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Production of Extracellular Traps against *Aspergillus fumigatus In Vitro* and in Infected Lung Tissue Is Dependent on Invading Neutrophils and Influenced by Hydrophobin RodA

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Abstract

*Aspergillus fumigatus* is the most important airborne fungal pathogen causing life-threatening infections in immunocompromised patients. Macrophages and neutrophils are known to kill conidia, whereas hyphae are killed mainly by neutrophils. Since hyphae are too large to be engulfed, neutrophils possess an array of extracellular killing mechanisms including the formation of neutrophil extracellular traps (NETs) consisting of nuclear DNA decorated with fungicidal proteins. However, until now NET formation in response to *A. fumigatus* has only been demonstrated *in vitro*, the importance of neutrophils for their production *in vivo* is unclear and the molecular mechanisms of the fungus to defend against NET formation are unknown. Here, we show that human neutrophils produce NETs *in vitro* when encountering *A. fumigatus*. In time-lapse movies NET production was a highly dynamic process which, however, was only exhibited by a sub-population of cells. NETosis was maximal against hyphae, but reduced against resting and swollen conidia. In a newly developed mouse model we could then demonstrate the existence and measure the kinetics of NET formation *in vivo* by 2-photon microscopy of *Aspergillus*-infected lungs. We also observed the enormous dynamics of neutrophils within the lung and their ability to interact with and phagocytose fungal elements *in situ*. Furthermore, systemic neutrophil depletion in mice almost completely inhibited NET formation in lungs, thus directly linking the immigration of neutrophils with NET formation *in vivo*. By using fungal mutants and purified proteins we demonstrate that hydrophobin RodA, a surface protein making conidia immunologically inert, led to reduced NET formation of neutrophils encountering *Aspergillus* fungal elements. NET-dependent killing of *Aspergillus*-hyphae could be demonstrated at later time-points, but was only moderate. Thus, these data establish that NET formation occurs *in vivo* during host defence against *A. fumigatus*, but suggest that it does not play a major role in killing this fungus. Instead, NETs may have a fungistatic effect and may prevent further spreading.


Introduction

*Aspergillus fumigatus* is the most important airborne fungal pathogen causing life-threatening infections in immunocompromised patients. Conidia, the asexually produced small fungal spores, are inhaled and reach the lung alveoli, where they are confronted with the first line of defence which is built up of resident alveolar macrophages and newly recruited neutrophil granulocytes (neutrophils). Conidia are thought to be killed by macrophages whereas hyphae are mainly attacked by neutrophils (reviewed in: [1–3]). However, recruited neutrophils are also able to phagocytose conidia directly [4,5] or prevent their germination as shown by Bonnett et al. [6]. Furthermore, the essential role of neutrophils in preventing invasive growth of *A. fumigatus* has recently been proven [7]. Nevertheless, the detailed mechanisms how these immune effector cells protect the human host against *A. fumigatus* are still a matter of debate.

The NAD(P)H oxidase in phagocytes is regarded to be essential for host defence against aspergillosis. This idea is supported by the fact that patients with chronic granulomatous disease are highly...
NETs against *A. fumigatus* In Vivo

Author Summary

The fungus *Aspergillus fumigatus* grows on decaying organic matter and produces large numbers of spores, called conidia, which are constantly inhaled by humans. This is harmless, because we have a functioning defence system of immune cells called neutrophil granulocytes, but people with too few or non-functioning neutrophils can die of *Aspergillus* infections. Neutrophils invade the lung, engulf/phagocytose and thereby kill conidia. Dying neutrophils can also throw their nuclear DNA on hyphal elements as NETs (Neutrophil Extracellular Traps) that are decorated with antimicrobial proteins. Thus, larger fungal amounts, including tissue-invading hyphae, can still be controlled. However, until today the formation of NETs has not been demonstrated in *Aspergillus*-infected lungs, the role of neutrophils for this process was unknown and whether the fungus has anti-NET defence strategies on its own was not clear. We demonstrate here the existence of NETs in *Aspergillus*-infected lungs, show that neutrophils produce these structures and that they phagocytose fungal elements within the lung tissue. Furthermore, we show that *Aspergillus* camouflages its spores by means of the surface protein hydrophobin RodA, which is able to strongly prevent NET formation by neutrophils. These studies shed new light on the dynamics and molecular mechanisms of this key process of host-pathogen interaction. Although these data establish that NET formation occurs in vivo during host defence against *A. fumigatus*, we suggest that NET formation does not play a major role in killing this fungus.

susceptible to fungal diseases, especially *Aspergillus* infections. Neutrophils of these patients show markedly deficient NAD(P)H oxidase activity [8]. The activation of NAD(P)H oxidase results in the formation of superoxide anions and other reactive oxygen intermediates (ROI) (reviewed in [1]). However although the catalase or yap1 and skn7 mutants display an increased sensitivity to ROIs in vitro, the detoxifying systems of *A. fumigatus* do not play any role in controlling the killing of *A. fumigatus* conidia by phagocytes in vitro [9].

Consequently, the production of ROI by the host may be important for control of *Aspergillus* on a level distinct from direct killing. This result is in agreement with recent findings that the granule proteins in neutrophils are primarily responsible for the killing process of microbes while ROI only function by activating vacuolar enzymes [10,11]. The contribution of NAD(P)H oxidase in killing conidia in macrophages, as shown for *A. fumigatus* [12], may be indirect by depolarising the phagocytic vacuole, leading to an influx of ions which results in the activation of digestion enzymes, as proposed by Segal [11]. The importance of vacuolar enzymes for fungal defence is also supported by the finding that knock-out mice lacking cathepsin G and elastase were found to be susceptible to *Aspergillus* infection [13].

Hence, the mechanism how the innate immune systems effectively counteracts spores and germings of *A. fumigatus* has to be further elucidated with a focus on killing mechanisms independent of direct ROI-mediated destruction. In the light of these observations the identification of extracellular fibres called neutrophil extracellular traps (NETs), which are produced as a final act of defence by dying neutrophils may be of major importance [14]. NETs are composed of chromatin covered with granular proteins which express antimicrobial activity. The process of NET formation depends on the induction of a ROI-mediated signaling cascade in neutrophils that ends up in the disintegration of the nuclear envelope and granular membranes [15]. After membrane rupture the NETs are formed by intracellular mixture of nuclear DNA with granular contents and then explosively released in a matter of seconds, a process that is associated with cell death. This unique sequence of events is also called NETosis (reviewed in [16]). NETs may mediate the trapping of conidia of *A. fumigatus* [17], as it has been shown for the yeast form and hyphal cells of *C. albicans* [18] and for *A. nidulans* [19].

Although NETs are an attractive model to explain defence against *A. fumigatus*, direct proof of their existence and importance in vivo is still lacking. The restoration of NAD(P)H oxidase activity in hematopoietic stem cells of a human CGD patient by gene transfer has been shown to re-establish NETosis in neutrophils derived from these cells in vitro and restore fungal defence against *A. nidulans* in the treated patient. However, the re-establishment of NET formation in this patient in vivo as basis for successful fungal defence could not be demonstrated directly and thus remained a matter of speculation [19]. Furthermore, the importance of NETosis for the defence against the clinically much more relevant *A. fumigatus* still lacks experimental proof. Finally, molecular determinants of fungal pathogens that control or induce the production of NETs by binding neutrophils are so far entirely unknown.

To get a better understanding of these issues, we set out to comprehensively study whether the different morphotypes of *A. fumigatus* have the potential to induce NETs in vitro. Furthermore, we aimed at shedding light on molecular mechanisms involved. Finally we wanted to clarify, whether NETs are really formed in *Aspergillus*-infected lungs and whether this is dependent on newly arriving neutrophils.

Results

Human neutrophils produce extracellular traps when encountering different *A. fumigatus* morphotypes

To analyse whether *A. fumigatus* induced the production of NETs by human neutrophils, different morphotypes of *A. fumigatus*, i.e. resting or swollen conidia and hyphae, were co-incubated with human neutrophils for different time periods. Confocal images of cultures stained with propidium iodide and calcofluor white during co-incubation showed, that freshly isolated non-prestimulated neutrophils produced typical NET structures against all morphotypes within three hours (Figure 1 and Figure S1). NET formation started with a rapid enlargement of fungal contacts of neutrophils before final NET formation, we also observed that neutrophils phagocytose fungal elements as NETs (Neutrophil Extracellular Traps) that are produced as a final act of defence by dying neutrophils ([19], Figure S1). NET formation in this patient in vivo as basis for successful fungal defence could not be demonstrated directly and thus remained a matter of speculation [19]. Furthermore, the importance of NETosis for the defence against the clinically much more relevant *A. fumigatus* still lacks experimental proof. Finally, molecular determinants of fungal pathogens that control or induce the production of NETs by binding neutrophils are so far entirely unknown.

NETs against *A. fumigatus* In Vivo

Author Summary

The fungus *Aspergillus fumigatus* grows on decaying organic matter and produces large numbers of spores, called conidia, which are constantly inhaled by humans. This is harmless, because we have a functioning defence system of immune cells called neutrophil granulocytes, but people with too few or non-functioning neutrophils can die of *Aspergillus* infections. Neutrophils invade the lung, engulf/phagocytose and thereby kill conidia. Dying neutrophils can also throw their nuclear DNA on hyphal elements as NETs (Neutrophil Extracellular Traps) that are decorated with antimicrobial proteins. Thus, larger fungal amounts, including tissue-invading hyphae, can still be controlled. However, until today the formation of NETs has not been demonstrated in *Aspergillus*-infected lungs, the role of neutrophils for this process was unknown and whether the fungus has anti-NET defence strategies on its own was not clear. We demonstrate here the existence of NETs in *Aspergillus*-infected lungs, show that neutrophils produce these structures and that they phagocytose fungal elements within the lung tissue. Furthermore, we show that *Aspergillus* camouflages its spores by means of the surface protein hydrophobin RodA, which is able to strongly prevent NET formation by neutrophils. These studies shed new light on the dynamics and molecular mechanisms of this key process of host-pathogen interaction. Although these data establish that NET formation occurs in vivo during host defence against *A. fumigatus*, we suggest that NET formation does not play a major role in killing this fungus.
investigated the process by live cell fluorescence imaging. These analyses allowed to precisely reconstruct the kinetics of the reaction (Figure 3A and Video S1). Normally, neutrophils rapidly phagocytosed conidia, as described [4]. Interestingly, the large hyphal structures, that could not be internalised, were covered and ensheathed by multiple neutrophils. The rate of NET production was dependent on the chosen ratio between neutrophils and fungal elements (data not shown). At a ratio of 1:1 only a minority of cells in a population (12.4±9.5%) were finally observed to disintegrate and undergo NETosis, which was clearly visible by rapid staining of externalised DNA by the nucleic acid dye propidium iodide in the supernatant (Figure 3A and Video S1). Thus, NETosis was not

Figure 1. NET formation by human neutrophils co-incubated with resting and swollen conidia or hyphae of *A. fumigatus*. CLSM fluorescence, bright field and overlay images showing NET formation of human neutrophils after co-incubation with *A. fumigatus*. Extracelluar DNA was stained with propidium iodide (red), conidia and hyphae with calcofluor white (blue). Microscopic pictures were taken after 3 hours. Neutrophils were co-incubated with resting conidia (A), swollen conidia (B) and hyphae (C). All scale bars represent 20 μm length.

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an invariant response pathway of dying neutrophils and its frequency was further influenced by the E/T ratio. Often only a sub-fraction of neutrophils was able to generate NETs, while the majority of cells remained alive. Nevertheless, even when only few cells were observed to undergo NETosis these could produce NETs of considerable size. A minority of cells died without signs of NET formation (data not shown). The latter was evident from bright red nuclear staining of condensed cells (Figure 3B, black arrows) or swollen cells with dilute cytoplasmic staining (Figure 3B, white arrows).

Figure 2. Scanning electron microscopy (SEM) micrographs of conidia and hyphae trapped in NETs. NET formation of human neutrophils after co-incubation with A. fumigatus. Microscopic pictures were taken after 3 hours. Neutrophils were co-incubated with resting conidia (A), swollen conidia (B) and hyphae (C). All scale bars represent 5 μm length. Morphological structures are indicated by labelled arrows.
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Figure 3. Time-lapse widefield microscopy of NET formation by neutrophils co-incubated with swollen conidia of *A. fumigatus*. (A) Time series of NET formation by human neutrophils upon contact to swollen *A. fumigatus* conidia. Extracellular DNA was stained with propidium iodide (red), conidia and germ tubes with calcofluor white (blue-pink). Microscopy was carried out for 5 h after onset of co-incubation while single pictures were taken every 30 seconds (Video S1). (B) Co-incubation after 150 minutes. Black arrowheads indicate cells which had died without releasing DNA, white arrowheads point to cells undergoing preparation for NET release. The colours are the same as in (A). (C) DNase digestion of NETs after 180 minutes of co-incubation. White arrowheads indicate the preformed NET structures right before destruction by the enzyme (Video S2). The colours are the same as in (A). Where appropriate, real time is indicated in minutes. The DNase was added 7 min before onset of the visible NET digestion to the border of the microscopy chamber right before sealing. The size of scale bars is indicated directly. These movies are representative for at least 6 independent experiments that were performed.

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To further confirm that NETs produced against A. fumigatus consisted of DNA, we added DNase I to neutrophil-Aspergillus co-cultures containing prominent NET-structures (Figure 3C and Video S2). Within minutes after addition of DNase I NETs were completely disrupted, indicating that NETs observed in these systems were indeed composed of DNA (Figure 3C and Video S2).

**Phagocytosis and NET production by invading neutrophils in the lungs of Aspergillus-infected mice**

The data above suggested, that contact to A. fumigatus elements, especially growing hyphae, triggered NET formation by human neutrophils, as previously described for conidia alone [17]. However, like with the study by Jaillon [17] this observation was purely based on *in vitro* experiments. Thus, although NET-structures have been observed in tissue wounds *in vivo* before [14,20] or in lungs infected with Candida albicans [21], it is not known, whether they also exist in lungs recently infected with A. fumigatus and also the kinetics of NET formation *in vivo* has not been characterised, yet [19]. As direct imaging of NETs in the lungs of humans is not possible [19], we newly developed a mouse model of early invasive aspergillosis (Figure 4A, and Video S3) allowing us to clarify, whether NETs really occur during defence against an acute A. fumigatus infection *in vivo*.

We intratracheally injected swollen conidia, that were stained with calcfluor white, into wild type C57/Bl6 mice or mice with a targeted insertion of EGFP into the lysozyme locus (Lys-EGFP), thus harboring green neutrophils [22]. Pilot experiments had demonstrated that swollen conidia, which represent the *Aspergillus* morphotype associated with the onset of invasive growth, produced prominent NET-structures *in vitro*. After 7–10 h, mice were killed and their lungs were analysed for fluorescent cells, fungal elements as well as NETs by 2-photon microscopy (Video S3). These analyses demonstrated the formation of large fungal clusters with outgrowing hyphae and attached host cells associated with alveoli (Figure 4B and Video S8). Clearly also structures closely resembling the NETs we had observed before *in vitro* (Figure 1 and 3) were present within infected lung tissue (Figure 4B–D). The structures were especially enriched in areas with bulk associations of multiple fungal elements (Figure 4C and D) while in control animals, which only received PBS we did not observe these structures (Figure 4E). DNase digestion of these structures was possible.

Neutrophils could be observed to be highly motile within these lung-slice preparations (Figure 5A and Video S4) and we measured average migration velocities of almost 10 mm/min with more than 50% of cells migrating (data not shown). Such migration parameters are very similar to values measured for neutrophils *in vivo* [23,24], suggesting that our approach allowed the measurement of near natural neutrophil motility in vital lung tissue. Importantly, we could also observe neutrophils phagocytosing individual conidia in these living lung slices (Figure 5B, arrowheads and Video S3) leading to the localisation of conidia inside of neutrophils (Figure 5C and Video S9) and their transportation with the migrating cells over larger distances. Neutrophils could also be observed carrying swollen conidia with small hyphal segments over large distances in a collective effort (Figure 5G, arrowhead, Video S6), similar to what we had observed before *in vitro* (Figure 3 and Video S10). This was also highly reminiscent of the pattern of 2-D phagocytosis which we previously described in an *in vitro* system [4]. Sometimes individual motile neutrophils were observed migrating along the curvature of alveoli, potentially scanning the environment (Figure 5E, arrowhead, Video S7) for infection.

These data strongly suggested the rapid production of NETs against an infection with A. fumigatus *in vivo*. However an important question was, whether neutrophils were required for NET formation. It could be clearly shown that neutrophils massively invaded the lung shortly after infection with A. fumigatus (Figure 6A). To address their importance for NET formation, we depleted neutrophils in mice by injection of anti Gr-1 monoclonal antibodies as reported [25]. 24 h later, animals were infected with A. fumigatus and investigated as described above. The depletion of neutrophils strongly inhibited their immigration into the lungs of infected mice. When the Gr-1 depletion was done in Lys-EGFP mice there were hardly any NET-structures detectable by staining with the DNA-specific dye Sytox Orange and no green neutrophils were patrolling the tissue (Figure 6B) despite the presence of prominent fungal clusters in the lung. A quantification of NETs in neutrophil-depleted compared to untreated mice further underscored this finding (Figure 6C). Since, however, the natural infectious particles are not swollen but rather resting conidia, we also quantified the NET formation in response to an infection with this airborne form of the fungus in untreated mice. Here, NET formation was less prominent than with swollen conidia, but still clearly detectable (Figure 6C). The almost complete lack of NET structures in neutrophil-depleted mice despite the presence of large fungal masses was prominent, thus showing for the first time a direct connection between the availability of infiltrating neutrophils in the lung and the local development of NET structures.

**The frequency of NET formation is morphotype- and strain dependent**

To further characterise NET formation quantitatively, we analysed the DNA content of the supernatant of co-cultures of A. fumigatus with freshly isolated, unstimulated neutrophils using propidium iodide. Further confirming our imaging data (Figures 1 and 2), NET production in the supernatants was highest when hyphae were co-incubated with neutrophils and considerably lower with swollen and in particular resting conidia (Figure 7A). The addition of both DNase I or the NADP(H) oxidase inhibitor DPI (diphenyldihydroindolium) led to a reduced amount of fluorescence indicating reduction in the generation of NETs (Figure 7A). Even more, the addition of DPI abolished NET formation completely which supports the finding that NET formation depends on the production of ROI [15]. The relative decrease of the number of neutrophils during the co-incubation experiments (E/T ratios of 1:10 instead of 1:5) with resting or swollen conidia and hyphae resulted in less NET formation, whereas ratios of 1:1 resulted in higher fluorescence signals and thus increased NET formation (data not shown). Furthermore, NET formation also depended on the surface structure of the pathogen because latex beads did not trigger significant NET formation (Figure 7A and Figure S1E) and the measured low background fluorescence was obviously caused by neutrophils which had undergone lysis after 3 h of incubation.

To further study the ability of different fungal strains to trigger NET formation, we employed different mutant and wild type strains of A. fumigatus in NET-forming assays *in vitro*. As shown in Figure 7B, NET formation also depended, at least in part, on the strain analysed. NET induction triggered by the DAL wild type strain was lower than that observed with the ATCC46645 wild type strain. Interestingly, a polycysteine synthase (*pksP*) mutant strain did not trigger significantly different fluorescence and thus NET production by neutrophils compared with the respective wild type strain ATCC46645. This indicated that dihydroxynaphthalene melanin, which is lacking in the *pksP* mutant, does not influence
NET formation, although this cell wall component is able to suppress ROI-production in neutrophils [26,27].

Hydrophobin RodA influences NET formation

Nevertheless, cell wall components are the first structures of the fungal pathogen encountered by invading phagocytes and thus they should play a role in shaping the immune response against *A. fumigatus*. Since it has recently been shown that hydrophobin RodA, the major surface component of *A. fumigatus* conidia, renders them immunologically inert, thus not triggering adaptive immune responses [28], we raised the question whether RodA was able to suppress NET formation as key antifungal immune response of the innate arm of cellular immunity. Hydrophobin RodA is present on resting conidia, in reduced amounts on swollen conidia and lacking on hyphae [29]. Therefore, we analysed the ΔrodA mutant lacking the hydrophobin RodA surface layer of swollen and resting conidia [30]. Confirming an important role of hydrophobin RodA for this process, NET formation was significantly increased when neutrophils encountered swollen and resting ΔrodA mutant conidia as compared to wild type conidia (Figure 7B). NET formation induced by resting conidia of the ΔrodA mutant was even stronger than the increased NET formation induced by any of the hyphal forms investigated in parallel which suggested, that hydrophobin RodA was a major factor for silencing the NET-function of neutrophils. This also indicated, that resting conidia do express a NET-inducing element that is shielded by hydrophobin RodA, as described before for the induction of adaptive immune responses [28]. NET formation was almost identical when hyphae of wild type and ΔrodA mutant hyphae were compared suggesting, that a potentially strong NET-inducer that is present on resting conidia and normally shielded by RodA is lost during hyphal development. Consistently, addition of purified hydrophobin RodA to ΔrodA mutant conidia reduced the NET formation (Figure 7B). Furthermore the chemical removal of the rodlet layer of DAL wild type resting conidia by hydrofluoric acid (HF) treatment, which also kills conidia, lead to a significant increase of NET formation (Figure 7B), whereas the level of NET formation stayed the same after HF treatment of resting conidia of the ΔrodA mutant. Obviously also dead conidia trigger NET formation and thus it appears unlikely that an actively secreted product rather than a fixed surface structure mainly activates NET formation. In addition, when RodA was already genetically removed in the ΔrodA mutant, HF-treatment did not further enhance NET formation by resting conidia. Taken together, these data indicate that RodA helps *Aspergillus* to evade NET induction thus constituting the first molecularly defined pathway in *A. fumigatus* for escape from this central response of neutrophils to fungal infection.

Killing of *A. fumigatus* by neutrophils

Despite the clear induction of NET formation we did not observe an influence of NET formation on killing of *A. fumigatus* resting and swollen conidia *in vitro*. *A. fumigatus* conidia were co-incubated with freshly isolated, unstimulated human neutrophils and CFUs of the fungus were determined at different time points. As shown in Figure 8A, after 180 min about 35% of both swollen and resting conidia were killed. This killing rate was in the range found in previous studies, in which killing rates of around 50% of all conidia were observed after 160 min [9]. Addition of DNase I and DPI did not affect the killing of conidia (Figure 8B). Therefore, it seems unlikely that NET formation contributes to killing of conidia in this system. To elucidate whether killing can mainly be explained by phagocytosis, we added cytochalasin D, which disrupts actin filaments and thus inhibits phagocytosis, to conidia-neutrophil co-incubation experiments. Cytochalasin D effectively inhibited the killing of *A. fumigatus* conidia by naive neutrophils (Figure 8B). So we suggest that the killing of conidia is mainly caused by phagocytosis and thus not by NET formation.

Consistently, the ΔrodA conidia were killed at almost the same rate as the parental wild type conidia (DAL strain), although the induction of NET formation differed significantly between the two strains. The addition of 0.07 µg hydrophobin RodA did not influence the killing of ΔrodA in comparison to untreated ΔrodA conidia (Figure 8C) significantly. Taken together, these data indicate that NET formation does not directly affect killing of conidia in this system *in vitro*.

To unravel the role of NETs in killing *A. fumigatus* hyphae we measured the respiration rate of hyphae after different time periods of co-incubation with neutrophils. Since conventional CFU determination is almost impossible for the hyphal growth form of filamentous fungi, the analysis of the oxygen consumption rate served as an indirect parameter for cell viability. The first significant differences in oxygen consumption of hyphae after co-incubation with neutrophils were detected after 9 h and increased further at later time points (up to 12 h) (Figure 9) in comparison to untreated controls. The addition of DNaseI or DPI almost completely abolished the detrimental effect of the neutrophils. These findings suggest that NETs do reveal antifungal activity against fungal hyphae, which, however, occurs with a certain time lag at later stages.

Discussion

Here, we demonstrate that both human and murine neutrophils produce neutrophil extracellular traps (NETs) in response to the human-pathogenic fungus *A. fumigatus*. Typical NET-structures which have already been described for other pathogens were observed by fluorescence and electron microscopy during co-incubation of neutrophils with *A. fumigatus* mycelium and conidia. Both fungal morphotypes were embedded in NETs consisting of smooth fibres and globular domains as first described by Brinkmann et al. [14] and others [reviewed in [16]]. The DNA intercalating dye propidium iodide stained NETs strongly. EM revealed that neutrophils engulf *A. fumigatus*...
NETs against A. fumigatus In Vivo

Figure 5. Neutrophil motility and interaction with fungal elements in living lung slices. Lys-EGFP mice were infected and acute lung slices were prepared as described in Figure 4. Subsequently, time-lapse 2-photon microscopy was used to generate movies of cells migrating in these lung slices. (A) A still image of a movie showing individual neutrophils (green). DNA (red) of tissue fungal elements (blue). Tracks of migrating cells are shown in white. (B) Image of the tracks alone (Video S4). (B) Many neutrophils (green) can be seen migrating within the tissue and internalising conidia (light blue) within a neutrophil (green). See also Video S5. (D) Multiple neutrophils (green) cooperate to transport a hypha (White arrowhead, hypha is light blue. The area of the red shown enlarged from the bottom and as 3-D rendering from the side to demonstrate the internalisation of a conidium (light blue) within a neutrophil (green). See also Video S9.

Besides experimental in vitro data, we provide the first direct observation of NETs in neutrophils infected with A. fumigatus and show that these structures form within 3-4 hours after exposure to the first immigrating neutrophils. The recent paper by Urban et al. identified NETs in lungs fixed 24 h after infection with C. albicans, thus not allowing to investigate the early kinetics of this response and also precluding analysis of cell migration in the infected site. Also the role of immigrating neutrophils was not addressed in this study [21]. Our study is thus an important step forward in being the first to demonstrate the existence of NETs in A. fumigatus-infected lungs and highlighting the importance of newly arriving neutrophils for their generation. This information is critical for a complete understanding of neutrophil defence during fungal attack. A recent study impressively demonstrated that the lack of functional NADPH oxidase in neutrophils of a patient suffering from chronic granulomatous disease inhibited the production of NETs in response to A. fumigatus in vitro. Re-introduction of a functional enzyme by gene therapy rescued the NET-phenotype in vitro and enabled the patient to eradicate a therapy-resistant invasive aspergillosis [19]. However, due to technical limitations the study did not directly demonstrate the existence of NETs in the infected patient lung nor could it demonstrate that the infiltration of functional neutrophils was essential for their formation. Our study closes this gap in our knowledge and provides the first direct hint to neutrophil-derived NET formation in response to A. fumigatus infection in vivo.

Our data also show the explosive release of the NET DNA, which occurs within a few seconds, while the preceding process, that prepares a neutrophil for the final NET-release, lasts up to 3 hours. The release kinetic and the fact that the structures we observe in vitro are highly sensitive to DNase I-mediated destruction well agree with recently published data [13] and further confirm that we were visualising true NETosis. It is interesting to note that only a subpopulation of neutrophils actually ended up producing NETs, although this was also dependent on the chosen E/T ratio. Often, a majority of cells either stayed alive or underwent normal necrotic or apoptotic cell death as detected by entry and permanent residence of nuclear dyes in cells but not the explosive release of DNA. This was despite the fact that most if not all neutrophils brieﬂy touched or stayed in close contact with fungal elements in these assays. Also our analyses of neutrophils migrating in live lung-tissue underscored, that only a minority of neutrophils secrete NETs. Although we frequently observed NET structures closely associated with fungal masses in lung-slices, we also observed large numbers of highly motile neutrophils in between.

What ultimately decides, whether a neutrophil performs NETosis or other types of responses after contacting fungal elements, remains unclear. It is, however, conceivable, that control mechanisms exist that limit the production of NETs because external DNA, especially in the form of nucleosomes as present in the NET structures [14,21], is potentially harmful. Nucleosomes can be taken up by DNA-specific B cells that can then make anti-nuclear-antibodies (ANAs) because they get help from T cells specific for the histone component of nucleosomes [33]. ANAs are found in many autoimmune diseases such as systemic lupus erythematosus [33,34] and often mediate the pathologies associated with the disease. A NET-inhibiting mechanism driven by the amount of external DNA is an attractive concept. This would, however, imply that neutrophils possess a mechanism that allows them to measure the amount of external DNA, inhibiting their further production of NETs if this amount is too high. Indeed, Toll Like Receptor 9 is a well known receptor for dsDNA [35] and very recently, new receptors for intracellular DNA have been identified [36–38] that might serve such a function. It would thus be interesting to study animals mutant for such proteins for their ability to generate NETs.

The novel mouse model for investigating NETs and invading neutrophils in live lung tissue introduced here proved to be a very helpful approach. We can demonstrate structures in living lung tissue that closely resemble the NETs observed before in vitro by confocal microscopy and scanning electron microscopy. As we show, the migration parameters of cells in our experiments are in accordance with previously published data on neutrophils observed in true intravital setups in various organs [23,24,39] and also our own experience for neutrophil migration in vivo. This supports, that the tissue slice approach maintains near-natural cellular behaviour. As it is currently not foreseeable, how true intravital 2-photon microscopy deeply within the breathing lung can be technically achieved, this new approach opens a promising new avenue for the investigation of lung-associated immune responses.

Moreover, we have identified here a novel molecular mechanism by which A. fumigatus conidia escape neutrophil attacks via NETs. Fungal hydrophobin RodA, which very recently was identified as being important to protect conidia from recognition by the adaptive immune response [28], now also shows its potency in protecting conidia from triggering NET formation. However, the molecular mechanisms how hydrophobin RodA achieves this
Figure 6. NET formation in vivo is dependent on the presence of newly immigrating neutrophils. (A) Mice were intratracheally injected with either swollen *A. fumigatus* conidia or PBS. 7 h after infection, the number of neutrophils in the bronchoalveolar lavage of these mice was measured by FACS. (B) Lys-EGFP mice were treated with the neutrophil depleting anti Gr-1 antibody RB6-8C5 24 h before infection with *A. fumigatus*. A lung slice of such a mouse analysed 10 h after infection shows almost no green cells (arrowhead in the magnification of the area boxed in green.
shows one of the very rare cells in this slice) and no NET-like structures (note only punctate red staining for DNA of nuclei) in areas of fungal masses (blue). (The area of the red square is magnified on the right) The image is representative for 3 animals that were analysed. (C) Quantification of NET formation in Gr-1- and mock-depleted mice infected with swollen conidia as well as untreated mice infected with resting conidia. Shown is a representative result of 3 independent experiments performed. For each condition 20 fungal clouds >20 μm were scored for the presence of NET structures. The 3 images are reference pictures for the type of structure scored with ×+/++.

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Strains and media

Aspergillus fumigatus wild type strains ATCC 46645 (ATCC), DAL [42] as well as the mutant strains pksP [26], and AroDΔ [30] were employed. The strains were cultivated in RPMI 1640 w/o glutamine (Lonza, Wuppertal, Germany) medium with 5% (v/v) heat inactivated FCS (PAA, Colbe, Germany).

Cultivation conditions

For microscopical analysis by both fluorescence microscopy and scanning electron microscopy (SEM) analysis A. fumigatus was cultivated over night in RPMI with 5% (v/v) heat inactivated FCS at 37°C on cover slips in a wet chamber. For determining colony forming units (CFUs) and the quantification of extracellular DNA, hyphae (16 h), swollen conidia (2 h) and resting conidia were incubated in 96 well plates (Brand) in 100 μl RPMI with 5% (v/v) heat inactivated FCS at 37°C.

Isolation of neutrophils

Human neutrophils were isolated from peripheral blood of healthy donors according to the protocol of Wozniok et al. [43]. After a gradient centrifugation of the blood in “Polymerphrep”Ko (Axis Shield, UK) at 550×g, neutrophils were collected and purified by erythrocyte lysis with ACK buffer. Then, the granulocytes were washed with HBSS buffer and diluted in RPMI media with 5% (v/v) heat inactivated FCS.

Scanning electron microscopy

Starting with 1×10⁶ conidia, A. fumigatus was grown on cover slips in 100 μl RPMI media with 5% (v/v) heat inactivated FCS for 16 h. To generate swollen conidia, resting conidia were preincubated in RPMI media for 2 h before. Resting conidia and swollen conidia were co-incubated with 2×10⁵ neutrophils. The cell culture/conidium mixture was incubated at 37°C. After 180 min co-incubation, a sample was drawn and washed with 0.1 M cacodylate buffer pH 7.2 (Serva, Germany) and then fixed with 2.5% (v/v) glutaraldehyde cacodylate buffer three times for 45 min. The samples were then washed with cacodylate buffer, dehydrated in a graduated ethanol series, critical-point dried (BAL-TEC CPD030, Balzer, Liechtenstein), coated (BAL-TEC SCD 005) and analysed with a Carl Zeiss SMT (Oberkochen, Germany) scanning electron microscope. Due to the fragility of the NET-structures, disturbance of the media in each step were kept to a minimum to preserve the cellular structures.

Immunofluorescence and confocal laser scanning microscopy (CLSM)

100 μl RPMI media with 5% (v/v) heat inactivated FCS on cover slips were inoculated with 1×10⁶ A. fumigatus conidia. Then, 2×10⁵ neutrophils were added and the cover slips were incubated at 37°C. After different time points (0, 60, 120 and 180 min) the media were extracted and 10 μl of a solution containing 1 μg/ml propidium iodide / 100 μg/ml calcofluor white (Sigma, Deisenhofen, Germany) were added to the cover slips and inverted on a microscopic slide. Fluorescence microscopic analysis was performed with an Axiovert 200 M/LSM 5 live confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Fluorescence
signals were detected using a 415–480 nm band pass filter for calcofluor white and a 560–675 nm band pass filter for propidium iodide. Images were obtained using the ZEN 2008 software (Zeiss).

Live imaging of NETosis and NET-destruction

After 3–4 h of pre-incubation in RPMI 1640 (Biochrom, Germany) supplemented with 5% (v/v) FCS at 37°C a total of 1×10⁶ swollen A. fumigatus conidia were stained with calcofluor...
Figure 8. Survival of resting and swollen conidia of A. fumigatus strains after co-incubation with neutrophils. The number of CFU determined for conidia without co-incubation (T0) was set as 100% survival. CFUs of A. fumigatus conidia were determined as described in materials and methods. Survival of swollen (grey bars) and resting conidia (white bars) are depicted. (A) Survival of A. fumigatus strain ATCC46645 during co-incubation with neutrophils over time (T0, T60, T120 and T180). Asterisks indicate significant difference (*p < 0.05 or **p < 0.01) in survival in comparison to the time point T0 for each morphotype. (B) Survival of A. fumigatus strain ATCC46645 after co-incubation with neutrophils and in the presence of DNase I or DPI. Asterisks indicate significant difference (*p < 0.05 or **p < 0.01) in survival in comparison to the time point T180 after NETs against A. fumigatus In Vivo.
neutrophil co-incubation. The survival rate did not increase significantly by the addition of DNase I or the NAD(P)H-oxidase inhibitor DPI. Only the addition of cytochalasin D increased the survival of A. fumigatus conidia. (C) Analysis of the survival of the A. fumigatus strain pk5P and its parental wild type strain ATCC46645 showed no significant differences in killing. Also the deletion mutant ArodA and its parental wild type strain DAL revealed no difference in killing. The addition of the conidial hydrophobin RodA (0.07 µg [w/w]) did not influence the survival of the Aspergillus fumigatus mutant strain ArodA and the wild type strain DAL during co-incubation with neutrophils.

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Figure 9. Determination of the atmospheric molecular oxygen consumption of A. fumigatus hyphae after co-incubation with neutrophils for 3 to 12 h. Hyphae (black bars) were co-incubated with neutrophils for different periods of time. Untreated, hyphae not co-incubated with neutrophils served as controls (stippled bars). The change of the oxygen saturation in the medium (in %) over time (h) was plotted. Asterisks indicate significant difference (*p < 0.05 or **p < 0.01) in the change of oxygen saturation in comparison to the control. In some experiments DNase I or DPI was added to the co-incubation and the control.

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white (Sigma) for 15 min at a final concentration of 50 µg/ml. These conidia were then co-incubated with 2x10^5 freshly isolated human neutrophils in a laboratory-made microscopy chamber containing 200 µl RPMI 1640 supplemented with 5% (v/v) FCS and 10 µl of a 10 µg/ml propidium iodide solution as described before [4]. Fluorescence and cell behaviour were monitored simultaneously at 37°C at two frames per minute using an Olympus BX61 microscope with a 60×UMPLFL W/IR/NA 0.9 lens, together with the cellR software (version 2.1) from Olympus Biosystems (Munich, Germany). For the DNase assay, the co-incubation of neutrophils and Aspergillus was carried out in a 96 well cell culture plate for three hours followed by calcofluor white and propidium iodide staining. After this time the co-incubation was pipetted into a laboratory made microscopy chamber and immediately before start of the time lapse microscopy 10 µl of a DNase I solution ([1 U/µl] Qiagen, Germany) were added to the medium at the border of the chamber.

Kill assay based on colony forming units

The co-incubation of 1x10^6 swollen and resting conidia with 2x10^5 freshly isolated human neutrophils was carried out in 100 µl RPMI in 96 well microtiter plates (Brand, Germany). When indicated, NAD(P)H oxidase inhibitor DPI (16 µM) or DNase I (100 U/ml) were added. For inhibiting phagocytosis the neutrophils were preincubated with 10 µl g/ml cytochalasin D (Sigma Aldrich, Taulkirchen) for 20 min and then added to A. fumigatus conidia. After 180 min, 2x10^5 swollen and resting conidia with 50 U/ml DNase I were added. The co-incubation experiment was started with 2x10^5 fresh isolated, unstimulated neutrophils. After two different time points (from 3 to 12 h) 10 ml ice-cold water and 10 ml PBS were added, mixed for 60 s using a Vortex mixer and centrifuged for 15 min at 4000 rpm at 21°C (Centrifuge 5810R, Eppendorf, Hamburg). The pelleted mycelium was resuspended in 3 ml fresh RPMI with 5% heat inactivated FCS and applied to the sample chamber. Pure RPMI medium was set as 100% oxygen saturation.

Viability assay based on the determination of the oxygen consumption rate

Determination of the respiration rates of A. fumigatus hyphae were routinely performed with an oxygen monitor (YSI 5300, YSI Life Sciences, USA) equipped with polarographic Clark-type electrodes. The depletion of dissolved oxygen in RPMI medium with 5% heat inactivated FCS was measured for 10 minutes at 37°C under continuous stirring.

Samples were prepared as follows: 1x10^7 A. fumigatus conidia were grown for 16 h in 3 ml RPMI with 5% heat inactivated FCS at 37°C and 200 rpm. After centrifugation, the supernatant was discarded and 1 ml fresh RPMI with 5% heat inactivated FCS was added. The co-incubation experiment was started with 2x10^7 fresh isolated, unstimulated neutrophils. After two different time points (from 3 to 12 h) 10 ml ice-cold water and 10 ml PBS were added, mixed for 60 s using a Vortex mixer and centrifuged for 15 min at 4000 rpm at 21°C (Centrifuge 5810R, Eppendorf, Hamburg). The pelleted mycelium was resuspended in 3 ml fresh RPMI with 5% (v/v) heat inactivated FCS and applied to the sample chamber. Pure RPMI medium was set as 100% oxygen saturation.

Quantification of extracellular DNA

The co-incubation experiments of A. fumigatus conidia or mycelium with neutrophils in black 96 well plates for 3 h was carried out as described above. In some experiments, 16 µM DPI, 100 U/ml DNase I, and 0.07 µg purified RodA was added to the
2-Photon microscopy in infected lungs

Swelling leading to the onset of germination in conidia was carried out by a 7 h pre-incubation step in RPMI 1640 (Biochrom) supplemented with 5% FCS (v/v) at 37°C. A total of 5×10⁶ swollen A. fumigatus conidia were stained with calcofluor white (Sigma) for 15 min at a final concentration of 50 µg/ml. For infection these conidia were applied intratracheally into female C57/Bl.6 mice (8–10 weeks old, Harlan, Germany) resuspended in total volume of 100 µl PBS after filtration through a 70 µm cell strainer. 7–10 h later the infected animals were sacrificed by an overdose of isoflurane and the lungs were filled in situ with prewarmed low-melting agarose (2% w/v, Promega, Germany). After solidification for 30 minutes at 4°C the right lung lobe was prepared and cut horizontally along the midline with a vibratome (752M Vibroslice, Campden Instruments, UK). The upper half of the lung was then transferred into a Petri dish filled with PBS heated to 37°C and supplemented with Sytox Orange (Invitrogen, USA) at a final concentration of 5µM. 2-photon microscopy was performed using an upright Axio Eximier stage equipped with a 20×NA1.0 water dipping lens (Zeiss). For imaging, different areas along the dissection were scanned down to 400 µm depth using an illumination wavelength of 800 nm detecting green (530 nm) and red (580 nm) fluorescence, as well as the Second harmonic generation (SHG)-signal and the blue calcofluor fluorescence (at 400–470 nm emission) with the external non-descanned detectors (NDD). SHG detects fibrillar structures such as proteins of the extracellular matrix by their emission of light at half of the wavelength used for illumination. The frame rate for movies was up to 12 frames/minute at a fixed focal depth. See also Video S3 for an explanation of the method. The movie was made based on the 3-D structure of a real mouse lung using the GNU-licensed software Blender (www.blender.org).

Quantification of NET formation in infected lungs

To estimate the importance of neutrophils in an in vivo NET formation animals were treated i.p. with 100 µg anti-Gr-1 antibody (clone RB6-8C5) 24 hours prior to i.t. infection with 10⁶ calcofluor white stained WT conidia. 7 hours later the infected lungs were prepared, stained with Sytox Orange (5µM in PBS) and observed for fungal masses with a diameter ≥20 µm by 2-photon microscopy. These structures were microscopically scored for NET formation in 3 categories: (−) no NETs detectable, (+) single NET fibres attached to the fungal cloud and (++) distinct NETs surrounding the fungal material. 20 fungal clouds were checked for NET appearance per investigated lung.

Statistics

The Student’s t-test was used for significance testing of two groups. For the measurement of NET formation (Figure 7A) we compared the fluorescence values for hyphae of ATCC46645 with hyphae treated with DNase I as well as with DPI. In addition, the values of swollen and resting conidia were tested for significant difference. All significant differences are labeled with an asterisk (*p<0.05; **p<0.01). For the investigation of the strain-dependent difference in NET formation (Figure 7B) resting conidia of the DAL strain were compared with resting conidia after HF treatment. In all killing experiments (Figure 8) a Student’s t-test was applied.

For all in vitro experiments blood samples of four different donors were used: two female and two male donors. For the determination of CFU five technical replicates were applied, for quantification of NET formation eight technical repetitions were used. For the quantification of respiration rates, all experiments were repeated three times.

Supporting Information

Figure S1 NET formation by human neutrophils in vitro. Freshly isolated human neutrophils were co-incubated with conidia of Aspergillus fumigatus, that had before been swollen in RPMI for 150 min. Extracellular DNA was stained with propidium iodide (red), conidia and germ tubes with calcofluor white (blue-pink). Microscopy was carried out for 5 h after onset of co-incubation while single pictures were taken every 30 seconds. Indicated is a scale bar and real time in minutes. Please note the explosive release of a NET (intensive red colour around a central neutrophil) between 189–191 minutes into the experiment.

Video S1 Kinetic of NET formation by human neutrophils in vitro. After 180 minutes of neutrophil-Aspergillus co-incubation a well established NET is visible. At the beginning of the movie (0 min) DNase I was added to the medium. At 7 minutes digestion of the NET started and was finished (no NET structure detectable) by 9.5 minutes into the experiment. Indicated is a scale bar and real time in minutes.

Video S2 Kinetic of NET degradation by DNase I in vitro. After 180 minutes of neutrophil-Aspergillus co-incubation a well established NET is visible. At the beginning of the movie (0 min) DNase I was added to the medium. At 7 minutes digestion of the NET started and was finished (no NET structure detectable) by 9.5 minutes into the experiment. Indicated is a scale bar and real time in minutes.

Video S3 Investigating NET formation in the murine lung. A movie showing the sequence of events leading to the formation of NETs in murine lungs and their analysis by time-lapse 2-photon microscopy in situ.

Video S4 Neutrophils migrate through an Aspergillus-infected lung slice. A Lys-EGFP mouse was infected with swollen A. fumigatus conidia and 7 h later the lung was prepared as described in movie 3. The sequence shows several neutrophils (green) migrating within lung tissue (blue). Red staining is from the DNA-specific dye Sytox Orange showing cell nuclei cut open by the preparation. Please note the active motility of cells within the tissue. Indicated is a scale bar and real time in minutes.

Video S5 Neutrophils phagocytose conidia of Aspergillus fumi- gatus in an infected lung slice. A Lys-EGFP mouse was treated as
described for movie 4. The sequence shows highly active neutrophils (green) migrating through the lung tissue (blue) either carrying engulfed conidia (light blue) or being caught in the act of phagocytosis. Nuclei and NETs in the area are stained red (Sytox Orange). The average velocity of neutrophils in this experiment was 9.8 μm/min and 54% of cells were migrating at any time point (activity). Indicated is a scale bar and real time in minutes.

Found at: doi:10.1371/journal.ppat.1000873.s006 (3.62 MB MOV)

**Video S6** Neutrophils transport hyphae of *Aspergillus fumigatus* within an infected lung slice. A Lys-EGFP mouse was treated as described for movie 4. The sequence shows several neutrophils (green) cooperating in the transport of a hypha (light blue) from the centre part (starting at minute 9 of the movie) to the left, where a larger accumulation of other neutrophils is located. Please also note the general accumulation of large numbers of neutrophils in a swarm-like manner into the entire area, which is a typical feature of these cells in *vivo*. The dark blue colour indicates lung tissue and red indicates DNA (Sytox Orange). Indicated is a scale bar and real time in minutes.

Found at: doi:10.1371/journal.ppat.1000873.s007 (3.35 MB MOV)

**Video S7** Neutrophils cross epithelial borders and migrate on the alveolar surface an infected lung slice. A Lys-EGFP mouse was treated as described for movie 4. The sequence shows several neutrophils (green) migrating within the lung slice. At 12 minutes into the movie a single cell is seen (at 5 o’clock) crossing the epithelial border of an alveolus (blue) and migrating on its surface. Red shows DNA of nuclei (Sytox Orange). Indicated is a scale bar and real time in minutes.

Found at: doi:10.1371/journal.ppat.1000873.s008 (1.41 MB MOV)

**Video S8** Neutrophils internalise conidia in living lung slices. A Lys-EGFP mouse was treated as described for movie 4. The movie shows a single neutrophil (green) with a conidium (light blue) inside of the cell. To demonstrate that the conidium is really inside, the entire cell has been reconstructed from a Z-stack and is rotated.

Found at: doi:10.1371/journal.ppat.1000873.s009 (7.96 MB MOV)

**Video S9** Swollen hyphae germinate in the lung and induce NET formation. A wild type mouse was treated as described for Video S4. The movie shows a series of Z-slices through a large fungal mass with clearly detectable outgrowing hyphae as well as NET-structures at the periphery of the mass and the borders of alveoli. Afterwards, the whole Z-stack is 3-D rendered and rotated to demonstrate the 3-D appearance of the fungal ball relative to the lung structures. Dimensions of the stack are given at the bottom.

Found at: doi:10.1371/journal.ppat.1000873.s010 (4.38 MB MOV)

**Video S10** Neutrophils cooperate to transport hyphal fragments into larger accumulations. Isolated neutrophils were incubated together with hyphae in the presence of the blue DNA dye Hoechst in a laboratory made incubation chamber and analysed by live cell microscopy. The movie shows a number of free neutrophils associate with a small hyphal fragment and transport it to an area, where a larger hypha is already associated with many other neutrophils. Similar phenomena can be observed in acute lung slices. Real time is given at the bottom of the movie.

Found at: doi:10.1371/journal.ppat.1000873.s011 (6.55 MB MOV)

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**Author Contributions**

Conceived and designed the experiments: OK AAB MG. Performed the experiments: SB OK MH VA SN AT. Analyzed the data: SB OK MH JPL AAB MG. Contributed reagents/materials/analysis tools: AJ JPL. Wrote the paper: OK AAB MG.

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