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TLRs Govern Neutrophil Activity in Aspergillosis

Silvia Belloccio,* Silvia Moretti,* Katia Perruccio,* Francesca Fallarino,* Silvia Bozza,* Claudia Montagnoli,* Paolo Mosci,* Grayson B. Lipford,† Lucia Pitzurra,* and Luigina Romani2*

Polymorphonuclear neutrophils (PMNs) are essential in initiation and execution of the acute inflammatory response and subsequent resolution of fungal infection. PMNs, however, may act as double-edged swords, as the excessive release of oxidants and proteases may be responsible for injury to organs and fungal sepsis. To identify regulatory mechanisms that may balance PMN-dependent protection and immunopathology in fungal infections, the involvement of different TLR-activation pathways was evaluated on human PMNs exposed to the fungus *Aspergillus fumigatus*. Recognition of *Aspergillus* and activation of PMNs occurred through the involvement of distinct members of the TLR family, each likely activating specialized antifungal effector functions. By affecting the balance between fungicidal oxidative and nonoxidative mechanisms, pro- and anti-inflammatory cytokine production, and apoptosis vs necrosis, the different TLRs ultimately impacted on the quality of microbicidal activity and inflammatory pathology. Signaling through TLR2 promoted the fungicidal activity of PMNs through oxidative pathways involving extracellular release of gelatinases and proinflammatory cytokines while TLR4 favored the oxidative pathways through the participation of azurophil, myeloperoxidase-positive, granules and IL-10. This translated in vivo in the occurrence of different patterns of fungal clearance and inflammatory pathology. Both pathways were variably affected by signaling through TLR3, TLR5, TLR6, TLR7, TLR8, and TLR9. The ability of selected individual TLRs to restore antifungal functions in defective PMNs suggests that the coordinated outputs of activation of multiple TLRs may contribute to PMN function in aspergillosis. *The Journal of Immunology,* 2004, 173: 7406–7415.

Invasive fungal infections have become the major cause of infectious morbidity and mortality in patients with qualitative or quantitative defects of polymorphonuclear neutrophil (PMN)1 function, graft-vs-host disease, and receipts of steroids for prevention and therapy (1). Consistent with their role, PMNs are the predominant immune cells in the acute stage of most fungal infections and play a crucial role in determining the type of pathology associated with fungal infections in different clinical settings (2, 3). In this regard, PMNs may act as double-edged swords, as they are essential for pathogen eradication but an excessive release of oxidants and proteases may be responsible for injury to organs and fungal sepsis (4). This implicates that, for an efficient control of fungal pathogens, tight regulatory mechanisms are required to balance protection and immunopathology.

Oxidant generation is not sufficient to mediate fungal killing by PMNs without complementary nonoxidative mechanisms mediated by granule constituents (5, 6). A clear distinction exists among the different granules of PMNs. Specific and secretory, myeloperoxidase (MPO)-negative granules are considered to play important roles in initiating the inflammatory response. Accordingly, the MPO-negative granules mainly mediate extracellular release of their protein contents, such as gelatinases (7). In contrast, azurophil MPO-positive granules are viewed as particularly active in the digestion of phagocytosed material (7). Although during phagocytosis PMNs are known to release only small amounts of oxidants in the surroundings (8), the findings that products of the oxidative stress may impede the phagocytic-dependent clearance of inflammatory products (9) and that host proteins rather than those of engulfed microbes are found to be the targets of oxidative reactions in PMNs (10, 11) suggest that reactive oxygen intermediates (ROIs) production may adversely affect the host ability to oppose the inflammatory pathology.

Consistent with their role in immune surveillance, human (12, 13) and murine (14) PMNs express TLRs, a family of conserved, mammalian cellular receptors that mediate cellular responses to structurally conserved pathogen-associated microbial products (15). All TLRs activate a core set of stereotyped responses, such as inflammation (16), although the intricacies of how TLRs signal have provided the molecular basis for tailoring the immune responses to pathogens (17). Understanding this dichotomy may ultimately lead to innovative approaches to the treatment of diseases associated with altered innate immunity.

As TLRs have been implicated in host defense against fungi (2, 18), the present study was undertaken to characterize the functional activity of TLRs in the PMNs response to *Aspergillus fumigatus*, a frequent cause of pneumonia and death in bone marrow-transplanted (BMT) patients (3, 19). To this purpose, the involvement of different

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1 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; MPO, myeloperoxidase; ROI, reactive oxygen intermediate; BMT, bone marrow transplant; ZYM, zymosan; LTA, lipoteichoic acid; FLAG, flagellin; DCF, dichlorodihydrodifluorescein diacetate; SOD, superoxide dismutase; MFI, mean fluorescence intensity; MMP, matrix metalloproteinase; AZL, azide; HEP, heparin; GLIO, glitoxin; DEX, dexamethasone; HPRT, hypoxanthine phosphoribosyltransferase.

2 Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy; and †Coley Pharmaceutical Group, Wellesley, MA 02481

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Address correspondence and reprint requests to Dr. Luigina Romani, Microbiology Section, Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy. E-mail address: lromani@unipg.it

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TLR-activation pathways was evaluated in murine and human PMNs exposed to the fungus in the presence of different TLR ligands. The ability of each individual TLR to activate specialized antifungal effectors on PMNs in response to conidia and hyphae of Aspergillus suggests a possible involvement of TLRs in governing the function of PMNs in aspergillosis.

Materials and Methods

Animals
Female, 8- to 10-week-old C57BL/6 mice were from Charles River Laboratories (Calco, Italy) or The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of homozygous TLR2-, TLR4-, and TLR9-deficient mice, raised on a C57BL/6 background (14), were bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia (Perugia, Italy). Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Microorganisms, culture conditions, and infections
The origin, characteristics, and culture conditions of the strains of A. fumigatus used in this study have already been described (14). Conidia were harvested by extensive washing of the slant culture with 5 ml of 0.025% Tween 20 in saline. For generation of hyphae, resting or swollen conidia were allowed to germinate (>98% germination) by incubation in Sabouraud broth (~20 and 6 h, respectively) at 37°C. For Aspergillus infection, conidia were given intranasally for 3 consecutive days (2×10^7/20 μl saline/injection) to mice immunosuppressed with cyclophosphamide (150 mg/kg i.p.) a day before. Mice were anesthetized by i.p. injection of 2.5% avertin (Sigma-Aldrich, St. Louis, MO). For the quantification of fungal growth in the lungs, the chitin assay was used (20). For histological analysis, sections (3-4 μm) of paraffin-embedded tissues were subjected to H&E staining (20).

TLR ligands
Zymosan (ZYM, as a TLR2 stimulus) from Saccharomyces cerevisiae, lipopolysaccharide (LTA, as a stimulus for TLR2 and TLR6) from Staphylococcus aureus and polysine-polycytidylic acid (poly(I:C)), as a TLR3 stimulus) were purchased from Sigma-Aldrich. Ultra-pure LPS (as a TLR4 stimulus) from Salmonella minnesota Re 595 was from Labogen (Milan, Italy). Flagellin (FLAG, as a TLR5 stimulus) was from Alexis (Vinci, Italy). R848 (a stimulus for TLR7 and TLR8) and CpG oligonucleotides 2006 (CpG-ODN, as a TLR9 stimulus) of proven immunostimulatory sequences were obtained as described (21, 22). Unopsonized ZYM was used to avoid complement receptor activity. To assess for the TLR specificity of selected TLR ligands, assays were done with the human embryonic kidney cell line HEK293 stably transfected with human TLR2 or TLR4 (23). Ultra-pure LPS induced IL-8 production (161±12 pg/ml) in HEK293/TLR4 but not in HEK293/TLR2. ZYM induced IL-8 production (222±19 pg/ml) in HEK293/TLR2 but not in HEK293/TLR4 whereas LTA promoted IL-8 production in HEK293/TLR2 only, although to a low extent (84±12 pg/ml).

Cell purification
Human PMNs were obtained from the heparinized whole blood of healthy donors or BMT patients, who gave their written informed consent, by lysis with ammonium chloride and fractionation by Ficoll Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) centrifugation. The purity of PMNs preparations was >97%, as determined by Giemsa morphology. BMT patient cells received haploidentical, T cell-depleted peripheral blood cells, and underwent conditioning with total-body irradiation followed by administration of thiopeta, antithymocyte globulins, and fludarabine, as described (24). Postgrafting immune suppressive treatment was given for graft-vs-host disease prophylaxis. All postgrafting blood samples used in the present study showed 100% donor chimera, assessed as described (24). Murine CD11b^+Gr-1^+ PMNs were positively selected with magnetic beads (Miltenyi Biotec, Bologna, Italy) (14) from the peritoneal cavity of uninfected wild-type or TLR-deficient mice 8 h after the i.p. injection of 1 ml of endotoxin-free 10% thioglycolate solution. The PMN’s recovery was equivalent (between 15 and 18×10^6 cells/mouse) between wild-type, TLR4-, and TLR9-deficient mice and slightly decreased (between 10 and 12×10^6/mouse) in TLR2-deficient mice. RT-PCR analysis confirmed the absence of the relevant TLR mRNA in TLR-deficient PMNs, either unexposed or after the exposure to Aspergillus conidia and hyphae. Endotoxin was depleted from all solutions with Detoxi-gel (Pierce, Rockford, IL) and/or antagonized by 5 μg/ml polymyxin B added to the cultures. On

FACS analysis, Gr-1^- PMNs were >98% pure and stained positive for the CD11b myeloid marker. No differences in cell viability (between 95 and 98% by the dye exclusion test) were observed on PMNs recovered from the different types of mice.

Phagocytosis, antifungal effector activity, and cultures
For phagocytosis, PMNs were incubated at 37°C with unopsonized Aspergillus conidia for 60 min and the percentage of cells with internalized conidia (% internalization) was calculated on Giemsa-stained preparations (14). For fungicidal activity, PMNs were incubated with unopsonized fungal cells (at an effector to fungal cell ratio of 1:5) for 120 (conidia) or 60 min (hyphae) at 37°C. For the conidialcidal activity, the percentage of CFU inhibition (mean±SE), referred to as conidialcidal activity, was determined as described (20). To assess the damage to the hyphae, viability staining with the fluorescence probe FUN-1 (Molecular Probes, Leiden, The Netherlands) was examined in a Fluorescence Microplate Reader (Titertek II; Flow Laboratories, Milan, Italy) at 485 nm excitation/620 nm emission (25). As controls, hyphae were incubated without cells and were treated with 96% ethanol. For staining of degranulated PMNs, the cytospin preparations were subjected to methylene blue (for azurophil granules) or eosin (for nonazurophil granules) staining following standard procedures (14). The effects of TLR stimulation on PMN functions were assessed by preincubating PMNs with LPS (as a different TLR ligands (unopsonized ZYM (1, 10, and 100 μg/ml), LTA (1, 10, 100 μg/ml), poly(I:C) (10, 50, 100 μg/ml), LPS (1, 10, 100 μg/ml), FLAG (0.1, 1, 10 μg/ml), R848 (100, 300, 900 μM) and CpG ODN 2006 (0.01, 0.1, 1 μM)) before the addition of fungi, as detailed above. Photographs were taken using a high resolution Microscopy Color Camera Axiocam Color, using Axiolvision Software Release 3.0 (Carl Zeiss, Milan, Italy). For cytokine determination, PMNs were stimulated with the different TLR ligands in Iscove’s medium with no serum but with polymixin B for 24 h.

Detection of respiratory burst
PMN production of ROIs was done by either measuring the oxidation of dichlorodihydrofluorescein diacetate (DCF; 5 μM), a cell-permeable compound that becomes fluorescent upon oxidation by hydrogen peroxide and other reactive oxygen species (26), by flow cytometry or quantifying the release of superoxide anion (O2-) in the culture supernatants through the measure of the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c (14). For flow cytometry, the results were expressed as median fluorescence intensity (MFI). For O2^- release was measured in a Microplate Reader Bio-Rad 550 Bio-Rad, Rome, Italy) and the results were expressed as nanomoles O2^-/10^6 cells.

Zymography
Gelatin zymography was performed as described (27). Gelatinase activity was detected as white lysis bands against a blue background and quantitative evaluation of the gelatinolytic activity was performed by scanning the gel using a Bio-Rad Gel DOC 1000 imaging densitometer (Bio-Rad). Gelatinolytic activity of matrix metalloproteinase 9 (MMP9) was determined by scanning the lysis band in the 72-4D area.

MPO assays
Western blotting was performed as described (28). Briefly, samples (10^6 cell equivalent) were probed with rabbit polyclonal anti-human MPO Ab (Calbiochem, San Diego, CA) (1:2000) and visualized using ECL (Amersham Pharmacia Biotech). MPO activity was determined by the oxidation of 3,3’,5,5’-tetrathylbenzidine by H2O2, and light absorption was measured at a 655 nm wavelength. Units of MPO activity (per 10 min) were calculated from a standard curve that used the peroxidase (Sigma-Aldrich) enzyme as the standard enzyme. MPO data were expressed as U/10^6 cells.

Inhibition of PMN antifungal effector function
The selective inhibitors of PMN function included azide (AZI; Sigma-Aldrich), a potent and specific inhibitor of MPO (29–31), heparin (HEP; Sigma-Aldrich), a compound that binds to α-defensins and inhibits their function (32) and glitoxin (GLIO; Sigma-Aldrich), a fungal metabolite that inhibits NADPH oxidase of human PMNs (33). PMNs were preincubated for 15 min at 37°C with 0.1 mM AZI, a concentration known to inhibit MPO activity without altering other PMN functions (31) before being added to resting or germinated conidia. GLIO (1 μg/ml) or HEP (0.5 μM) were added immediately before incubating PMNs with fungi. For degranulation, cells were treated for 18 h with 15 mM strontium chloride (Sigma-Aldrich), a compound devoid of cellular toxicity (34). The treatment did not affect the cell viability or the ability to produce ROIs (data not available).
shown). PMNs were exposed to 0.5 μM dexamethasone (DEX; Laboratorio Farmacologico Milanese, Varese, Italy) and/or TLR ligands for 120 min before the addition of fungi.

Assessment of neutrophil viability and apoptosis
PMNs were exposed to different TLR ligands for 120 min before the addition of conidia and hyphae for a further 120 min. Apoptosis was quantified by staining with the human Annexin VFITC kit (Benden Med System Diagnostic, Vienna, Austria) and analyzed by flow cytometry. Four hours of stimulation was used based on preliminary experiments and according to previous studies (26).

Cytokine assays
The levels of cytokines in culture supernatants were determined by Kit ELISA (R&D Systems, Space Import-Export, Milan, Italy). The detection limits (picograms per milliliter) of the assays were <32 for TNF-α and <3 for IL-10.

RT-PCR
Total RNA was extracted (TRIzol; Invitrogen Life Technologies, Milano, Italy) from peripheral PMNs after the exposure to unopsonized Aspergillus resting conidia or hyphae for 60 min, as suggested by initial experiments. Synthesis and PCR of cDNA were done as described (14). The forward and reverse PCR primers used for murine or human TLRs and hypoxanthine phosphoribosyltransferase (HPRT) were as described (14). The products were subjected to 32 cycles of PCR amplification at 94°C/30 s, 58°C/30 s, and 72°C/2 min. The synthesized PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.

Analysis of p38 and NF-κB activation
The activation of p38 and NF-κB on murine PMNs exposed for 30 min at 37°C to Aspergillus conidia or hyphae was done as described (23). Blots of cell lysates were incubated with rabbit polyclonal Abs recognizing either the unphosphorylated form of p38 MAPK, or the double-phosphorylated (Thr180/Tyr182) p38 MAPK (Cell Signaling Technology, Celbio, Milano, Italy), or Abs specific for the Rel A, 65 kDa DNA binding subunit of human NF-κB (Zymed Laboratories, South San Francisco CA), followed by HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology), as per manufacturer’s instructions. Blots were developed with the Enhanced Chemiluminescence detection kit (Amersham Pharmacia Biotech, Codogno Monzese, Milano, Italy). Bands were visualized after exposure of the blots to Kodak RX film. To ensure similar protein loading in each lane, the phosphoblots were stripped and the membranes were reprobed with Abs against p38 and NF-κB.

Statistical analysis
The Student t test or ANOVA and Bonferroni’s test were used to determine the statistical significance of differences in experimental groups was defined as p < 0.05. The data reported were pooled from five to eight experiments, unless otherwise specified. In vivo groups consisted of four to six animals. The in vivo data are from one representative experiment of five.

Results
TLR mRNA induction and activation in PMNs upon exposure to Aspergillus conidia or hyphae
To evaluate whether the exposure to A. fumigatus may affect the levels of TLR mRNA and TLR-dependent signaling in PMNs, freshly isolated PMNs were assessed for the presence of TLR mRNA by RT-PCR upon exposure to unopsonized conidia or hyphae at different time points. No mRNAs for TLR1, TLR3, TLR5, and TLR7 were observed on human and murine PMNs after the exposure to conidia or hyphae at any time point (data not shown). In contrast, after 60 min of exposure, the levels of TLR9 and TLR4 mRNA were increased upon the exposure to conidia or hyphae and those of TLR2 mRNA upon exposure to conidia on both human and mouse cells were exposed to the different TLR ligands, at different concentrations, and assessed for phagocytosis, fungicidal activity, and quality of antifungal effector mechanisms, such as oxidant production and degranulation. Although PMNs are considered to exert maximal antifungal activity against extracellular hyphae (36), they may also phagocytose and kill resting conidia (20, 37), an activity that may be of benefit to the host in conditions of functional impairment of conidiocidal activity of alveolar macrophages (38) or after swelling of conidia (39). We found that the

FIGURE 1. Kinetics of TLR mRNA induction in PMNs upon exposure to A. fumigatus. A, Peripheral PMNs were exposed for 15, 60, and 120 min to unopsonized resting conidia and hyphae before the assessment of TLR mRNA by RT-PCR. B, Peritoneal murine PMNs were exposed for 60 min. DNA levels were normalized against the HPRT gene. None, Cells exposed to the medium alone. C, Activation of NF-κB and p38 on murine PMNs exposed to Aspergillus conidia and hyphae. The assay was done by probing the cell lysates with specific anti-phospho-38 and anti-NF-κB Abs. Shown are the data from one representative experiment of three.
phagocytosis of conidia was impaired by LTA and poly(I:C) but unaffected by ZYM, LPS, FLAG, R848, and CpG-ODN (Fig. 2A). With respect to the fungicidal activity, only ZYM, LPS, or CpG-ODN either significantly increased (ZYM) or left unaffected the conidiodical and the hyphal damage activities, whereas LTA, poly(I:C), FLAG, and R848 all variably decreased both antifungal effector activities (Fig. 2A). Interestingly, a sustained and prolonged (still present after 20 h of pulsing) killing activity was observed upon exposure to LPS, partly to CpG-ODN but not to ZYM (data not shown). A dose dependency was observed for the above effects but, interestingly, opposite effects were also observed (CpG-ODN) upon increasing the concentrations (Fig. 2B). Therefore, these data suggest that the induction of TLR2, TLR4, and TLR9 expression upon exposure to fungi positively correlates with the induction of antifungal effector functions on PMNs. Interestingly, the pattern of TLR expression may not predict the responsiveness to the relevant TLR ligands.

On assessing the relative contribution of oxidative and nonoxidative mechanisms on the PMNs ability to kill Aspergillus conidia and hyphae, we found that the conidiodical activity was markedly inhibited by GLIO, partially by AZI or by degranulation and unaffected by HEP. In contrast, the ability to damage hyphae was greatly inhibited by AZI and degranulation, and unaffected by GLIO and HEP (Fig. 3). No direct adverse effects on fungal cells were observed with these compounds (data not shown). Therefore, as in macrophages (29, 30), the conidiodical activity of PMNs mainly proceeds by parallel MPO-dependent and -independent pathways while the hyphal damage is dependent on the MPO-dependent system and granule constituents.

To assess the effects of TLR stimulation on these pathways, we measured both the cell-associated (MFI) and the extracellular (O₂⁻) ROI production and degranulation in response to conidia and hyphae in the presence of the different TLRs agonists. We found that ROI production was increased in response to conidia and hyphae and was modified by the different ligands. The intracellular ROI production against conidia was particularly increased by ZYM, inhibited by LTA and poly(I:C), and unaffected by the other TLR ligands. ZYM also increased the extracellular ROI release in response to hyphae along with poly(I:C) and LPS, while LTA decreased it (Fig. 4A). A similar pattern of production, although to a lesser degree, was observed upon stimulation with the TLR ligands alone (data not shown). With respect to degranulation, we found that, in line with previous studies (40), both conidia and hyphae induced PMN degranulation, although to a greater extent by hyphae for the azurophil degranulation. Degranulation of both types of granules were potentiated by poly(I:C), greatly decreased by LTA, and unaffected by FLAG, R848, and CpG-ODN. Interestingly, ZYM appears to mainly promote the nonazurophil degranulation while LPS mainly the azurophil degranulation (Fig. 4B).

To further confirm the differential effects of TLR stimulation on the pattern of degranulation against the fungus, the levels of MMP9, a gelatinase found mainly in nonazurophil granules (7) and MPO, a marker of azurophil granules (7), were measured in culture supernatants of PMNs stimulated with conidia and hyphae and/or TLR ligands. According to the notion that peroxidase-negative granules mainly mediate extracellular release of their protein contents (7), we found that the active form of MMP9 was abundantly released in response to the fungus and to conidia more than hyphae. Quantification of zymograms by densitometric scanning revealed that MMP9-related gelatinolytic activity was significantly increased upon stimulation with ZYM or poly(I:C) only. In contrast, the release of MPO occurred more in response to hyphae than conidia and was increased by poly(I:C) and LPS. Both productions were decreased by LTA, R848, and CpG-ODN (Fig. 4C).

Together, these data indicated that the expression of the antifungal effector functions of PMNs may occur through the involvement of distinct members of the TLR family, each likely activating

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**FIGURE 2.** Antifungal activities of PMNs. A, Purified human PMNs from peripheral blood were pretreated with ZYM (10 μg/ml), LTA (10 μg/ml), poly(I:C) (50 μg/ml), LPS (10 μg/ml), FLAG (1 μg/ml), R848 (300 nm), and unmethylated CpG oligonucleotide (CpG, 0.06 μM) or different concentrations of the above TLR ligands (B) for 120 min before the assessment of effector functions after a subsequent incubation with resting conidia (120 min) or hyphae (60 min). For phagocytosis and fungidical activity, see Materials and Methods. B, Values are the mean ± SE of samples taken from five independent experiments. *, p < 0.05, with and without (None) TLR ligands.

**FIGURE 3.** Effects of various inhibitors on PMN antifungal effector functions. Peripheral blood PMNs were preincubated for 15 min at 37°C with 0.1 mM AZI before being added to resting or germinated conidia. GLIO (1 μg/ml) or HEP (0.5 U/ml) were added immediately before incubating PMNs with fungi. For degranulation, cells were treated for 18 h with 15 mM strontium chloride. Each treatment did not directly affect viability of fungi. *, p < 0.05, with or without (None) inhibitors.
specialized antifungal effector functions (Table I). ZYM appears to promote the fungicidal activity of PMNs through oxidative pathways involving primarily nonazurophil granules while LPS appears to favor the oxidative pathways through the participation of azurophil granules. However, ZYM, more than LPS, favors the extracellular degranulation. Both pathways were not significantly affected by CpG-ODN, whereas, along with the phagocytosis, they were greatly inhibited by LTA.

**TLRs modulate PMN apoptosis and necrosis**

As PMNs become apoptotic once they have undergone phagocytosis and oxidase products are implicated in the process (41), we measured PMN apoptosis and necrosis after exposure to conidia or hyphae in the presence of the different TLR ligands. Both apoptosis and necrosis were observed upon exposure to the fungus and, interestingly, apoptosis was significantly (p < 0.05) increased by exposure to hyphae and necrosis in response to conidia (from 17% of apoptotic cells in unstimulated PMNs to 25 and 15% in response to hyphae and conidia, respectively; from 4% of necrotic cells of unstimulated PMNs to 13 and 6% in response to conidia and hyphae, respectively). Different effects were observed with the different TLR ligands, being that the necrosis was markedly (>2-fold) fostered by ZYM and poly(I:C), slightly (<2-fold) by R848 and CpG-ODN, and unaffected by LPS. The PMN death was unaffected by LPS even after 20 h of incubation (data not shown).

Interestingly, apoptosis was greatly (>3-fold) increased by LTA, despite the low ROI production observed, a finding that may disprove a general role for ROIs in the induction of apoptosis. These data suggest that an action on PMN survival and mechanism of death is a further mechanism through which TLRs may govern immunopathology associated with the clearance of fungi.

**TLRs affect cytokine production by PMNs**

As PMNs release cytokines in response to fungi (2), we analyzed the production of TNF-α and IL-10 following stimulation with the different TLR ligands. The production of TNF-α was observed in response to each stimulus, although to a variable degree. However, the concomitant production of IL-10 was only observed in response to LTA and LPS (Fig. 5). Therefore, the balance between proinflammatory and anti-inflammatory cytokine production is differentially affected by the different stimuli.

**TLRs govern clearance of the fungus and histopathology in vivo**

Because both TLR2 and TLR4 have been implicated in the response of human PMNs to LPS (12) and ODN may also signal in a TLR9-independent manner on human PMNs (42), we resorted to TLR2-, TLR4-, and TLR9-deficient mice to assess the fungicidal activity of PMNs together with parameters of inflammatory pathology in the lungs of infected mice. The conidiocidal activity was greatly reduced in TLR4-deficient PMNs, partially reduced in TLR2-deficient PMNs, and slightly increased in PMNs from TLR9-deficient mice, while the hyphal damage activity was impaired in TLR4-deficient PMNs only and actually significantly increased in TLR9-deficient PMNs (Fig. 6A). Therefore, despite some redundancy in TLRs usage, TLR4 signaling is essential for the antifungal effector activity of PMNs against both conidia and hyphae of *Aspergillus*.

The assessment of the fungal burden in the lungs of TLR-deficient mice with aspergillosis revealed that the fungal burden was higher in TLR2- or TLR4-deficient and lower in TLR9-deficient as compared with wild-type mice (Fig. 6A). In contrast, histological examination of the lungs revealed that the inflammatory pathology was greatly reduced in TLR2- or TLR9-deficient mice but not in TLR4-deficient mice. The nonazurophil degranulation was virtually absent in TLR2-deficient PMNs, while the azurophil degranulation was either decreased in TLR4-deficient PMNs or, interestingly, increased in TLR9-deficient PMNs (Fig. 6B).

Together, these data suggest that the control of the infection and resolution of the inflammatory pathology are tightly regulated processes upon which TLRs exert disparate activity. The inflammatory pathology associated with the clearance of the fungus appeared to be associated with the TLR2-dependent activation of the oxidant pathway and nonazurophil degranulation and largely independent of azurophil degranulation.

**TLR agonists restore the antifungal activity of defective PMNs**

The elevated morbidity and mortality due to fungal sepsis in bone marrow transplantation or corticosteroid-treated patients (3)
prompted us to evaluate whether the impaired PMN function induced by corticosteroid treatment or observed in bone marrow transplantation could be rescued by selective TLR agonists. To this purpose, the antifungal effector activity was assessed on PMNs from either healthy donors upon treatment with DEX in vitro or from BMT patients at a different time posttransplantation. Consistent with the finding that ROI production by PMNs was unaffected by DEX (data not shown), the ability to damage hyphae more than the conidiocidal activity was impaired by the steroid. However, the defective activity could be rescued by signaling through TLR2, TLR4, and TLR9 (Fig. 7A). In BMT patients, the conidiocidal activity of PMNs was low in the early period posttransplant but was gradually recovering during the subsequent period (Fig. 7B), despite a similar degree of phagocytic ability (from 65% at 30 days to 80% at 180 days). Here again, the antifungal activity was fostered by signaling through TLR2, TLR4, and TLR9.

**Discussion**

The present study shows that TLRs, by activating the antifungal state of PMNs through distinct mechanisms, play an important role in governing the functions of PMNs in fungal infection and the associated inflammatory pathology. Variable expression of TLRs has been reported on human PMNs (12, 13). Nevertheless, the ability of TLR agonists to activate PMN functions suggests that TLRs are important pattern recognition receptors for PMNs (13). TLR2, TLR4, and TLR9 all have been implicated in host defense against *A. fumigatus* (2). We found here that these as well as other TLRs mediate the recognition and response to *Aspergillus* by PMNs in a morphotype-specific manner. The various fungal forms...
FIGURE 7. TLR agonists restore antifungal activity of human PMNs treated with dexamethasone (DEX) or derived from BMT patients. A, PMNs were exposed to 0.5 μM DEX and/or TLR ligands (see legend to Fig. 2 for details) for 120 min prior the addition of resting or germinated conidia. *, p < 0.05, with and without (None) DEX; **, p < 0.05, TLR ligands vs no TLR ligands (DEX alone). B, PMNs, from at least two BMT patients for each time point after transplant, were exposed to resting conidia in the presence of the different TLR agonists. The results are the mean ± SE of the different samples, each tested twice. *, p < 0.05, with and without (None) TLR ligands. **, p < 0.05; PMNs at 60 or 180 days vs PMNs at 30 days.

not only elicit a different response but also differ in their susceptibilities to cellular microbicidal mechanisms. Coordination of responses for fungicidal activity by PMNs is complex, requiring interactive effects of multiple PMN products (4). In line with the finding that deficiency of both the oxidative and nonoxidative systems impairs the killing of the fungus (43, 44) and may predispose to the infection (30), we found here that, similar to macrophages (43), the oxidative mechanisms mainly operate in the destruction of conidia, while the oxidative burst in combination with nonoxidative mechanisms is responsible for hyphal damage by PMNs. It is of interest that the fungal metabolite GLIO, a putative virulence factor, that specifically inhibits NF-κB (45) and NADPH oxidase (33), greatly impaired the conidocidal activity of PMNs, leaving unaffected the ability to damage hyphae.

Similar to what was observed with murine PMNs (14), ZYM stimulated and LPS prolonged the fungicidal activity of PMNs. However, different mechanisms of antifungal activity appeared to be at work. ZYM promoted the fungicidal activity of PMNs through the elicitation of the oxidative pathway and the involvement of granules producing MMP9 while LPS favored the oxidative pathway through the involvement of azurophil granules releasing mainly MPO. As the quantity and specificity of delivery of toxic PMN products ultimately determine the relative efficiency of fungicidal activity vs inflammatory cytotoxicity to host cells (5), it follows that qualitative different responses originated upon TLR2 or TLR4 signaling on PMNs, although the contribution of additional receptors (such as the β-glucan and mannose reccep-
mechanism that fails to provoke a proinflammatory response (54). It is therefore very interesting that the anti-inflammatory state induced by LTA is associated with fostered apoptosis, while the proinflammatory state associated with TLR2, and partly TLR7 and TLR9 stimulation, involves fostered PMN necrosis. Although LPS promoted PMN survival (55), it did not affect PMN survival in response to Aspergillus. However, the concomitant production of IL-10 induced by LPS may tip the balance toward an anti-inflammatory state. Therefore, by regulating PMN survival and type of death or, alternatively, because of the differential action on cell apoptosis and necrosis observed upon their activation, TLRs may directly impact on type of inflammatory pathology associated with PMN recruitment and activation in fungal infections. These results reinforce the concept that TLR dysfunction may play a role in the proinflammatory response and pathophysiology of polymicrobial sepsis (17, 56).

Although the fact that TLR signaling may result in contrasting outputs in different types of effector cells (57), including in response to Aspergillus (14, 18, 58–60), provides additional complexity to the system, it is reasonable to believe that manipulation of TLRs by selective agonists might provoke divergent sequences and magnitudes of functional responses, so that diverse outcomes ultimately may transpire (Table 1). Although much remained to be learned as to whether TLR ligands exert their effects directly through TLR signaling or through other indirect mechanisms, the ability of selected TLR ligands to restore the defective antifungal activity of PMNs from BMT patients or to antagonize the steroid-induced immunosuppression suggests that the coordinated outputs of activation of multiple TLRs may govern the function of PMNs in aspergillosis.

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