Involvement of Toll-Like Receptor 2 in Experimental Invasive Pulmonary Aspergillosis

Viviane Balloy,1 Mustapha Si-Tahar,1 Osamu Takeuchi,2 Bruno Philippe,3 Marie-Anne Nahori,4 Myriam Tanguy,5 Michel Huerre,5 Shizuo Akira,2 Jean-Paul Latgé,3 and Michel Chignard1*

Unité de Défense Innée et Inflammation, Inserm E336, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France;1 Department of Host Defense, Research Institute For Microbial Diseases, Osaka University, Osaka, Japan;2 Unité des Aspergillus, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France;3 Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 28, rue du Dr Roux, 75015, Paris, France;4 and Unité de Recherche et d’Expertise Histotechnologie et Pathologie, Institut Pasteur, 25, rue du Dr Roux, 75015 Paris, France5

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Aspergillus fumigatus, an opportunistic fungal pathogen, causes severe and usually fatal invasive pulmonary aspergillosis in immunocompromised hosts. Interestingly, Drosophila cells lacking the Toll protein are prone to A. fumigatus infection. In the current study, we looked for the involvement of Toll-like receptor 2 (TLR2) in the recognition of A. fumigatus by analyzing the in vivo and ex vivo responses of immunocompromised TLR2−/− and TLR2+/+ mice to this fungus. Upon intratracheal administration of conidia, survival and tumor necrosis factor alpha (TNF-α), interleukin-12, and macrophage inhibitory protein-2 alpha concentrations in the airspaces of TLR2−/− mice were significantly lower than those of TLR2+/+ animals. In vitro analysis of TNF-α production by conidia-challenged alveolar macrophages from TLR2−/− revealed a significant deficiency in comparison with macrophages from TLR2+/+ mice. Infected TLR2−/− mice also have a higher respiratory distress and a higher pathogen burden than TLR2+/+ mice. These data demonstrate that TLR2 plays a significant role in the defense of the host against A. fumigatus infection.

Aspergillus fumigatus is recognized as the most prevalent airborne fungal pathogen and causes severe and usually fatal invasive pulmonary aspergillosis (IPA) in immunocompromised hosts. IPA has become a leading cause of death, due to an increasing number of immunocompromised patients and an increase in the severity of immunosuppressive therapies. Classical risk factors are corticotherapy, cytotoxic chemotherapy, and transplantation accompanied by immunosuppressive therapy, and neutropenia remains the most important predisposing factor. Available data indicate that the major armaments of host defense against IPA belong to the innate immune system (for a review, see references 3, 16, 17, 30, and 33), such as alveolar macrophages and recruited neutrophils (1, 32).

These two cell types of leukocytes that constitute the first line of defense of the host are able to sense invading microorganisms through different types of receptors, especially through the newly described family of Toll-like receptors (TLR) (3, 16, 17, 40, 42). Scrutiny of the human and mouse genomes has revealed the existence of not less than 10 different TLR. These receptors are involved in the recognition of various microbe-derived patterns of molecules. Early studies have focused on the role played by TLR2 and TLR4 and on their archetypal ligands, i.e., lipoproteins and lipoteichoic acid from gram-positive bacteria on the one hand and lipopolysaccharides from gram-negative bacteria on the other hand. To date, many other ligands have been identified for TLR2 and TLR4, as well as for TLR3, TLR5, TLR7, TLR8, and TLR9 (for a review, see references 3, 16, 17, 40, and 42). All appear key receptors of the innate immune system as their activation initiates a signaling cascade leading to NF-κB nuclear translocation and the induction of different proinflammatory genes (3, 16, 17, 40, 42). Thus, TLR appear to be directly involved in the fight of infections. For instance, TLR2-deficient mice show higher susceptibility than wild-type mice to infection by the gram-positive bacteria Staphylococcus aureus (38) or Streptococcus pneumoniae (15). More importantly, it has been reported that a mutation in the TLR2 gene may predispose human beings to life-threatening bacterial infections (23).

Interestingly enough, in a pioneering work, Lemaître et al. (21) reported that the Toll protein (after which the name TLR was coined) in Drosophila is implicated in the defense against A. fumigatus. The fact that Drosophila Toll protein is activated during fungal or gram-positive infections (22) led us to postulate that TLR2 along with gram-positive bacteria may sense A. fumigatus and as such may play a protective role in IPA. This hypothesis was supported by two early observations, i.e., (i) TLR2 is also activated by zymosan, a yeast cell wall component (41), and (ii) there is a suspicion of an involvement of TLR2 in a model of chronic fungal asthma (4). Since then, several in vitro studies using cells from the myeloid lineage have shown that TLR2 is involved in the sensing of A. fumigatus (5, 24, 27, 28). Taking advantage of mice genetically deficient in TLR2 (39), the present study analyzes the role of TLR2 in IPA by specifically evaluating in vivo responses in detail and in vitro responses of alveolar macrophages.
MATERIALS AND METHODS

Materials. Ketamine was from Merial (Lyon, France), xylazine and enrofloxa-
cin from Bayer (Puteaux, France), tetracycline hydrochloride from Roussel Di-
mant (Paris-La Défense, France), and vinblastine from Lilly (Saint-Cloud, France). For culture medium, fetal calf serum (FCS) was purchased from Hy-
clone (Logan, UT) and penicillin and streptomycin from Gibco (Paisley, United
Kingdom). Acylacetone was from Sigma-Aldrich (Saint-Quentin Fallavier, France). Finally, β-glucosaminohydrochloride and 4-(dimethylamino)-benzalde-
hyde were both from Merck (Darmstadt, Germany). Kits for the immunoenzy-
matometric detection of the galactomannan antigen of A. fumigatus were obtained
from Bio-Rad (Marnes la Coquette, France).

Preparation of A. fumigatus conidia. A clinical isolate of A. fumigatus (Green
strain CBS 144.89) was maintained on 2% malt extract agar slants at 22°C. Conidia
were recovered from cultures grown for 7 days by washing the slant
with a phosphate-buffered saline (PBS)-0.1% Tween 20 solution and
gently scraping. Conidia were then washed by centrifugation (5 min at 10,000 ×
g) and suspended in a PBS-0.1% Tween 20 solution. Conidium concentrations
were evaluated by measurement of the optical density of the suspension at 600
nm, with a 0.6 optical density corresponding to 2 × 10⁶ conidia/ml. The suspen-
sion was then diluted in order to allow the delivery of mice to the desired
concentration under a 50-μl volume.

Animal experiments. Homozygous mutant mice for TLR2 (TLR2<sup>−/−</sup>) gener-
ated by gene targeting as described previously (39) were back-crossed eight times
with C57BL/6 to ensure a similar genetic background. TLR2<sup>−/−</sup> homozygous
homozygous littermates were also back-crossed eight times with C57BL/6. For
preparation of the experimental model of IPA, and for the backcross, 7-week-old
C57BL/6 mice were provided by the Centre d’Elevage R. Janvier, Le Genest.
Saint-Isle, France. For the in vivo experiments, 7-week-old male mice were
depleted of neutrophils by an intravenous administration of the antineoplastic
agent vinblastine (5 mg/kg of body weight) 66 h before infection (1). At the time
of conidium administration, depletion of blood-circulating neutrophils was 100%
and remained as such for 48 h. Mice were given drinking water ad libitum
containing 50 μg/ml tetracycline hydrochloride during 4 days before infection.
Then, 6 h before infection and every 24 h thereafter, mice were administered
by the subcutaneous route 0.25 mg enrofloxacin. Mice were cared for in con-
cordance with Pasteur Institute guidelines in compliance with European animal
welfare regulation. For intratracheal administration, antibiotic-treated mice were anes-
thetized by intramuscular administration of 1 mg ketamine and 0.2 mg xylazine
to facilitate conidia inhalation and until normal breathing resumed. This proto-
col allowed highly reproducible infection of the whole lung (personal data).

Assessment of the basal respiratory function. Conscious mice were placed in
a whole-body barometric plethysmographic chamber (Buxco Electronics, Sha-
ron, CT) to analyze their basal respiratory capacity over time. The system mea-
sures both the magnitude and the slope of the chamber pressure. The basal
respiratory capacity of each individual mouse was estimated by recording the
enhanced pause pressure expressed as Penh according to the manufacturer’s
instructions and as previously reported (20) and which increase is an indicator
of deterioration changes in airway mechanics.

Collection of BALF and measurement of immunoreactive TNF-α, IL-12, and
MIP-2a content. Mice were euthanized by the intraperitoneal administration
of pentobarbital (12 mg/mouse). Tracheas were cannulated, and lungs were washed
eight times with 0.5 ml PBS to provide 4 ml of bronchoalveolar lavage fluid
(BALF). There were no significant differences in the total volume of PBS infused
into the lungs or in the volume recovered after the lavage procedure among any
experimental groups. Cell-free BALF obtained after centrifugation (300 × g for
15 min) was used for tumor necrosis factor alpha (TNF-α) measurement by an
enzyme immunometric assay as previously described (31). Interleukin-12 (IL-12)
and macrophage inhibitory protein-2 alpha (MIP-2α) were quantified by specific
enzyme-linked immunosorbent assay kits from Biosource (Nivelles, Belgium)
and R&D System Europe (Lille, France), respectively.

Determination of the in vitro TNF-α production by alveolar macrophages.
BALF PBL cells were collected from naïve TLR2<sup>−/−</sup> and TLR2<sup>−/−</sup> mice and pooled by
group. Collected cells were counted (Coulter Electronics, Margency, France)
and centrifuged at 300 × g for 15 min. Cells were resuspended at 10⁶/ml of RPMI
1640 supplemented with 0.1% FCS and 2 mM glutamine. Aliquots of 200 μl were
dispensed into 96-well tissue culture plates for a 1-h adhesion step at 37°C. Wells
were then washed to remove nonadherent cells, and remaining adherent cells
(∼2 × 10⁶/well) were immediately incubated at 37°C with A. fumigatus conidia
for 6 h at the microbe to cell ratio of 1:1. Culture supernatants were then
assayed for TNF-α concentrations. It is of note that for the in vitro experiments
with alveolar macrophages, swollen conidia were prepared by incubating 10⁶
conidia/ml in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine at
37°C for 2 h. The level of killing of resting conidia by alveolar macrophages in
vitro after a 6-h incubation is very low, in the range of 6%. In contrast, swollen
conidia are more sensitive to killing than resting conidia (29). For that reason,
the in vitro activation of alveolar macrophages by A. fumigatus was investigated
using swollen conidia.

Histology. Lungs, kidneys, liver, brain, and spleen were collected at 24 and 48
h after intratracheal infection with 3 × 10<sup>6</sup> conidia. Organs were fixed in
3.7% neutral-buffered formaldehyde, embedded in paraffin, and cut into 5-μm-thick
sections. Sections were then stained with hematoxylin-cosin stain for tissue
examination and with methenamine silver for fungus detection according to
the method of Sinha et al. (35). Upon light microscopic examination, conidia and
hyphae were counted on 10 different sections per lung mouse at a magnification
of ×1,000. One hyphus was defined as a filamentous structure whose length was
>10 μm, including a branching structure which derived from a maternal hyphus.
By contrast, transversal sections of hyphae as well as conidia were not included.
Accordingly, the total count of 30 fields at a magnification of ×1,000 allowed us
to compare the ability to develop germinative structures from conidia considered
to be the pathogenic form of the fungus.

Measurement of chitin in lungs. Lungs and kidneys of mice were homogenized
in 5 ml distilled water containing 0.05% Tween 20, frozen, and then homogenized.
Total concentrations were hydrolyzed in 1 ml HCl (6 N) and AlCl₃ (500 μl).
Reaction was neutralized by the addition of 1 ml NaOH (8 N). Samples were
centrifuged (1,500 × g, 10 min, 20°C). Standards consisting of 200 μl glu-
cosamine (50 to 200 μg/ml H2O) or 200 μl of supernatant from each tissue
preparation were added to 200 μl buffer containing 25 volumes NaClO₂ (1.5 M)
and 1 volume acylacetone (4%) heated at 100°C for 20 min and cooled in
water. A total of 1.4 ml of ethanol 95% was added. A fresh solution of 4-(di-
methylamino)-benzaldehyde (1.6 g in 60 ml HCl and 95% [vol/vol] ethanol) was
made, and 200 μl was added to each tube. Optical density was measured at 520
nm after 45 min. Chitin content was measured in glucosamine equivalents (14).

Measurement of galactomannan in lungs and blood. Galactomannan antigen
(34, 36) was detected by means of a commercially available kit. Serum samples
were treated as recommended by the manufacturer. For the quantification of
galactomannan in lung tissues, aliquots (300 μl) of lung homogenates prepared
for the chitin assay were centrifuged at 1,500 × g for 10 min at 4°C and
galactomannan concentration was evaluated in supernatants as for serum. The
ratio of absorbance sample/absorbance threshold serum was calculated for each
test point. Then, ratio values were converted in concentrations expressed in
ng/ml by considering the ratio value of 1 as being a concentration of 1 ng/ml, as
recommended by the manufacturer.

Statistical analysis. Survival data were analyzed by means of log-rank com-
parisons of Kaplan-Meier estimates. Kaplan-Meier survival curves, followed by the
Wilcoxon test using JMP 5.0 software (SAS, Cary, NC). Other results are expressed as
means ± standard errors of the means (SEM). Differences between the data were ana-
yzed by Student’s unpaired t test. A P value of <0.05 was considered significant.

RESULTS

Mortality following intratracheal administration of A. fumi-
gatus conidia. We first analyzed the survival of immunocom-
petent and vinblastine-immunocompromised C57BL/6 mice
upon intratracheal administration of conidia from A. fumiga-
tus. Vinblastine treatment induced a sustained neutropenia
(1, 20) which has for consequence a reduced innate host defense
against the fungi (32). Upon administration of 10⁶ conidia,
all vinblastine-treated mice survived. By contrast, when 10⁷
conidia were given, all immunocompromised mice died within
5 days while all the immunocompetent ones survived long
term. Neutropenia induced by the antitumor antibiotic
rub6-C5 (8) has the same consequences in terms of animal
survival (data not shown).

Having established the model, two groups of either
TLR2<sup>−/−</sup> or TLR2<sup>−/−</sup> mice, both immunocompromised upon

whether alveolar macrophages were collected from naïve or vinblastine-treated mice, they did not differ in their capacity to produce TNF-α. The induction of TNF-α by A. fumigatus in BALF was significantly reduced for TLR2−/− mice (P = 0.0005). A. fumigatus triggers a significant respiratory distress only in the absence of TLR2, as Penh values for uninfected TLR2+/+ and TLR2−/− mice were 0.60 ± 0.06 (n = 4) and 0.52 ± 0.06 (n = 4), respectively.

Analysis of the pathogen growth. No conidia or hyphae were observed upon microscopic examination in kidneys, liver, brain, or spleen either at 24 h or at 48 h after conidium administration. As expected, conidia and hyphae were present in lungs at 24 h but with no apparent difference between TLR2+/+ and TLR2−/− mice. Analysis of the lungs from both TLR2+/+ and TLR2−/− mice at 48 h (Fig. 4) showed diffuse and plurifocal lesions of bronchiolitis with segmental destruction of the bronchiolar wall and perivascular involvement. Some foci of necrosis were seen around the bronchi and the vessels in both groups, but the lesions of bronchiolitis and necrosis appeared to be more severe in infected TLR2−/− mice.

Analysis of the TNF-α, IL-12 and MIP-2α in BALF and of the in vitro TNF-α production by alveolar macrophages incubated with conidia. TNF-α, IL-12, and MIP-2α concentrations were quantified in the BALF 24 h after intratracheal administration of 3 × 10⁶ conidia or their vehicle. As shown in Fig. 2, A. fumigatus triggered the synthesis of the three tested mediators but the recovered concentrations were significantly reduced in TLR2−/− compared to TLR2+/+ mice. Alveolar macrophages collected from either TLR2−/− or TLR2+/+ naïve mice were incubated for 6 h in the presence or absence of swollen conidia at a 1/1 ratio. This resulted in the induction of TNF-α synthesis for both cell types. Nonetheless, this induction was significantly reduced for TLR2−/− compared to TLR2+/+ macrophages (Fig. 3). It is of note that whether alveolar macrophages were collected from naïve or vinblastine-treated mice, they did not differ in their capacity to produce TNF-α in response to conidia (data not shown).

Assessment of the basal respiratory function. Analysis of the basal respiratory function of infected mice showed a significant difference between the two groups as early as 6 h after conidium administration, with Penh values of 0.82 ± 0.07 and 0.51 ± 0.09 (n = 4; P < 0.05) for TLR2−/− and TLR2+/+, respectively. These data indicated that the pulmonary infection by A.
than in infected TLR2\(^{+/+}\) mice (Fig. 4A to D). There were a few infiltrates characterized by few or no polymorphonuclear cells; macrophages and lymphocytes were observed around the bronchi and the vessels. Silver staining (Gomori-Grocott procedure) revealed typical features of experimental IPA (Fig. 4E and F). It especially allowed us to observe that the fungal infection mainly colocalized with the necrotic areas by comparison of silver (Fig. 4E and F) and hematoxylin-eosin (Fig. 4A and B) stainings. A higher magnification of the silver-stained sections (Fig. 4G and H) evidenced numerous hyphae within the parenchyma. Counting revealed a higher number of hyphae in TLR2\(^{−/−}\) than in TLR2\(^{+/+}\) mice, with values of 13.1 ± 0.7 versus 10.8 ± 0.6 per section (means ± SEM of 30 sections from three mice; \(P < 0.05\)), respectively. To obtain another quantification of the invasive hyphal form of \(A\). \textit{fumigatus}, we evaluated the lung burden of chitin, a component of the hyphal wall that is absent from mammalian cells. Significant differences were observed at 24 and 48 h between the two groups of mice, with the highest values of chitin (in glucosamine equivalents) being found in infected TLR2\(^{−/−}\) mice (Fig. 5). Moreover, the concentration of galactomannan, which is released by the growing fungus, was evaluated in lungs and blood at 24 and 48 h (Fig. 5). Except for the blood at 24 h, higher significant concentrations were found in TLR2\(^{−/−}\) than in TLR2\(^{+/+}\) mice.

**DISCUSSION**

The present data demonstrate the participation of TLR2 to the host defense against the opportunistic fungus \(A\). \textit{fumigatus}. The strongest evidence is given by the survival difference between TLR2\(^{−/−}\) and TLR2\(^{+/+}\) mice upon induction of IPA. We also analyzed the production of TNF-\(\alpha\) by TLR2\(^{−/−}\) mice. Indeed, TNF-\(\alpha\) is one of the main cytokines produced by cells of the macrophage lineage after TLR2 activation (10, 39) and is a critical primary mediator in the initiation of pulmonary innate immunity in experimental pneumonia (12, 17). Moreover, it is known that TNF-\(\alpha\) production is crucial for the regulation of IPA, as its neutralization by specific blocking antibodies in an experimental murine model results in an increased mortality associated with an increased fungal burden (26). This may be also relevant for the human pathology as two reports indicated that a clinical treatment with an anti-TNF-\(\alpha\) antibody is associated with IPA (9, 44). As a result, we observed that the lack of TLR2 apparently renders alveolar macrophages less responsive to \(A\). \textit{fumigatus}. Indeed, TNF-\(\alpha\) production was reduced in TLR2\(^{−/−}\) compared to TLR2\(^{+/+}\) both...
in vivo and in vitro. Along with TNF-α production, we also detected reduced concentrations of IL-12, a cytokine responsible for the optimal development of antifungal immunity in mice with IPA (7), and of MIP-2α, a ligand of CXCR2 which plays an essential role in host defense against *A. fumigatus* (25). Thus, a TLR2-mediated responsiveness of macrophages, and also most probably of other resident cells such as epithelial and dendritic cells, is susceptible to account for the control of the lung infection by *A. fumigatus* as attested by (i) the observation of a larger number of hyphae in lungs, (ii) the presence of a larger amount of chitin in lungs, and (iii) the detection of a higher concentration of galactomannan in the lungs and serum of TLR2−/− compared to TLR2+/+ mice. Interestingly, our findings are consistent with those of a previous study showing that TNF-α production by *A. fumigatus*-challenged peritoneal macrophages (24) and lungs (2) of MyD88−/− mice is almost absent. MyD88 being a critical adaptor molecule for the intracellular signal transduction induced by the TLR family (3, 26). In conclusion, our study shows that TLR2 plays a significant role in a murine model of experimental IPA. It remains to be established the nature of the pathogen-associated molecular pattern(s) expressed by *A. fumigatus* that triggers the activation of the TLR2-associated signaling pathway leading to the induction of the innate immune response.

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