MAJOR ARTICLE

Species-Specific Recognition of *Aspergillus fumigatus* by Toll-like Receptor 1 and Toll-like Receptor 6

Ivana Rubino,[1] Alix Coste,[2] Didier Le Roy,[1] Thierry Roger,[1] Katia Jaton,[2] Michael Boeckh,[4] Michel Monod,[3] Jean-Paul Latgé,[5] Thierry Calandra,[1] and Pierre-Yves Bochud[1]

[1]Department of Medicine, Infectious Diseases Service, [2]Institute of Microbiology, and [3]Department of Dermatology, University Hospital and University of Lausanne, Switzerland; [4]Fred Hutchinson Cancer Research Center, Seattle, Washington; and [5]Department of Parasitology and Mycology, Pasteur Institute Paris, France

Background. *Aspergillus fumigatus* causes invasive aspergillosis, a potentially fatal infection in oncohematological patients. Innate immune detection of *A. fumigatus* involves Toll-like receptor (TLR) 4 and TLR2, which forms a heterodimer with either TLR1 or TLR6. The role of those coreceptors in *Aspergillus* sensing is unknown.

Methods. Cytokine production was measured in bone marrow–derived macrophages (BMDMs) from wild-type (WT) and TLR-deficient mice after incubation with a WT and an immunogenic RodA-deficient (ΔrodA-47) strain of *A. fumigatus* and in lungs from these mice after intranasal mold inoculation. *Aspergillus fumigatus*–mediated NF-κB activation was measured in HEK293T cells transfected with plasmids expressing mouse or human TLRs.

Results. Bone marrow–derived macrophages from TLR1- and TLR6-deficient mice produced lower amounts of interleukin 12p40, CXCL2, interleukin 6, and tumor necrosis factor α than BMDMs from WT mice after stimulation with *A. fumigatus*. Lungs from TLR1- and TLR6-deficient mice had diminished CXCL1 and CXCL2 production and increased fungal burden after intranasal inoculation of ΔrodA *A. fumigatus* compared with lungs from WT mice. ΔrodA strain-mediated NF-κB activation was observed in HEK293T cells expressing mouse TLR2/1, mouse TLR2/6, and human TLR2/1 but not human TLR2/6.

Conclusions. Innate immune detection of *A. fumigatus* is mediated by TLR4 and TLR2 together with TLR1 or TLR6 in mice and TLR1 but not TLR6 in humans.

Aspergillus fumigatus is a ubiquitous mold that can cause invasive aspergillosis (IA), a potentially lethal infection in oncohematological patients. With an incidence rate ranging 5%–15%, IA is one of the most frequent infections in patients undergoing intensive myeloablative chemotherapy for acute leukemia or allogenic hematopoietic stem cell transplantation [1]. Despite the availability of newer antifungal drugs, IA is still associated with a high mortality (30%–60%) [2]. Understanding the pathogenic mechanisms of IA may have important consequences for the prevention and management of this infection in patients at risk.

Strategically located at the host-pathogen interface, Toll-like receptors (TLRs) are essential components of the innate immune system. They constitute a family of at least 12 transmembrane proteins localized either on the cell surface (TLR1, 2, 4, 5, and 6) or within endocytic vesicles (TLR3, 7, 8, and 9) of mammalian cells. The extracellular domain of TLRs is characterized by the presence of leucine-rich repeats that detect specific microbe-associated molecular patterns [3]. Their intracellular domain interacts with signaling molecules, leading to the activation of transcription factors and the release of soluble mediators such as cytokines and chemokines that initiate and shape adaptative immune response [4, 5].

The involvement of TLR2 and TLR4 in the innate immune detection of *A. fumigatus* was suggested by several studies measuring cytokine production from bone marrow–derived macrophages (BMDMs) from

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Correspondence: Pierre-Yves Bochud, MD, Department of Medicine, Infectious Diseases Service, Centre Hospitalier Universitaire Vaudois (CHUV), Rue du Bugnon 48, 1011 Lausanne (Suisse), Switzerland (pierre-yves.bochud@chuv.ch).

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wild-type (WT) and TLR-deficient mice [6–8], a study measuring transfection of TLR plasmids into different cell-lines [9], and in vivo models of IA [10, 11]. However, these experiments were not univocally confirmed [9, 12, 13] and did not assess the possible role of TLR1 and TLR6, known as TLR2 coreceptors [14–16].

Recent studies show that *A. fumigatus* RodA protein, which constitutes interwoven rodlet fascicles on the outermost cell wall of conidia, prevents innate immune recognition and could in part explain incongruity so far reported in literature [17, 18]. Aimanianda et al showed that removal of this protein from *A. fumigatus* either chemically, genetically (by using a mutant deficient in RodA, ΔrodA), or biologically (by germination) enhanced immune activation [17]. Human dendritic cells and murine alveolar macrophages stimulated by dormant conidia (DOC) from ΔrodA *A. fumigatus* produced large amounts of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 1β (IL-1β), whereas the same cells stimulated by DOC from WT *A. fumigatus* produced almost undetectable amounts of these cytokines [17].

In this study, we used the immunogenic rodletless mutant strain of *A. fumigatus* (ΔrodA-47) to investigate the role of TLR1, TLR2, TLR4, and TLR6 in the immune responses to *A. fumigatus*. This strain was used together with WT *A. fumigatus* in cells from knockout mice as well as HEK293T cells transfected with TLR plasmids. We confirmed the role of TLR2 and TLR4 in the innate immune detection of this pathogen in both mice and humans and showed that this detection involves both TLR1 and TLR6 in mice and TLR1 but not TLR6 in humans. Moreover, we analyzed the role of TLR1 domains in the detection of this pathogen in humans and also the functional effects of polymorphisms located in the coding region of TLR1 and previously associated with an increased risk of developing IA in immunocompromised patients [19].

MATERIALS AND METHODS

Mice

Mice deficient in TLR1, TLR2, TLR3, TLR4, and TLR6 (all from C57BL/6 background) were described elsewhere [20–25]. Wild-type female C57BL/6 mice purchased from Charles River Laboratories were used as controls. All mice were bred and housed with filtered air under standard conditions. All animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations number 876.6 and 877.6) and performed according to the institution's guidelines for animal experiments.

Cells and Reagents

Human HEK293T cells (American Type Culture Collection No. CRL-1573) were cultured in cellular grade Petri dishes in Dulbecco's modified Eagle medium (Invitrogen). Bone marrow–derived macrophages and bone marrow–derived dendritic cells (BMDCs) were obtained from femoral and tibial bone marrow cells and cultured in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with monocyte and granulocyte-monocyte colony-stimulating factor, respectively [26]. All media were supplemented with 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, 100 IU/mL of penicillin, 100 μg/mL of streptomycin (all from Invitrogen) and 10% heat-inactivated fetal calf serum (Sigma–Aldrich), and cells were maintained in a humidified incubator at 37°C and 5% carbon dioxide. Adherent cells were harvested on day 5 (HEK293) and day 7 (BMDMs and BMDCs), enumerated, and used for the experiments.

Stimulations were performed using 100 ng/mL of *Salmonella minnesota* ultra pure lipopolysaccaride (LPS) (List Biological Laboratories), 1–10 μg/mL palmitoyl-cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-OHtrifluoro-acetate salt (Pam_3CSK_4; EMC microcollections), 100 ng/mL of macrophage-activating lipopeptide-2 (MALP-2) (Invivogen), and 10 μg/mL polyinosine-polycytidylic acid (Invivogen) DOC and germinated conidia (GC) from *A. fumigatus* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS) 144-89 (WT) and DOC from *A. fumigatus* ΔrodA CBS 144-89 at multiplicity of infection of 1. Dormant conidia were obtained from 6-day cultures grown on Sabouraud agar plate at 37°C. Germinated conidia were obtained by incubating DOC for up to 8 hours in Sabouraud liquid medium (2% glucose and 1% mycopeptone) at 37°C [17]. Conidia were killed by exposure to 2% paraformaldehyde (PFA) (4°C, overnight).

Cytokine and Chemokine Quantification

Bone marrow–derived macrophages from WT and TLR-deficient mice were incubated with WT and ΔrodA strains of *A. fumigatus* and subsequently analyzed for cytokine expression and secretion after 4 hours and 24 hours, respectively. This timing resulted in near maximal stimulation of cellular responses. Total RNA was isolated from BMDMs using the RNeasy kit (Qiagen). Reverse transcription of 1 μg of RNA was performed using the ImProm II Reverse Transcription System kit (Promega). Quantitative polymerase chain reaction (PCR) was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (PE Applied Biosystems) and primer pairs listed in Table 1. All samples were tested in triplicate as described elsewhere [27]. Gene-specific expression was expressed relative to the expression of HPRT in arbitrary units (AU) (Table 1). Collected cell-culture supernatants were processed for enzyme-linked immunosorbent assay (ELISA) quantification of mouse interleukin 12p40 (IL-12p40) and CXCL2 (R&D System).

In Vivo Infection Model

For infection assays, female mice aged 8–12 weeks (18–22 g) were inoculated intranasally twice (6 hours apart) with 20 μL

Table 1. Oligonucleotides Used in Reverse-Transcription Polymerase Chain Reaction Analyses

	Forward (5′→3′)	Reverse (5′→3′)
Human		
HPRT	GAACGTCTTGCTCGAGATGTG	CCAGCAGGTCAGCAAAGAATT
TLR2	GCCTCTCCAAGGAAGAATCC	TCCTGTTGTTGGACAGGTCA
TLR1	GGGTCAGCTGGACTTCAGAG	AAAATCCAAATGCAGGAACG
TLR4	AAGCCGAAAGGTGATTGTTG	CTGAGCAGGGTCTTCTCCAC
TLR6	GAACATGATTCTGCCTGGGT	GCTGTTCTGTGGAATGGGTT
Mouse		
Hprt	GTTGGATACAGGCCAGACTTTGTTG	GATTCAACTTGCGCTCATCTTAGGC
Il12b	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
Cxcl1	CCGCTCGCTTCTCTGTGC	CTCTGGATGTTCTTGAGGGAATC
Cxcl2	CCAACCACCAGGCTACAG	CTTCAGGGTCAAGGCAAAC
Aspergillus fumigatus cytochrome B	TTGTATTCTTCATGCCTAACGCA[a]	CGGAACAATAGCAGGTGGAGTT

[a] probe: AGGTGATAGTGAAAATTATGTTATGGCTAATCCAATGC.

phosphate-buffered saline plus 0.05% of Tween 80 (Sigma-Aldrich), either alone or mixed with 10^8 colony-forming units (CFUs) of DOC. One day after inoculation, mice were euthanized by carbon dioxide. Collected lungs were homogenized in 2 mL of phosphate-buffered saline with a Medic Tools tissue homogenizer. Chemokine expression and fungal burden were quantified by CFU counts and quantitative real time PCR (RT-PCR). Colony-forming units adjusted to the weight of tissue were calculated by plating several dilutions of tissue homogenates onto Sabouraud agar plates. The remaining homogenate was used for quantitative PCR of cytochrome B measurement as described above and using primer pairs listed in Table 1.

DNA Expression Vectors

Wild-type and mutant human TLR1 (hTLR1) constructs were kindly provided by T. R. Hawn (University of Washington, Seattle [28]); human $TLR1_{ext}/TLR6_{int}$ and $TLR6_{ext}/TLR1_{int}$ plasmids were provided by R. I. Tapping (University of Illinois, Urbana [29]). Other plasmids were published elsewhere (human TLR 2 [hTLR2], human TLR6 [hTLR6] and human CD14 [hCD14] [30, 31]). Murine plasmids were purchased from Invivogen (mouse TLR1 [mTLR1], mouse TLR6 [mTLR6], and mouse CD14 [mCD14]) or described elsewhere (mouse TLR 2 [mTLR2] [32]). Endothelial cell-leukocyte adhesion molecule (ELAM) luciferase reporter plasmid and transfection control pRL-TK plasmid were purchased (Promega). Plasmids were amplified in *Escherichia coli*, purified using the EndoFree Plasmid kit (Qiagen).

Luciferase Assay

The day before transfection, HEK293T cells were seeded at 2×10^4 cells per well in 96-well plates (Costar). Transient transfection with 50 ng of ELAM plasmid, 5 ng of pRL-TK plasmid, and 10 ng of each plasmid present in the mix was performed using Effectene transfection reagent (Qiagen). Equal DNA quantity was obtained by adding appropriate amounts of empty plasmid. Toll-like receptor plasmid expression was confirmed by RT-PCR. Twenty-four hours after transfection, cells were stimulated for 4 hours and lysed in passive lysis buffer. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) on a FLUOstar Omega luminometer (BMG Labtech).

Statistical Analysis

The unpaired Student *t* test was used for statistical analysis of experiments. Differences were considered statistically significant at $P < .05$.

RESULTS

Murine TLR1 and TLR6 Mediate Immune Responses to *A. fumigatus*

To determine the role of mTLR1 and mTLR6 in the innate immune detection of *A. fumigatus*, we analyzed the production of IL-12p40, CXCL2, IL-6, and TNF-α by BMDMs from WT, TLR1-, TLR2-, TLR3-, TLR4-, and TLR6-deficient mice in response to *A. fumigatus*. Bone marrow–derived macrophages were incubated with a WT strain of *A. fumigatus* (CBS 144-89) and a strain deficient in the RodA coating protein (ΔrodA CBS 144-89), known for its strong immunogenic properties. The production of IL-12p40, CXCL2, IL-6, and TNF-α was measured by RT-PCR and ELISA. Wild-type BMDMs stimulated with DOC from the ΔrodA strain of *A. fumigatus* produced increased IL-12p40, CXCL2, IL-6, and TNF- α protein (Figure 1) and messenger RNA (mRNA) (Supplementary Figure 1 *A–D*) levels compared with those stimulated with DOC from the WT strain. Interleukin 12p40, CXCL2, IL-6, and TNF-α production induced by both WT and ΔrodA strains of *A. fumigatus*

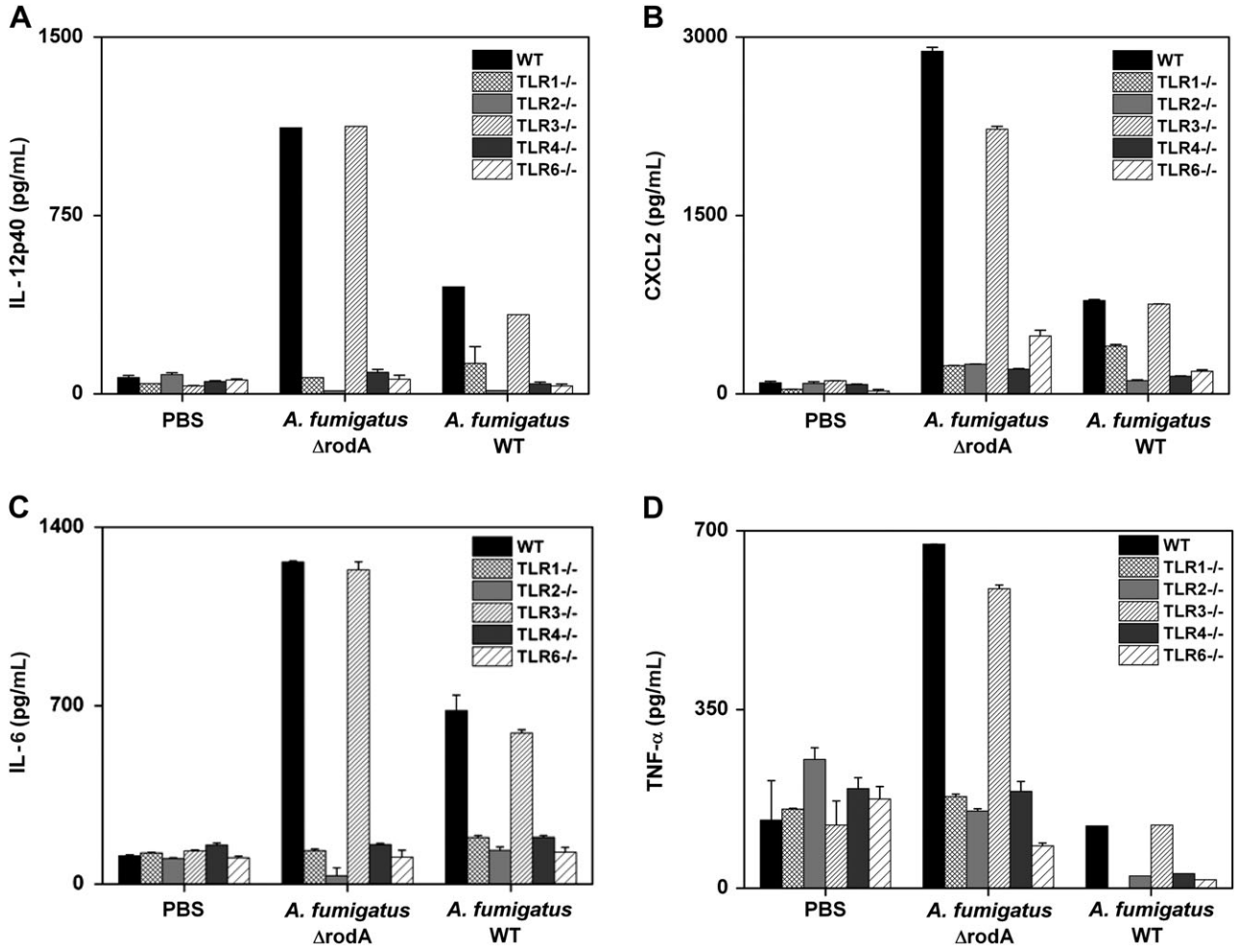

Figure 1. Murine Toll-like receptor (TLR) 1, TLR2, TLR4, and TLR6 are required to produce interleukin 12p40 (IL-12p40), CXCL2, interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) in response to wild-type (WT) and ΔrodA *Aspergillus fumigatus*. Bone marrow–derived macrophages from WT, TLR1$^{-/-}$, TLR2$^{-/-}$,TLR3$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$ mice were incubated for 24 hours with 2% PFA ΔrodA *A. fumigatus* (multiplicity of infection [MOI], 1) and WT *A. fumigatus* (MOI, 1). IL-12p40 (*A*), CXCL2 (*B*), IL-6 (*C*), and TNF-α (*D*) secretion was quantified by enzyme-linked immunosorbent assay. Data are means ± standard deviations of triplicates from 1 experiment representative of 3 experiments. Abbreviations: PBS, phosphate-buffered saline; PFA, paraformaldehyde.

was decreased in BMDMs deficient in TLR1 and almost abolished in BMDMs deficient in TLR2, TLR4, and TLR6, but not TLR3, compared with WT BMDMs (Figure 1). As a control, the ability of WT and TLR-deficient cells to produce these cytokines was assessed by stimulation with specific TLR agonists: LPS (TLR4 ligand), Pam_3CSK_4 (TLR1/2 ligand), and MALP-2 (TLR6/TLR2 ligand) (data not shown). Similar results were obtained in another experiment measuring IL-6 production by BMDCs, instead of BMDMs, from TLR-deficient mice after *A. fumigatus* stimulation (Supplementary Figure 1 *E–F*).

To assess the role of TLR1 and TLR6 during the germinating phase of the mold, which is mimicked by deletion of the rodlet layer, we compared the immune responses after stimulation with GC and DOC from the WT strain of *A. fumigatus* as well as DOC from the ΔrodA strain. Bone marrow–derived macrophages stimulated with both GC from the WT strain and DOC from the ΔrodA strain produced increased levels of IL-12p40 and CXCL2 compared with those stimulated with DOC from the WT strain (Figure 2). Furthermore, BMDMs deficient in TLR1 and TLR6 showed impaired production of IL-12p40 and CXCL2 after stimulation with all 3 different forms of *A. fumigatus* (Figure 2). Altogether, these data suggest that the innate immune detection of *A. fumigatus* in mice involves TLR1, TLR2, TLR4, and TLR6 and that the ΔRodA strain of *A. fumigatus* has an activation pattern similar to that of WT GC.

To further assess the role of TLR1 and TLR6 in the innate immune detection of *A. fumigatus*, we used an in vivo infection model of *A. fumigatus* pneumonia. CXCL1 and CXCL2 mRNA expression, as well as *A. fumigatus* burden, was measured in lungs from WT, TLR1-, TLR2-, TLR4-, and TLR6-deficient mice

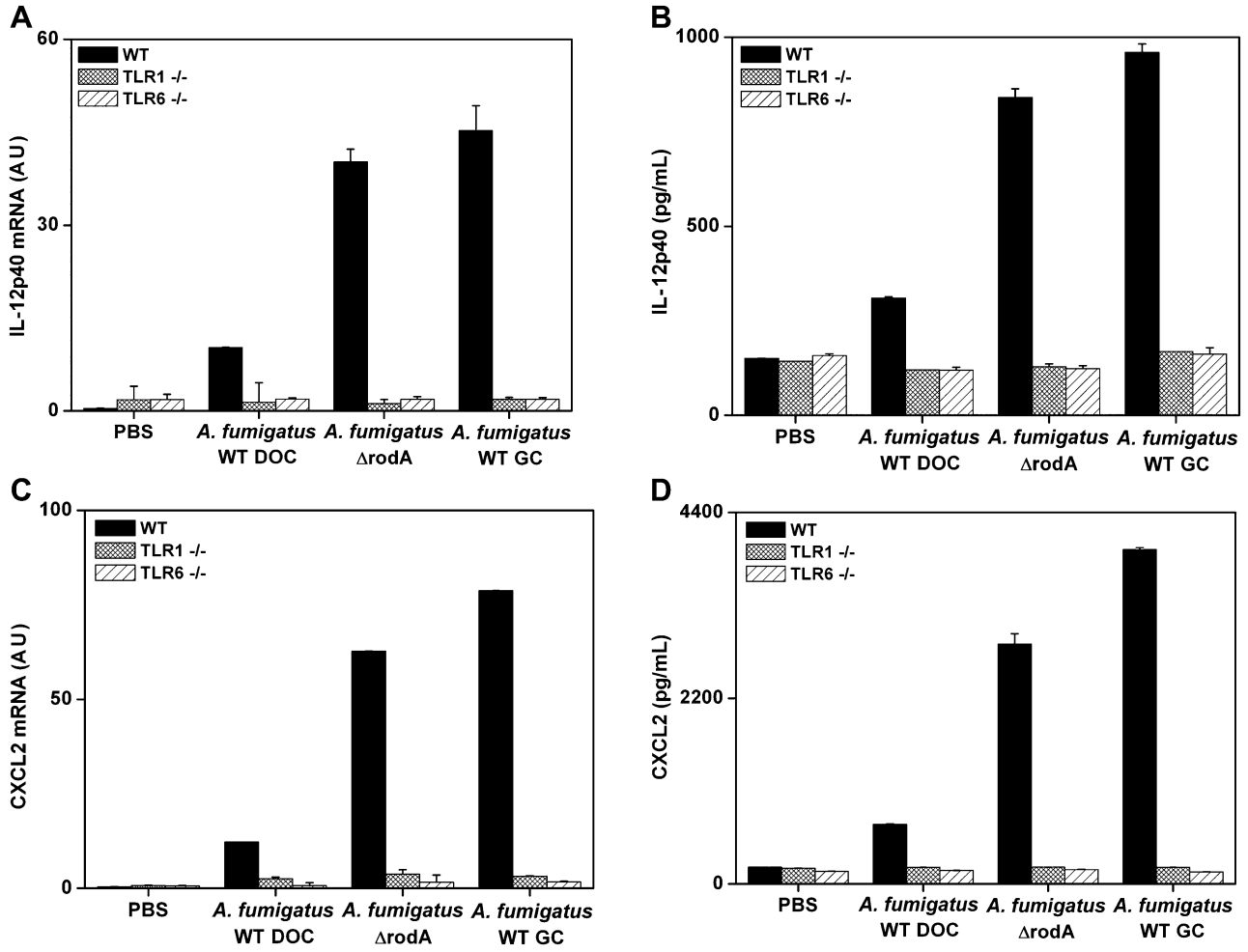

Figure 2. Mouse Toll-like receptor (TLR) 1 and TLR6 are required to express and secrete interleukin 12p40 (IL-12p40) and CXCL2 in response to wild-type (WT) and ΔrodA *Aspergillus fumigatus* dormant (DOC) conidia and WT *A. fumigatus* germinated conidia. Bone marrow–derived macrophages from WT and TLR1- and TLR6-deficient mice were incubated for 4 hours (*A, B*) or 24 hours (*C, D*) with 2% PFA ΔrodA *A. fumigatus* DOC, WT *A. fumigatus* DOC, and germinated conidia (GC) at a multiplicity of infection of 1. Expression of IL-12p40 and CXCL2 was measured by reverse-transcription polymerase chain reaction (*A, B*), and secretion was quantified by enzyme-linked immunosorbent assay (*C, D*). Data are means ± standard deviations of triplicates from 1 experiment representative of 3 experiments. Abbreviations: AU, arbitrary units; mRNA, messenger RNA; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

24 hours after intranasal inoculation of ΔrodA *A. fumigatus*. In lungs from WT mice, the infection strongly increased CXCL1 and CXCL2 mRNA levels (Figure 3*A* and 3*B*), whereas the mold was nearly undetectable as revealed by CFU counts and RT-PCR detecting *A. fumigatus* cytochrome B gene (Figure 3*C* and 3*D*). In lungs from TLR1-, TLR2-, TLR4-, and TLR6-deficient mice, the expression of CXCL1 and CXCL2 mRNA was dramatically reduced (Figure 3*A* and 3*B*), whereas large amounts of the mould were found in lung homogenates (Figure 3*C* and 3*D*). Similarly, lungs from TLR1- and TLR6-deficient mice infected with the WT (instead of the ΔrodA) strain of *A. fumigatus* showed an increased fungal burden compared with lungs from WT mice (Figure 3*E*). The presence of conidia in lungs was confirmed by staining of frozen tissue sections with Blankophor (not shown) [33]. To determine the influence of TLRs on mortality, TLR1- and TLR6-deficient mice as well as WT mice (4 each) were infected with WT and ΔrodA *A. fumigatus* as described above and followed up for 30 days. Survival rate was 100% in TLR1- and TLR6-deficient mice as well as in WT mice. Altogether, these results suggest that the absence of TLR1, TLR2, TLR4, and TLR6 causes impaired production of inflammatory mediators in response to the mold and delayed fungal clearance. However, the absence of TLR1 or TLR6 does not induce mortality in this model.

Mediation of Immune Responses to *A. fumigatus* by Human TLR1 but Not TLR6

To compare the role of human and mouse TLR1 and TLR6 in the innate immune detection of *A. fumigatus*, HEK293T cells were transiently transfected with vectors expressing human

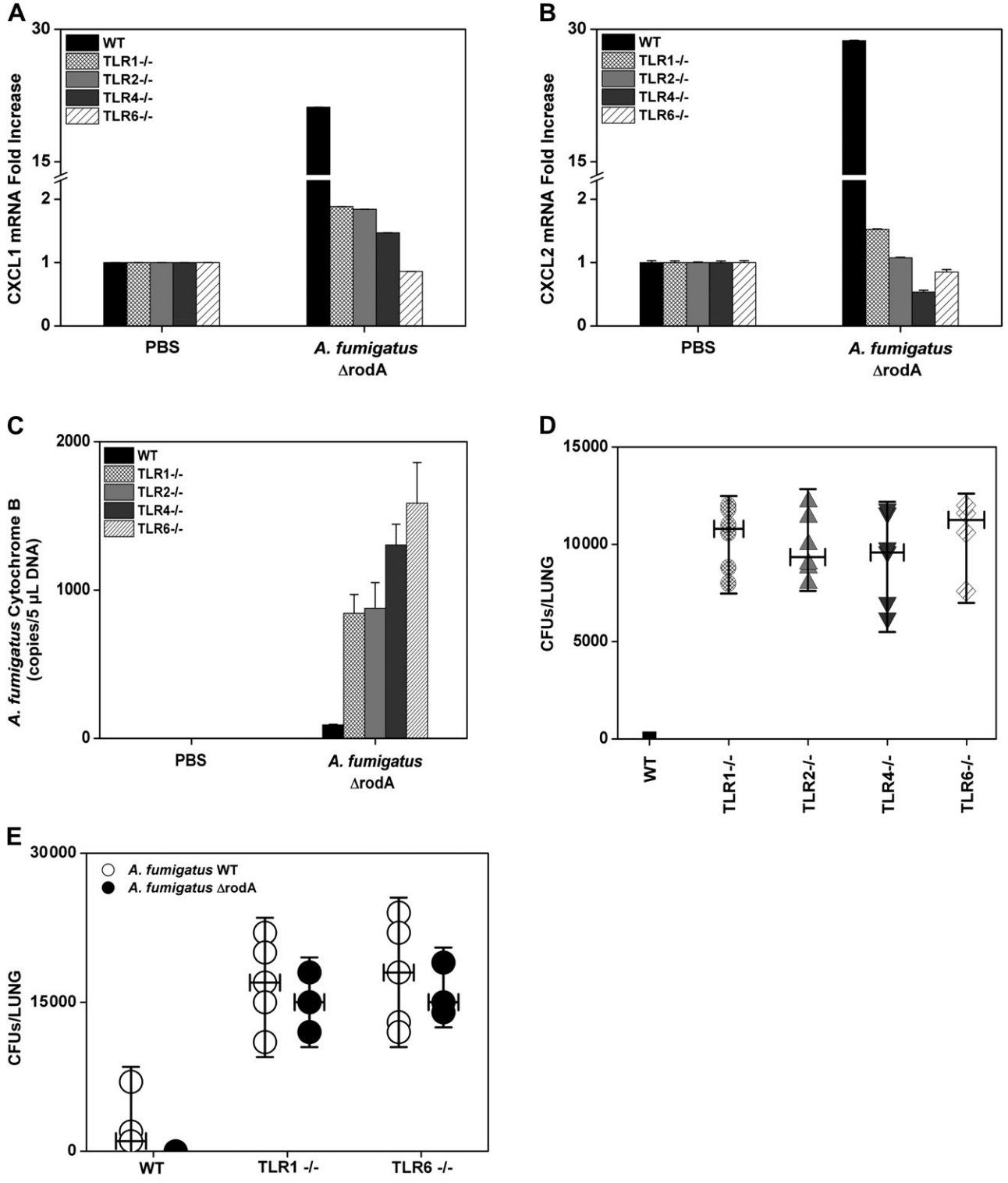

Figure 3. Toll-like receptor (TLR) 1-, TLR2-, TLR4-, and TLR6-deficient mice show impaired chemokines expression in response to *Aspergillus fumigatus* and increased fungal burden in lungs compared with wild-type (WT) mice. Wild-type, TLR1$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR6$^{-/-}$mice were infected twice, 6 hours apart, with 10^8 colony-forming units (CFUs) of ΔrodA (*A*–*E*) or WT (*E*) *A. fumigatus*. Twenty-four hours after infection lungs were collected and tested for CXCL1 and CXCL2 messenger RNA (mRNA) expression by reverse-transcription polymerase chain reaction (RT-PCR) (*A*, *B*). Lung homogenates were plated onto Sabouraud agar plates for CFU counts (*C*, *E*) and analyzed for quantification of *A. fumigatus* cytochrome B copy number by RT-PCR (*D*). Abbreviation: PBS, phosphate-buffered saline.

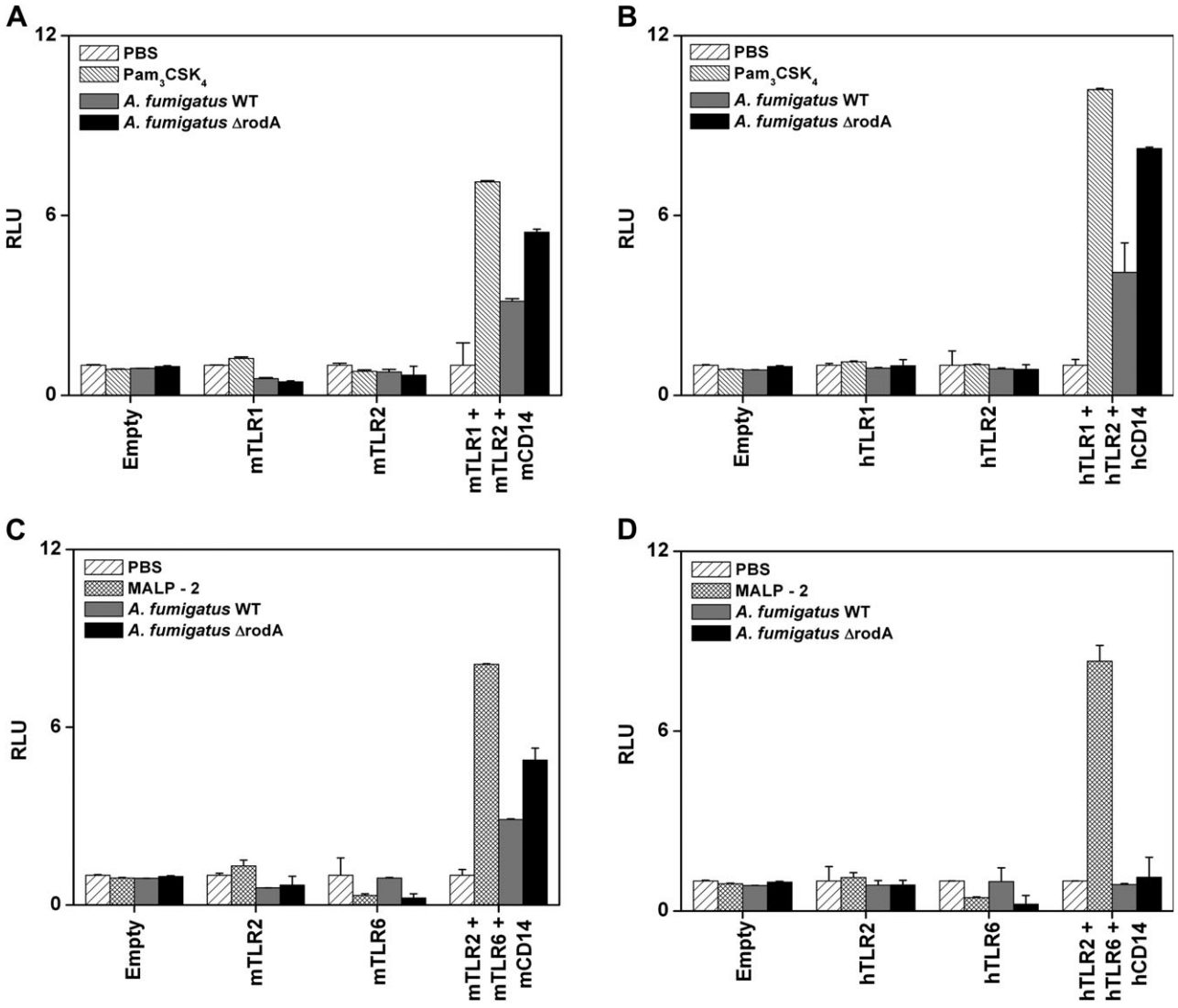

Figure 4. Innate immune response to wild-type (WT) and ΔrodA *Aspergillus fumigatus* is mediated by murine Toll-like receptor (TLR) 2/TLR1 (mTLR2/mTLR1) or TLR2/TLR6 (mTLR2/mTLR6) heterodimers and human TLR2/TLR1 (hTLR2/hTLR1). HEK293T cells were transfected with murine and human TLR1 and TLR2 (*A, B*) or murine and human TLR2 and TLR6 (*C, D*) alone or together with murine (mCD14) and human CD14 (hCD14). Cells were cotransfected with the ELAM luciferase and pRL-TK plasmids. The next day, cells were incubated for 4 hours with phosphate-buffered saline (PBS), Pam_3CSK_4 (1 μg/mL) (*A, B*), MALP-2 (100 ng/mL) (*C, D*). Wild-type or ΔrodA *A. fumigatus* (multiplicity of infection, 1) results are expressed as the ratio of luciferase to *Renilla* luciferase activity (relative luciferase units [RLU]). Data are means ± standard deviations of triplicates from 1 experiment representative of 3 experiments. Abbreviations: ELAM, endothelial cell-leukocyte adhesion molecule; MALP-2, macrophage-activating lipopeptide-2.

and mouse TLR1 and TLR6 (each in combination with TLR2 and CD14 from the corresponding species), together with a NF-κB luciferase reporter (ELAM) and a transfection control (pRL-TK). The day after transfection, cells were incubated for 4 hours with WT or ΔrodA *A. fumigatus* or appropriate controls, and luciferase activity was measured. Both strains of *A. fumigatus* induced NF-κB activation in cells expressing the mTLR2/mTLR1) (Figure 4*A*) and the hTLR2/hTLR1 (Figure 4*B*) combination, as well as the mTLR2/mTLR6 combination (Figure 4*C*) but not the hTLR2/hTLR6 combination (Figure 4*D*). Cells expressing either the human or mouse TLR2/TLR1 combination were able to respond to Pam_3CSK_4 (TLR2/TLR1 ligand) but not to MALP-2 (TLR2/TLR6 ligand) (Figure 4*A* and 4*B*). Cells expressing the human or mouse TLR2/TLR6 combination showed NF-κB activation in response to MALP-2 but not to Pam_3CSK_4 stimulation (Figure 4*C* and 4*D*). These results suggest that detection of *A. fumigatus* in mice requires the presence of TLR2 together with TLR1 or TLR6, whereas detection in humans relies on TLR2 along with TLR1 but not TLR6.

To further ascertain the role of TLR1 and TLR6 in the detection of *A. fumigatus* in human, HEK293T cells were

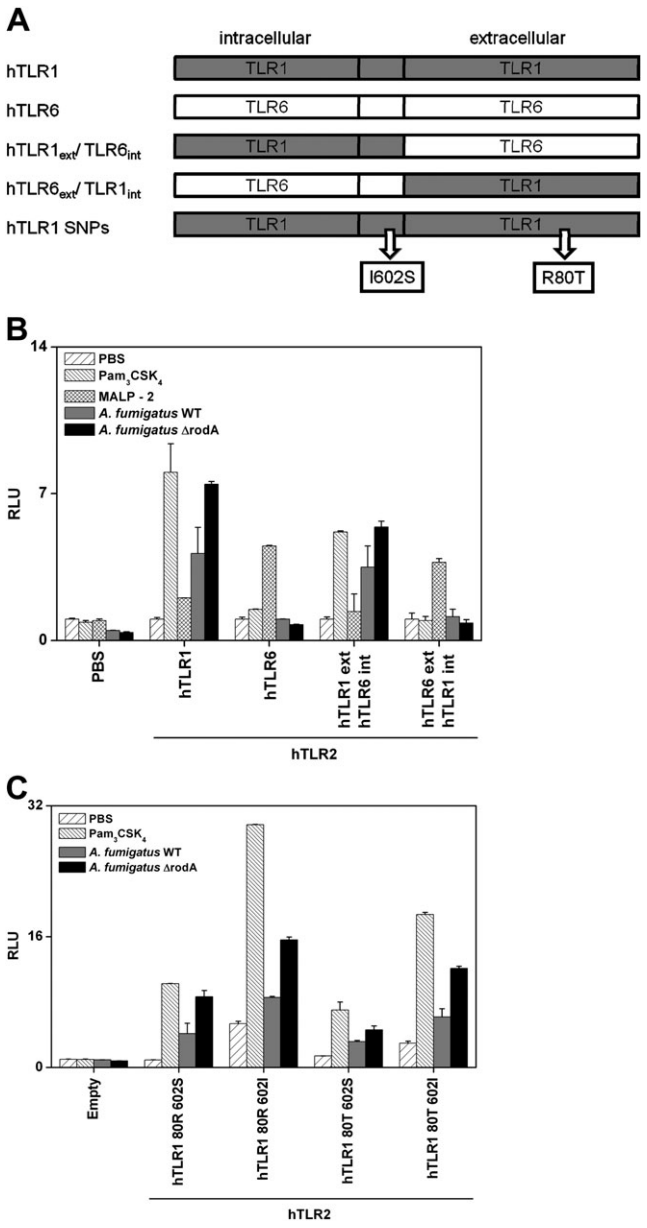

Figure 5. Extracellular domain of human Toll-like receptor (TLR) 1 (hTLR1) and G1805T (S602I) and G239C (R80T) polymorphism can modulate NF-κB activation on stimulation with wild-type (WT) and ΔrodA *Aspergillus fumigatus*. *A*, Wild-type hTLR1 and human TLR6 (hTLR6) plasmids carry human complementary DNA (cDNA) cloned into a construct with V5 epitope TAG (pEF6-V5His-TOPO, Invivogen). Chimeric plasmids carry hTLR1 extracellular and transmembrane domains merged with TLR6 intracellular domain ($hTLR1_{ext}/hTLR6_{int}$) or hTLR6 extracellular and transmembrane domains merged with TLR1 intracellular domain ($hTLR6_{ext}/hTLR1_{int}$). Human TLR1 haplotype plasmids carry a WT version of TLR1 from human cDNA cloned into a construct with V5 epitope TAG (pEF6-V5His-TOPO; Invivogen) that has been genetically modified using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis; Stratagene) to reproduce all possible haplotypic combinations of G1805T (S602I), located in the transmembrane junction, and G239C (R80T), located in the ectodomain. *B, C*, HEK293T cells were transfected with different mixes of plasmids, as indicated. *B,* In all conditions, cells were transfected with endothelial cell-leukocyte adhesion molecule transfected with hTLR2 in combination with chimeric constructs carrying the extracellular domain from hTLR1 with the intracellular domain from hTLR6 ($hTLR1_{ext}/TLR6_{int}$) or the extracellular domain from hTLR6 with the intracellular domain of hTLR1 ($hTLR6_{ext}/hTLR1_{int}$) (Figure 5*A* and 5*B*). Wild-type and ΔrodA *A. fumigatus* induced NF-κB activation in cells expressing chimeric TLRs carrying the extracellular domain from TLR1 ($hTLR1_{ext}/TLR6_{int}$) but not in those carrying the extracellular domain from TLR6 ($hTLR6_{ext}/hTLR1_{int}$) (Figure5*A* and 5B). As a control, Pam_3CSK_4 induced NF-κB activation in cells expressing chimeric TLRs carrying the extracellular domain from TLR1 ($hTLR1_{ext}/hTLR6_{int}$), and MALP-2 induced NF-κB activation in those carrying the extracellular domain from TLR6 ($hTLR6_{ext}/hTLR1_{int}$), as described elsewhere [29]. Altogether, these data show that TLR1, but not TLR6, mediates the innate immune detection of *A. fumigatus* in humans and that the extracellular domain of human TLR1 is required for this detection.

Because TLR1 is involved in the innate immune response to *A. fumigatus* in humans, we investigated the functional role of 2 common genetic polymorphisms located in the coding region of TLR1, G1805T (S602I; minor allele frequency [MAF], 0.25 in whites) and G239C (R80T; MAF, 0.06 in whites), which have been previously associated with susceptibility to IA [19] and/or with impaired TLR1 function [28, 34].

Figure 5 continued. (ELAM) and pRL-TK together with empty vector or human TLF1 (hTLR2) and human CD14 (hCD14). As reported, cells have been complemented also with chimeric plasmids $hTLR1_{ext}/hTLR6_{int}$ or $hTLR6_{ext}/hTLR1_{int}$ or WT hTLR1 or hTLR6, as control. *C*, In all conditions, cells were transfected with ELAM and pRL-TK together with empty vector or hTLR2 and hCD14. As reported, cells have been also transfected with plasmids carrying WT hTLR1 (80R 602S) and all hTLR1 haplotypes (80R 602I, 80T 602S; 80T 602I). One day after transfection, cells were incubated for 4 hours with phosphate-buffered saline (PBS), Pam_3CSK_4 (1 μg/mL), MALP-2 (100 ng/mL), or ΔrodA *A. fumigatus* (multiplicity of infection 1), as indicated. NF-κB activation was measured by the luminescent activity of a NF-κB firefly luciferase reporter plasmid (relative luciferase units [RLU']. Data are means ± standard deviations of triplicates from 1 experiment representative of 3 experiments. Supplementary Figure 1. Mouse Toll-like receptor (TLR) 1, TLR2, TLR4, and TLR6 are required to express interleukin 12p40 (IL-12p40), CXCL2, interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) in response to wild-type (WT) and ΔrodA *Aspergillus fumigatus*. Bone marrow–derived macrophages (BMDMs) (*A–D*) and bone marrow–derived dendritic cells (BMDCs) (*E–F*) from WT, $TLR1^{-/-}$, $TLR2^{-/-}$, $TLR3^{-/-}$, $TLR4^{-/-}$, and $TLR6^{-/-}$ mice were incubated for 4 hours with 2% PFA ΔrodA *A. fumigatus* (multiplicity of infection [MOI], 1) and WT *A. fumigatus* (MOI, 1). For BMDMs, expression of IL-12p40 (*A*), CXCL2 (*B*), IL-6 (*C*), and TNF-α (*D*) was measured by reverse-transcription polymerase chain reaction (RT-PCR). For BMDCs, expression (*E*) and secretion (*F*) of IL-6 was measured by RT-PCR and enzyme-linked immunosorbent assay, respectively. Data are means ± standard deviations of triplicates from 1 experiment representative of 3 experiments. Abbreviations: AU, arbitrary units; mRNA, messenger RNA; PBS, phosphate-buffered saline.

HEK293T cells were transfected with hTLR1 plasmids containing the 4 haplotypic combinations of G1805T (S602I) and G239C (R80T) (Figure 5*A* and 5*C*). HEK293T transfected with plasmids carrying the 602I allele showed higher levels of NF-κB activation at baseline and after stimulation with WT and ΔrodA *A. fumigatus* compared with those carrying the 602S allele. Cells transfected with plasmids carrying the 80T allele revealed lower levels of NF-κB activation after stimulation with WT and ΔrodA *A. fumigatus* compared with those carrying the 80R allele. These results suggest that hTLR1 variants modulate innate immune responses to *A. fumigatus*.

DISCUSSION

In this study, we investigated the role of TLRs in the innate immune detection of *A. fumigatus* in mice and humans. Previous studies suggested a role for TLR2 and TLR4 in the detection of this pathogen [6, 8, 12], but these observations were not univocally confirmed [9, 12]. Moreover, limited information was available on the role of TLR1 and TLR6, which both dimerize with TLR2 for signaling [14–16]. We showed that the detection of *A. fumigatus* is mediated by TLR1 and TLR6 in mice and TLR1 but not TLR6 in humans, together with TLR2 in both species. Our data also confirm the role of TLR4 in both mice and humans.

Immune studies of *A. fumigatus* are complicated by the evolving composition of its cell wall during its growth, thereby conferring different immunological properties to the pathogen [35]. Investigators used different growth stages of the fungus (ie, resting conidia [8, 12], swollen conidia [12], or hyphae [6, 8, 12]). Resting conidia produce a rodlet layer, which constitutes a mechanical protection from immune cells, leading to weak, almost undetectable, innate immune responses [17]. The weak immunogenicity of the fungus, at least during this early step, may explain why some studies failed to detect the involvement of TLR2 [9], TLR4 [9, 12], and MyD88 [9, 13]. In the present study, we used a mutant form of *A. fumigatus* (ΔrodA), which does not produce this masking layer and mimics the more immunogenic, germinated form of the fungus [17]. Dormant conidia from this immunogenic strain produced similar, but stronger, immune responses than the WT strain.

Species-specific differences in TLR ligand detection have been described elsewhere. Mice but not humans can detect taxol through TLR4 [36], trilauroylated peptides through TLR2 [37], and LPS from *Leptospira* through TLR4 [38]. Phylogenetic studies suggest that the different TLRs arose from ≥1 common ancestor by gene duplications events and subsequent deletions and substitutions [39, 40]. It was proposed that the TLR1 family (including TLR1, TLR2, TLR6, and TLR10) underwent more species-specific changes during evolution compared with other families [41]. Human TLR1 and TLR6 are closely located on chromosome 4 [39], suggesting a gene duplication event. As for the other TLRs, the TIR signaling domains of TLR1 and TLR6 have more sequence homology (96% in paralogs and 80% in orthologs) than the leucine-rich repeat (LRR) ligand discriminating domains (71% and 46%, respectively) [42]. This suggests that the adaptive pressure shaped the ligand binding site to improve the recognition and the defense against host-specific pathogens. This is consistent with our transfection experiments using TLR1/TLR6 chimeric plasmids, showing that TLR1, but not TLR6, was able to detect *A. fumigatus* in humans, and that this specificity was conferred by the TLR1 external LRR-containing, but not internal TIR-containing, domain.

The multiplicity of pattern-recognition receptors (PRRs) targeting the mold would suggest that the innate immune recognition is redundant and that deficiency in a single PRR would only account for a moderate effect. However, consistent with previous observations [43], our data show that the absence of a single PRR or adaptor protein leads to a relatively strong impairment of cytokine production on *A. fumigatus* stimulation and/or an important increase in the fungal burden after infection. In fact, the activation of several PRRs (either simultaneously or at different times of the infection) may be necessary for appropriate immune responses. This strategy may be adopted by the innate immune system to avoid continuous and deleterious activation of inflammatory responses to a pathogen to which we are exposed on a daily basis, such as *A. fumigatus*.

It is not known whether this innate immune signal requires a single ligand or multiples ligands from *A. fumigatus* nor whether different TLRs are activated by the same or by distinct ligands. Some ligands were shown to be detected by both TLR1/2 and TLR2/6 heterodimers (such as *Ureaplasma parvum* lipoprotein and *Chlamydia trachomatis* elementary bodies [44, 45]), but others are restricted to TLR1/2 (triacylated lipopeptides and lipoarabinomannan [23, 46]) or TLR2/6 (diacylated lipopeptides [22]) heterodimers. However, identification of *A. fumigatus* exact ligand(s) may be limited by the ability to effectively isolate components of the pathogen's cell wall and intracellular compartment.

Identification of the PRRs involved in the innate immune detection of *A. fumigatus* may have important consequences for patient management. Single nucleotide polymorphisms in the *TLR4* gene from hematopoietic stem cell transplantation donors have been associated with an increased susceptibility to IA in the recipient [47]. We examined the functional role of 2 *TLR1* single nucleotide polymorphisms located in the extracellular domain (G239C, R80T) and in the junction of the transmembrane and intracellular domain (G1805T, S602I) that were described in a report of a genetic study on susceptibility to IA [34]. Our work shows that TLR1

80T is associated with a lower NF-κB activation than TLR1 80R. This is consistent with the findings of the genetic study, showing that 80T is associated with an increased risk of IA [19]. Our experiments showed that TLR1 602I leads to higher NF-κB activation in response to *A. fumigatus* than TLR1 602S. This is consistent with other studies showing that TLR1 602I confers a better transmembrane expression of the receptor than TLR1 602S and is protective against leprosy [28, 34]. R80T is located in the highly conserved extracellular domain of TLR1 shown to be necessary for TLR1 ligand recognition. One can speculate that this polymorphism has an important impact on the folding of the LRR domain, leading to an impairment of ligand binding and/or TLR1 function, as already described for other TLRs [48, 49]. Our results may contribute to explain the genetic variability in susceptibility to IA and develop new preventing strategies in high-risk patients [19, 47].

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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References

1. Ben-Ami R, Lewis RE, Kontoyiannis DP. Invasive mould infections in the setting of hematopoietic cell transplantation: current trends and new challenges. Curr Opin Infect Dis **2009**; 22:376–84.
2. Trifilio S, Singhal S, Williams S, et al. Breakthrough fungal infections after allogeneic hematopoietic stem cell transplantation in patients on prophylactic voriconazole. Bone Marrow Transplant **2007**; 40:451–6.
3. Kumagai Y, Akira S. Identification and functions of pattern-recognition receptors. J Allergy Clin Immunol **2010**; 125:985–92.
4. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol **2007**; 7: 353–64.
5. Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. Cold Spring Harb Perspect Biol **2009**; 1:a001651.
6. Bellocchio S, Moretti S, Perruccio K, et al. TLRs govern neutrophil activity in aspergillosis. J Immunol **2004**; 173:7406–15.
7. Braedel S, Radsak M, Einsele H, et al. *Aspergillus fumigatus* antigens activate innate immune cells via Toll-like receptors 2 and 4. Br J Haematol **2004**; 125:392–9.
8. Meier A, Kirschning CJ, Nikolaus T, Wagner H, Heesemann J, Ebel F. Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. Cell Microbiol **2003**; 5:561–70.
9. Dubourdeau M, Athman R, Balloy V, et al. *Aspergillus fumigatus* induces innate immune responses in alveolar macrophages through the MAPK pathway independently of TLR2 and TLR4. J Immunol **2006**; 177:3994–4001.
10. Bellocchio S, Montagnoli C, Bozza S, et al. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol **2004**; 172:3059–69.
11. Bretz C, Gersuk G, Knoblaugh S, et al. MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. Infect Immun **2008**; 76:952–8.
12. Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM. Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. J Biol Chem **2002**; 277:39320–6.
13. Marr KA, Balajee SA, Hawn TR, et al. Differential role of MyD88 in macrophage-mediated responses to opportunistic fungal pathogens. Infect Immun **2003**; 71:5280–6.
14. Jin MS, Kim SE, Heo JY, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell **2007**; 130:1071–82.
15. Kang JY, Nan X, Jin MS, et al. Recognition of lipopeptide patterns by Toll-like receptor 2–Toll-like receptor 6 heterodimer. Immunity **2009**; 31:873–84.
16. Farhat K, Riekenberg S, Heine H, et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol **2008**; 83:692–701.
17. Aimanianda V, Bayry J, Bozza S, et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature **2009**; 460:1117–21.
18. Thau N, Monod M, Crestani B, et al. Rodletless mutants of *Aspergillus fumigatus*. Infect Immun **1994**; 62:4380–8.
19. Kesh S, Mensah NY, Peterlongo P, et al. TLR1 and TLR6 polymorphisms are associated with susceptibility to invasive aspergillosis after allogeneic stem cell transplantation. Ann N Y Acad Sci **2005**; 1062:95–103.
20. Adachi O, Kawai T, Takeda K, et al. Targeted disruption of the MyD88 gene results in loss of IL-1– and IL-18–mediated function. Immunity **1998**; 9:143–50.
21. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: Toll-like receptor 4 (TLR4)–deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol **1999**; 162:3749–52.
22. Takeuchi O, Kawai T, Muhlradt PF, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol **2001**; 13:933–40.
23. Takeuchi O, Sato S, Horiuchi T, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol **2002**; 169:10–14.
24. Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. Science **2003**; 301:640–3.
25. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **2001**; 413:732–8.
26. Roger T, Froidevaux C, Le Roy D, et al. Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. Proc Natl Acad Sci U S A **2009**; 106:2348–52.
27. Delaloye J, Roger T, Steiner-Tardivel QG, et al. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. PLoS Pathog **2009**; 5:e1000480.
28. Hawn TR, Misch EA, Dunstan SJ, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. Eur J Immunol **2007**; 37:2280–9.
29. Omueti KO, Beyer JM, Johnson CM, Lyle EA, Tapping RI. Domain exchange between human Toll-like receptors 1 and 6 reveals a region required for lipopeptide discrimination. J Biol Chem **2005**; 280:36616–25.

30. Bochud PY, Hawn TR, Aderem A. Cutting edge: a Toll-like receptor 2 polymorphism that is associated with lepromatous leprosy is unable to mediate mycobacterial signaling. J Immunol **2003**; 170: 3451–4.
31. Into T, Kiura K, Yasuda M, et al. Stimulation of human Toll-like receptor (TLR) 2 and TLR6 with membrane lipoproteins of *Mycoplasma fermentans* induces apoptotic cell death after NF-kappa B activation. Cell Microbiol **2004**; 6:187–99.
32. Roger T, David J, Glauser MP, Calandra T. MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature **2001**; 414:920–4.
33. Monod M, Jaccoud S, Stirnimann R, et al. Economical microscope configuration for direct mycological examination with fluorescence in dermatology. Dermatology **2000**; 201:246–8.
34. Johnson CM, Lyle EA, Omueti KO, et al. Cutting edge: a common polymorphism impairs cell surface trafficking and functional responses of TLR1 but protects against leprosy. J Immunol **2007**; 178:7520–4.
35. Bozza S, Perruccio K, Montagnoli C, et al. A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. Blood **2003**; 102:3807–14.
36. Lien E, Means TK, Heine H, et al. Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J Clin Invest **2000**; 105:497–504.
37. Grabiec A, Meng G, Fichte S, Bessler W, Wagner H, Kirschning CJ. Human but not murine Toll-like receptor 2 discriminates between tri-palmitoylated and tri-lauroylated peptides. J Biol Chem **2004**; 279:48004–12.
38. Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, et al. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. J Immunol **2005**; 175:6022–31.
39. Kruithof EK, Satta N, Liu JW, Dunoyer-Geindre S, Fish RJ. Gene conversion limits divergence of mammalian TLR1 and TLR6. BMC Evol Biol **2007**; 7:148.
40. Zhou H, Gu J, Lamont SJ, Gu X. Evolutionary analysis for functional divergence of the Toll-like receptor gene family and altered functional constraints. J Mol Evol **2007**; 65:119–23.
41. Roach JC, Glusman G, Rowen L, et al. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A **2005**; 102:9577–82.
42. Kubarenko A, Frank M, Weber AN. Structure-function relationships of Toll-like receptor domains through homology modelling and molecular dynamics. Biochem Soc Trans **2007**; 35:1515–18.
43. Garlanda C, Hirsch E, Bozza S, et al. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. Nature **2002**; 420:182–6.
44. Shimizu T, Kida Y, Kuwano K. Ureaplasma parvum lipoproteins, including MB antigen, activate NF-{kappa}B through TLR1, TLR2 and TLR6. Microbiology **2008**; 154:1318–25.
45. Bas S, Neff L, Vuillet M, et al. The proinflammatory cytokine response to *Chlamydia trachomatis* elementary bodies in human macrophages is partly mediated by a lipoprotein, the macrophage infectivity potentiator, through TLR2/TLR1/TLR6 and CD14. J Immunol **2008**; 180: 1158–68.
46. Tapping RI, Tobias PS. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. J Endotoxin Res **2003**; 9:264–8.
47. Bochud PY, Chien JW, Marr KA, et al. Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. N Engl J Med **2008**; 359:1766–77.
48. Hawn TR, Verbon A, Lettinga KD, et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to Legionnaires' disease. J Exp Med **2003**; 198: 1563–72.
49. Hidaka F, Matsuo S, Muta T, Takeshige K, Mizukami T, Nunoi H. A missense mutation of the Toll-like receptor 3 gene in a patient with influenza-associated encephalopathy. Clin Immunol **2006**; 119: 188–94.