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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 GTCGTT 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 Bellocchio et al. (3, 4). TLR9$^{-/-}$ mice paradoxically survived longer than wild-type (WT) mice

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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 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 50 μM β-2-mercaptoethanol, and 10% heat-inactivated FBS. *Escherichia 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 GpC 2137 and GpC 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 with 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 L8-80 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 MspI or HpaII. Restriction digestion was confirmed by 1% agarose gel electrophoresis of the treated and untreated samples. The sizes of the resulting digested DNA fragments were compared to a DNA ladder (NEB).

Mice. All mice were specific pathogen free and housed in the University of Massachusetts Medical Center animal facility. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The TLR9-deficient mice were obtained from Robert Finberg (UMass Medical School), who obtained the mice from Shizuo Akira (Osaka, Japan). The knockout mice were backcrossed 12 times to a C57BL/6 background.

Generation and stimulation of BMDCs. Bone marrow-derived dendritic cells (BMDCs) were obtained according to the protocol of Lutz et al. (20) and as in our previous studies (11, 22, 33). Briefly, mice were euthanized and bone marrow cells were harvested from the femurs and tibiae. After treatment with red blood cell lysis buffer, the remaining cells were suspended at a final density of 1×10^6 cells per ml in R10 media supplemented with 10% supernatant from J558L cells (as a source of granulocyte-macrophage colony-stimulating factor). Cells were seeded in non-tissue-culture-treated petri dishes and incubated at 37°C in air supplemented with 5% CO_2. The media were changed every 3 days. On day 8 or 9, cells were harvested and the BMDCs were positively selected on a magnetic column using CD11c-coated magnetic beads, as per the manufacturer's instructions (Miltenyi Biotech).

BMDCs (2×10^5 cells per well) were added to 48-well plates containing a final volume of 500 μl media (R10 with 10% J558L supernatant). BMDCs were transfected with DNA using 30 μg/ml of *N*-[1-(2,3-dioloyloxy)propyl-*N*,*N*,*N*-trimethylammonium methylsulfate] (DOTAP; Roche) according to the manufacturer's instructions. Briefly, DNA and DOTAP were mixed together in HEPES-buffered saline and incubated for 15 min at 25°C. The DNA-DOTAP complexes were then added to BMDCs and further mixed by pipetting. Treated cells were incubated for 24 h at 37°C in air supplemented with 5% CO_2. Supernatants were collected and cytokine production was analyzed for tumor necrosis factor alpha (TNF-α) and interleukin 12p70 (IL-12p70) by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, CA).

Isolation and stimulation of human pDCs. pDCs were isolated as described previously (32). Briefly, peripheral blood was obtained by venipuncture following informed consent from healthy volunteers using a protocol approved by the University of Massachusetts Medical Center Institutional Review Board. Blood was anticoagulated with heparin and diluted 1:1 with Hanks balanced salt solution (BioWhittaker, Walkersville, MD), and the peripheral blood mononuclear cells (PBMCs) were collected following Ficoll-Hypaque (Lymphoprep, Westbury, NY) density gradient centrifugation. Human pDCs were positively selected from the PBMCs using CD304-coated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

pDCs (5×10^4) were stimulated with DNA-DOTAP complexes in 96-well flat-bottom plates containing a final volume of 200 μl RPMI 1640 supplemented with 10% fetal calf serum and 10 ng/ml recombinant IL-3 (R&D Systems, Minneapolis, MN). After 20 h of culture in a 37°C/5% CO_2 incubator, supernatants were collected and human IFN-α levels were 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-κB) and HEK293 cells stably transfected with hTLR7 and an NF-κB luciferase construct (HEK/hTLR7/NF-κB) were a gift from the Eisai Research Institute (Andover, MA). Cells were maintained in tissue culture flasks containing Dulbecco's minimal essential media supplemented with 10% FBS. Cells were treated with the ODNs at the specified concentrations at a final volume of 150 μl/well and incubated at 37°C in air supplemented with 5% CO_2 for 18 h. Luciferase activity in cell lysates was measured using a Steady Glo luciferase assay system (Promega, Madison, WI) on a luminometer (Envision; Perkin Elmer).

Genome-wide scanning for GC content, CpG dinucleotides, and TLR9 stimulatory sequences. Both mouse- and human-like motifs were searched for in both DNA strands of the assembled contigs (AF.contigs.031704; release date 12 May 2007) downloaded from the Sanger ftp site (ftp://ftp.sanger.ac.uk/pub/pathogens/A_fumigatus). Sequences matching the following patterns were selected: human like, [CT][CT]GTCGTTN(0,4)GTCGTT; and mouse like, [CT][CT]GACGTTN(0,4)GACGTT. Residues in brackets indicate ambiguities acceptable in the specified positions, N indicates any nucleotide, and the numbers in parentheses indicate the number of times residues can appear in that position. Mismatches were allowed only at the eighth or the last position. GC content was calculated from the number of G's and C's found for one strand divided by the number of base pairs sequenced. CpG dinucleotides were found when searching both strands, and the frequency was calculated using two times the number of base pairs sequenced. The "fuzznuc" algorithm (EMBOSS program; http://bioweb.pasteur.fr/seqanal/EMBOSS) was used for each of the searches.

Statistical analysis. Means ± standard errors (SE) were analyzed by one-way or two-way analysis of variance (ANOVA) using a statistical software package (GraphPad Prism 4.02; GraphPad Software, San Diego, CA). Pairs of group means were then compared by Bonferroni's multiple comparison test. Statistical significance was defined as P of <0.05.

RESULTS

***A. fumigatus* contains unmethylated CpG DNA.** We sought 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′ . . . CCGG . . . 3′. However, they differ

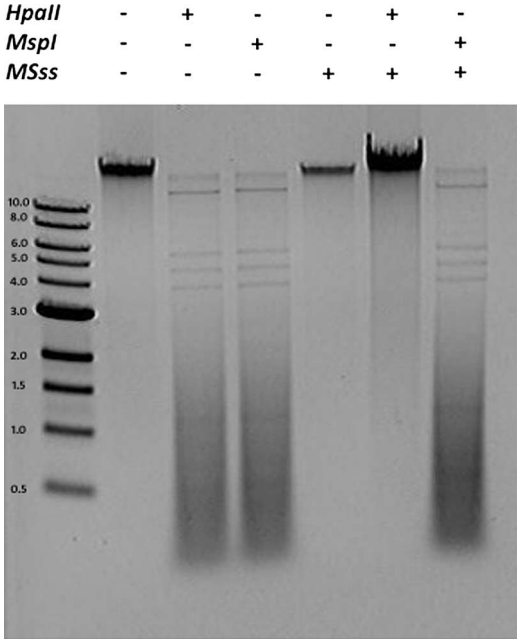

FIG. 1. *A. fumigatus* DNA contains unmethylated CpG sequences. Genomic DNA was isolated from *A. fumigatus* and left untreated or treated with the indicated enzymes. "+" indicates treatment; "−" indicates absence of treatment. The digest was then analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. The left lane shows a DNA ladder with numbers indicating the size of the DNA, in kb. The figure is representative of 12 individual experiments, each with similar results.

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

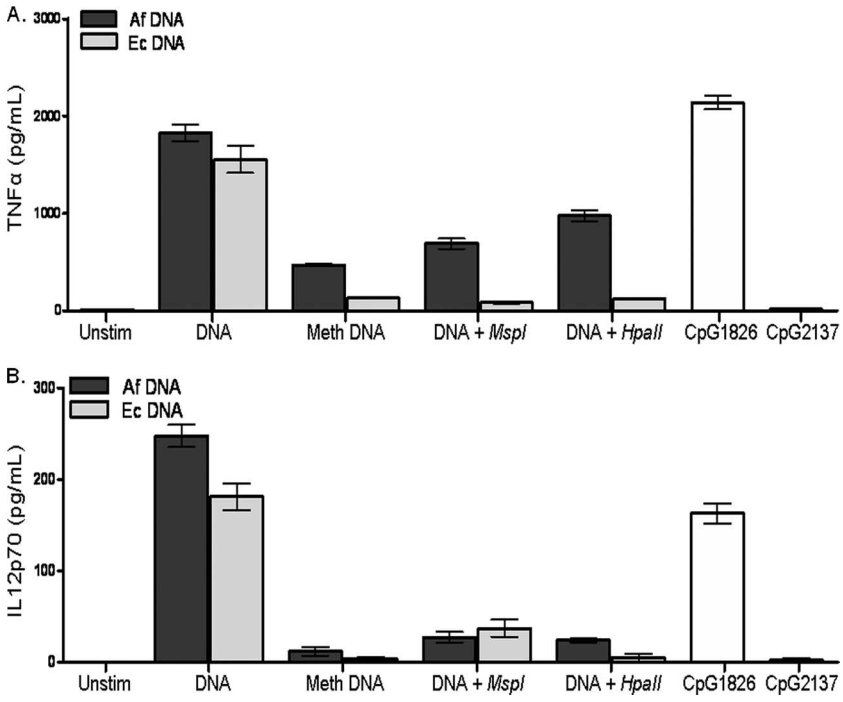

FIG. 2. Stimulation of murine BMDCs by *A. fumigatus* DNA. BMDCs were stimulated by transfecting 2.5 μg/ml of *A. fumigatus* DNA or *E. coli* DNA that was left untreated (DNA) or treated with the CpG methyltransferase M.SssI (Meth DNA), MspI, or HpaII. CpG 1826 at 3 μg/ml served as a positive control, while unstimulated cells and GpC 2137 were negative controls. After 24 h, supernatants were collected and TNF-α (A) and IL-12p70 (B) concentrations were determined by ELISA. Data are means ± SE of five individual experiments. P was <0.001 according to one-way ANOVA with a Bonferroni posttest for TNF-α and IL-12p70 stimulated by untreated *A. fumigatus* or *E. coli* DNA compared with any treatment.

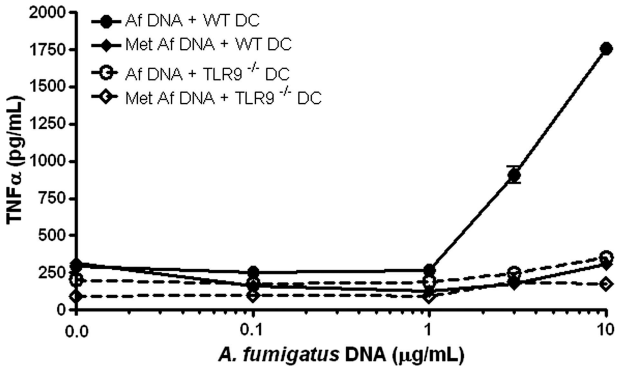

FIG. 3. *A. fumigatus* DNA stimulates TNF-α secretion in a TLR9-dependent manner. BMDCs from WT and TLR9$^{-/-}$ mice were transfected with the indicated concentrations of *A. fumigatus* DNA that was left untreated (Af DNA) or treated with the CpG methyltransferase M.SssI (Met Af DNA) and incubated at 37°C. After 24 h, supernatants were collected and TNF-α concentrations were determined by ELISA. The concentrations of TNF-α released from unstimulated WT and TLR9$^{-/-}$ BMDCs were 225 ± 40 pg/ml and 134 ± 18 pg/ml, respectively. Data are means ± SE of a representative (out of four) experiment performed in triplicate. *P* was <0.001 according to one-way ANOVA with a Bonferroni posttest for TNF-α stimulated by Af DNA in WT BMDCs compared with any other group at DNA concentrations of 3 and 10 μg/ml. DC, dendritic cells; KO, knockout.

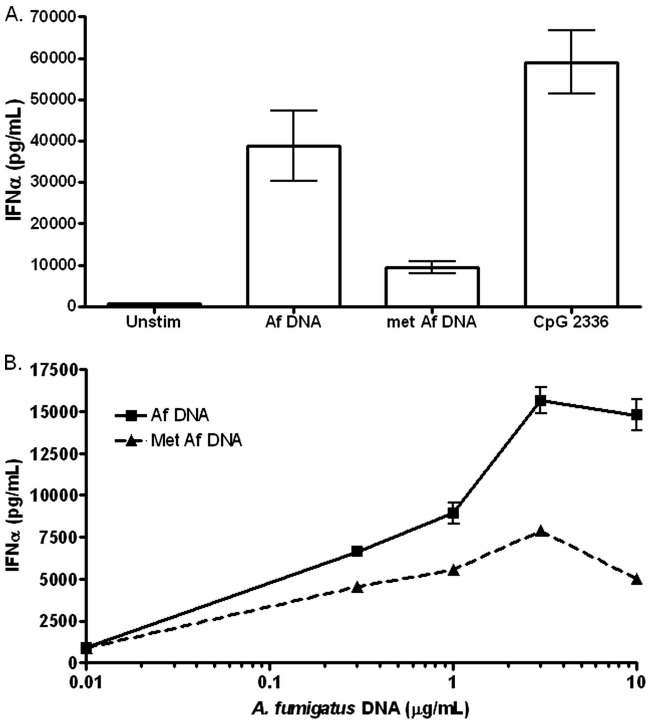

FIG. 4. Stimulation of human pDCs by *A. fumigatus* DNA. Human pDCs were isolated from PBMCs by positive selection using CD304^{+} magnetic beads. (A) pDCs were stimulated with 10 μg of *A. fumigatus* DNA which was either left untreated or methylated with the CpG methyltransferase M.SssI. Unstimulated and CpG 2336 (10 μg/ml)-stimulated pDCs served as negative and positive controls, respectively. After 18 to 24 h, supernatants were collected and analyzed by ELISA for IFN-α. Results are shown as means ± SE of seven individual donors. *P* was <0.001 comparing untreated and methylated DNA by one-way ANOVA with a Bonferroni posttest. (B) As in panel A, except a dose-response curve was performed comparing untreated and methylated *A. fumigatus* DNA. Data are means ± SE of a representative (out of three) experiment performed in triplicate. *P* was <0.001 comparing untreated and methylated DNA at concentrations of 1, 3, and 10 μg/ml by two-way ANOVA with a Bonferroni posttest.

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 fashion. 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 mAF5, 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 cotransfected 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 hTLR7 and an NF-κB-driven luciferase reporter gene (data not shown).

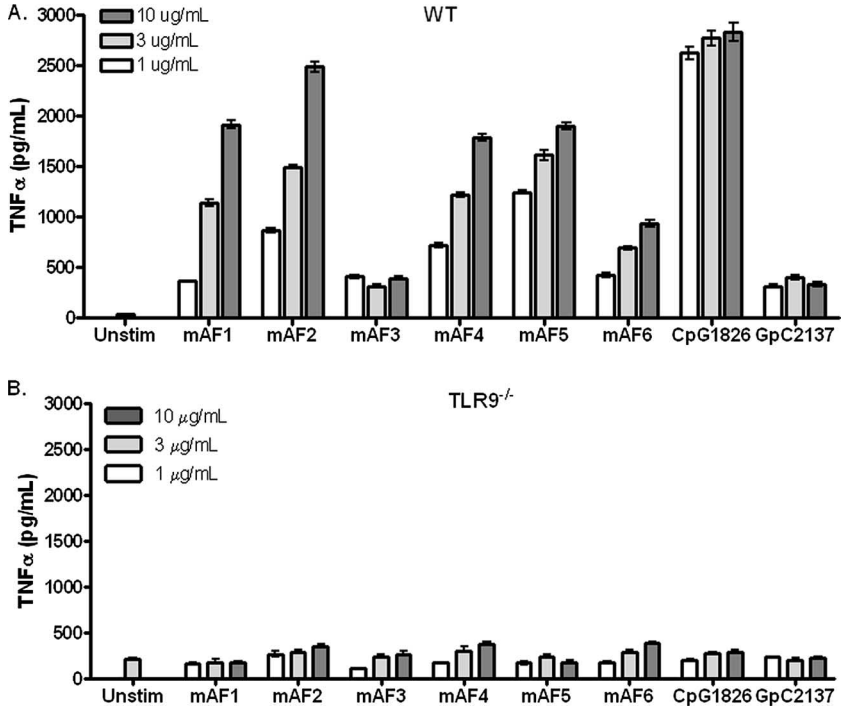

FIG. 5. Stimulation of murine BMDCs by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome. BMDCs from WT (top panel) and TLR9$^{-/-}$ (bottom panel) mice were stimulated with the indicated mouse-like CpG motifs (see Table S1 in the supplemental material). CpG 1826 served as a positive 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.

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

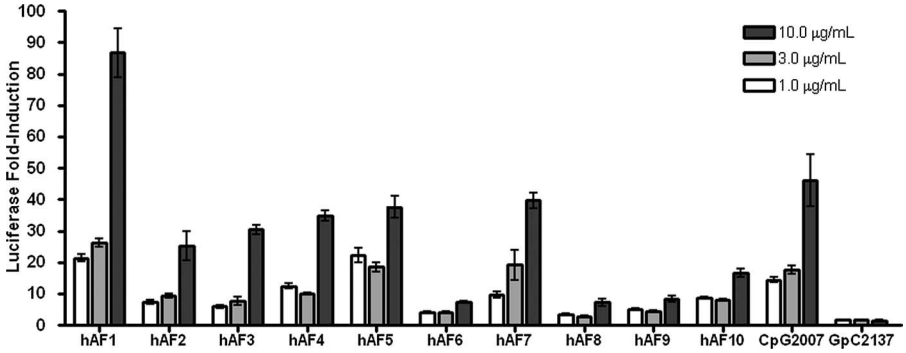

FIG. 6. Stimulation of hTLR9-transfected HEK293 cells by ODNs containing CpG-rich motifs present in the *A. fumigatus* genome. HEK293 cells stably coexpressing hTLR9 and the NF-κB-driven luciferase reporter construct were stimulated with the indicated human-like CpG motifs (see Table S1 in the supplemental 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-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 sequences 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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