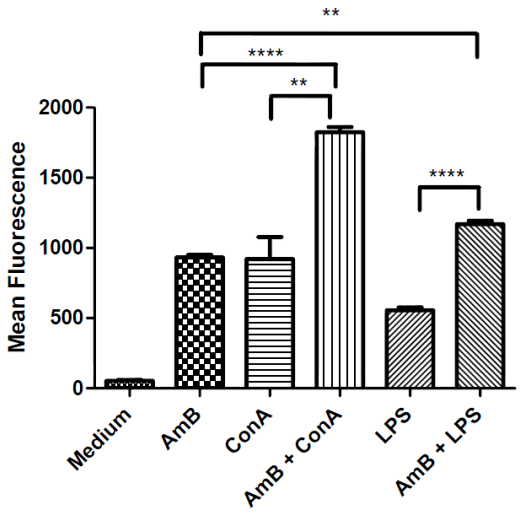
Figure 12. AmB Primes Bovine PBMCs for Enhanced Stimulation after Exposure to IL-18



A) and B) Bovine PBMCs were cultured with AmB (14.06 ng/ml) or X-VIVO medium for 24 hours at 37ºC in 10% CO_2. The cells were then cultured with rhu IL-18 (10 ng/ml) or X-VIVO medium for an additional 24 hours and analyzed by flow cytometry in IL-2Rα two-color FACS assays to determine the percentage of γδ T cells expressing IL-2Rα. In addition, the fluorescence intensity of the stain was measured. A) The percentage of γδ T cells expressing IL-2Rα after treatment. B) The mean fluorescence intensity of staining for IL-2Rα on γδ T cells after treatment. Error bars represent the standard error from a minimum of 3 independent experiments. C) PBMCs from 6 bovine calves were incubated with 28.13 ng/ml AmB or X-VIVO medium for 24 hours at 37ºC in 10% CO_2. 10 ng/ml rhu IL-18 or X-VIVO medium was then added for an additional 24 hours. Supernatants were collected and measured for concentration of IFN-γ by ELISA. Each sample was plated in triplicate, and error bars represent standard error. P-values for differences in means are: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 13. AmB Primes Bovine PBMCs for Enhanced Stimulation after Exposure to ConA or LPS

A.



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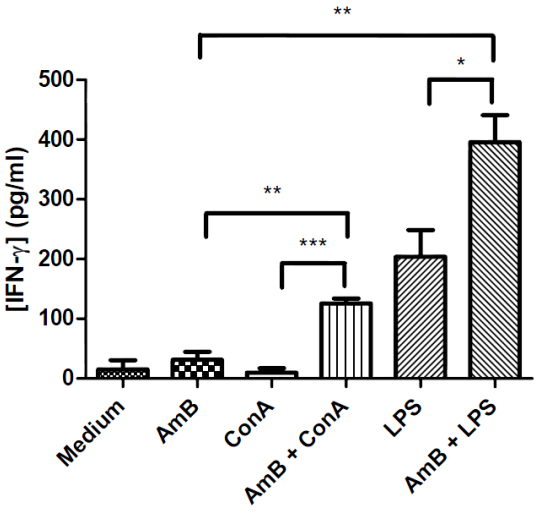


Bovine PBMCs were cultured at 37ºC in 10% CO_2 with AmB (21.09 ng/ml) or X-VIVO medium for 24 hours. ConA (50 ng/ml), LPS (10 μg/ml), or X-VIVO was then added to the cells for an additional 24 hours. The cells were analyzed by flow cytometry in IL-2Rα two-color FACS assays to determine the fluorescence intensity of the stain, measured. A) The mean fluorescence intensity of staining for IL-2Rα on γδ T cells after treatment. Error bars represent the standard error from a minimum of 3 independent experiments. B) Supernatant fluids were collected after incubation and measured for concentration of IFN-γ by ELISA. Each sample was plated in triplicate, and error bars represent the standard error. P-values for differences in means are: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

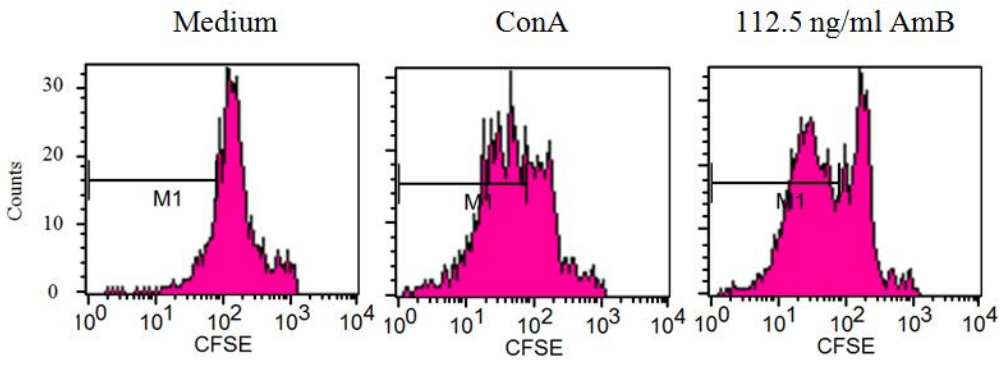
Amphotericin B caused proliferation of γδ T cells, which was detected in a 5-day CFSE assay and calculated based upon a decrease in CFSE staining at each cell division (Figure 14). The AmB-induced proliferation was dose-dependent, as lower doses caused few cell divisions above medium-treated cells, while doses of AmB above 112.5 ng/ml were slightly toxic to the cells and caused less proliferation to occur (Figure 14). These results were consistent with the dose curve related to AmB-induced IL-2Rα upregulation on γδ T cells (Figure 10), as doses higher than 112.5 ng/ml showed decreased activation, likely due to cytotoxicity at higher concentrations.

Amphotericin B Induces Activation of Human γδ T Cells

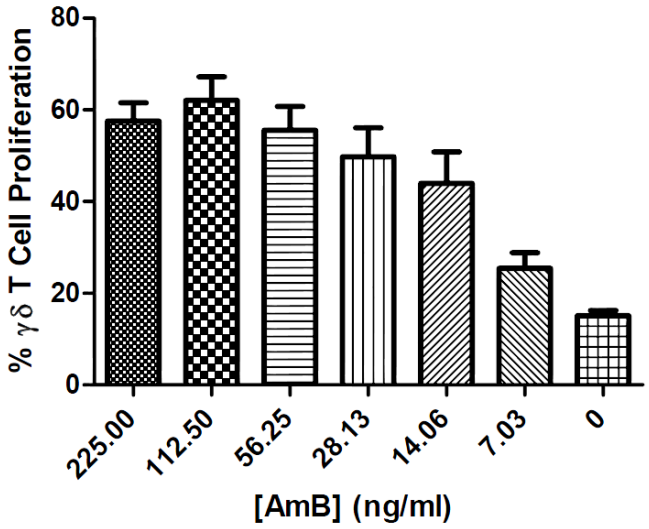
Human γδ T cells were analyzed for their response to AmB, and it was found that a dose-dependent activation of the cells was induced by the antifungal drug (Figure 15). Upregulation of CD69, on human γδ T cells was observed in a similar manner to IL-2Rα upregulation on bovine γδ T cells, indicating activation of these cells. However, the dosages needed for potent activation were much higher for human cells than bovine cells, as the lowest activating dose in humans (1.76 μg/ml) was well above the cytotoxic dose (0.225 μg/ml) in bovine cells (Figure 15). Additionally, CD69 expression on human γδ T cells caused by incubation with the agonists PMA and ionomycin was enhanced when the PBMCs were also cultured with AmB (Figure 16).

Figure 14. Amphotericin B Induces the Proliferation of Bovine γδ T Cells

A.

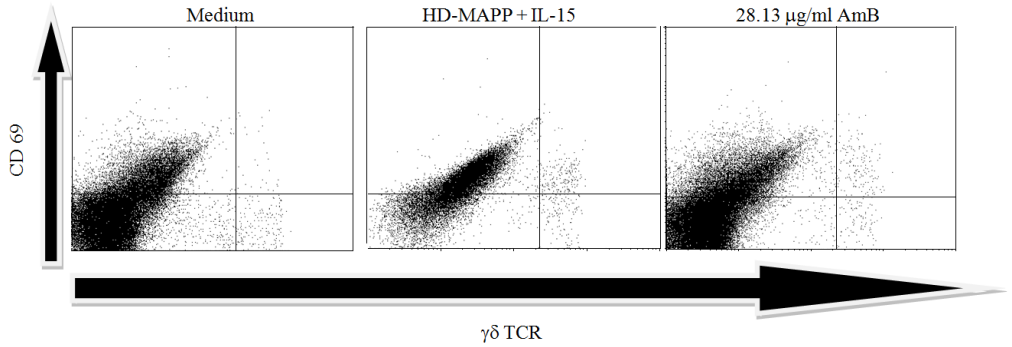


B.

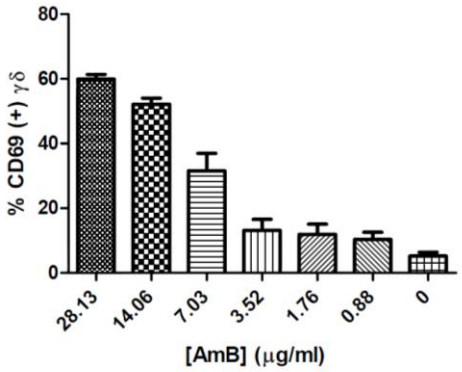


Proliferation of CFSE-labeled bovine PBMCs was measured in response to being cultured with several concentrations of AmB for 5 days at 37ºC in 10% CO_2. Responses by γδ T cells were determined using γδ TCR mAbs and a CFSE stain in two color FACS analyses. Data are representative of three independent experiments from six bovine calves. A) CFSE FACS plots demonstrating reduction in CFSE staining (indicating cell proliferation) on γδ T cells after treatment with medium alone, 1 μg/ml ConA, or 112.5 ng/ml AmB. B) Graph depicting the percentage of γδ T cells which proliferated at least once after treatment with different concentrations of AmB.

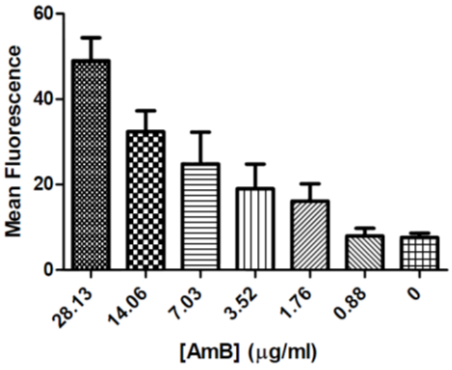
Figure 15. AmB Induces the Expression of CD69 on Human γδ T Cells



A.



B.

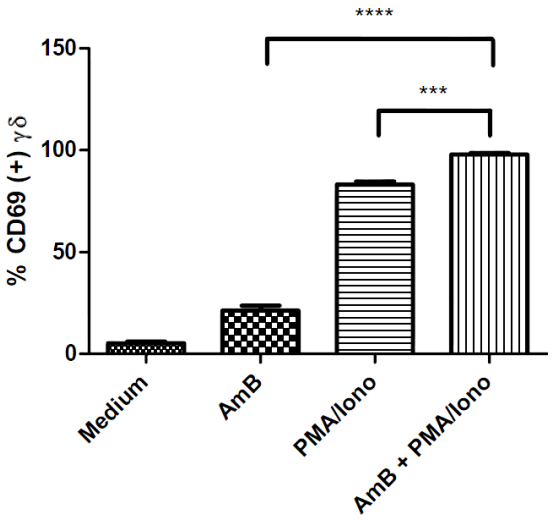


C.

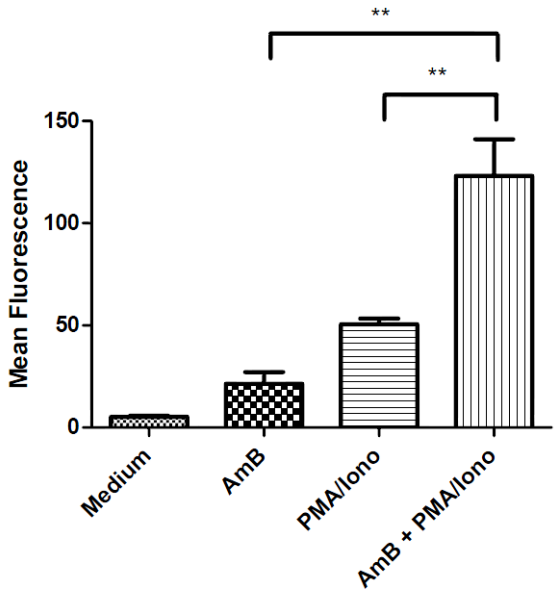
Freshly collected human PBMCs were cultured for 48 hours at 37ºC in 10% CO_2 with several concentrations of Amphotericin B and analyzed by flow cytometry in two-color FACS assays to determine the percentage of γδ T cells expressing CD69, and the fluorescence intensity of the stain was measured. A. The percentage of γδ T cells expressing CD69 after treatment with AmB as compared to X-VIVO medium alone. B. The mean fluorescence intensity of staining for CD69 on γδ T cells after treatment with AmB or X-VIVO medium. Error bars represent the standard error from a minimum of 3 independent experiments using PBMCs from four healthy human donors.

Figure 16. AmB Acts Synergistically with PMA and Ionomycin to Induce Expression of CD69 on Human γδ T Cells

A.



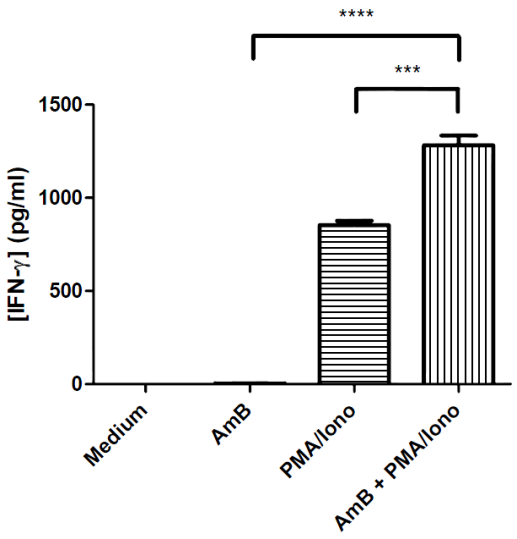
B.



Human PBMCs were cultured with Amphotericin B (7.03 μg/ml), PMA (1 ng/ml) and ionomycin (1 μg/ml), all three compounds, or medium only for 48 hours at 37ºC in 10% CO_2. The cells were analyzed by flow cytometry using a two-color FACS assay, and the percentage of γδ T cells expressing CD69 (A) and the mean fluorescence intensity of CD69 staining on γδ T cells (B) were determined for each treatment. Error bars represent the standard error from a minimum of 3 samples using PBMCs from four healthy human donors. P-values for differences in means are: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Detectable amounts of IFN-γ produced by human PBMCs could not be induced with AmB treatment alone at any dose as detected by ELISA (data not shown). However, the IFN-γ production elicited by the addition of PMA and ionomycin to human PBMCs was enhanced by the addition of a low concentration of AmB (Figure 17). Furthermore, intracellular staining for IFN-γ revealed that a small dose of AmB alone was sufficient to elicit the production of the cytokine by human γδ T cells, and the production of IFN-γ caused by PMA and ionomycin was enhanced by co-culturing the cells with AmB (Figure 18).

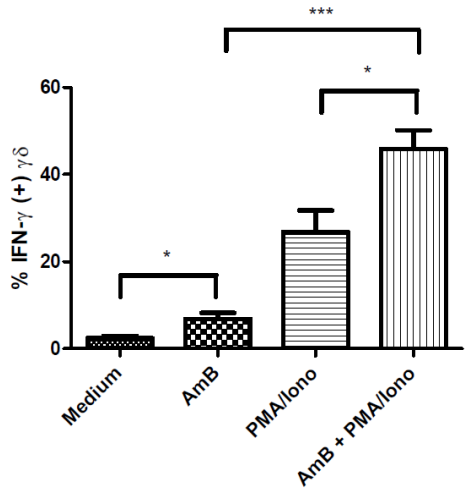
Figure 17. AmB Acts Synergistically with PMA and Ionomycin to Elicit Production of IFN-γ from Human PBMCs



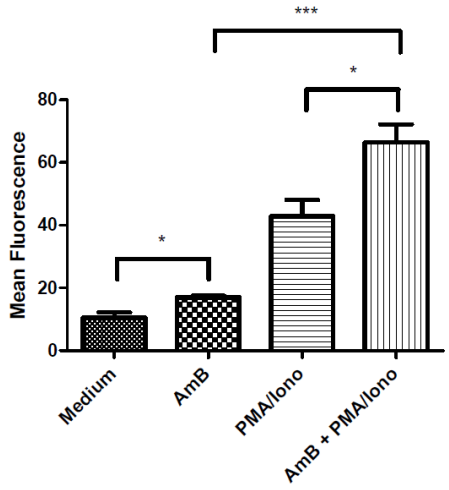
Freshly collected human PBMCs were cultured for 48 hours at 37ºC in 10% CO_2 with PMA (1 ng/ml) and ionomycin (1 μg/ml), Amphotericin B (7.03 μg/ml), all three agonists, or medium alone. Supernatants were collected, and the concentration of IFN-γ was determined by ELISA. Error bars represent the standard error, and samples were analyzed in triplicate using PBMCs from four healthy human donors. P-values for differences in means are: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 18. AmB Induces the Production of IFN-γ by Human γδ T Cells and Enhances the Response by Cells Exposed to PMA and Ionomycin

A.



B.



Human PBMCs from four healthy donors were cultured with PMA (1 ng/ml) and ionomycin (1 μg/ml), Amphotericin B (7.03 μg/ml), all three compounds, or medium alone for 18 hours at 37ºC in 10% CO_2. The cells were then treated with brefeldin A for 4 hours and stained extracellularly with an anti-γδ TCR stain. Saponin was added overnight after the cells were fixed with paraformaldehyde, and they were then stained with an intracellular IFN-γ antibody. The cells were subsequently analyzed by flow cytometry. A) The percentage of γδ T cells staining positive for intracellular IFN-γ after each treatment as determined by a two-color FACS assay. B) The mean fluorescence intensity of staining for intracellular IFN-γ after culture with or without the agonists. P-values for differences in means are: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

DISCUSSION

γδ T cells are an evolutionarily well-conserved subset of lymphocytes, and they are thought to be the precursors of modern B and αβ T cells (Ferrick 00; Richards 00). Although originally believed to have a similar function as αβ T cells in host defense, it has been thought that γδ T cells contribute more to the innate immune rather than the adaptive immune response. However, the exact roles of these cells have not been fully elucidated, and much has yet to be explored in terms of what constitute potential agonists for these cells. Once agonists are identified, it is important to examine whether manipulation of the γδ T cells for the benefit of the host can be achieved by utilizing such agonists to activate or suppress the function of the cells. One compound which has been found to activate this population of lymphocytes is a clinically approved antifungal drug, Amphotericin B (AmB). The effects of AmB on the immune system have been extensively studied in several cell types and in various infectious disease settings. However, the immunomodulatory effects of AmB on γδ T cells have not been described, and the work detailed here has initiated the elucidation of some of the aspects of the relationship between the antifungal drug and these lymphocytes.

When bovine PBMCs were cultured with amphotericin B, an upregulation of IL-2R on γδ T cells occurred, which is a priming event for activation (Figure 10A). The lowest dilution which consistently caused priming of γδ cells was 14.0625 ng/ml, while cytotoxicity was seen at concentrations of 225 ng/ml and higher (Figure 10B, C). The proliferation of bovine γδ T cells was also enhanced by the presence of AmB (Figure 14)

in a similar manner to the activity of ConA, a known T cell mitogen, in a dose-dependent manner. Although some studies suggest that AmB has an immunosuppressive effect on some cells (Hauser 83; Schindler 93; Kretschmar 01), the data presented here supports the stimulatory and proliferative effect of AmB on immune cells which has been described (Shirley 79A; Shirley 79B; Henry-Toulme 89; Kumar 00).

Similar results were observed when alternate formulations of AmB were utilized (Figure 11). Fungizone, another deoxycholate form of the drug, and AmBisome, a liposomal version, both caused upregulation of IL-2R on bovine γδ T cells. However, both alternate formulations had less activity than Sigma deoxycholate at equivalent concentrations. It is well established that liposomal formulations of AmB are less toxic than deoxycholate AmB (New 81; Ostro 89; Wong-Beringer 98; Robinson 99). The toxicity of the antifungal drug has been attributed to the ability of AmB to interact with cholesterol in the mammalian membranes (Kinsky 62; Cass 70; Andreoli 73; Hsuchen 73; Maddux 80), but it has also been suggested that the inflammatory response caused by administration (Sau 03; Simitsopoulou 05) and the utilization of certain signalling pathways (Bellocchio 05) may also contribute to the adverse reactions observed. Therefore, it is conceivable that the reduction in the upregulation of IL-2R on γδ T cells after exposure to AmBisome could be contributed to a difference in signalling or cytokine production from these cells. The reduction seen after exposure to Fungizone, another deoxycholate formulation similar to Sigma AmB, may also be for similar reasons, but it is unclear why this version does not induce similar responses from γδ T cells as the other deoxycholate form. Lot variability of Fungizone preparations could

account for this particular sample of Fungizone being less active than if another lot were to be tested. Sigma deoxycholate AmB was utilized in all further studies reported here, given that it had the most potent effect on bovine γδ T cells. It should be noted, however, that this report demonstrates that Fungizone did have immunostimulatory effects on γδ T cells at low concentrations, and this formulation of AmB is often added to media as an antifungal supplement. Therefore, studies aimed at elucidating the effector functions of immune cells that culture the cells in media supplemented with Fungizone could be flawed.

AmB at any concentration did not cause IL-2R upregulation on all of the γδ T cells in the PBMC preparation (Figure 10A). Therefore, it is likely that AmB is only active on a subset of the circulating bovine γδ T cell population. Additionally, monocytes were found to be necessary for the effect of AmB on bovine γδ T cells in vitro, indicating that these cells may be responsible for some of the signalling that is involved with the γδ T cell response after a host is exposed to AmB (data not shown). The relationship between monocytes and γδ T cells has been well-studied, and the idea that human monocytes can secrete molecules (Ueta 96; Roelofs 09) or present antigen (Miyagawa 01) after exposure to pathogens, causing the activation of γδ T cells, has been reported. Additionally, AmB has been shown to accumulate within human monocytes (Martin 94) and elicit production of cytokines from them (Rogers 99; Rogers 00; Rogers 02; Dotis 06). Therefore, our data indicating a requirement for monocytes in the AmB-induced activation of bovine γδ T cells is not surprising.

The data presented here reveal that very low doses of AmB can prime bovine PMBCs for increased responses to other mitogens (Figures 13 and 14). Previous work has described the ability of AmB to act synergistically with mitogens such as ConA or LPS (Seay 82; Walls 82; Mehta 85; Sarthou 86; Kumar 00) to activate immune cells from various species, but in these studies the cells were cultured with AmB and the mitogens concurrently. To determine the effects on γδ T cell activation, AmB was cultured with bovine PBMCs for 24 hours before additional agonists (ConA, LPS, or IL-18) were added for another 24 hours. The cells were not washed before the second agonists were added, and it was discovered that AmB can prime the cells for further activation by ConA, LPS, or IL-18 (Figures 13A, B and 14A, B) at very low doses. Additionally, the amount of IFN-γ production by bovine PBMCs in response to any of the three mitogens tested was greater when the cells were first primed with AmB (Figures 13C and 14C).

LPS is a TLR4 agonist, and deoxycholate AmB has been shown as having TLR2-stimulating capacities on human and murine cells (Sau 03; Bellocchio 05). Furthermore, results from previous work performed in our laboratory support the notion that TLRs are necessary for responses generated by AmB in an infectious disease setting in the mouse (Figure 7). Therefore, it is not unlikely that AmB primes bovine γδ T cells for activation via TLR2 stimulation, allowing the cells to respond more robustly when they are exposed to a TLR4 agonist (LPS) shortly thereafter. Alternatively, AmB could be effectively stimulating monocytes through TLR2, which could be necessary for subsequent γδ T cell activation.

A similar scenario may be occurring with responses to ConA, a TCR cross-linking molecule. AmB has been shown to inhibit murine T cell proliferation in response to ConA, but the drug was added after cells were exposed to ConA, and the authors found that the inhibitory effect was only relevant if AmB was added at early time points after ConA-stimulation (Kumar 00). It was noted that if AmB was administered four hours after ConA stimulation, the inhibitory effect was completely eliminated. The study also found a stimulatory effect of AmB on ConA-primed cells, but the effect was only seen in the presence of ibuprofen and catalase. The authors concluded that AmB stimulates macrophages which then produce H_2O_2, causing an inhibition of T cell proliferation due to interference with an early step in the TCR signalling pathway. Although that study found that AmB required additional compounds to cause proliferation of ConA-stimulated T cells, our results indicate that AmB is able to prime cells for activation by ConA (Figure 13). As stated previously, AmB is likely causing a priming event via a TLR, and it is plausible that those cells will be more responsive to mitogens because of the priming event. Therefore, the results indicating that the response of AmB-primed γδ T cells to the TCR cross-linking molecule ConA is greater than to ConA or AmB alone are reasonable. However, direct effects on γδ T cells were not determined, and the effect could be entirely dependent on the presence of monocytes. For example, the γδ T cells might be responding to increased TNF-α production by the monocytes exposed to AmB.

IL-18 (Okamura 95), ConA (Marcucci 84), and LPS (Blanchard 86) are all known to induce IFN-γ production from T cells, and the data presented here support the idea that AmB can enhance the effect of these mitogens on the production of IFN-γ by

bovine PBMCs (Figures 13C and 14C). Furthermore, it was discovered that AmB can elicit IFN-γ production from these cells even at very low doses without the aid of additional mitogens. The exposure of bovine PBMCs to a low dose of AmB, therefore, causes a small amount of IFN-γ to be produced by these cells, but it also primes them for a greater response to various agonists affiliated with the induction of the IFN-γ pathway. Studies have been performed that indicate administration of IFN-γ in combination with AmB may enhance the ability of the drug to combat fungal infections in mice (Joly 94; Clemons 01). If AmB is mediating an anti-fungal response via the IL-18/IFN-γ pathway, the addition of exogenous IFN-γ would be expected to enhance the effect, as was seen in those studies. However, the finding that AmB primes bovine PBMCs for the production of more IFN-γ in response to IL-18 is novel.

Amphotericin B affected human γδ T cells in a similar manner to what was observed when bovine γδ T cells were exposed to the drug. The expression of CD69, a marker found on human T lymphocytes, is an indication of activation of the cells (Cambiaggi 92; Ziegler 93); therefore, the ability of AmB to induce expression of the protein was determined. The drug had the capability to activate human γδ T cells in a dose-dependent manner (Figure 15), similar to the activation caused by HD-MAPP with IL-15, known activators of γδ T cells. The lowest concentration which caused activation of human γδ T cells was 1.76 μg/ml, and cytotoxicity was first seen at a concentration of 56.26 μg/ml. It should be noted that the lowest activating concentration of the drug in human cells was much greater than the highest (non-cytotoxic) amount causing activity in bovine cells (1.76 μg/ml versus 0.1125 μg/ml).

PMA and ionomycin are known activators of leukocytes, including T cells (Castagna 82); therefore, these agonists were utilized in conjunction with AmB to determine whether they could synergistically activate human γδ T cells. It was discovered that after 48 hours of culture with the agonists, the cells were stimulated, as indicated by their expression of CD69 (Figure 16). The low dosage of AmB utilized did not cause a high percentage of γδ T cells to express CD69 when added alone, although PMA/ionomycin did (Figure 16A). However, AmB in combination with PMA/ionomycin caused almost 100% of the γδ T cells to express CD69. Mean fluorescence values for staining of CD69 on γδ T cells show the synergistic effect more clearly (Figure 16B). Therefore, AmB can act together with other T cell activators for additional activation of human γδ T cells.

Because AmB alone was able to induce IFN-γ production from bovine PBMCs (Figures 13 and 14), its ability to do so from human cells was investigated. However, IFN-γ could not be detected by ELISA after human PBMCs were cultured with any concentration of AmB (data not shown). Therefore, human PBMCs were also cultured with PMA/ionomycin in addition to AmB for 48 hours to determine whether IFN-γ could be elicited from these cells. It was found that, similarly to expression of CD69, AmB acted synergistically with PMA/ionomycin to enhance IFN-γ production from human PBMCs, as detected by ELISA (Figure 17). Intracellular staining for IFN-γ was also performed to determine whether human γδ T cells could be induced to produce the cytokine. Surprisingly, a small dosage of AmB alone was able to promote IFN-γ production by the cells (Figure 18). Additionally, high amounts of the cytokine were

produced by γδ T cells after culture with PMA/ionomycin, but this effect could be enhanced in the presence of a small amount of AmB. Therefore, although IFN-γ production by human PBMCs could not be detected by ELISA after culture with AmB alone at any concentration, results obtained via intracellular staining, a more sensitive assay, support the idea that AmB can lead to the production of the cytokine from human cells without the aid of additional stimuli.

Previous work performed in the Jutila laboratory supports the idea that AmB can enhance clearance of bacterial pathogens (Figure 6A) and can cause the activation of dendritic cells (data not shown). Additionally, AmB was found to elicit IFN-γ from macrophages and dendritic cells exposed to *C. burnetii* (Figure 6B), which is in agreement with studies that have found AmB to enhance the ability of macrophages to combat fungal infections and augment their phagocytic capabilities (Lin 77; Aslanzadeh 91; Tohyama 96). It has also been shown that AmB can cause macrophages to release higher amounts of other cytokines, such as TNF-α and NO (Stein 87; Chia 89; Wolf 90; Tokuda 93; Yamaguchi 93; Hermann 94; Tohyama 96; Mozaffarian 97). Furthermore, IFN-γ presence was found to augment the AmB-induced production of both nitrite (Mozaffarian 97) and TNF (Chia 89) by murine macrophage cell lines. The effects of AmB on alveolar macrophages (Jahn 98; Rogers 99) and PMNs (Wolf 90; Roilides 98A; Roilides 98B; Gonzalez 01; Roilides 02) have been studied, and the results indicate that the antifungal drug can aid these cells in the clearance of fungal infections by eliciting an increase in the production of cytokines or other metabolites from them. Therefore, our data suggesting that AmB can activate and cause an increase in the production of IFN-γ

from macrophages and dendritic cells is in agreement with these studies with regard to the concept that the drug can affect immune cells and enhance their ability to combat infections.

Work performed in our laboratory has identified that AmB likely requires TLR4 function in the lung and TLR2 in the periphery for neutrophil recruitment (Figure 7). It has been shown previously that MyD88, CD14, and TLR2 are all essential for TNF-α production by human and murine macrophages and that TLR2 was needed for IL-1β production by the cells (Sau 03). Another study speculated that different formulations of AmB affect which TLR is associated with the effects of AmB on immune cells (Bellocchio 05). The study analyzed the pro-inflammatory response by *Aspergillus fumigatus*-infected murine PMNs in the presence of AmB deoxycholate or AmBisome. The results suggest that AmB deoxycholate activates TLR2-dependant signalling in the PMNs, whereas AmBisome (a liposomal formulation) was found to preferentially utilize TLR4 signalling. Our laboratory had used a deoxycholate formulation of AmB in the neutrophil recruitment studies (Figure 7), so the requirement for TLR2 in neutrophil recruitment in the periphery supports the study by Bellocchio, *et al.* in regard to the signalling occurring in neutrophils in the presence of AmB. As TLR2 was found to be important for macrophage-AmB interactions in another study using deoxycholate AmB (Sau 03), it would appear that TLR2 is the primary signalling receptor which deoxycholate AmB utilizes. However, our data suggest that TLR4 may play a role in the AmB-induced effect in the lung, and the study by Sau, *et al.* also found the AmB-induced TNF-α production by peritoneal macrophages to be partially TLR4-dependent (Sau 03).

As AmB is a microbial product, it is a possibility that endotoxin contamination could have played a role in the TLR responses observed. However, testing for endotoxin was performed via an LAL assay (as described in Materials and Methods), and all formulations utilized were found to be negative for levels of detectable endotoxin. Therefore, the AmB is presumed to be the TLR agonist, and the results supporting that it has tissue-specificity is novel.

This unique observation might be explained by the concept of lipid raft alterations by agonists. As AmB can interact with cholesterol on cell membranes, it has been suggested that the drug can alter cholesterol- and glycolipid-rich regions on the membranes of cells, termed lipid rafts (Sau 03). It has been shown that changing lipid raft formation on membranes can affect cell signal transduction (Carozzi 00). Lipid rafts are found on γδ T cells (Lu 11), and alterations in lipid rafts on T cells have been shown to change the response to bacterial antigens (Triantafilou 02). Therefore, it is possible that AmB can also alter lipid raft formation on γδ T cells and cause changes in signalling pathways. Additionally, it has been shown that the presence of disease (dyslipidemia—high cholesterol) caused lipid raft rearrangement on alveolar macrophages but not on peritoneal macrophages (Madenspacher 10). The study also noted that when lipid raft alterations occurred, an increase in the expression of TLR4 on the alveolar macrophages was observed. Therefore, a hypothesis as to why AmB utilizes different TLR signalling pathways in a tissue-specific manner might be explained by its effects on the lipid rafts of γδ T cells. AmB in the peritoneum may not alter lipid rafts on γδ T cells, similar to effect the dyslipidemia state has on the peritoneal macrophages. As such, AmB would utilize its

default receptor, which has been speculated to be TLR2 (Sau 03; Bellocchio 05) (Figure 7). However, in the lung, lipid raft rearrangement on γδ T cells may occur due to the presence of AmB, as was seen with alveolar macrophages during the dyslipidemia state. This phenomenon might cause TLR2 to be inaccessible to the AmB for signalling. Therefore, AmB would likely utilize its backup method of signalling (TLR4), which has been shown to occur in the absence of TLR2 (Bellocchio 05). Moreover, if AmB-induced lipid raft rearrangement also causes an increase in TLR4 expression on γδ T cells in the lung, as dyslipidemia was shown to do on alveolar macrophages (Madenspacher 10), more TLR4 would be available for signalling on γδ T cells in the lung than when AmB is absent. It should be noted that the study by Sau, *et al.* showing primarily TLR2-dependance of AmB for signalling utilized peritoneal macrophages and did not look at alveolar-derived cells (Sau 03). Therefore, the TLR4-dependence which appears to occur in the lung would not have been discovered.

The effects of AmB on a bacterial infection outside of the lung and peripheral settings were also determined (Figure 8). It was discovered that AmB-treated mice displayed a decreased abscess volume and dermonecrosis layer versus PBS-treated animals in a murine model of skin and soft-tissue infection with *Staphylococcus aureus*. As bacterial cells do not have sterols in their membranes, it is not probable that the drug is having a direct anti-bacterial effect. Rather, AmB is likely enhancing the effects of immune cells such as neutrophils, which have been shown to play a major role in the clearance of skin and soft tissue infections with *S. aureus* (Molne 00; Miller 11). The drug could also be affecting γδ T cells, as it was found that IL-17 production by these

cells was necessary for neutrophil recruitment in a murine model of cutaneous *S. aureus* infection (Cho 10). It has been demonstrated that LPS, a molecule found on the outside of Gram-negative bacteria, activates γδ T cells and that AmB can enhances this effect (Figure 13). Other studies support the idea that AmB can affect the immune response when various mitogens are present (Seay 82; Walls 82; Mehta 85; Sarthou 86). Additionally, it has been shown that γδ T cells respond to peptidoglycan, found in the membrane of Gram-positive bacteria (Hedges 05). Therefore, it is plausible that γδ T cells are responding to the peptidoglycan found on the *S. aureus* bacteria more robustly in the presence of AmB, causing an increase in γδ T cell activation, proliferation, and production of IL-17. In response to the higher amounts of IL-17 being present, additional neutrophils would likely be recruited to the area, which could account for the enhanced clearance of the *S. aureus* observed when AmB is present. However, these specific parameters were not directly measured.

The activity of AmB during immunization was studied previously in the Jutila laboratory, and it was discovered that a small amount of AmB added to a norovirus-like particle vaccine given to mice induced more IgG production from the animals versus those immunized with the vaccine alone (Figure 9). The levels of IgG production when AmB was utilized as an adjuvant were similar to those elicited by adding MPL, a widely used adjuvant, to the vaccine. Therefore, the results indicate that AmB could be utilized as an alternative adjuvant in vaccines.

AmB was shown to enhance the clearance of *Coxiella burnetii* (Figure 6A) and induce dendritic cell activation and neutrophil recruitment in a murine model of infection

(data not shown). Additionally, AmB augmented IFN-γ production from murine lung macrophages and dendritic cells exposed to *C. burnetii* (Figure 6B). As IFN-γ is an important cytokine involved in the clearance of intracellular bacteria, such as *Salmonella* spp. (Jouanguy 99) and *Coxiella* spp. (Dellacasagrande 02), these results indicate that AmB could be utilized in an infectious disease setting for enhanced clearance of a pathogen. The effects of Amphotericin B on bovine γδ T cells could be established at concentrations below those needed for activation of human cells (ng/ml versus μg/ml), and the effect could be elicited without the aid of additional agonists. Therefore, the adjuvant potential for AmB in a disease setting may be more relevant in cattle than in humans.

Bovine PBMCs produced IFN-γ after being cultured with a low concentration of AmB (Figure 12), and bovine γδ T cells were induced to proliferate (Figure 14) and were primed for activation (Figure 10) after exposure to the drug. As neonatal bovine calves can have up to 70% of their circulating lymphocytes be γδ T cells (Goodman 88; Hein 91; Hayday 00) and have not yet developed a fully-functional adaptive immune system, an ideal situation would be to administer AmB intravenously into newborn bovine calves as an adjuvant to modulate the γδ response to potential bacterial infection.

The adjuvant effects of AmB in mice were observed at doses between 1.0-20.0 μg/kg, which translates to 4.5-81.0 μg/kg in a human (Reagan-Shaw 08). The effective antifungal dose used in humans for treatment of fungal infections is 0.25-1.0 mg/kg, and it is well-established that these doses often are accompanied by adverse reactions. The data presented here illustrate that bovine cells are much more sensitive to the adjuvant

effects of AmB, and the amounts needed are below the cytotoxic doses seen in humans (μg/ml versus mg/ml). If similar dosing is utilized in calves as was done in mice, a 50 kg calf would be given 0.225-4.05 mg of AmB to induce the adjuvant effect, an amount well below the antifungal dose in humans. Additionally, as AmB is known to have a long half-life (Atkinson 78; Craven 79), one dose of the drug may be sufficient to elicit a potent adjuvant effect.

In summary, due to the extensive roles γδ T cells have in the immune system, specifically in the inflammatory response, it is of interest to identify agonists for these cells. The discovery that concentrations of AmB which are lower than those utilized during antifungal therapy prime bovine γδ T cells for activation and cause direct activation of human γδ T cells is novel. Additionally, it was discovered that the drug can act synergistically with other T cell agonists for a more robust response from these cells. AmB was also found previously to enhance clearance of a systemic bacterial infection and decrease the effects of a skin and soft tissue infection in mice. Furthermore, it was demonstrated that the antifungal drug can act as a potent vaccine adjuvant. The results presented here implicate AmB as a direct and indirect stimulator of murine, bovine, and human γδ T cells. As such, the potential for other clinical drugs as mitogens for these cells is a possibility. The mechanism by which AmB activates γδ T cells and the pathways involved in the response have yet to be elucidated, but preliminary data suggest that TLRs are involved and that IFN-γ production by γδ T cells plays an important role as well. Therefore, AmB has the potential to be administered so as to manipulate the γδ T cell response for the benefit of the host during infectious disease.

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