Activation of platelets by *Aspergillus fumigatus*

Potential role in the immunopathogenesis of aspergillosis

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Abstract

Aspergillus fumigatus is the most frequent cause of invasive mould infections worldwide. Platelets contribute to inflammation and promote thrombosis, characteristically seen in aspergillosis, and might be involved in both antifungal defense and histopathological process. In the following experiments in vitro activation of platelets by conidia, swollen conidia and hyphae from A. fumigatus was assessed by flow cytometry and enzyme immunoassays. THP-1 monocytes and human monocytes with and without platelets were cultured with hyphae from A. fumigatus, and the release of interleukin (IL)-8 was measured by enzyme immunoassay. A. fumigatus potently induced the expression of CD62p and CD63 and the release of CD40 ligand, RANTES and Dickkopf homolog-1 in platelets, with particularly enhancing effects of hyphae as compared with conidia. The hyphae-mediated activation of platelets further enhanced the release of IL-8 in both THP-1 monocytes and in human adherent monocytes. In conclusion we have found that A. fumigatus is a potent inducer of platelet-mediated inflammation, potentially promoting protective as well as harmful responses during aspergillosis.
Introduction

Aspergillosis is the most common mould infection worldwide, and Aspergillus fumigatus (A. fumigatus) accounts for more than 90% of the cases (6). In contrast to most human pathogens, which are encountered infrequently, A. fumigatus spores are inhaled on a daily basis and, occasionally, exposure to large numbers of conidia can occur. The first line host defense against Aspergillus infection is based on innate immunity mediated by monocytes/macrophages and neutrophils (11,12,17,18). The adaptive immune system responds to a pathogen only after it has been recognized by the innate immune system (11). While inadequate immune responses may predispose invasive disease, overly robust immune responses to A. fumigatus may result in a spectrum of human disease states ranging from allergic bronchopulmonary aspergillosis to invasive aspergillosis in the immunocompromised host (9). Despite better diagnostic tools and therapeutic advances, the infection is difficult to diagnose and treat and outcome of invasive aspergillosis is often fatal.

Several lines of evidence support a role for platelets in inflammation (10). Platelet-mediated inflammation has been demonstrated during various acute and chronic infections and it has been suggested that platelets contribute to antimicrobial defenses (5,8). Very little is known about the role of platelets in defense against Aspergillus infection. In this connection it is interesting that important risk groups for invasive aspergillosis, e.g. patients with chemotherapy-induced neutropenia and recipients of hematopoietic stem cell transplants (6,13), very often have concurrent thrombocytopenia in addition to neutropenia. Furthermore, it has been reported that liver transplant recipients with thrombocytopenia have a considerably higher incidence of fungal infection than non-thrombocytopenic patients (3). There are some reports on the interaction between A. fumigatus and platelets, showing inhibition of fungal
growth potentially involving release of known platelet-derived microbial peptides as well as
direct physical interaction between platelets and conidia or hyphae (4,16).

*Aspergillus fumigatus* is angioinvasive, leading to intravascular thrombosis and
dissemination of the fungus through the bloodstream (1,2,21). In view of the well known role
of thrombocytes in vascular thrombosis in general, it is also possible that thrombocytes
contribute to the vascular damage and thrombosis which is a hallmark of invasive
aspergillosis.

To further study the possible role of platelets in the immune response and
pathogenesis of *Aspergillus fumigatus* we have examined the effect of conidia and hyphae on
relevant platelet-related inflammatory mediators. We have also examined the ability of
*Aspergillus*-exposed platelets to modulate inflammatory responses in monocytes.

### Materials and methods

**Preparation of *Aspergillus* conidia and hyphae**

*A. fumigatus* (ATCC MYA 1163) was grown on Sabouraud agar at 28°C for seven days. The
agar contained penicillin (12 mg/L) and streptomycin (40 mg/L) to prevent bacterial
contamination. Conidia were harvested by gently scraping the medium and solubilizing in
phosphate-buffered saline (PBS) with 0.05% Tween. The suspension was filtered through 25
µm Easy Pressure Syringe Filter Holder (Gelman laboratory, New York, NY) with
polypropylene separators (10 µm). The conidial concentration was determined by counting in
a Bürker chamber. Live conidia were stored at 4°C with a weekly turnover to ensure best
possible viability, and diluted to correct concentrations in RPMI 1640 (PAA laboratories,
Pasching, Austria) prior to use. In some experiments the conidia were incubated at 37°C, 5%
CO₂ for 6 or 18 hours to generate swollen conidia and hyphae, respectively.
Preparation and stimulation of citrated platelet rich plasma (PRP)

Preparation of citrated PRP, obtained from healthy, volunteered individuals, was performed as previously described (15). PRP (6.0 x 10^8 platelets/mL) was incubated at 22°C with 10 µM SFLLRN (synthesized at The Biotechnology Centre of Oslo, Oslo, Norway), Tris-buffered saline (TS, pH 7.4; 20 mM Tris and 150 mM NaCl), resting and swollen conidia at a concentration of 6.0 x 10^7/mL, hyphae grown from the same conidial concentration, or a combination of SFLLRN and the conidia or hyphae. Some experiments were done with hyphae separated from platelets with 0.4 µm cell culture inserts (BD Falcon™, Franklin Lakes, NJ). At different time points, aliquots were removed and centrifuged at 13,000 g for 5 minutes to obtain platelet (and Aspergillus)-free plasma (PFP) which was stored at -80°C until cytokine measurements or used for further in vitro experiments in THP-1 monocytes and adherent monocytes (see below).

Preparation and stimulation of THP-1 and adherent monocytes

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 (PAA Laboratories, Pasching, Austria) with or without PRP, hyphae from *A. fumigatus* in PRP as described above, and hyphae from *A. fumigatus* in the same concentration at 37°C and 5% CO₂. In a separate set of experiment, THP-1 monocytes were also incubated with PFP from un-stimulated and *A. fumigatus* (hyphae)-activated (120 minutes) PRP (see above).

In a separate set of experiments, PBMC (5 x 10^6 cells/mL), isolated from heparinized blood from six healthy volunteers through Isopaque-Ficoll (Lymphoprep; Nycomed Pharma, Oslo, Norway) gradient centrifugation, were incubated in RPMI with 10% fetal calf serum (FCS; PAA Laboratories) in 12 well cell culture cluster (Costar, Corning, NY) at 37°C, 5% CO₂ for 45 minutes, washed carefully three times in phosphate-buffered saline (PBS) at 37°C.
to obtain adherent monocytes, verified through microscopy. Stimulation and culturing of
adherent monocytes with hyphae from *A. fumigatus* were done as described for THP-1
monocytes with adjusted platelet and hyphae concentrations to yield the same ratios.

After 4 hours, cell and hyphae free supernatants were harvested and stored at -80°C
until further analysis.

**Flow cytometry**

The labeled platelets were analyzed using a FACS Calibur flow cytometer (Becton Dickinson,
San Diego, CA) as described (7). Briefly, light scatter and fluorescence channels were set at
logarithmic gain, and platelets were gated based on forward and side scatter properties as well
as fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against CD41 or
CD61 (BD Pharmingen, San Jose, CA). Platelet activation was checked by the well-
documented surface exposure of the integral α-granule membrane protein P-selectin (CD62P)
and the lysosomal integral membrane protein CD63 using FITC-conjugated monoclonal
antibodies against CD62P and CD63 (both from BD Pharmingen), respectively. Isotype
control antibodies were used as appropriate, and positive cells were defined by a fluorescence
intensity gate containing only 1% of the events observed with the unspecific antibody.

Altogether, 10 000 positive events were analyzed each time and the CXP software (Beckman
Coulter, Miami, FL) were used for data processing.

**Enzyme immunoassays (EIAs)**

Concentration of regulated on activation, normal T cell expressed and secreted
(RANTES/CCL5), the tumor necrosis factor (TNF) superfamily member LIGHT (TNFSF14),
interleukin-8 (IL-8/CXCL8), monocyte chemotactic protein-1 (MCP-1/CCL2), and Dickkopf
homolog-1 (DKK-1) were measured by EIAs from R&D Systems. Soluble CD40L (sCD40L)
was measured with EIA from Bender Medsystems (Vienna, Austria). The intra- and inter-assay coefficient of variation were <10% for all assays.

**Ethics**

Informed consent from blood collection was obtained from all individuals. The study was approved by the local ethical committee.

**Statistical analyses**

Data are presented as means±SEM unless otherwise stated. The Wilcoxon matched pairs test was used in the paired situation. Coefficients of correlation were calculated by the Spearman rank test. Probability values (2-sided) were considered significant at p<0.05.

**Results**

*A. fumigatus* induces platelet-activation

When investigating the ability of *A. fumigatus* to induce platelet activation, several significant findings were made (Figure 1-3). First, resting conidia, swollen conidia and in particular hyphae markedly induced an increase in surface expression of CD62P as assessed by flow cytometry. In fact, the hyphae-induced expression of CD62P was similar to the effect of the thrombin receptor agonist SFLLRN, and the combination of these two stimuli showed additive effect on CD62P expression. Second, a similar pattern was seen for the expression of the lysosomal integral membrane protein CD63, although no effect of resting conidia was observed. Third, in contrast to the effect of conidia and hyphae on the membrane expression of CD62P and CD63, only hyphae induced a significant increase in the release of RANTES and CD40L with a particularly pronounced effect on CD40L. Fourth, we have recently showed that platelets upon activation may release significant amount of DKK-1, an important regulator of the wingless (Wnt) signaling pathways that are involved in inflammation and...
vascular development (22), and notably, hyphae, but not conidia, induced a marked release of DKK-1 upon platelet activation. Finally, while the combined stimulation of SFLLRN and swollen conidia or hyphae showed enhancing effect on CD40L, the combination of SFLLRN and resting or swollen conidia attenuated the SFLLRN-induced release of RANTES and DKK-1. The release of TNFSF14 (LIGHT) was very low in all experiments with no significant effects of conidia or hyphae neither alone nor in combination with SFLLRN (data not shown).

Aspergillus is known to produce secondary metabolites that potentially could induce platelet activation. However, experiments (n=3) with cell culture inserts showed that direct contact between platelets and Aspergillus hyphae is necessary to achieve increased expression of CD62P and CD63 (Figure 3).

**Effect of hyphae-activated platelets on chemokine release in THP-1 monocytes**

Platelets are known to induce inflammatory responses in adjacent cells such as monocytes/macrophages (10). To further elucidate the inflammatory interaction between A. fumigatus and platelets, we therefore examined the ability of hyphae-activated platelets to induce release of the inflammatory chemokines IL-8 and MCP-1 in THP-1 monocytes. While there was no release of IL-8 in un-stimulated THP-1 cells or cells that were co-cultured with un-stimulated PRP, hyphae induced a modest and significant release of IL-8 in THP-1 monocytes after culturing for 4 hours (Figure 4). More importantly, this hyphae-mediated increase in IL-8 release was markedly enhanced when THP-1 cells were incubated with hyphae and PRP, suggesting that the interaction between hyphae and platelets promotes inflammatory responses in THP-1 monocytes (Figure 4). The increase of IL-8 in the co-culture experiments could potentially reflect enhanced release from Aspergillus activated platelets. However, neither conidia nor hyphae from A. fumigatus induced any detectable amounts of IL-8 (detection limit of the assay was 2 pg/mL) when cultured with PRP for 120...
minutes (data not shown). In contrast to the enhancing effect on IL-8 release in THP-1 cells after co-culture with hyphae and PRP, PFP from PRP that had been stimulated with hyphae for 4 hours did not induce any significant increase in IL-8 release after incubation for additional 4 hours with THP-1 monocytes (1.8 ± 0.7 pg/mL versus 3.6 ± 1.7 pg/mL, un-stimulated and PFP exposed THP-1 cells, respectively, n=2). This finding suggests that the inflammatory response in THP-1 monocytes may be dependent on direct contact between monocytes, platelets and hyphae. Finally, in contrast to the effect on IL-8, hyphae and PRP did not induce any release of MCP-1 when co-cultured with THP-1 monocytes (20 ± 5.9 pg/mL versus 25 ± 7.6 pg/mL, THP-1 + PRP and THP-1 + PRP + hyphae, respectively; n=6; p=0.3), suggesting that the Aspergillus/platelet induced inflammatory response in THP-1 monocytes has some degree of selectivity.

Effect of hyphae-activated platelets on chemokine release in human adherent monocytes

Similar to THP-1 cells, when human adherent monocytes (n=6) were incubated with hyphae and PRP, there was a significant increase in IL-8 release comparing cells that were incubated with hyphae or PRP alone. In fact, the IL-8 response was even more pronounced in adherent human monocytes than in THP-1 cells, further supporting the in vivo relevance of the inflammatory interaction between hyphae, platelets and monocytes. Moreover and similar to THP-1 cells, hyphae and PRP had no effect on MCP-1 release in adherent human monocytes (22 ± 6.3 pg/mL versus 20 ± 5.5 pg/mL, monocytes + PRP and monocytes + PRP + hyphae, respectively; n=6; p=1.0).

Discussion

Previous in vitro studies have suggested that platelets may be involved in the immune response against Aspergillus fumigatus, but their role in aspergillosis is still unclear. In the
present study we report several new findings concerning the interaction between platelets and

*Aspergillus* with potential relevance to the role of platelets in aspergillosis.

Our findings indicate that *A. fumigatus* induces activation of α-granules and lysosomal

granules resulting in enhanced expression of membrane-bound (i.e., CD63 and CD62P) as

well as soluble (i.e., RANTES, CD40L and DKK-1) mediators. Although RANTES, CD40L

and DKK-1 are released from α-granules, the release mechanisms of CD40L differ from that

of the two other molecules (15). Thus, the ability of *A. fumigatus* to enhance the release of all

these mediators, underscores its capability to promote platelet activation in a broad way.

Moreover, hyphae from *A. fumigatus* induced platelet responses to the same extent as

thrombin receptor activation, and for some of the responses (e.g., CD63 and CD62P) co-

activation with hyphae markedly enhanced the SFLLRN-mediated responses. These findings

indicate that *A. fumigatus* is a potent activator of platelets, which may act synergistic with

thrombin activation.

Moulds like *A. fumigatus* differ from most other pathogens in their ability to switch

between different morphological phenotypes inducing different immune response patterns

(19). The present study clearly shows that conidia and hyphae differ in the ability to activate

platelets. Thus, while hyphae potently induced platelet activation as assessed by all the

measured parameters (except for LIGHT release), there was no significant release of

RANTES, CD40L or DKK-1 when platelets were exposed to conidia. Moreover, resting

conidia attenuate the release of RANTES, CD40L and DKK-1 in SFLLRN-activated platelets,

while hyphae had the opposite effect. Although the responses were more modest than for

hyphae, swollen and even resting conidia induced up-regulation of membrane-bound CD62P

and CD63 (induced only by swollen conidia), suggesting that release of soluble mediators and

up-regulation of membrane-bound markers may be somewhat differently regulated upon

exposure to conidia. Hyphae, which are the fungal morphotype responsible for invasive
disease, are likely to come in contact with platelets during infection. However, it is also conceivable that inhaled conidia may encounter platelets in both interstitial tissue and alveolar spaces in the presence of inflammation and hemorrhage which occur frequently during *Aspergillus* infection (20).

Monocytic phagocytes play an important role in the innate immune response to *A. fumigatus* by ingesting and inactivating conidia. It is therefore interesting that we could demonstrate that hyphae activated platelets may enhance the inflammatory response in monocytes as measured by the release of the inflammatory chemokine IL-8. We found no enhancing effect on IL-8 release when platelets were replaced by cell-free platelet relasate in the co-culture experiments with THP-1 monocytes, suggesting that direct contact between monocytes, hyphae and platelets is necessary. This finding may support previous studies by Weyrich et al. showing that platelet-mediated release of IL-8 in monocytes is dependent on direct contact between activated platelets and monocytes (24).

Our findings in human monocytes suggest that the inflammatory interaction between platelets, monocytes and *Aspergillus* also occur in vivo, potentially contributing to the inflammatory responses seen in invasive aspergillosis. Hence, RANTES, released from platelets upon exposure to hyphae, may induce monocyte arrest in an inflamed endothelium (23). The co-localization of platelets, monocytes and hyphae in inflamed vessel walls may promote inflammatory responses which may be relevant in *Aspergillus* infection where one of the histopathological hallmarks is angioinvasion, thrombosis and vascular events. It has previously been reported that the histological patterns of tissue injury in invasive pulmonary aspergillosis differ between neutropenic and non-neutropenic patients (20). Thus, inflammatory necrosis is the predominant pattern in non-neutropenic patients, while angioinvasion is most frequent in neutropenic patients. Since most neutropenic patients also have an often profound thrombocytopenia, normal platelet numbers may not be necessary for
A novel finding in the current study was that hyphae from *A. fumigatus* promoted a platelet-mediated release of DKK-1. DKK-1 is a major regulator of the Wnt-signaling pathway, a complex cascade of mediators which have been shown to be involved in a wide range of physiological and pathophysiological responses such as inflammation, angiogenesis and regulation of cell survival (14). Recently, we reported that DKK-1 also may play a major role in platelet-dependent endothelial activation and inflammation (22), and Wnt signaling has been shown to be involved in the pathogenesis of various disorders ranging from cancer to atherosclerosis and septicemia (14,22). It is tempting to hypothesize that DKK-1 and the Wnt pathway also could be involved in the *A. fumigatus*-induced inflammation and vascular pathology. Our findings should encourage further studies on the role of Wnt signaling pathway and DKK-1 in responses to *Aspergillus* infection.

In the current study we show that *A. fumigatus* is a potent inducer of platelet-mediated inflammation, potentially promoting further inflammatory responses in monocytes. These findings may reflect a role for platelets in the protective immune response against *Aspergillus* infection. On the other hand, inflammatory necrosis, thrombosis and fungal invasion of blood vessels are well known histopathological hallmarks in patients with invasive infection caused by *A. fumigatus*, and the *Aspergillus*-induced activation of platelets with release of inflammatory mediators, may well contribute to these harmful consequences of invasive aspergillosis. The specific importance of platelets for the pathogenesis of *Aspergillus* infection may differ between patient groups according to the presence or absence of thrombocytopenia. We believe that further studies are warranted to elucidate the possible importance of platelets in *Aspergillus* infection.


Figure Legends

Figure 1. Expression of CD62P (A) and CD63 (B) in platelets upon stimulation with conidia (left column), swollen conidia (middle column) and hyphae (right column) from *Aspergillus fumigatus* as assessed by flow cytometry after incubation for 120 minutes.

Figure 2. The relative change in the expression of CD62P (A) and CD63 (B) on platelet surface and the release of CD40L (C), RANTES (D) and DKK-1 (E) in platelets stimulated with SFLLRN (10 µM) alone or in combination with conidia (left column), swollen conidia (middle column) or hyphae (right column) from *Aspergillus fumigatus*. The expression of CD62P and CD63 was assessed by flow cytometry (percentage of positive platelets), and the release of CD40L, RANTES and DKK-1 was measured by EIA in platelet-free supernatants after incubation for 120 minutes. The release of CD40L, RANTES, and DKK-1 in un-stimulated cells were 2.5 ± 0.35 ng/mL, 9.1 ± 0.8 pg/mL, and 0.1 ± 0.02 pg/mL, respectively. Data is given in mean ± SEM (n=6). *p<0.05 versus un-stimulated (us). †p<0.05 versus SFLLRN.

Figure 3. Expression of CD62P (A) and CD63 (B) in un-stimulated (us) platelets (left column) compared with platelets stimulated with hyphae (middle column) from *Aspergillus fumigatus* and hyphae separated from platelets with cell culture inserts (right column) as assessed by flow cytometry after incubation for 120 minutes.

Figure 4. The release of IL-8 from THP-1 monocytes after culturing for 4 hours with or without un-stimulated PRP, hyphae from *Aspergillus fumigatus* or a combination thereof.
Data is given in mean ± SEM (n=11). *p<0.05 versus un-stimulated THP-1 monocytes (Us).

†p<0.05 versus THP-1 monocytes + hyphae and THP-1 monocytes + PRP.

Figure 5. The release of IL-8 from human monocytes after culturing for 4 hours with or without un-stimulated PRP, hyphae from *Aspergillus fumigatus* or a combination thereof.

Data is given in mean ± SEM (n=6). *p<0.05 versus un-stimulated monocytes (Us), versus monocytes + hyphae and versus monocytes + PRP.