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Michel Chignard, Viviane Balloy, Jean-Michel Sallenave, Mustapha Si-Tahar. Role of Toll-like receptors in lung innate defense against invasive aspergillosis. Distinct impact in immunocompetent and immunocompromized hosts.. *Clinical Immunology*, 2007, 124 (3), pp.238-43. 10.1016/j.clim.2007.05.004 . pasteur-00193763

**HAL Id: pasteur-00193763**

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Submitted on 6 Dec 2007

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**Role of Toll-like receptors in lung innate defense against invasive aspergillosis. Distinct impact in immunocompetent and immunocompromized hosts.**

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Running title: Immunological status and Toll-like receptors

## Abstract

Toll-like receptors are key to pathogen recognition by a host and to the subsequent triggering of an innate immune response. Experimental and clinical evidence show that defects in Toll-like receptors or in signaling pathways downstream from these receptors renders hosts susceptible to various types of infection, including aspergillosis. Patients receiving an immunosuppressive regimen, including corticosteroid therapy or cytotoxic chemotherapy, are also susceptible to infections. *Aspergillus fumigatus* is an opportunistic pathogen that infects the lungs of immunosuppressed hosts. Here, we review the evidence that experimental inactivation of various Toll-like receptors and of their signaling pathways may worsen cases of invasive pulmonary aspergillosis. Moreover, the literature clearly indicates that the type of immunosuppression is very important, as it influences whether or not Toll-like receptors contribute to infection. The involvement of Toll-like receptors, based on the immunological status of the patient, should be considered if an immunosuppressive treatment must be administered.

Key words:

Infection, immunosuppression, TLR, lung, *Aspergillus*.

## I - Contribution of Toll-like receptors (TLRs) to the innate immune response of the host

In recent years, the TLR family has emerged as a major group of pathogen-recognition receptors that play a central role in the induction of immune responses. Humans express ten TLRs involved in the recognition of various pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), a major component of Gram-negative bacteria and detected by TLR4, as well as lipopeptides produced mainly by Gram-positive bacteria and detected by TLR2. Other interactions include those between dsRNA and TLR3, flagellin and TLR5, ssRNA and TLRs7 and 8, and CpG DNA and TLR9 (1-3). Ligand-TLR interactions, except those involving TLR3, trigger the binding of at least one adaptor molecule, MyD88, to the intracellular domain of the TLR. This is the first step of a signaling cascade that leads to the activation of various nuclear factors and, in particular, of NF- $\kappa$ B which induces the production of a large array of inflammatory molecules such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , IL-6, and IL-12. The signaling pathway is analogous to the one triggered by IL-1 $\beta$ . Upon stimulation, MyD88 recruits IRAK-4 to TLRs through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6 which indirectly enhances activity of the I $\kappa$ B kinase (IKK) complex. Once activated, the IKK complex induces phosphorylation and subsequent degradation of I $\kappa$ B, which leads to nuclear translocation of transcription factor NF- $\kappa$ B (1). Engagement of various TLRs or interactions between multiple TLRs may result in different cellular responses, allowing a certain degree of specificity for host defense against various pathogens. Interestingly enough in a pioneering work, Lemaitre *et al.* (4) reported that the Toll protein (after which the name TLR was coined) in *Drosophila* is implicated in the defense against the fungus *Aspergillus fumigatus*. This review details the interactions between members of the mammalian TLR family and *A. fumigatus*, in distinct immune contexts.

## II - The innate immune response to *A. fumigatus*

*A. fumigatus* is an airborne fungus. Its conidia are small enough to reach the alveoli easily during breathing. In immunocompromised patients, conidia germinate, and hyphae develop and invade the parenchyma. This leads to tissue destruction and respiratory failure characteristic of invasive pulmonary aspergillosis (IPA), a life-threatening disease with a death rate higher than 60% (5). Mortality from IPA has increased several-fold during the last 20 year period, a finding that is explained by the use of aggressive and intensive chemotherapy, the use of immunosuppressive drugs, and HIV/AIDS pandemic (5). Neutropenia is the most important risk factor, and transplantation, especially lung and bone marrow transplantation, is another increasingly significant risk factor (6). The increased risk of IPA in patients undergoing transplantation is related to different immune defects including neutropenia in the pre-engraftment phase and the use of antirejection therapy, including corticosteroids (6).

A plethora of molecules, particularly cytokines and chemokines produced either by monocytes/macrophages or by epithelial cells, are involved in defense against *A. fumigatus*. Many recent reviews describe these mediators and the respective role of these cells in host defense against this pathogen (7-9). Thus, one of the most important roles is probably played by TNF- $\alpha$  that stimulates the antifungal effector function of neutrophils and macrophages and induces the expression of a number of other protective cytokines including IFN- $\gamma$ , IL-1, IL-6 and IL-12 (10, 11). Nonetheless, rather than extensively covering this aspect, this review concentrates on the ‘cellular sensing’ process used by TLRs to recognize *A. fumigatus*.

## II-1 TLRs and the *in vitro* response to *A. fumigatus*

Interactions between TLRs and *A. fumigatus* were discovered a few years ago, and are far from being completely understood. The findings from various studies are summarized in Table 1. The first report on this subject was published in 2001. This report ignited a controversy about whether TLR2 and/or TLR4 was involved in the interaction. Wang *et al.* (12) challenged human monocytes *in vitro* with hyphal fragments, and demonstrated the involvement of TLR4, but not of TLR2. By contrast, the following year, a well-conducted study using peritoneal macrophages from TLR2-/- mice and transfected human embryonic kidney (HEK) cells expressing either human TLR2 or TLR4 indicated that optimal signaling responses to *A. fumigatus* required TLR2, and that TLR4 was not essential in both mouse and human cells (13). Reconciling these opposing findings, Meier *et al.* (14) used transfected HEK cells expressing TLRs1 to 10 to show that TLR2 and TLR4, and not other TLRs,

recognized *A. fumigatus*. In agreement with the latter, Netea *et al.* (15) indicated that conidia and hyphae activated murine peritoneal macrophages through TLR2 and TLR4. Furthermore, Netea *et al.* (15) demonstrated that macrophages responded differently to conidia and hyphae. It appears that whereas TLR2 recognizes both conidia and hyphae, TLR4 only detects conidia. Moreover, TLR2 activation by hyphae induces the production of IL-10. Thus, the authors speculated that the phenotypic switching during germination is an important escape mechanism for *A. fumigatus*, enabling it to evade host defense (15, 16). This is the consequence of the expression by conidia and by hyphae of different cell wall components (17) constituting PAMPs that are most probably differentially recognized by TLR2 and 4. These findings also suggest that the host recognizes at least two TLR ligands expressed by *A. fumigatus*. However, the PAMPs produced by this fungus and sensed by TLRs have not been described. This underlines the importance of this specific area where more research is needed. In summary, *in vitro* studies suggest that TLR2 is involved in the recognition of *A. fumigatus*. The contribution of TLR4 remains to be more firmly established.

## II-2 Which cell type participates in the innate immune response to *A. fumigatus*?

Polymorphonuclear neutrophils (PMN) are typical cells of the innate immune system. Their importance in host defense against IPA has been demonstrated experimentally. Mice rendered neutropenic by chemotherapy and inoculated with conidia of *A. fumigatus* die within three days, whereas all nontreated mice receiving the same inoculum survive (Fig. 1A). Alveolar macrophages are also key cells of the antifungal innate immune system. This is demonstrated *in vivo* by specific, chemically-induced depletion of these cells (Fig. 1B). TLRs contribute to macrophage recognition of *A. fumigatus*, as described above, but TLRs are also important pattern-recognition receptors for PMN (18). PMN express all the known TLRs, except TLR3. Specific TLR ligands trigger or prime cytokine release and superoxide generation in PMN, particularly important for the fungicidal activity of these cells (9). Findings from *in vitro* experiments suggest that TLRs control PMN activity against *A. fumigatus* (19). More precisely, signaling through TLR2 promotes fungicidal activity and release of proinflammatory cytokines. TLR4-dependent signaling also favors fungicidal activity, but triggers production of the anti-inflammatory cytokine IL-10. In addition to macrophages and PMN, dendritic cells recognize *A. fumigatus* (20, 21). Similar to macrophages and PMN, *A. fumigatus* recognition by dendritic cells, whether of mouse (21) or human (22) origin, depends on the presence of TLR2 and TLR4.

The respiratory epithelial cell, a nonprofessional immune cell, is believed to actively participate in innate defense against pathogens (23). These epithelial cells express all known TLRs (24-27) and produce various cytokines and chemokines needed for recruitment of cells of the innate defense, including PMN (28). However, the epithelial cell response seems to be less intense than that of leukocytes: the interaction between *A. fumigatus* and epithelial cells leads to the production of comparatively modest levels of IL-6 and IL-8 (29). This may be explained, in part, by different compartmentalization of the TLRs in epithelial cells than in leukocytes. Unlike leukocytes, respiratory epithelial cells do not express various pattern-recognition receptors involved in *A. fumigatus* sensing on their cell surfaces, including TLR4 (27).

### II-3 TLRs and the *in vivo* response to *A. fumigatus*

In addition to existing contradictions in the literature regarding *in vitro* findings, there is a current debate about the involvement of TLRs in the innate defense against *A. fumigatus*, *in vivo*. Whereas fungus burden and survival read-outs have shown that TLR4 and MyD88 are protective *in vivo*, in specific gene-deficient mice, TLR2 was shown to have more subtle effects (30). TLR2<sup>-/-</sup> mice immunosuppressed by cyclophosphamide treatment (a chemotherapeutic drug that lowers white blood cell numbers) exhibited a higher fungus burden in the lungs than did wild-type mice. However, their survival rates were similar to those of wild-type mice. Thus, unlike the findings of *in vitro* studies, Bellocchio *et al.* (30) showed that *in vivo* TLR4, but not TLR2, participated in host defense against *A. fumigatus*. By contrast, Balloy *et al.* showed that TLR2 is important *in vivo*: TLR2<sup>-/-</sup> mice rendered neutropenic by treatment with vinblastine (an anticancer drug) had a higher fungal burden, but a lower survival rate than wild-type mice following intratracheal administration of *A. fumigatus* (31). There are no obvious explanations for the discrepancy between the studies of Bellocchio *et al.* (30) and Balloy *et al.* (31). Nonetheless, it is known that host immune responses can be influenced by the immunosuppression regimen, the background genotype of mice, and the inoculum delivery (32). Interestingly enough, two differences between the foregoing studies are notable, namely the chemical agent used to induce neutropenia and the administration of the inoculum. Bellocchio *et al.* (30) used cyclophosphamide to induce neutropenia and administered  $3 \times 10^7$  conidia intranasally on each of three consecutive days.

Balloy *et al.* (31) induced neutropenia with vinblastine and challenged mice with a single intratracheal inoculum of  $3 \times 10^6$  conidia. It is of note that another investigation reported the putative involvement of TLR2 in a chronic fungal asthma murine model infected with *A. fumigatus* (33), supporting Balloy *et al.*'s findings.

### III. Immunosuppression and TLRs

Whereas MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup>, and/or TLR4<sup>-/-</sup> neutropenic mice do not survive *A. fumigatus* pulmonary infection, immunocompetent mice do (30, 31, 34). Therefore, TLR2 and/or TLR4 are essential in the absence of PMN, but not in the healthy host. This suggests that TLRs expressed by PMN are not as important *in vivo* as other pathogen-recognition receptors expressed by PMN. One such critical pathogen-recognition receptor may be Dectin-1, a  $\beta$ -glucan receptor. It is expressed by PMN (35) and is involved in the sensing of *A. fumigatus* (36-38). Accordingly, when PMN are absent or their number are very low such as during chemotherapy-induced neutropenia, TLR2 and/or TLR4 expressed by resident lung cells may be the most effective sensing receptors.

Whereas several immunosuppressive drugs may result in similar host susceptibility to infection, this may occur through different mechanisms. Experimentally, animals that were immunosuppressed by cytotoxic chemotherapy or corticosteroid therapy died through different mechanisms (39). In animals immunosuppressed by chemotherapy, death was a consequence of infection and increased pathogen burden in peripheral organs. In other animals immunosuppressed by corticosteroid therapy, there was an unexpected, excessive influx of PMN into the lungs and restricted fungus development (39). Though TLR2 protects against IPA in mice administered chemotherapy (31), it does not seem to contribute to the recognition of *A. fumigatus* in mice given corticosteroid therapy. The survival of these corticotherapy-treated mice was independent of TLR2 expression (Fig. 2). This may be explained, in part, by *in vitro* studies showing that conidia are recognized by both TLR2 and TLR4, whereas hyphae are only recognized by TLR2 (15). Hyphae are clearly present during neutropenia (39); therefore, TLR2 is involved. During corticosteroid therapy, the development of germ tubes by germinating conidia is limited (39). Presumably, the consequence is that TLR4 is the major actor in this specific context, with TLR2 having little or no effect. The



effect of corticosteroid therapy in TLR4<sup>-/-</sup> and MyD88<sup>-/-</sup> mice infected with *A. fumigatus* has not been studied, but it would be interesting to know the consequences for experimental IPA.

#### IV. Clinical relevance and conclusion

Our understanding of how the immune system recognizes pathogens has greatly increased in recent years, due to the discovery of TLRs. They play a central role in the detection of PAMPs and in the initiation of effective innate and adaptive immune responses (1, 2, 3). There is accumulating evidence, based on *in vitro* and *in vivo* studies, that supports a role for TLRs in *A. fumigatus* sensing. Several clinical reports confirm that TLRs contribute to the pathophysiology of infectious diseases and that TLR gene polymorphisms are associated with predisposition to aspergillosis. A recent study indicated that single nucleotide polymorphisms of TLR1 and TLR6 are associated with susceptibility to IPA after allogeneic stem cell transplantation (40). By contrast, no association has been found between IPA and two classical TLR4 polymorphisms, Asp299Gly and Thr399Ile, thus questioning the relevance of the *in vitro* findings indicating a role of TLR4 in the recognition of *A. fumigatus*. Hence, the human research studies support the concept that, by acting as a heterodimer with either TLR1 or TLR6 (41, 42), TLR2 is a major component of the host recognition of *A. fumigatus*. Nonetheless, as discussed above, there is evidence suggesting that the involvement of TLRs during *A. fumigatus* infection is influenced by the immunological status of the host.

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Figure 1.

The role of PMN and alveolar macrophages in invasive pulmonary aspergillosis.

Mice were infected intratracheally with  $1 \times 10^7$  (A) or  $1 \times 10^6$  (B) conidia of *A. fumigatus* and their survival was recorded. PMNs were depleted in mice (A and B) through the intravenous administration of vinblastine (43). Macrophages were depleted in mice (B) through the intratracheal administration of a liposome-encapsulated clodronate suspension (43). Mice with PMN-depletion alone overcame a small inoculum of *A. fumigatus* conidia (B), but PMN were required if a larger inoculum was administered (A).

Figure 2.

Absence of TLR2 involvement in corticosteroid-treated mice.

Corticosteroid-treated mice were infected intratracheally with two concentrations of *A. fumigatus* conidia and their survival was recorded. Unlike findings in mice immunosuppressed by neutropenia (31), *A. fumigatus* recognition is not TLR2 dependent in mice that are immunosuppressed by corticotherapy.