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Toll-Like Receptor 9-Dependent Immune Activation by Unmethylated CpG Motifs in Aspergillus fumigatus DNA

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Phagocytic defenses are critical for effective host defenses against the opportunistic fungal pathogen Aspergillus fumigatus. Previous studies found that following challenge with A. fumigatus, Toll-like receptor 9 (TLR9) knockout mice survived longer than wild-type mice. However, the mechanism responsible was not defined. Here we demonstrate that A. fumigatus contains unmethylated CpG sequences, the natural ligands for TLR9. A. fumigatus DNA and synthetic CpG-rich oligodeoxynucleotides (ODNs) containing sequences found in the A. fumigatus genome potently stimulated the production of proinflammatory cytokines in mouse bone marrow-derived dendritic cells (BMDCs) and human plasmacytoid dendritic cells. The response was decreased when the fungal DNA was treated with a CpG methylase or with CpG-specific endonucleases. A role for TLR9 was demonstrated as cytokine production was abolished in BMDCs from TLR9-deficient mice. Moreover, transfection of HEK293 cells with human TLR9 conferred responsiveness to synthetic CpG-rich ODNs containing sequences found in A. fumigatus DNA. Taken together, these data demonstrate that TLR9 detects A. fumigatus DNA, resulting in the secretion of proinflammatory cytokines, which may contribute to the immune response to the pathogen.

Aspergillus fumigatus is a widely distributed opportunistic fungal pathogen. Exposure generally occurs when airborne spores (conidia) are inhaled into the lungs or sinuses (27). While inhalation of conidia rarely causes disease in the normal host, immunocompromised persons are prone to develop invasive aspergillosis (9). Despite new additions to the antifungal armamentarium, mortality rates in those with established disease exceed 50 percent (9). Clinical and experimental studies have demonstrated that the innate immune responses of phagocytes are essential for effective host defenses against A. fumigatus (27, 31).

A critical component of innate defenses is the ability of phagocytes to recognize structurally unrelated and evolutionarily conserved microbial constituents referred to as “pathogen- (or pattern-) associated molecular patterns” (PAMPs) (1). Among the receptors that recognize PAMPs are the mammalian Toll-like receptors (TLRs), a family of at least 12 members that initiate signaling pathways through a series of adaptor proteins, including MyD88, TIRAP/MAL, TRIF, and TRAM. This results in activation of downstream signaling pathways and the subsequent production of inflammatory cytokines and the initiation of adaptive immune responses. Some TLRs, including TLR1, TLR2, TLR4, TLR5, and TLR6, are primarily expressed on the plasma membrane where they recognize specific molecules on the surfaces of microbes, including fungi (1, 18). Others, including TLR3, TLR7, TLR8, and TLR9, are found in intracellular compartments and signaling responses require ligand internalization (1, 16).

TLR9 is activated by DNA rich in unmethylated CpG motifs, whereas DNA lacking such sequences is either inert or inhibitory (17). Studies using CpG-containing oligodeoxynucleotides (ODNs) have defined sequences that stimulate immune responses (14). The optimal CpG motif appears to be GACGTT for mice and GTACGT for humans. The number of CpG motifs and the spacing of the motifs also influence immunostimulatory capacity. A-class CpG-containing ODNs strongly stimulate plasmacytoid dendritic cell (pDC) alpha interferon (IFN-α) responses but poorly induce B-cell proliferation (14). In contrast, ODNs of the B class are weak inducers of pDC IFN-α but strong inducers of B-cell proliferation. C-class ODNs have intermediate effects.

In the human, TLR9 is found mainly on pDCs and B cells. In contrast, the cellular distribution of TLR9 in the mouse is much broader and includes myeloid dendritic cells, pDCs, monocytes, macrophages, and B cells. Clinical trials of CpG DNA, used as an adjuvant in vaccines and as part of treatment for infectious diseases and neoplasms, are ongoing (12). Originally, TLR9 was thought to be specific for bacterial and viral DNA (both of which are rich in unmethylated CpG motifs). However, recent studies have suggested a role for TLR9 in the recognition of eukaryotic DNA. Mammalian DNA, when complexed with anti-DNA antibodies, is a potent self-antigen for TLR9 and may play a role in promoting systemic lupus erythematosus and other autoimmune diseases (2). Moreover, TLR9-dependent stimulation of murine DC by malarial DNA has been demonstrated (24).

A possible role for TLR9 in host defenses against fungal infections was suggested by Belliocchio et al. (3, 4). TLR9−/− mice paradoxically survived longer than wild-type (WT) mice...
following challenge with Candida albicans hyphae and A. fumigatus conidia. Compared with WT mice, the TLR9−/− mice had an organ fungal burden that was significantly lower and a shift in Th1/Th2 reactivity in favor of Th2 cells. Moreover, the TLR9−/− mice infected with A. fumigatus had a markedly reduced inflammatory response in the lungs. These data led us to hypothesize that fungal DNA contains unmethylated CpG motifs capable of stimulating TLR9 and thereby influencing the host response to fungal challenge. Here we demonstrate that DNA obtained from A. fumigatus stimulates TLR9-dependent responses in human and murine cells.

MATERIALS AND METHODS

Reagents. Reagents, unless otherwise stated, were obtained from Sigma- Aldrich (St. Louis, MO). Dulbecco’s minimal essential media and RPMI 1640 media were obtained from Gibco (Invitrogen, Carlsbad, CA). Low-endotoxin fetal bovine serum (FBS) was obtained from Tissue Culture Biologicals (Tulare, CA). R10 media consisted of RPMI 1640 supplemented with 100 μM penicillin, 100 μM streptomycin, 2 mM l-glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FBS. Excherichia coli K-12 DNA containing undetectable levels of endotoxin was purchased from Invivogen (San Diego, CA). ODNs (see Table S1 in the supplemental material) based upon the sequences found in the A. fumigatus genome were synthesized on a phosphothiorate backbone by Alpha DNA (Canada). Stimulatory CpG 1826, CpG 2007, and CpG 2336 ODNs and control CpG 2317 and CpG 2243 ODNs with phosphothiorate linkages were purchased from Coley Pharmaceuticals (Wellesley, MA).

Genomic DNA preparation. DNA was obtained from two strains of A. fumigatus. Strain AF293 was obtained from the Fungal Genetic Stock Center (University of Missouri, Kansas City, MO). The genome of this strain has been sequenced (23). The other strain of A. fumigatus used was a clinical isolate (21). The data shown are from DNA from strain AF293, except for Fig. 2, for which DNA from the clinical isolate was utilized. In preliminary studies, the two sources of DNA stimulated similar quantities of cytokines (data not shown). A. fumigatus was grown on minimal liquid media consisting of yeast extract supplemented with 2.5% glucose and 3.4 g/liter yeast nitrogen base supplemented with ammonium sulfate and amino acids at 37°C for 40 to 48 h. Fungi were harvested through a nylon mesh filter and washed with Tris-sodium-EDTA (0.05 M Tris-HCl, pH 8.0, 0.150 M NaCl, 0.1 M EDTA, pH 8) (28). Subsequently, hyphae were freeze-dried and ground to a powder, and the DNA was extracted with chloroform/isopropanol. The DNA was treated with 20 μg/ml of RNase B and 20 μg/ml of proteinase K and further extracted with ethanol. Extracted fungal DNA was purified over a cesium chloride gradient by centrifugation at 30,000 rpm (Beckman Ultracentrifuge L-80 M) for 40 h at 20°C. Purified DNA collected from the gradient was extracted with saturated N-butanol and further extracted by ethanol extraction. DNA was then tested for the presence of glucans and endotoxin using a Limulus amebocyte lysate test (Cape Cod Associates). Endotoxin levels were found to be ≤0.03 endotoxin units/μg of DNA.

Enzyme treatment of DNA. Isolated genomic A. fumigatus DNA was subjected to CpG methylation using the CpG methyltransferase M.SssI (NEB). The methylation reaction was performed following the manufacturer’s instructions. In addition, DNA that was either methylated or untreated was subjected to cleavage at CpG sequences by restriction enzyme digestion with the isoschizomers HpaII and MspI. These two endonucleases cleave DNA containing unmethylated CpG DNA and an NF-κB-driven luciferase construct (HEK/hTLR9/NF-kB) and HEK293 cells stably transfected with hTLR7 and an NF-κB-driven luciferase construct (HEK/hTLR7/NF-kB) were a gift from the Eisiai Research Institute (Andover, MA). Cells were maintained in tissue culture flask cultures containing Dulbecco’s minimal essential media supplemented with 10% FBS. Cells were treated with the ODNs at the specified concentrations with a final volume of 200 μl RPMI 1640 supplemented with 10% fetal calf serum and 10 mg/ml recombinant IL-3 (R&D Systems, Minneapolis, MN). After 20 h of culture in a 37°C/5% CO2 incubator, supernatants were collected and measured using a commercially available ELISA kit (Bender MedSystems module set; Burlingame, CA). Samples were assayed in duplicate at dilutions that fell within the range of the standard curves.

Luciferase assay. HEK293 cells stably transfected with human TLR9 (hTLR9) and an NF-κB-driven luciferase construct (HEK/hTLR9/NF-kB) were used to determine if genomic DNA extracted from A. fumigatus contained unmethylated CpG motifs. DNA was treated with HpaII and MspI. These two endonucleases cleave DNA containing the sequence 5′...CGCG...3′. However, they differ
in that HpaII only cleaves unmethylated CpG sequences whereas MspI cleaves both methylated and unmethylated CpG sequences. Both HpaII and MspI cleaved A. fumigatus DNA (Fig. 1). To demonstrate the specificity of the cleavage reaction, the DNA was treated with the CpG methyltransferase M.SssI prior to restriction enzyme digestion. Methylation abolished the capacity of HpaII but not MspI to cleave the DNA. These results demonstrate that A. fumigatus DNA contains unmethylated CpG sequences.

A. fumigatus induces TNF-α and IL-12p70 production in mouse BMDCs. In order to determine if A. fumigatus DNA was immunostimulatory, we measured TNF-α and IL-12p70 release following incubation of the DNA with BMDCs. Cells treated with the fungal DNA potently stimulated production of both cytokines, with concentrations observed similar to those seen following stimulation with E. coli DNA and the synthetic ODN CpG 1826 (Fig. 2). However, when the cells were treated with enzymatically methylated A. fumigatus DNA, the cytokine response was nearly completely abolished. Similarly, treatment of the DNA with the CpG-specific endonucleases HpaII and MspI potently reduced cytokine production.

A. fumigatus DNA stimulates TNF-α secretion in a TLR9-dependent manner. Given the role of TLR9 as an intracellular sensor of unmethylated CpG-rich DNA, we next compared cytokine responses of BMDCs derived from WT and TLR9−/− mice following stimulation with A. fumigatus DNA. In the BMDCs from WT mice, native DNA stimulated TNF-α release in a dose-dependent manner (Fig. 3). Methylated DNA also induced TNF-α production, although lower levels were
detected than with untreated DNA. However, neither untreated nor methylated *A. fumigatus* stimulated TNF-α release from TLR9−/− BMDCs.

**DNA from *A. fumigatus*** stimulates the production of type I IFN in human pDCs. Immunostimulatory CpG-rich motifs differ between mice and humans (8). Therefore, we next determined whether *A. fumigatus* DNA would also stimulate human pDCs, a cell type which highly expresses TLR9 (30). *A. fumigatus* DNA stimulated IFN-α production in human pDCs obtained from seven different donors (Fig. 4A) in a dose-dependent fashion (Fig. 4B). As was observed with murine dendritic cells, cytokine production was significantly decreased if the DNA was methylated.

**ODNs containing CpG-rich motifs present in the *A. fumigatus* genome stimulate signaling and cytokine responses in a TLR9-dependent manner.** Next, we took advantage of the nearly complete (98.0%) deciphering of the *A. fumigatus* genome to determine the GC content of the genome and to search for putative immunostimulatory CpG-rich motifs in *A. fumigatus* DNA. The GC content of the sequenced portion of the *A. fumigatus* genome was determined to be 49.8%. Moreover, the percentage of CpG dinucleotide sequences was 5.35%. Using the conservative search criteria described in Materials and Methods, 23 and 87 potential murine and human immunostimulatory motifs were identified, respectively (see Table S1 in the supplemental material). Samples of these ODNs were then synthesized on a phosphothiorate background and tested for their capacity to stimulate murine BMDCs and human TLR9-transfected HEK293 cells.

The six synthesized ODNs containing murine-like motifs stimulated BMDCs derived from WT mice to secrete TNF-α, although there was considerable variation in their stimulatory capacities (Fig. 5A). The most potent of the ODNs, mAF2 and mAF3, stimulated TNF-α concentrations that were nearly as high as that seen with CpG 1826. In contrast, cytokine levels stimulated by mAF3 and mAF6 were barely above the background. None of the ODNs stimulated cytokine release from TLR9−/− BMDCs above background levels (Fig. 5B).

Next, ODNs representing human-like CpG motifs were assessed for their capacity to stimulate HEK293 cells stably co-transfected with hTLR9 and an NF-κB-driven luciferase reporter gene. All ODNs tested induced luciferase activity, with the level of activity generally increasing in a dose-dependent manner (Fig. 6). As was observed with the murine-like motifs, the stimulatory capacities of the individual ODNs were quite variable. hAF1, which occurs nine times in the *A. fumigatus* genome, was at least as potent as the positive control, CpG 2007. The control ODN, GpC 2137, did not induce the activation of the TLR9-transfected cells. The ODNs tested in Fig. 6 did not induce significant luciferase activity in HEK293 cells stably transfected with hTLR9 and an NF-κB-driven luciferase reporter gene (data not shown).
DISCUSSION

Innate immunity plays a critical role in microbial defenses by allowing the host to rapidly respond to a challenge and by providing a bridge to adaptive immunity. The strong association of invasive aspergillosis with qualitative and quantitative disorders of phagocyte function suggests that innate recognition of *Aspergillus* spp. is of particular importance in defending against these ubiquitous fungi. Previous studies by our laboratory and others have demonstrated that following incubation of phagocytes with *A. fumigatus* conidia and hyphae, TLR2- and TLR4-dependent signaling cascades are stimulated, leading to proinflammatory cytokine production (6, 19, 21, 25, 26). Here, we demonstrate a putative contribution of another TLR, TLR9, to defenses against aspergillosis.

Whereas the ligands for TLR2 and TLR4 on *A. fumigatus* remain undefined, several lines of evidence establish unmethylated CpG-rich fungal DNA as the ligand for TLR9. First, using restriction endonucleases and DNA methylases, the presence of unmethylated CpG sequences (the natural ligand for TLR9) in *A. fumigatus* DNA was demonstrated. Second, stimulation of murine DC from TLR9−/− mice with *A. fumigatus* DNA resulted in greatly impaired cytokine production.

![Graph showing TNF-α production in WT and TLR9−/− BMDCs](image1)

**FIG. 5.** Stimulation of murine BMDCs by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome. BMDCs from WT (top panel) and TLR9−/− (bottom panel) mice were stimulated with the indicated mouse-like CpG motifs (see Table S1 in the supplemental material). CpG 1826 served as a positive control, while unstimulated (Unstim) cells and GpC 2137 were negative controls. After 24 h, supernatants were collected and TNF-α concentrations were determined by ELISA. Data are means ± SE of a representative (out of three) experiment performed in triplicate.

![Graph showing TNF-α production in WT and TLR9−/− BMDCs](image2)

**FIG. 6.** Stimulation of hTLR9-transfected HEK293 cells by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome. HEK293 cells stably coexpressing hTLR9 and the NF-κB-driven luciferase reporter construct were stimulated with the indicated human-like CpG motifs (see Table S1 in the supplemental material). CpG 2007 served as a positive control, while GpC 2137 was a negative control. After 18 h, luciferase activity was determined in the cell lysates. Results are expressed as n-fold induction over unstimulated cells. Data are means ± SE of a representative (out of four) experiment performed in triplicate.
compared with that seen with WT DC. Nevertheless, the possibility that some stimulation induced by the A. fumigatus DNA was due to mechanisms independent of TLR9 cannot be excluded. DC from TLR9−/− mice did secrete some residual TNF-α after treatment with A. fumigatus DNA. Similarly, some residual cytokine secretion was observed after stimulation of WT DC with methylated DNA. This residual response could be due to other intracellular DNA sensors, including DAI (DNA-dependent activator of IFN-γ-regulatory factors) (29) and an as-yet-unidentified sensor dependent on TANK-binding kinase 1 (10).

In a survey of 15 bacterial species, the immunostimulatory capacity of bacterial DNA samples directly correlated with the frequency of CpG dinucleotides (7). In that study, the CpG frequency ranged from 1.44% to 12.21%. Our analysis of the A. fumigatus genome determined the frequency of CpG dinucleotides to be 5.35%, similar to that of bacterial DNA. Mammalian DNA is thought to be less immunostimulatory than bacterial DNA because the frequency of the CpG motif is suppressed. In addition, mammalian, but not bacterial, DNA is highly methylated (1). Although mammals and fungi share membership in the eukaryotic kingdom, we found that CpG motifs in A. fumigatus DNA had a low level of methylation. Thus, the pattern of degradation of A. fumigatus DNA following treatment with HpaII, an endonuclease specific for unmethylated CCGG sequences, was similar to the pattern seen after treatment with MspI, an endonuclease which cleaves both methylated and unmethylated CCGG sequences. Consistent with these findings, A. fumigatus DNA stimulated dendritic cell cytokine responses comparable to those stimulated by E. coli DNA, which has a CpG frequency of 7.27%.

An in silico genome-wide analysis of the fungal DNA demonstrated the abundant presence of CpG-rich motifs of the type predicted to be stimulatory for mouse and human TLR9. The analysis was conservative and additional stimulatory motifs likely exist within the genome. Synthetic ODNs containing CpG-rich motifs found in A. fumigatus DNA stimulated mouse and human cells. Interestingly, of the synthetic ODNs tested, the one that was most stimulatory had a sequence which appeared the most often in the A. fumigatus genome. The potential for this ODN to be used as an immunostimulant in humans should be considered given that it stimulated human cells as well as a CpG-rich ODN that is undergoing clinical trials.

The relative contribution of A. fumigatus DNA to the immunology of human aspergillosis remains to be determined. Future studies will examine the conditions under which DNA is released from the fungal cell, an event that presumably would be a prerequisite for an interaction with TLR9 to occur. In addition, in its tissue-invasive hyphal phase, A. fumigatus is mostly an extracellular pathogen and TLR9 is located intracellularly in the endosomal compartments. Nevertheless, disparities between WT and TLR9−/− mice with regards to their susceptibilities to aspergillosis (3) suggest that the interaction of A. fumigatus DNA and TLR9 occurs in vivo. In addition, a recent study examined variants in TLR genes in a population of Italian children with hematological malignancies (15). The frequency of the C allele of the TLR9 T-1486C polymorphism was significantly higher in patients with invasive mold infections than in patients without invasive fungal infections. Furthermore, a recent study (5) found an association of the TLR9 T-1237C polymorphism with allergic bronchopulmonary aspergillosis, a form of aspergillosis mainly found in patients that suffer from asthma and cystic fibrosis.

An inflammatory response is beneficial to the host if it enables the host to contain or eliminate the pathogen. However, the immune response can be detrimental if it results in damage to host tissues. The finding that TLR9−/− mice survived longer than WT mice following challenge with A. fumigatus suggests that, at least in some circumstances, the response to A. fumigatus DNA favors the pathogen rather than the host. The nature of the immune response might be important too. In addition to direct inflammatory effects resulting from stimulation of proinflammatory cytokines, including type I IFNs, CpG-rich DNA biases toward Th1-type responses (13). Finally, while our studies focused upon A. fumigatus, it is likely that DNA from other medically important fungi also contain unmethylated CpG-rich motifs capable of stimulating immune responses.

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REFERENCES


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