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Toll-like receptors: Recent advances, open questions and implications for aspergillosis control

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Aspergillus fumigatus is a pathogenic mould that can cause severe and life-threatening infections in immunocompromised patients. Apart from novel and improved antifungals, additional strategies are required to protect patients at risk from developing invasive aspergillosis. Given the problems in diagnosis of this disease, important perspectives lie in attempts to elicit and strengthen a protective immunity. The innate immune system is the first line of defence against *A. fumigatus*. Phagocytes engulf and kill inhaled conidia, but also closely communicate with the adaptive immune system. Recognition of invading microbes is mediated by pattern recognition receptors (PRRs), and Toll-like receptors (TLR) 2 and TLR4 have been implicated in the immune response to *A. fumigatus*. The analysis of this process is hampered by the fact that *A. fumigatus* infections are inevitably coupled to germination resulting in the appearance of different fungal morphotypes, like conidia and hyphae. While conflicting data still exist on the relative importance of TLR2 and 4 in recognition of distinct *A. fumigatus* morphotypes, recent evidence suggests that certain TLR agonists can be used to divert the immune response towards an optimal fungicidal activity in the absence of detrimental inflammatory consequences.

Keywords *Aspergillus fumigatus*, toll-like receptors, dectin-1

Introduction

Breathing leads to the uptake of a wide variety of particles from the environment, among them various microorganisms including fungal spores. While most inhaled microorganisms are harmless, others, like *Aspergillus fumigatus* conidia, are potentially dangerous and have to be rapidly recognized and eliminated. It has been estimated that every day humans inhale several hundred *A. fumigatus* conidia that, due to their physical properties, are able to reach the alveoles. The fact that invasive aspergillosis (IA) is restricted to severely immunocompromised patients reflects the efficient surveillance of the lung by the innate immune system. Alveolar macrophages are primarily responsible for the elimination of inhaled conidia, as they are able to track down and kill these spores. Macrophages are additionally able to recruit neutrophils to the site of infection that have been shown to attack and eliminate conidia and hyphae [1,2]. Phagocytes also communicate with the adaptive immune system, thereby determining whether an inflammatory response is triggered or not.

Since inhalation of *A. fumigatus* conidia is a common, daily event, the immune system has to be able to distinguish a normal exposure from a dangerous infection, and a key element in this process appears to be represented by macrophages. The ability of phagocytes to recognize pathogenic microorganisms largely depends on their expression of various PRRs, which recognize conserved pathogen-associated molecular patterns (PAMPs). The TLR family represents an important subset of the PRRs and has been the subject of intense research in recent years [3]. While the list of PAMPs is growing, it became obvious that certain TLRs are able to recognize several, biochemically distinct PAMPs [4].

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Several recent studies provided evidence that TLRs are not the only PRRs engaged in recognition and phagocytosis of *A. fumigatus* and other fungal pathogens. Pentraxin-3 (PTX3) is a soluble PRR that binds to galactomannan on the surface of *A. fumigatus* conidia. PTX3-binding results in an enhanced rate of phagocytosis by alveolar macrophages and PTX3-deficient mice are highly susceptible to *A. fumigatus* infections [5]. The dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) has been shown to mediate binding and internalization of *A. fumigatus* conidia by dendritic cells. DC-SIGN is a type II C-type lectin and conidial binding can be blocked by *A. fumigatus* galactomannan or mannan preparations derived from other fungi [6]. Dectin-1 has been identified as the major receptor for ß1-3 glucan on dendritic cells and macrophages [7] and several studies demonstrated a pivotal role for dectin-1 in the recognition and internalisation of different fungi (for recent review see [8]).

Attempts to pinpoint the relative importance of the various TLRs for recognition of *A. fumigatus* have been undertaken by several groups. *In vitro* experiments using transfected cells and isolated macrophages from TLR-deficient mice provided evidence for an involvement of either TLR2 and/or TLR4. Sometimes controversial results were obtained, due to different experimental set-ups and the types of macrophages used. However, the analysis became even more complicated by the fact that germination of *A. fumigatus* is an integral part of the infectious process. This morphological transformation, leading from inhaled resting conidia to swollen conidia, germlings and finally hyphae, results in exposure of different surface structures, being mainly proteinaceous in resting conidia and rather rich in carbohydrates in all other morphotypes. Moreover, recent findings demonstrated a stage-specific surface exposure of certain cell wall carbohydrates, like ß1-3 glucan [9–12], and production and exposure of additional PAMPs by secretion and fungal lysis provides another level of complexity.

Although the relative importance of distinct PRRs still has to be determined, infection experiments in TLR-deficient mice provided convincing evidence for an involvement of TLR2 and TLR4 in the host response to *A. fumigatus* [13,14]. Recent evidence furthermore suggests that certain TLR ligands are able to divert the immune response, thereby enhancing the fungicidial activity while reducing deleterious inflammatory cytotoxicity. Activation of certain TLRs and modulation of their signalling might therefore provide a possibility to strengthen the immune response in patients at risk to develop systemic *A. fumigatus* infections.

Recognition of fungal pathogens by Toll-like receptors

In humans the TLR family consists of ten functional members [3]. Interaction of TLRs with relevant PAMPs activates a cytosolic signalling cascade leading to the translocation of NF-κB into the nucleus and finally resulting in the production of cytokines and chemokines. Some TLRs seem to be able to recognize several, biochemically distinct PAMPS [4] and the ability of certain TLRs to form functional heterodimers might contribute to this diversity [15,16]. The most promiscuous receptors known so far are TLR2 and TLR4, and both seem to be particularly important for the recognition of PAMPs localized on microbial surfaces. The relevance of TLR2 and TLR4 for the immune response against fungal pathogens has been analysed in some detail for *Candida albicans* and *Cryptococcus neoformans*. Netea and co-workers analysed disseminated candidiasis in a mouse model and found a higher fungal burden in TLR4-deficient mice [17], whereas mice lacking TLR2 turned out to be more resistant [18]. Both kinds of mice showed normal levels of TNFα, a cytokine indicative for a proinflammatory immune response. However, blocking TLR2 in human mononuclear cells led to a significant inhibition of TNFα production [17]. The resistance of *Candida*-infected TLR2-deficient mice was attributed to the reduced production of IL-10 and a reduced survival of regulatory T cells, suggesting a deleterious role for TLR2 signalling in systemic *Candida* infections in mice [18]. However, the importance of TLR2 and TLR4 in the immune response to systemic *Candida* infections is still a matter of discussion. Villamón and co-workers found that TLR2-deficient mice are more susceptible to *Candida* infection [19], while Bellocchio *et al*. reported that TLR2-deficiency has no impact on the survival of mice infected with *Candida albicans* yeasts or hyphae [13]. Very recently, Murciano *et al*. reported that TLR4 is dispensible in a murine model of hematogenously disseminated *Candida* infection [20]. Glucuronoxylomannan, a component of the capsule of *Cryptococcus neoformans*, induces signalling through TLR4 and TLR2 [21], but inhibits TNFα-release from human monocytes [22]. In a mouse model of *Cryptococcus* infection, lack of TLR2 and 4 had only a minor impact, whereas a major role in the immune response to *Cryptococcus* was attributed to MyD88 [23].

Relevance of different morphotypes during *A. fumigatus* infections

During *A. fumigatus* infection germination of inhaled conidia is an integral part of the infectious process and consequently the immune system has to recognize and defeat different *A. fumigatus* morphotypes (Fig. 1). Apart from the obvious morphological transformation from small spores to elongated hyphae, there are more, sometimes subtle changes in the surface structure which may nevertheless have severe consequences for the immunological recognition. Therefore a detailed analysis first requires a precise definition of those *A. fumigatus* morphotypes that have to be individually analysed. No standardized protocols exist so far for the generation and isolation of the different *A. fumigatus* morphotypes, and therefore this is no trivial task. In the following, we propose definitions for four *A. fumigatus* morphotypes that are important during infection: resting conidia, swollen conidia, germlings and hyphae.

(1) *Resting conidia* have a diameter of about 2–3 μm and are covered by a characteristic hydrophobic sheath mainly consisting of proteins like hydrophobin A [24]. This surface layer seems to be important for long term survival of conidia under variable environmental conditions. Hence, intact resting conidia usually represent the starting point of infection.
(2) *The environment* in the lung supports conidial activation resulting in the osmotic swelling of the spore. *Swollen conidia* still have a round appearance, but an enlarged diameter of approx. 5 μm, and therefore they can easily be distinguished from resting conidia by conventional microscopy. The spherical growth also results in the fragmentation and disintegration of the hydrophobic surface layer, thereby exposing the underlying fungal cell wall, which mainly consists of carbohydrates [25].
(3) *The hall* mark of the germination process is the polar outgrowth of the germ-tube. Electron microscopic studies suggest that certain surface molecules and structures are only found on the surface of the first 10–20 μm of the germ-tube, but not on hyphae [26]. This morphotype, referred to as germling, differs from hyphae also by the fact that it can still be internalized by phagocytosis. As a pragmatic definition germlings can be considered as swollen conidia with an attached germ-tube of maximal 30 μm.
(4) *Hyphae* are elongated and often branched filaments. In order to properly analyse their role during infection, it is important to ensure that preparations of this morphotype are devoid of conidia and conidiophores.

An early step during germination is the loss of the characteristic echinulate morphology of resting spores resulting in small and smooth conidia [26]. These spores have a phenotype similar to that described for a hydrophobin A and a *pksP* mutant [24,27]. Hydrophobin A is the major constituent of the hydrophobic surface layer, while PksP is an essential enzyme for synthesis of dihydroxynaphthalene (DHN)-like melanin. Conidia of both mutants have a smooth surface and deletion of the *pksP* gene additionally results in a characteristic white colour [27]. Recent data suggest that small conidia with a smooth surface that appear early in the germination process, and interestingly also

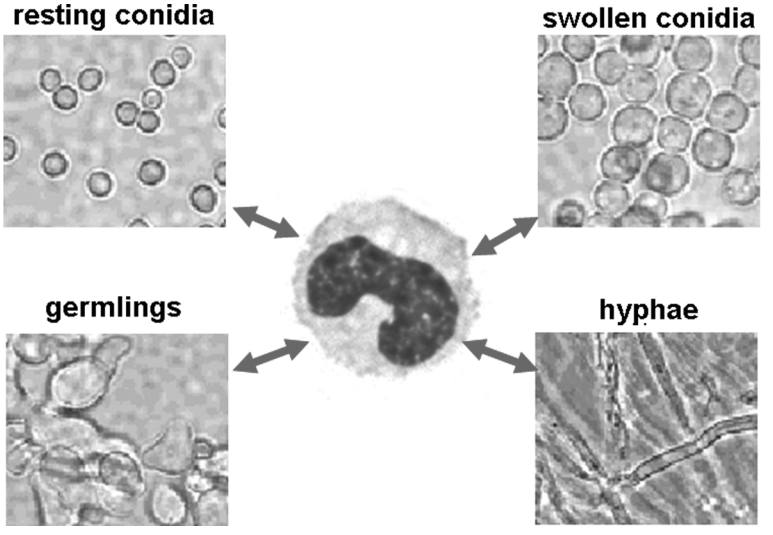

Fig. 1 Macrophages must be able to recognize different *Aspergillus fumigatus* morphotypes.

resting *pksP* mutant conidia display large amounts of ß1-3 glucan on their surface [43]. This combination of a small size and a high amount of ß1-3 glucan on their surface clearly distinguishes these spores from resting and swollen wild-type conidia and suggests that they may represent a fifth relevant *A. fumigatus* morphotype.

TLR-mediated recognition of *A. fumigatus*

In recent years recognition of *A. fumigatus* by TLRs has been analysed by several groups. To compare and discuss the available data, we decided to focus on the production of TNFα, a parameter analysed in all studies as a major read-out for an inflammatory response. *A. fumigatus* induced TNFα production by murine macrophages of peritoneal and alveolar origin was first reported by Taramelli *et al*. [28]. Conidia and hyphae, whether killed or alive, were found to stimulate both kinds of macrophages equally well, although differences in the kinetics of the response were noticed. In line with these data, *A. fumigatus* conidia were independently found to trigger a Th1-type cytokine response in human blood mononuclear cells [29].

In the following years the ability of different *A. fumigatus* morphotypes to activate isolated macrophages has been the subject of several reports (Table 1). The finding of a MyD88-independent TNFα response to hyphae initially argued against a relevance of TLRs in the immune response to *A. fumigatus* [30], since this adaptor protein is an essential requirement for signalling of all TLRs, except TLR3. This finding, however, was challenged by two reports using MyD88-deficient macrophages [9,31] and by several other studies using TLR-deficient macrophages [11,31–34] or a mouse model of infection [13,14]. All these studies implicated either TLR2 and/or TLR4 in the immune response to conidia and/or hyphae and TLR2 and 4 were also shown to recognize PAMPs that are released by *A. fumigatus* during growth under *in vitro* conditions [35]. However, despite the amount of available data, the relative importance of TLR2 and TLR4 for the interaction of *A. fumigatus* with macrophages remains controversial.

Using human adherent monocytes and blocking antibodies Wang *et al*. [32] found that hyphae trigger an inflammatory response through TLR4, but not TLR2. We reported that conidia and hyphae activate murine macrophages through TLR4, and to a lesser extent TLR2 [33], while others found a requirement for TLR2, but not for TLR4 [31] or a mainly TLR4-dependent response to conidia, but not to hyphae [34]. The latter result led to the interesting hypothesis that lack of TLR4-ligands on hyphae might reflect an immune evasion strategy [34]. Such an inability to recognize and combat the most dangerous *A. fumigatus* morphotype would constitute a serious and surprising gap in the immune surveillance of this common microbial challenge. *A. fumigatus* has only recently become a serious pathogen and this happened merely as a consequence of modern medicine, therefore it seems unlikely that this fungus had been able to evolve sophisticated mechanisms to interact with the human immune system. An immune evasion as described by Netea and co-workers could nevertheless take place if the hyphal surface of *A. fumigatus* was by coincidence devoid of relevant PAMPs. This, however, appears unlikely in light of the recent identification of large amounts of the potent PAMP ß1-3 glucan on the hyphal surface [10,12]. Alternatively, *A. fumigatus* hyphae might actively suppress an immune response and in fact, secretion of certain toxins, like the

Table 1 Recognition of *Aspergillus fumigatus* morphotypes through TLR2 and TLR4 as published recently. HMDMΦ: human monocyte-derived macrophages; pMΦ: murine peritoneal macrophages; PMN: human polymorphonuclear neutrophils; BMDMΦ: murine bone marrow-derived macrophages; AMΦ: murine alveolar macrophages; Hy: hyphae; RC: resting conidia; SC: swollen conidia; n.d.: not determined

Reference	Macrophage	*A. fumigatus* morphotype	TLR2	TLR4
Wang *et al*., 2001 [32]	HMDMΦ	Hy	−	++
Mambula *et al*., 2002 [31]	pMΦ	RC	++	−
	pMΦ	SC	−	++
	pMΦ	Hy	++	−
Netea *et al*., 2003 [34]	pMΦ	RC	+	++
	pMΦ	Hy	+	−
Meier *et al*., 2003 [33]	pMΦ	RC	+	++
	pMΦ	Hy	+	++
Bellocchio *et al*., 2004 [13]	PMN	RC	+	++
	PMN	Hy	+	++
Hohl *et al*., 2005 [9]	BMDMΦ	RC	+	n.d.
Steele *et al*., 2005 [11]	AMΦ	Hy	++	n.d.

ribotoxin mitogillin, was shown to be restricted to growing hyphae [36].

Aspergillus fumigatus is responsible for the vast majority of cases of invasive aspergillosis, although conidia of other non-pathogenic *Aspergillus* species usually outnumber those of *A. fumigatus* in the air. Since many *Aspergillus* spores have a size comparable to that of *A. fumigatus* [37] they should in principle be able to reach the lung. Therefore it is likely that alveolar macrophages permanently encounter conidia of non-pathogenic Aspergilli without triggering a significant immune response. In a first report, the pathogenic species *A. fumigatus* and *A. flavus* were found to induce TNFα production, whereas *A. terreus* and *A. niger* were less efficient in this respect [28]. While this suggested a differentiated immune response to Aspergilli depending on their pathogenic potential, no significant difference was observed in a more recent study between a pathogenic *A. fumigatus* and a non- or only weakly pathogenic *A. niger* strain [33]. The ability to initiate an infectious process is likely to be the crucial parameter determining whether an immune response is mounted or not.

The relevance of TLR-mediated signals for the outcome of *A. fumigatus* infections has so far been analysed by two studies in a murine model of systemic aspergillosis. Without immunosuppression both, TLR4- and TLR2-deficient mice turned out to be resistant to *A. fumigatus* infections [13,14]. In one report, immunosuppression rendered TLR4- and MyD88-deficient mice susceptible to *A. fumigatus* infections, while TLR2-deficient mice survived like the wild type [13]. The latter mice, however, had a higher fungal burden in the lung demonstrating an impact of TLR2, which was subsequently confirmed in the second study demonstrating a higher susceptibility of TLR2-deficient mice [14].

TLRs do not only recognize PAMPs present on microbial surfaces, but additionally detect intracellular PAMPs, like DNA motifs or RNA species not found in human cells. TLR9 is known to be the receptor for unmethylated CpG DNA motifs [38] that are commonly thought to be restricted to bacteria and DNA viruses. Surprisingly, *A. nidulans* was also shown to be virtually devoid of genomic cytosine methylation [39], and it is probable that the same is true for *A. fumigatus*. With this in mind, it is likely that during infection activation by unmethylated CpG motifs occurs after lysis of *A. fumigatus* cells. Consistently, *A. fumigatus* conidia and hyphae have been shown to signal through TLR9 on murine polymorphonuclear neutrophils [13], but the same authors found TLR9-deficient mice to be resistant to *A. fumigatus* infections even after immunosuppression [13].

Recognition of *A. fumigatus* by dectin-1

The implication of both TLR2 and TLR4 in the control of *A. fumigatus* infections is in line with other evidence suggesting an intimate relationship between these two TLRs in response to several PAMPs [4]. In other fungi, different forms of mannan have been identified as ligands for TLR2 and/or TLR4, e.g., phospholipomannan for TLR2 and TLR4 [40], mannan for TLR4 [41] and glucuronoxylomannan for TLR4 [21]. While the relevant *A. fumigatus* TLR ligands are still unknown, ß1-3 glucan recently turned out to be an important fungal PAMP that is recognized by the C type-lectin-like receptor dectin-1 [8]. Signalling via dectin-1 has recently been shown to contribute substantially to the inflammatory response to *A. fumigatus* [9,11,12]. Interestingly, ß1-3 glucan, as the relevant PAMP, has been reported to be absent or hardly detectable on resting spores [9,11,12], whereas it was found in large quantities on the surface of swollen conidia. In two reports ß1-3 glucan was additionally found on the surface of hyphae [10,12], whereas one group reported that soluble dectin-1 does not bind to the hyphal surface [11]. The stage specific display of ß1-3 glucan confines the role of dectin-1 to the defence of certain *A. fumigatus* morphotypes. As outlined above, the immune response to activated *A. fumigatus* cells is strong, because their presence signals a beginning or ongoing infectious process. The high amount of surface accessible ß1-3 glucan found on the corresponding *A. fumigatus* morphotypes could provide a first molecular explanation for this phenomenon.

Interestingly, it has been observed that dectin-1 and TLR2 collaboratively induce a proinflammatory response to ß1-3 glucan rich fungal particles [42] and dectin-1 was additionally been shown to trigger the production of reactive oxygen species, a response primed by TLR activation [42]. Evidence for a co-operation between dectin-1 and TLR2 in the proinflammatory response to *A. fumigatus* has recently been reported [9,12] and our data suggest that both receptors are also engaged in the phagocytosis of *A. fumigatus* conidia [43]. Although several lines of evidence suggest a cross-talk between dectin-1 and TLR2 in the response to *A. fumigatus*, it is still unclear where and how these receptors interact.

TLR-mediated recognition of different *A. fumigatus* morphotypes: obstacles and pitfalls

For many reasons it appears mandatory to reinvestigate and verify the relative importance of TLR2 and TLR4 for the recognition of certain *A. fumigatus* morphotypes. Two reasons have been suggested to explain the conflicting data obtained in several studies: the different *A. fumigatus* strains used and the different sources of macrophages (e.g., peritoneal versus alveolar, human versus murine). Macrophages from different hosts or different anatomical sites may express different levels of important PRRs, like TLRs or dectin-1, and these expression levels may additionally be influenced by the culture conditions used. The mouse strain used to isolate TLR4-deficient macrophages represents another subtle, but potentially significant aspect, which has not been analysed in detail yet. Some studies were performed using mice with a natural point mutation in the *tlr*4 gene, while others used mice with a genetically engineered *tlr*4 deletion. Although this may provide an explanation for certain discrepancies, another major problem that is often overlooked is the preparation of the fungal material used for stimulation. If live fungal cells are used in such experiments any change in morphology occurring within the time frame of the experiment has to be taken into account. Separate analysis of different *A. fumigatus* morphotypes requires availability of homogenous preparations that have to retain their characteristic features throughout the experiment. Isolation of resting conidia and hyphae is a minor problem, whereas isolation of transient morphotypes, like swollen conidia and germlings, can be a serious experimental challenge. Since we do not have procedures to isolate pure subpopulations out of a heterogenous culture, we completely rely on the use of homogenous *A. fumigatus* cultures. A synchronous germination process is therefore a prerequisite for the isolation of distinct morphotypes. In our hands, *Aspergillus* minimal medium or the commonly used serum-free tissue culture medium result in heterogeneous cultures comprising all different morphotypes, whereas yeast-glucose medium, filtered with a cut-off of 8kDa, works well for this purpose. An elegant mean to obtain pure populations of swollen conidia has recently been introduced by Hohl and co-workers. These authors demonstrated that the antifungal Voriconazol can be used to arrest germination at a stage of swollen conidia [9].

Another simple but important obstacle in the attempts to compare the immune response to different fungal preparations is the quantification of the applied material. In some reports the number of fungal elements was used as a parameter. Then, however, it has to be taken into account that small resting conidia are compared with relatively large hyphae or hyphal fragments. Normalization by fungal surface would be optimal, but is difficult to achieve. An alternative and pragmatic compromise is the standardisation by fungal dry mass. If sufficient material is available, this parameter can easily be determined and correlates directly with the fungal surface.

Due to their length, hyphae are difficult to handle and, for practical reasons, several groups used hyphal fragments instead, which are usually generated by shearing [32–34]). In light of recent results, this could be problematic, since molecules at the ends of the fragments, which are normally buried within the cell wall are suddenly exposed. If viable hyphae are analysed they have to be washed before use to remove secreted *A. fumigatus* molecules, which may have a serious impact on the response of macrophages [44].

The production of cytokines like TNFα is a common read-out to analyse the immune response of macrophages. Usually this requires an incubation time of 12–20 h. However, since serum-containing tissue culture medium strongly supports germination, fungal elements have to be killed in such experimental set-ups to preserve their morphological characteristics. Different means have been used to kill *A. fumigatus* cells, e.g., antifungals (e.g., amphotericin B), formaldehyde, ethanol, thimerosal and heat, but little is known about the consequences of these treatments with respect to the availability of surface exposed PAMPs.

Toll-like receptors and the control of *A. fumigatus* infections

In the immunocompetent host inhaled conidia are permanently killed by alveolar macrophages, a process which requires reactive oxygen species [45] and lysosomal acidification [46]. Degradation is therefore the usual fate of inhaled conidia and exposure to their antigens is a common event. Mechanisms must therefore exist to distinguish such a daily exposure from a potentially dangerous infectious process, which requires recruitment of neutrophils and an inflammatory response. This may be achieved by recognition of certain PAMPs that are exclusively exposed on the surface of activated fungal cells, like swollen conidia, germlings or hyphae and may therefore serve as markers for a beginning infection, and recent evidence suggest that ß1-3 glucan is a first example of such a PAMP [9–11].

To combat activated fungal cells that could cause a systemic infection, macrophages and tissue cells have to recruit and activate effector cells, like neutrophils and

NK cells [47]. In the murine system recruitment of neutrophils by *A. fumigatus* has been shown to depend on TLR2 and TLR4 [33] and the ability of murine neutrophils to kill conidia and produce reactive oxygen species can be modulated by TLR ligands, like LPS, lipoteichoic acid and zymosan [13]. Several lines of evidence indicate that a Th1-like immune response is required to clear infections by *A. fumigatus* and other fungi. Consistently, antigen-specific proliferation of IFNγ-producing T cells has been observed in patients surviving an invasive aspergillosis [48] and protection of naive mice has been obtained by adoptive transfer of Th1 cells [49].

A host may respond to an *A. fumigatus* infection by either developing a Th1-like or Th2-like response. A Th1 response results in the production of proinflammatory cytokines and the recruitment and activation of neutrophils at the site of infection. The resulting massive attack of the immune system may eliminate the infectious microorganisms, but may additionally cause substantial collateral damage. In contrast, production of Th2-like cytokines supports a humoral immune response, but Th2 cells are additionally required to dampen a proinflammatory response after resolution of infection and to return to homeostasis.

A Th1-like response usually leads to clearance of *A. fumigatus* infection, while hosts exhibiting a dominant Th2-like response are more likely to develop a progressive IA [50]. Dendritic cells have been identified as key players able to direct an immune response in both directions [51]. An inflammatory response can eliminate invading fungi, but can additionally result in tissue destruction and inflammatory lung pathology. Any attempt to strengthen an inflammatory immune response must therefore try to avoid its severe and unintended side effects. Regulatory T cells have been shown to control effector cells and to limit deleterious effects to the host [52]. TLRs, which are present on these T cells and on many other immune effector cells, are attractive targets to trigger and modulate a tailor-made immune response. Ligation of TLRs usually leads to the production of pro-inflammatory cytokines, but in response to certain PAMPs and under distinct conditions TLRs may also trigger an anti-inflammatory response. The type of TLR stimulation during the initial phase of an immune response seems to determine the polarization of the adaptive immune response [50]. The ability to orchestrate a fine-tuned participation of different TLRs and PAMPs might therefore provide a way to generate a protective Th1-like response without causing deleterious effects to host tissues.

Recognition through TLR2 unambiguously leads to the production of TNFα and in principle TLR2 can induce a Th1-like response [53]. TLR2 signalling might also temporarily favour an inflammatory response, since TLR2 ligands can induce proliferation of regulatory T cells that, in turn, loose their ability to control effector cells during proliferation [54]. However, the synthetic TLR2 ligand Pam_3Cys has been shown to induce a Th2-biased immune response [55] and TLR2-mediated activation of regulatory T cells and production of the anti-inflammatory cytokine IL-10 has been observed during *Candida albicans* infection [18]. These and other data suggest that under certain circumstances TLR2-signalling can inhibit a protective inflammatory response.

TLR4 is not required for clearance of *A. fumigatus* infections in immunocompetent mice, while it is crucial for survival after immunosuppression [13]. *In vitro* data suggest that TLR4 explicitly triggers a protective Th1-like response in *A. fumigatus* infections, which is in line with the finding that stimulation of TLR4 has a strong Th1-like polarization capacity [56]. The presence of TLR7, TLR8 and TLR9 ligands strongly enhances this effect, suggesting that diverse TLR ligands have the capacity to favour a protective Th1 response to *A. fumigatus*. Interestingly, thymosin α1 a naturally occurring thymic peptide was also shown to promote a protective Th1-like response to *A. fumigatus* by signalling through TLR9 [57].

A novel perspective for modulating the immune response to *A. fumigatus* came from a recent study on the impact of amphotericin B on TLR signalling during *A. fumigatus* infections [2]. For many years deoxycholate amphotericin B had been the first antifungal of choice for treatment of *Aspergillus* infections despite its toxicity. Lipid-based formulations of amphotericin B have later on been developed to reduce this toxicity. Deoxycholate amphotericin B was shown to be a ligand for TLR2 and TLR1 causing production of TNFγ and other inflammatory cytokines [58,59]. In bone marrow-transplanted mice treatment with liposomal amphotericin B, but not with deoxycholate amphotericin B, diverted signalling from TLR2 to TLR4, thereby inducing a protective Th1-like response [2]. Liposomal amphotericin B stimulated the fungicidal activity of neutrophils in a TLR4-dependent manner, while attenuating the inflammatory cytotoxicity of deoxycholate amphotericin B. The anti-inflammatory and TLR4-dependent effect was also observed in the presence of amphotericin-free liposomes. Taken together, these data provide a first model for a successful manipulation of the TLR response to *A. fumigatus* towards an optimized fungicidal effect in the absence of a deleterious inflammatory cytotoxicity.

Outlook

Although many interesting data on different aspects of the immune response to *A. fumigatus* have been published in recent years, we still have to go a long way to understand the relevant processes on the molecular level. The identification of the *A. fumigatus* TLR2 and TLR4 ligands is an important issue that may help to understand and solve the discrepancies we face today. The ability of various TLRs to recognize biochemically diverse ligands is a fascinating but also disturbing phenomenon. Whether this is due to impurities in the currently used ligand preparations or indeed reflects an amazing functional flexibility of these receptors remains to be clarified. The interference of signalling processes triggered, e.g., through dectin-1 and TLR2 is a fascinating new aspect that deserves additional efforts to achieve an understanding of the underlying molecular cross-talk. Finally, we need to understand the consequences of the complex signalling events that determine the polarity of the resulting immune response and we have to know how to interfere with these processes in order to improve the perspective of patients suffering from invasive aspergillosis.

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