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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Indications that the Antimycotic Drug Amphotericin B Enhances the Impact of Platelets on *Aspergillus*

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ABSTRACT Platelets are currently thought to harbor antimicrobial functions and might therefore play a crucial role in infections, e.g., those caused by *Aspergillus* or mucormycetes. The incidence of invasive fungal infections is increasing, particularly during the coronavirus disease 2019 (COVID-19) pandemic, and such infections continue to be life-threatening in immunocompromised patients. For this reason, the interaction of antimycotics with platelets is a key issue to evaluate modern therapeutic regimens. Amphotericin B (AmB) is widely used for the therapy of invasive fungal infections either as deoxycholate (AmB-D) or as a liposomal formulation (L-AmB). We showed that AmB strongly activates platelets within a few minutes. AmB concentrations commonly measured in the blood of patients were sufficient to stimulate platelets, indicating that this effect is highly relevant *in vivo*. The stimulating effect was corroborated by a broad spectrum of platelet activation parameters, including degranulation, aggregation, budding of microparticles, morphological changes, and enhanced adherence to fungal hyphae. Comparison between the deoxycholate and the liposomal formulation excluded the possibility that the liposomal part of L-Amb is responsible for these effects, as no difference was visible. The induction of platelet activation and alteration by L-AmB resulted in the activation of other parts of innate immunity, such as stimulation of the complement cascade and interaction with granulocytes. These mechanisms might substantially fuel the antifungal immune reaction in invasive mycoses. On the other hand, thrombosis and excessive inflammatory processes might occur via these mechanisms. Furthermore, the viability of L-AmB-activated platelets was consequently decreased, a process that might contribute to thrombocytopenia in patients.

KEYWORDS amphotericin B, complement system, human platelets, innate immunity

Although in terms of public awareness little attention is paid to the impact of invasive fungal infections (IFIs), they contribute substantially to human morbidity and mortality. The increasing incidence of IFIs in recent decades is largely due to an increased number of immunocompromised individuals; e.g., recipients of stem cell or organ transplantations and patients with hematological malignancies or severe neutropenia are at particular risk for invasive mycoses (1–3). Despite medical progress, the still-high mortality rates among affected patients and emerging resistances to antimycotic drugs show the urgent need for new therapeutical approaches, which might be achieved by deeper insights into the mechanisms of antifungal host defense and its interactions with antimycotics. In this respect, the quickly reacting elements of innate immunity are of particular interest.

Blood platelets are small anucleate cell fragments and the second most abundant cellular elements in the circulation (4, 5). In recent years, a growing body of evidence has

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described platelets as versatile players in the immune continuum in response to infectious and noninfectious diseases (6–8). Platelets show a broad armamentarium of molecules with immune functions that are present on membranes or stored in granules, including pattern recognition receptors (PRRs), Fc and complement receptors, cytokines/chemokines, and antimicrobial peptides (reviewed in references 9–11). Consequently, platelets are involved in a multitude of processes, thereby building bridges between innate and adaptive immunity, hemostasis, inflammation, and thrombosis (4, 7). Platelets sense pathogens or pathogen-immunoglobulin complexes by various surface receptors and, upon activation, release the contents of their granules, change the pattern of their surface molecules, and shed proinflammatory microparticles (6, 10). Secreted platelet microbicidal peptides (PMPs) were shown to exert direct activity against various pathogens (10, 12–14). Furthermore, the function of platelets to bridge innate and adaptive immunity exerts a multitude of indirect effects on antimicrobial defense (8, 10, 11).

Rising evidence for a close mutual interference between platelets and the complement system has evolved in the last decade (15, 16). Complement is a multifunctional, tightly regulated protein cascade of innate immunity and combines functions of homeostasis with a multitude of modulating effects within innate and adaptive immunity (17, 18). Complement exerts host defense mechanisms by three major principles (19): (i) invading pathogens are opsonized to target them for ingestion and killing by phagocytes; (ii) inflammatory anaphylatoxins recruit neutrophils and macrophages to the sites of infection and stimulate their activity; and (iii) the terminal complement C5b-9 complex forms lytic pores in the membrane of pathogens (19).

Platelets and complement activate and regulate each other by different mediators. Stimulated platelets activate complement by P-selectin (CD62P) and chondroitin sulfate; conversely, complement C1q and C3a activate platelets (16, 20, 21). The complex interactions between platelets and complement have both beneficial functions in host defense and detrimental effects, such as excessive inflammation, thrombocytopenia, and thrombosis, if regulation goes out of control (15, 16).

The polyene macrolide amphotericin B (AmB) belongs to the most frequently used group of antimycotics worldwide. It shows the broadest spectrum of activity and a very low resistance potential; furthermore, the low cost of conventional AmB therapy is particularly important for developing countries (22). For these reasons, AmB was chosen for this investigation.

Binding of AmB to ergosterol of the fungal cell membrane leads to membrane disintegration (22–24). Lipid formulations such as liposomal AmB (L-AmB) substantially reduced the problematic adverse effect of nephrotoxicity in comparison to AmB deoxycholate (AmB-D) (22).

Previous reports stated that AmB affects the physiology of platelets (25, 26). In the present work, we aimed to expand and deepen knowledge about the effects of AmB on platelet activation and to investigate putative consequences for antifungal defense in invasive aspergillosis. A further aim was to include potential implications of the complement system as a hub between innate and adaptive immunity.

RESULTS

L-AmB induces activation of human platelets in a time- and dose-dependent manner. In antifungal therapy with L-AmB, plasma concentrations sometimes reach 90 μg/mL AmB (27, 28), and levels in close proximity to the infusion site might be even higher (the maximal concentration of the administered solution is 2 mg/mL [28]). Therefore, concentrations between 1 and 500 μg/mL were initially tested to monitor the effect of L-AmB on human platelets. Platelet activation was measured by flow cytometry, applying the surface exposure of P-selectin (CD62P). Significant stimulation of platelets with release of alpha granules was induced by 3 μg/mL L-AmB (Fig. 1A). The effect reached its maximum with concentrations between 10 and 50 μg/mL; at higher concentrations of L-AmB, the CD62P signal decreased again (Fig. 1A). As concentrations higher than 50 μg/mL are not commonly reached in patients, we focused on the lower range in the experiments described below.

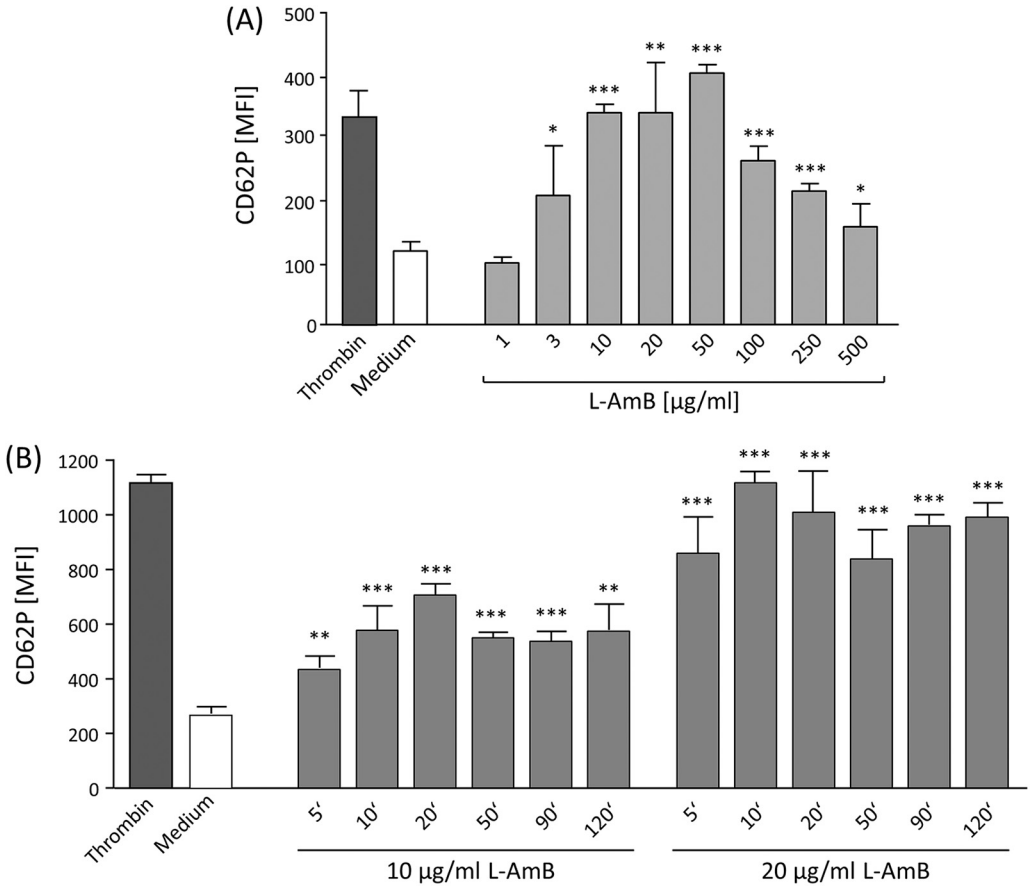

FIG 1 Induction of activation of human platelets by L-AmB. Human platelets were incubated for 30 min with increasing concentrations of L-AmB (A) or for 5 to 120 min with 10 and 20 μg/mL L-AmB (B). Incubation with thrombin and with medium was used as positive and negative controls, respectively. Platelet activation was measured by flow cytometry, applying a CD62P-specific antibody. MFI, mean fluorescence intensity. Experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown. The CD62P signal of samples was compared to that of the medium control by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Concentrations of 10 and 20 μg/mL L-AmB were used in kinetic studies. In these experiments, induction of platelet activation was shown to be a very rapid process. As early as 5 min after incubation with the antimycotic, the CD62P signal on the platelet surface was significantly increased (Fig. 1B).

L-AmB and AmB-D show comparable capacities to activate platelets. To determine whether AmB itself or the liposomal formulation is responsible for the platelet-activating capacity of L-AmB, human platelets were incubated for 30 min with increasing concentrations of L-AmB and AmB-D. Platelet activation was measured by flow cytometry, using CD62P as a marker for release of alpha granules and CD63 for secretion of dense granules.

Stimulation of platelets with increased levels of CD62P was detected by both L-AmB and AmB-D. A significant presence of CD62P was measurable at a concentration of 6 μg/mL of both formulations, and the degree of platelet activation increased continuously until 50 μg/mL (Fig. 2A). A significant degranulation of dense granules was induced by L-Amb and AmB-D even at the lowest concentration of 1 μg/mL, as marked by an enhanced level of CD63, and again, this level rose with higher concentrations of both AmB formulations (Fig. 2B).

These findings indicate that the stimulation of platelets is facilitated by the molecular structure of amphotericin B regardless of its formulation. Furthermore, AmB appears to induce a profound activation of platelets, including the release of both alpha and dense granules.

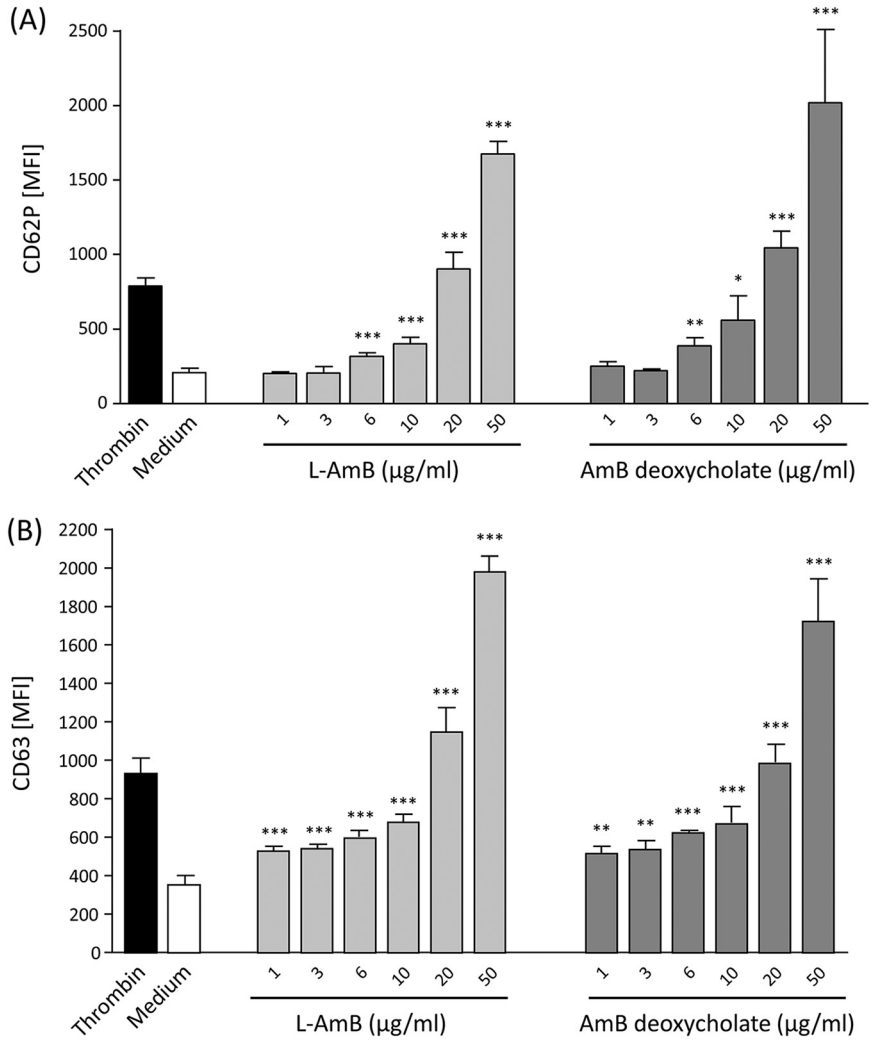

FIG 2 Comparison of liposomal AmB and AmB deoxycholate in their activating effect on platelets. Human platelets were incubated for 30 min with increasing concentrations of liposomal AmB and amphotericin B deoxycholate. Incubation with thrombin and with medium was performed as positive and negative controls, respectively. Platelet activation was measured by flow cytometry, using CD62P as a marker for the release of alpha granules (A) and CD63 as a marker for the release of dense granules (B), and expressed as mean fluorescence intensity (MFI). All experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown here. The MFI of samples was compared to that of the respective medium control by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Activation of platelets by amphotericin B results in increased aggregation. Stimulation of platelets frequently leads to formation of platelet aggregates. To verify this hypothesized effect of L-AmB, human platelets were incubated in RPMI 1640 medium or with 3 μg/mL and 10 μg/mL L-AmB for 1 to 4 h. Activation and aggregation of platelets were monitored by confocal fluorescence microscopy.

In medium, most platelets remained in a quiescent state, and only a few small aggregates could be detected (Fig. 3). In comparison, platelets showed activation by L-AmB concentrations of 3 μg/mL and 10 μg/mL (Fig. 3). Beginning aggregation of platelets was visible after 1 h of incubation for both concentrations, and large aggregates of activated platelets could be detected within 4 h. This effect was most pronounced for platelet incubation with 10 μg/mL L-AmB (Fig. 3), a concentration that is commonly reached in plasma during antifungal therapy.

Amphotericin B stimulates platelet binding to fungal hyphae and promotes antimicrobial activity of platelets. In another set of experiments, we examined if activation of platelets by amphotericin B also leads to enhanced platelet binding to fungal

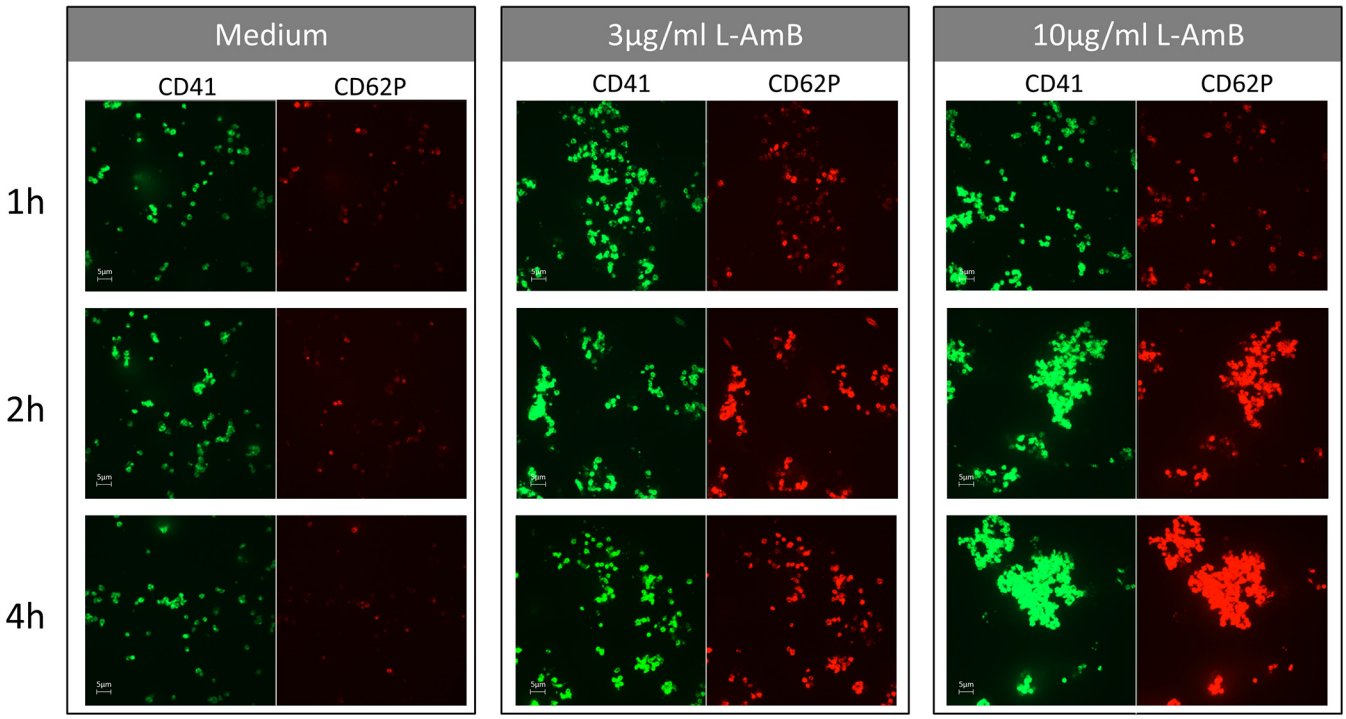

FIG 3 Platelet aggregation induced by L-AmB. Human platelets were incubated with medium, 3 μg/mL L-AmB, or 10 μg/mL L-AmB for 1 to 4 h. Platelet activation and aggregation were monitored by confocal fluorescence microscopy, using a FITC-labeled anti-CD41 antibody (green) to visualize all platelets and a phycoerythrin (PE)-labeled anti-CD62P antibody (red) to detect activation and aggregation. Representative fields of view are shown.

hyphae. Human platelets were incubated in medium or with increasing concentrations of L-AmB for 30 min, added to *Aspergillus fumigatus* hyphae derived from an overnight culture, and further incubated for 60 min. Binding of platelets was visualized by confocal microscopy.

While platelets incubated in pure culture medium failed to adhere to fungal hyphae (Fig. 4A and B), enhanced binding was seen after incubation with L-AmB even at very low concentrations, such as 1 μg/mL (Fig. 4A). Platelets stimulated by L-AmB developed a high capacity to bind to the fungus and sometimes even covered the largest part of the hyphae (Fig. 4A and B).

Stimulation of platelets by amphotericin B might also result in an elevated antimicrobial capacity due to the release of platelet microbicidal peptides (PMPs). To exclude the antifungal effect by AmB, we used the bacterium *Escherichia coli* to monitor this putative effect. *E. coli* grown overnight in Tryptic Soy Broth (TSB) medium was incubated with RPMI medium, 10 μg/mL L-AmB, quiescent platelets (Pt), or Pt plus 10 μg/mL L-AmB (ratio of *E. coli* to Pt = 1:1,000). Suspensions of *E. coli* were plated on nutrient agar, and CFU were counted. As expected, medium and AmB did not interfere with bacterial growth. Platelets incubated in medium showed the capacity to reduce bacterial growth, and this effect was significantly enhanced in the presence of L-Amb (Fig. 4C). These findings clearly indicate that stimulation of platelets by AmB induces an increased release of PMPs, which most likely also affects growth of fungal hyphae by adherence of platelets.

Amphotericin B triggers microparticle budding from platelets. Activation of platelets may cause budding of microparticles from their surface. These particles are known to activate leukocytes and endothelial cells, and they appear to play an important role in inflammatory processes (reviewed in references 6 and 10). To detect putative shedding of microparticles by platelets stimulated by AmB, human platelets were incubated with increasing concentrations of L-AmB and AmB-D for 30 min. Formation of platelet-derived microparticles was monitored by flow cytometry and confocal microscopy.

Both formulations of AmB induced significant formation of microparticles at concentrations of 1 to 10 μg/mL; at higher concentrations, the budding declined (Fig. 5A).

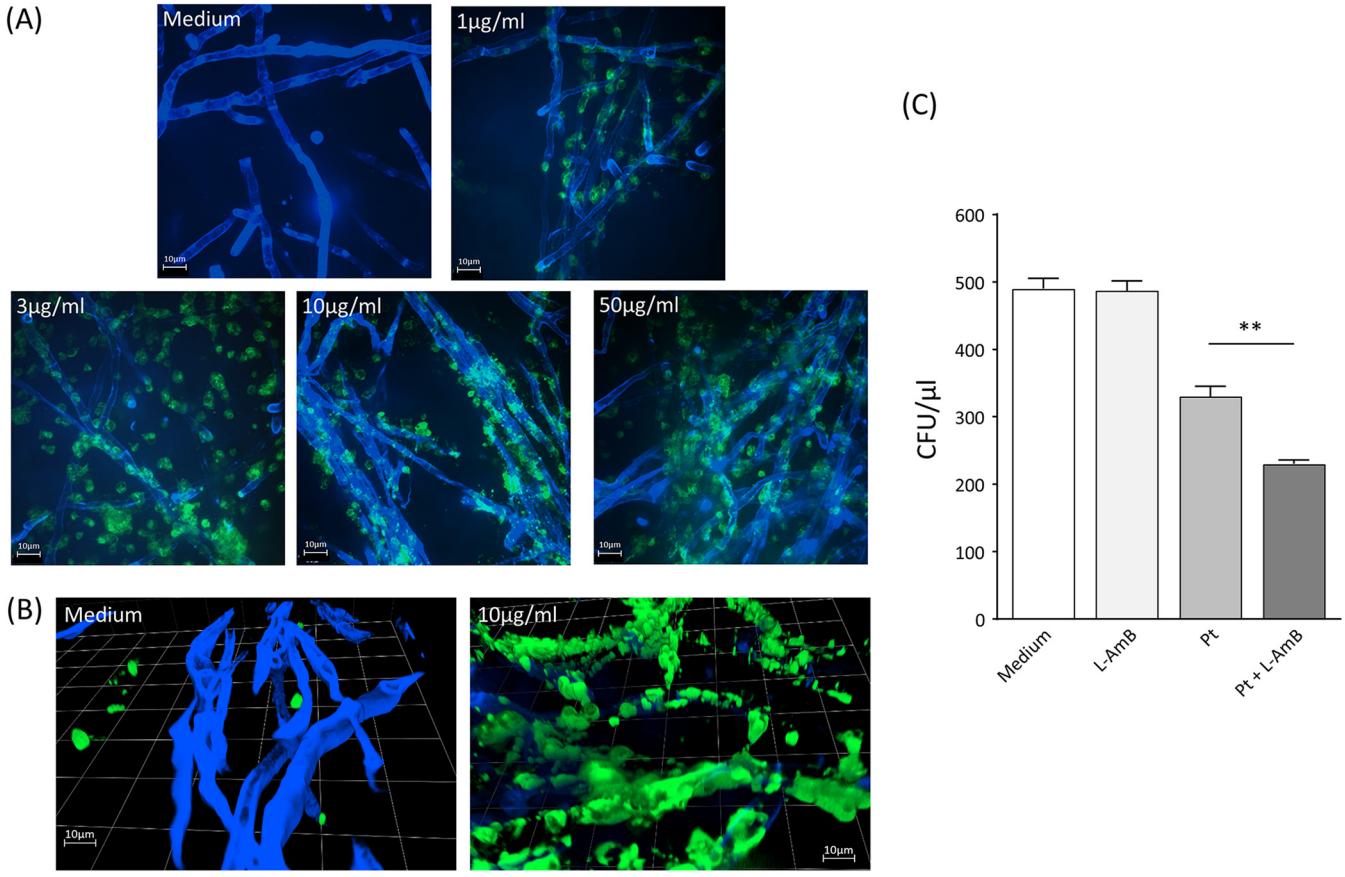

FIG 4 L-AmB stimulates binding of platelets to fungal hyphae and antimicrobial activity. (A and B) *Aspergillus fumigatus* was grown overnight. Human platelets were incubated for 30 min with either medium or increasing concentrations of liposomal AmB, added to the fungal hyphae, and incubated for another 60 min. After removal of unbound platelets, hyphae were stained with calcofluor white stain (blue) and the platelets with a FITC-labeled anti-CD41 antibody (green) for confocal microscopy. Two-dimensional (A) and three-dimensional (B) views of representative samples are shown. (C) *Escherichia coli* bacteria were incubated for 90 min with medium, 10 μg/mL L-AmB, quiescent platelets (Pt), or Pt + 10 μg/mL L-AmB. The number of bacteria was determined by plating and counting of CFU. The experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown. CFU of samples with Pt and Pt + 10 μg/mL L-AmB were compared by one-way ANOVA. **, $P < 0.01$.

These findings were confirmed by confocal microscopy. Platelets incubated in medium showed no microparticle formation, while a high number of these particles were shed by platelets in the presence of 10 μg/mL L-AmB (Fig. 5B). This effect might support the immune response against invading fungi but also contribute to excessive inflammatory processes.

Amphotericin B-induced platelet activation induces alterations in the plasma membrane and in platelet morphology. Further experiments revealed that stimulation of platelets by AmB induces a broad spectrum of activation parameters. Besides the effects described above, stimulation of platelets by L-AmB also resulted in the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. This effect includes a flip-flop mechanism that moves PS from the inner membrane leaflet to the outside of the platelet surface upon activation and contributes to the procoagulant activity of stimulated platelets (29). Binding of FITC (fluorescein isothiocyanate)-labeled annexin V to PS (29, 30) was measured by flow cytometry after incubation of human platelets in medium and with increasing concentrations of L-AmB. A significantly increased exposure of PS was detected at a concentration of 1 μg/mL, and the amount of PS continuously rose at higher L-AmB concentrations (Fig. 6A). Furthermore, a change of platelet morphology to a more globular shape without the typical development of filopodia could be visualized in 2- and 3-dimensional confocal microscopy upon incubation of platelets with both L-AmB and AmB-D (Fig. 6B).

Stimulation of platelets by amphotericin B triggers their interaction with the complement system. As there is growing evidence that an interplay between platelets and the complement system has important roles in the immune network (15, 16), we

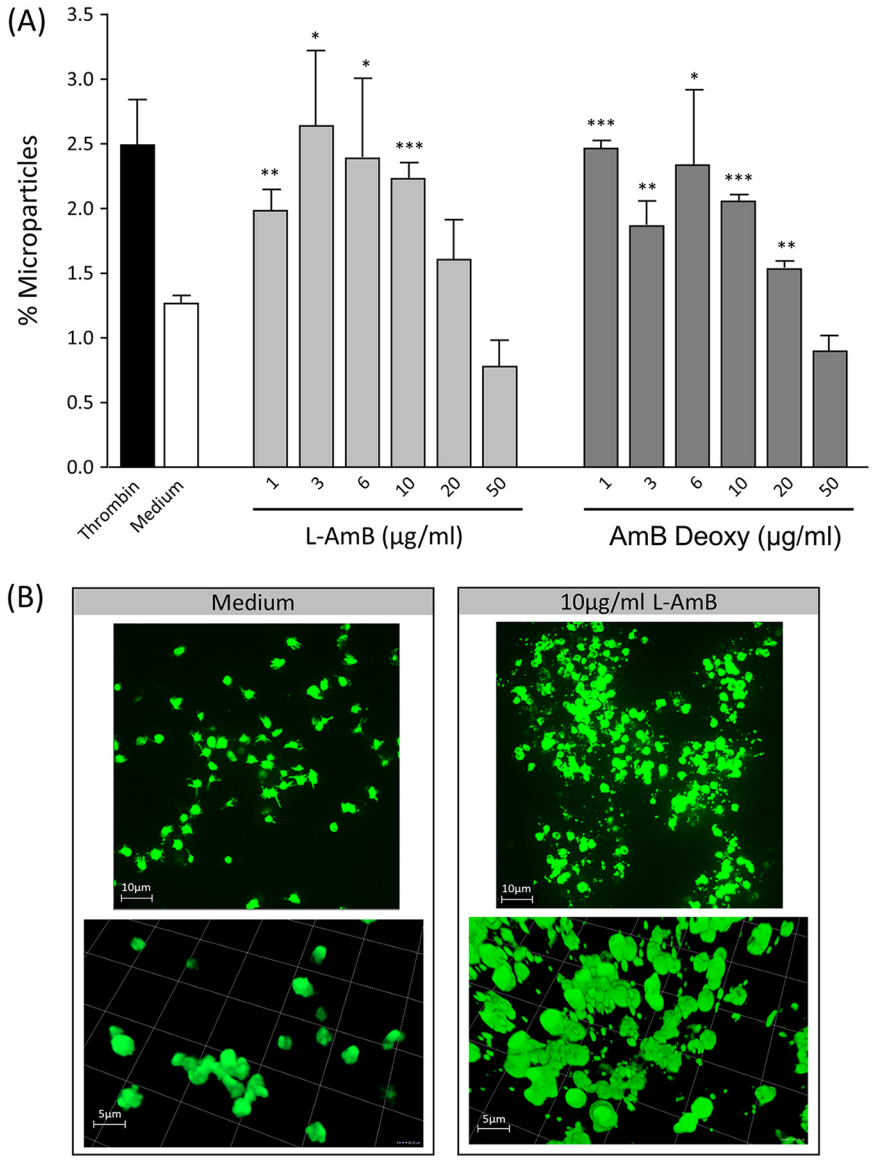

FIG 5 Triggering of platelet-derived microparticles by AmB. Platelets were incubated for 30 min with thrombin, medium, or increasing concentrations of liposomal or deoxycholate amphotericin B. (A) Newly formed microparticles positive for the platelet marker CD41 were gated in the samples according to their size and quantified by flow cytometry, and results are expressed as a percentage of all CD41-positive events. All experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown. The MFI of samples was compared to that the respective medium control by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. (B) A FITC-labeled anti-CD41 antibody was used to visualize platelets and platelet-derived microparticles via confocal microscopy; representative fields of view are shown.

took a closer look at the hypothesis that stimulation of platelets by AmB also results in enhanced interaction with complement. Stimulation of platelets proceeds via various pathways and does not necessarily induce activation of the complement system. For instance, previous studies showed that platelets stimulated by fungal galactosaminogalactan (GAG), but not by thrombin, induced complement activation and deposition of complement factors on the platelet surface (31).

Human platelets were preincubated for 30 min with thrombin, medium, or increasing concentrations of L-AmB or AmB-D. After further incubation for 60 min in the presence of human serum as a complement source, deposition of complement factor C3c and formation of the complement C5b-9 complex on platelets were monitored by flow cytometry.

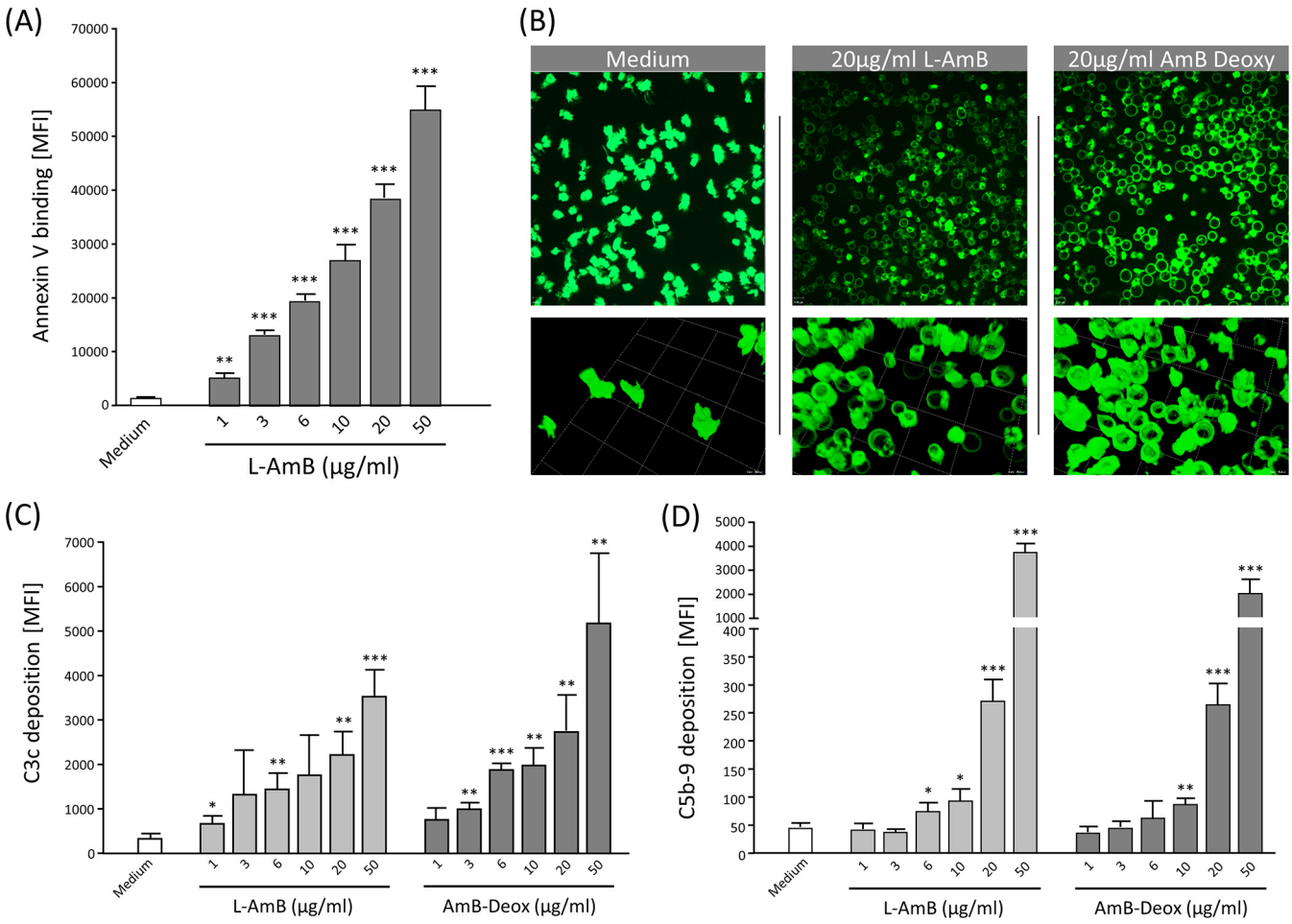

FIG 6 AmB-induced alterations in platelet plasma membrane, morphology, and interaction with the complement system. (A) Platelets were incubated for 30 min with medium or increasing concentrations of L-AmB or AmB-D. Exposition of phosphatidylserine on platelets was measured by binding of annexin V. (B) Platelet morphology after incubation for 90 min with medium, 20 μg/mL L-AmB, or 20 μg/mL AmB-D was visualized by two-dimensional (top) and three-dimensional (bottom) confocal microscopy. (C and D) Platelets were preincubated for 30 min with medium or increasing concentrations of liposomal or deoxycholate AmB. After addition of serum and further incubation for 60 min, deposition of C3c (C) and formation of the C5b-9 complex (D) on platelets were quantified. Annexin V, C3c, and C5b-9 on platelets were measured by flow cytometry, and results are expressed as mean fluorescence intensity (MFI). All experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown. MFI of samples was compared to that of the respective medium control by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

The experiments clearly revealed that platelets stimulated by L-AmB and AmB-D show the capacity to activate the complement cascade (Fig. 6C and D). Significant deposition of the complement fragment C3c on the platelet surface was detected after incubation with minor concentrations of 1 μg/mL L-AmB and 3 μg/mL AmB-D (Fig. 6C). The amount of the deposited C3c increased upon application of higher concentrations of both AmB formulations (Fig. 6C). Similarly, the exposure of the terminal C5b-9 complex was detected on platelets after their activation by both formulations of AmB (Fig. 6D), indicating that the complete complement cascade proceeds on the platelet surface. Large amounts of C5b-9 were found at AmB concentrations of 50 μg/mL. In comparison, activation of platelets by thrombin did not result in enhanced complement deposition (Fig. 6C and D), confirming previous results (31).

Amphotericin B induces loss of platelet viability and enhanced platelet interaction with granulocytes. The capacity of AmB to activate platelets might also affect their viability and their interaction with neutrophil granulocytes (polymorphonuclear leukocytes [PMNs]). To detect these effects, human platelets were incubated in medium or increasing concentrations of liposomal AmB. Platelet viability was quantified by staining with a Live/Dead dye and subsequent measurement of fluorescence by flow cytometry. To monitor the interaction with neutrophils, preincubated platelets

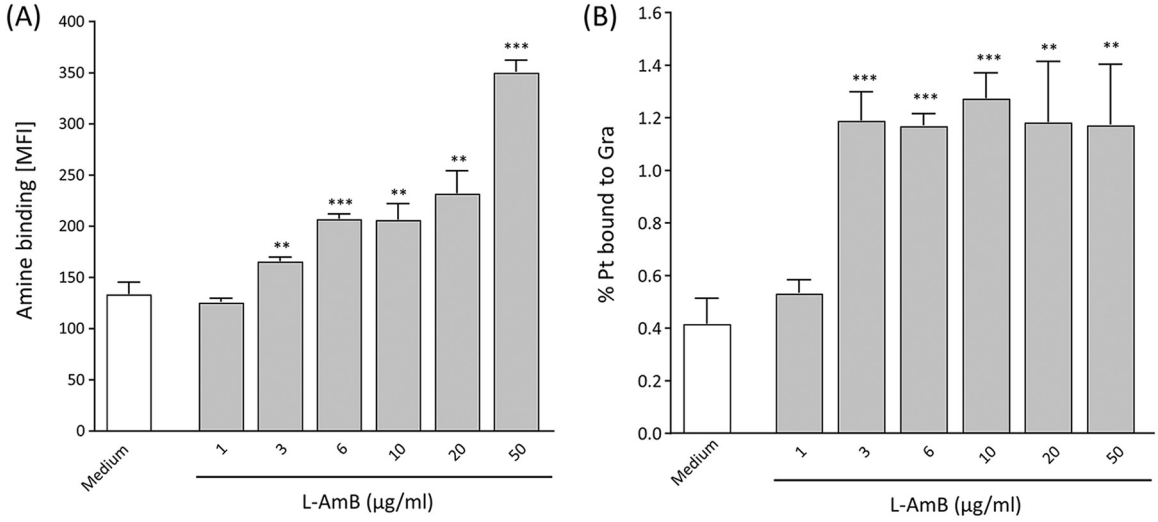

FIG 7 AmB-induced loss in platelet viability and changes in interaction with granulocytes. Human platelets were incubated for 30 min with medium or increasing concentrations of L-AmB. (A) Platelet viability was quantified by use a Live/Dead viability stain and analysis by flow cytometry. (B) Granulocytes were added to the platelets, and the percentage of platelets bound to granulocytes was quantified after 30 min by flow cytometry. All experiments were performed in triplicate and repeated at least 3 times with different platelet donors; representative results are shown. The MFI of samples was compared to that of the respective medium control by one-way ANOVA. **, $P < 0.01$; ***, $P < 0.005$.

were further incubated with human PMNs for 30 min. The percentage of CD41-positive platelets adhering to granulocytes was quantified by flow cytometry.

A significant increase of fluorescence, as indicated by the Live/Dead dye, was measured at a concentration of 3 μg/mL L-AmB, indicating early damage of the platelet plasma membrane. Incubation with higher concentrations resulted in a progressive decrease of platelet viability (Fig. 7A).

Furthermore, platelets activated by L-AmB showed enhanced binding to neutrophil granulocytes. The percentage of platelets adhering to PMNs significantly increased after 30 min of incubation with L-AmB concentrations of 3 to 50 μg/mL (Fig. 7B), which are commonly present in standard antifungal therapy with this drug.

DISCUSSION

Invasive fungal infections (IFIs) still contribute substantially to human morbidity and mortality in spite of medical advances. In recent decades, IFIs have become a global health problem, with continuously increasing numbers of cases affecting millions of individuals worldwide (32, 33). Ironically, medical progress represents one reason for this fact. New therapeutic regimens, immunosuppressive agents such as corticosteroids, chemotherapies against malignant diseases, and modern medical techniques have allowed the survival of premature infants and saved the lives of an uncountable number of patients with severe diseases. On the other hand, these advances have resulted in a rising number of individuals with immunodeficiencies, caused by prematurity, solid organ or hematologic transplantations, cancer therapy, HIV infection, or other conditions, which leave them particularly prone to invasive fungal infections (1, 32, 34). Furthermore, emerging resistances and a stagnating number of new antifungal agents contribute to the increasing number of IFIs (2, 3, 35–37). These facts show the urgent need for new therapeutical approaches, and deeper insights into antifungal host defense and its interplay with antimycotics will be crucial for meeting these needs. As IFIs are rapidly progressing diseases, the quickly reacting elements of innate immunity are of particular importance.

In this study, we focused on blood platelets and the complement system. While platelets were formerly considered merely key elements in hemostasis, there is growing evidence for their diverse contributions to host defense. They express a broad spectrum of receptors, such as pattern recognition receptors (PRRs) for recognition of

pathogen-associated molecular patterns as well as Fc and complement receptors (9, 10, 38, 39). Alpha granules of platelets contain a large variety of biologically active molecules, including PMPs and cytokines/chemokines, which are released upon platelet activation (11). Thus, platelets are very versatile elements that act as bridges between innate and adaptive immunity, inflammation, hemostasis, and thrombosis in a delicate balance (7). As shown by a number of working groups, platelets interact with cellular (B and T cells, neutrophils, macrophages, and endothelial cells) and humoral elements of the immune system (10, 15, 16).

A close mutual interplay of platelets with the complement system was discovered over the last 20 years (15, 16). Complement is an evolutionary ancient system with key roles in homeostasis and innate immunity and with complex connections to hemostasis, inflammation, and adaptive (B and T cells) host defense (17, 18). Three activation pathways lead to a common terminal pathway, and three mechanisms are used against invading pathogens: deposited complement fragments on pathogens (opsonization) mark them for phagocytosis, chemoattractant anaphylatoxins activate phagocytes and recruit them to the sites of infection, and the terminal C5b-9 complex forms lytic pores in membranes of pathogens (17, 19).

Various working groups have reported a close interplay of platelets and complement: They activate and regulate each other by different mediators such as P-selectin, chondroitin sulfate, and the complement factors C1q and C3a (15, 16). The pathway of platelet activation appears to be important, as not every stimulating agent or activation pathway results in complement activation (31). Different kinetics of clearance of bacteria, depending on opsonization and subsequent binding of platelets, indicate a close collaboration in host defense (40).

In this work, we studied the effects of amphotericin B (AmB) on platelets and possible implications for the complement system. We chose this antifungal agent because it remains the most frequently used antimycotic worldwide and is still considered the gold standard in the treatment of IFIs with the broadest spectrum of activity (22, 41). Both AmB deoxycholate (AmB-D) and liposomal AmB (L-AmB) were examined, as AmB-D may be the only option in resource-limited settings (22, 42). In the course of the coronavirus disease 2019 (COVID-19) pandemic, the number of invasive fungal infections such as COVID-19-associated pulmonary aspergillosis (CAPA) and, particularly, of COVID-19-associated mucormycosis (CAM), has been increasing (43, 44), and AmB was strongly recommended in cases of mucormycosis (42, 43, 45). Furthermore, AmB shows the lowest resistance potential among all known antimycotic agents (22).

Our studies clearly revealed that AmB shows a profound activating effect on human platelets with a broad spectrum of activation parameters (Fig. 1 to 3, 5, and 6A and B), independently of its formulation (Fig. 2). However, the exact activation pathway remains to be determined.

Despite investigations over more than 50 years, the precise mechanism of action of AmB is still not completely understood. Scientists agree that AmB causes disintegration of the fungal lipid membranes by interaction with ergosterol; however, several models of action (ion channel, surface adsorption, and sterol sponge models) are discussed in the literature (reviewed in references 22 and 24). AmB also binds to cholesterol in the plasma membrane of mammalian cells, and we hypothesize that platelet activation by AmB is also initiated by this interaction.

In patients, AmB serum concentrations reach about 3 μg/mL (AmB-D) and about 30 μg/mL (L-AmB) (27). After repeated administration of 5 mg/kg of body weight/day L-AmB, peak levels of 90 μg/mL AmB were measured (27, 28), and even higher concentrations are found near the infusion site. In our experiments, a significant and increasing degranulation of alpha granules was measured at concentrations between 3 and 50 μg/mL L-AmB; at higher concentrations, the signal decreased again (Fig. 1A), which is probably due to a cytotoxic effect (see below). Kinetic studies revealed that AmB-induced platelet activation is a rapid process, proceeding within a few minutes (Fig. 1B).

AmB-induced secretion of alpha and dense granules results in their fusion with the

platelet plasma membrane and in release of their respective contents, which entails a multitude of potential consequences. ADP and serotonin from dense granules are known as platelet activators and are involved in platelet aggregation; moreover, serotonin was shown to affect hyphal growth of *Aspergillus* species *in vitro* (46–48). Alpha granules contain a rich variety of biologically active substances, including immunomodulatory molecules (11). Among them, a number of chemokines, such as platelet factor 4 (PF4 [CXCL4]), neutrophil-activating peptide-2 (NAP-2 [CXCL7]), CXCL1, and CXCL5, are described as attracting and activating PMNs (9, 11, 49–52). Furthermore, P-selectin and CD40L (CD154), localized to alpha-granule membranes in quiescent platelets, are translocated to the platelet surface upon activation and bind to their corresponding receptors on PMNs, PSGL-1 and CD40, respectively. Our experiments indeed showed a fast and significant increase in binding of platelets to PMNs after their stimulation by AmB (Fig. 7B). This might have detrimental as well as beneficial effects in invasive fungal infections. Clearing of activated platelets by neutrophils and monocytes might lead to amplified thrombocytopenia, which could increase the risk of bleeding. On the other hand, our studies also revealed that platelets stimulated by AmB show increased aggregation (Fig. 3) and therefore may increase the risk of thromboses, which represent a pathological hallmark in the clinical course of mucormycoses (53).

Experiments with *Aspergillus fumigatus*, which is the most frequent cause of invasive aspergillosis, revealed that unstimulated platelets fail to adhere to fungal hyphae, while even low AmB concentrations stimulated platelets to bind to and cover large parts of *A. fumigatus* filaments (Fig. 4A and B). By this mechanism, degranulation and release of PMPs and serotonin at the fungal surface could cause a direct attack on the pathogen and potentially also enhance the antimycotic activity of AmB (12, 48, 54, 55). Moreover, binding of neutrophils to adhered platelets and further attraction of PMNs by released chemokines (see above) could recruit an increased number of neutrophils to the hyphae to exert their antifungal activity via oxidative (reactive oxygen species [ROS]) and nonoxidative mechanisms (56).

AmB was also found to induce alterations in the platelet plasma membrane. Exposure of phospatidylserine on the outer membrane leaflet is a common feature of platelet stimulation and contributes to procoagulant activity (Fig. 6A). A remarkable finding was the change of morphology to a globular shape without the typical development of filopodia (Fig. 6B). Furthermore, AmB significantly increased the shedding of platelet-derived microparticles (Fig. 5). These particles, which expose CD62P, CD154, and procoagulant glycoproteins, are reported to modulate coagulation, inflammation, and adaptive immunity even at sites distant from the location of activation (10, 57). At higher concentrations of AmB, shedding of microparticles decreased again (Fig. 5A), and this finding correlated with a decline of degranulation (Fig. 1A; also, see above) and platelet viability (Fig. 7A). AmB is known to cause hematological toxicities, including thrombocytopenia, and probably binding of AmB to cholesterol, and the resulting membrane perturbation is the main reason for its toxicity for platelets and other human cells (24, 58, 59).

Platelet stimulation by AmB also resulted in activation of the complement system (Fig. 6C and D). Platelet integrity is maintained by a spectrum of membrane-bound (CD46, CD55, and CD59) and fluid-phase (15, 16) complement regulators, and shedding of C5b-9-bearing microparticles might be a further regulatory mechanism (15). However, AmB concentrations of 20 μg/mL or above induced high levels of C5b-9 on the platelet membranes, which probably contributes to the detected loss of viability (Fig. 6D).

In invasive fungal infections, the stimulation of platelets by AmB and subsequent complement activation may exert beneficial as well as harmful effects. Complement and platelets exert a concerted antimicrobial action and form bridges to numerous elements of innate and adaptive immune defense (attraction and activation of PMNs and other immune cells; see above), thereby supporting the antimycotic impact of AmB. Possible detrimental consequences include thrombocytopenia and bleeding resulting from rapid removal of activated platelets, enhanced coagulation with thrombosis, and tissue damage by excessive inflammation, and all of these effects are also known to

occur in IFIs (15, 16, 60). Thus, platelets and complement represent double-edged swords that need to be tightly controlled, and many questions about this complex immune network remain to be answered.

MATERIALS AND METHODS

Antibodies, media, and chemicals. All antibodies as well as annexin V were purchased from BioLegend (San Diego, CA, USA) except the C3c antibody, which was from Dako (Glostrup, Denmark), and the C5b-9 antibody, which was from Hycult (Uden, The Netherlands). The Live/Dead fixable cell stain kit was from Thermo Fisher Scientific (Waltham, MA, USA), and Sabouraud and TSB medium were from BD Diagnostic Systems (Franklin Lakes, NJ, USA). Liposomal amphotericin B (Ambisome) was purchased from Gilead (Foster City, CA, USA); amphotericin B deoxycholate, thrombin, and calcofluor white stain were from Sigma-Aldrich (St. Louis, MO, USA).

RPMI 1640 medium (R6504; Sigma-Aldrich) was supplemented with 19.8 g/L glucose and 34.5 g/L MOPS (morpholinepropanesulfonic acid) and adjusted to pH 7.0 with NaOH. Sabouraud glucose medium and agar were purchased from BD Diagnostic Systems (Heidelberg, Germany).

AmB-D and L-Amb were initially dissolved in dimethyl sulfoxide (DMSO). Stock solutions were further diluted in RPMI 1640 medium to the required concentrations. Medium controls always contained corresponding concentrations of DMSO.

Preparation of platelet-rich plasma and serum. Human platelets were collected from healthy blood donors with informed consent (approved by the local ethics committee; no. AN5170 328/4.1 342/5.2) and prepared as concentrates by thrombocytapheresis with an Amicus cell separator (Baxter, Vienna, Austria) by the Department of Immunology and Blood Transfusion (Medical University of Innsbruck, Austria). The platelet number was routinely determined with a hemocytometer and adjusted to a concentration of 1.2×10^9 to 1.4×10^9/mL as described previously (61).

To obtain human serum, whole blood of healthy volunteers was collected without anticoagulants and centrifuged for 15 min at 1,500 $\times$ *g* at room temperature (RT). Serum from at least 5 donors was pooled and stored at −80°C for further use.

Fungal isolate and cultivation. A clinical isolate of *A. fumigatus* (A22) from a lung biopsy specimen of an immunosuppressed patient with respiratory insufficiency was used for the experiments (62). The fungus was grown on Sabouraud agar plates for 3 to 5 days at 37°C until sporulation was clearly visible. Spores were harvested by rinsing with phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma-Aldrich), washed with PBS, consecutively filtered through a 45-μm and a 10-μm cell strainer (BD Diagnostics System), and kept at 4°C.

Analysis of platelet degranulation, viability, and complement activation. Platelet concentrates were incubated with RPMI 1640 medium, 0.1 U thrombin, or AmB (either L-AmB or AmB-D) at 37°C, followed by fixation in 1% formalin for 30 min at RT.

To monitor activation, the samples were washed by centrifugation at 1,066 $\times$ *g*, incubated for 30 min with the indicated antibodies, washed again, and subsequently analyzed by flow cytometry (BD FACSVerse; BD Diagnostics). Platelets were gated by forward and sideward scatter properties and by using a labeled antibody against the platelet marker CD41. Secretion of alpha granules and dense granules during platelet activation results in surface exposure of CD62P (P-selectin) and CD63, respectively, and was detected by corresponding conjugated monoclonal antibodies.

Quantification of phosphatidylserine exposure on the platelet surface was used to further study activation. For this assay, platelets were incubated with FITC-conjugated annexin V according to the manufacturer's instructions and subsequently analyzed by flow cytometry.

Platelet viability was monitored by using a commercial Live/Dead fixable cell stain according to the manufacturer's instructions. The amine stain reacts only with outer cell surface proteins of living cells but can penetrate the damaged membranes of dead cells, resulting in intense staining of both the interior and exterior amines; quantification was performed by flow cytometry.

To monitor complement activation by platelets after incubation with AmB, 40% (vol/vol) human serum as the complement source or PBS as the control was added to the samples for 60 min. The samples were fixed with 1% formalin for 30 min (RT), washed with PBS by centrifugation at 1,100 $\times$ *g*, and incubated for 30 min (RT) with fluorescence-labeled specific antibodies to measure C3c deposition or C5b-9 formation on platelets. The C3c antibody detects both the C3c fragment of the C3 protein and the C3c part of C3b. The C5b-9 antibody binds to a C9 neoantigen of the terminal complement complex; this neoantigen is absent from native C9 prior to complex formation. Quantification was performed by flow cytometry.

Analysis of platelet-derived microparticles and aggregation. To assess budding of platelet-derived microparticles, the platelets were incubated with medium, thrombin, or various concentrations of L-AmB or AmB-D for 30 min, as described above, and fixated with 1% formalin. The microparticles were detected by flow cytometry by gating of the adequate size (0.5 to 1 μm) and binding of labeled CD41 antibody. Furthermore, aggregation of platelets and formation of microparticles were visualized by confocal microscopy employing a confocal microscope (PerkinElmer).

Platelet interaction with fungal hyphae. *A. fumigatus* hyphae were grown overnight in RPMI medium on round coverslips in 24-well cell culture plates. Platelets were incubated with medium or increasing concentrations of L-Amb for 30 min, washed, subsequently added to the washed fungal hyphae, and incubated for another 60 min. After removal of unbound platelets, hyphae were stained with calcofluor white stain; platelets were tagged with a FITC-labeled antibody against the platelet marker CD41.

After fixation in 1% formalin, unbound antibody was removed by washing; samples were embedded with Mowiol for visualization by confocal or fluorescence microscopy.

Isolation of PMNs. Fresh blood was from healthy human donors after obtaining informed consent; this study was approved by the Ethics Committee of the Medical University of Innsbruck, Austria (ECS1166/2018; 14 November 2018). Isolation of neutrophil granulocytes was performed as described previously (63). Briefly, a density gradient centrifugation applying Histopaque-1077 (Sigma Life Science) and RPMI medium was run at 570 × *g* for 25 min without a break. Supernatant was discarded, and the liquid was washed with PBS buffer and centrifuged at 500 × *g* for 10 min. Erythrocyte lysis was performed by adding distilled water to the cell pellet for 20 s, followed by addition of the same amount of 2× PBS. After centrifugation (500 × *g* for 5 min), the lysis procedure was repeated until the pellet appeared white. PMNs were suspended in RPMI 1640 medium containing 0.1% glucose. Purity was determined by light microscopy.

Statistical analysis. All assays were performed with platelets from at least three different donors, in triplicate. Results are presented as means and standard deviations from representative experiments. Statistical analyses were performed with GraphPad Prism 7 software, using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison test to check statistically significant values relative to controls. In addition, unpaired *t* tests were done to evaluate significant differences between two specified groups. *P* values of <0.05 were considered statistically significant.

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