Deoxynucleic Acids from Cryptococcus neoformans Activate Myeloid Dendritic Cells via a TLR9-Dependent Pathway


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The mechanism of host cell recognition of Cryptococcus neoformans, an opportunistic fungal pathogen in immunocompromised patients, remains poorly understood. In the present study, we asked whether the DNA of this yeast activates mouse bone marrow-derived myeloid dendritic cells (BM-DCs). BM-DCs released IL-12p40 and expressed CD40 upon stimulation with cryptococcal DNA, and the response was abolished by treatment with DNase, but not with RNase. IL-12p40 production and CD40 expression were attenuated by chloroquine, bafilomycin A, and inhibitory oligodeoxynucleotides (ODN) that suppressed the responses caused by CpG-ODN. Activation of BM-DCs by cryptococcal DNA was almost completely abrogated in TLR9 gene-disrupted (TLR9−/−) mice and MyD88−/− mice, similar to that by CpG-ODN. In addition, upon stimulation with whole yeast cells of acapsular C. neoformans, TLR9−/− BM-DCs produced a lower amount of IL-12p40 than those from wild-type mice, and TLR9−/− mice were more susceptible to pulmonary infection with this fungal pathogen than wild-type mice, as shown by increased number of live colonies in lungs. Treatment of cryptococcal DNA with methylase resulted in reduced IL-12p40 synthesis by BM-DCs. Furthermore, using a luciferase reporter assay, cryptococcal DNA activated NF-κB in HEK293 cells transfected with the TLR9 gene. Finally, confocal microscopy showed colocalization of fluorescence-labeled cryptococcal DNA with CpG-ODN and the findings merged in part with the distribution of TLR9 in BM-DCs. Our results demonstrate that cryptococcal DNA causes activation of BM-DCs in a TLR9-dependent manner and suggest that the CpG motif-containing DNA may contribute to the development of inflammatory responses after infection with C. neoformans. The Journal of Immunology, 2008, 180: 4067–4074.

Cryptococcus neoformans (Cn) is an opportunistic fungal pathogen and frequently causes fatal meningoencephalitis in patients with impaired immune responses, such as AIDS. It is an intracellular microorganism known to grow in macrophages, and has certain escape mechanisms that avoid killing by these cells (1). The host defense against Cn is largely mediated by cellular immunity (2) and CD4+ T cells play a critical role in eradicating the infection (3, 4). The fate of this infection is greatly influenced by the Th1-Th2 cytokine balance: polarized Th1 immune response leads to protection, while biased synthesis of Th2 cytokines renders hosts prone to infection (5). Such balance is critically regulated by a variety of immune cells at an early stage after invasion of Cn into lung tissues, which includes dendritic cells (DCs), macrophages, NK cells, NKT cells, γδ T cells, and even neutrophils (6–10).

Invasion of microbial pathogens into tissues results in the development of inflammatory responses, which are initiated by recognition of pathogen-associated molecular patterns via pattern recognition receptors, such as TLRs (11). Using mice genetically lacking MyD88 or TLR2 (MyD88−/− or TLR2−/− mice), it was revealed that MyD88 plays a critical role while TLR2 plays a relatively limited role in the response to Cn (12). In another study (13), TLR2−/− mice as well as MyD88−/− mice succumbed to the infection, which was associated with reduced production of TNF-α, IL-2, and IFN-γ. In contrast, in our study (14), TLR2 and TLR4 did not seem to be involved in the host-protective response to this infection. In contrast, TLR4 was reported to sense glucuronoxylomannan, a major component of the polysaccharide capsule of Cn, in Chinese hamster ovary cells (15). Thus, the role of

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Deoxynucleic Acids from Cryptococcus neoformans Activate Myeloid Dendritic Cells via a TLR9-Dependent Pathway

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Cryptococcus neoformans (Cn) is an opportunistic fungal pathogen and frequently causes fatal meningoencephalitis in patients with impaired immune responses, such as AIDS. It is an intracellular microorganism known to grow in macrophages, and has certain escape mechanisms that avoid killing by these cells (1). The host defense against Cn is largely mediated by cellular immunity (2) and CD4+ T cells play a critical role in eradicating the infection (3, 4). The fate of this infection is greatly influenced by the Th1-Th2 cytokine balance: polarized Th1 immune response leads to protection, while biased synthesis of Th2 cytokines renders hosts prone to infection (5). Such balance is critically regulated by a variety of immune cells at an early stage after invasion of Cn into lung tissues, which includes dendritic cells (DCs), macrophages, NK cells, NKT cells, γδ T cells, and even neutrophils (6–10).

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TLR2 and TLR4 in recognition of Cn remains to be fully understood, and some other TLR may be involved when considered along with the previous findings that the host-protective response was impaired in MyD88−/− mice (12, 13).

The unmethylated CpG motif-containing DNA from prokaryotic microorganisms such as bacteria and viruses is known to trigger host immune responses by interacting with TLR9 (16–18), which is distributed in the endosomal compartments (19, 20). Such interaction causes activation of signaling pathways mediated by an adaptor molecule, MyD88, leading to the synthesis of proinflammatory cytokines and expression of costimulatory molecules by macrophages and DCs (20, 21). In previous studies (22, 23), DNA from protozoa was reported to stimulate B cell proliferation and macrophage cytokine synthesis in a TLR9-dependent manner, although they are eukaryotic microorganisms. These findings raise a possibility that DNA from Cn may be involved in the activation of host immune responses, as in the case of protozoa. In agreement with this hypothesis, DNA from some fungi, such as Schizosaccharomyces pombe and Paracoccidioides brasiliensis, is also reported to stimulate a battery of immune responses (24, 25).

The main hypothesis of the present study was that DNA from Cn can induce the activation of DCs. The results showed that cryptococcal DNA stimulated bone marrow-derived DCs (BM-DCs) to synthesize cytokines and to express costimulatory molecules. These actions were completely dependent on the expression of TLR9. Furthermore, cryptococcal DNA was taken into the endosomal compartment in a manner similar to the synthetic CpG-oligodeoxynucleotide (ODN) and triggered a signaling pathway for the activation of NF-κB via TLR9.

Materials and Methods
Mice
TLR9−/−, MyD88−/−, Dectin-1−/−, and TRIF−/− mice were generated, as described previously (18, 26–28). Homozygous mice were backcrossed to C57BL/6 mice for more than eight generations. Male or female mice at 6–10 wk of age were used for the experiments and wild-type (WT) C57BL/6 mice/6 mice were used as controls. All of the mutant mice were kept under specific pathogen-free conditions at Oriental Biosciences (Kyoto, Japan), the Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, and Kyusyu University, respectively. The experiments were conducted according to the institutional guidelines and were approved by the institutional ethics committees.

Microorganisms
The acapsular strain of Cn, designated as Cap67 (a gift from Dr. S. M. Levitz, Boston University, Boston, MA) and its parent strain, designated as B3501 (a gift from Dr. K. Chang, National Institutes of Health, Bethesda, MD) were used. Serotype A-encapsulated strains of Cn, designated as YC-11 and YC-13, were established from patients with pulmonary cryptococcosis (29). The yeast cells were cultured on potato dextrose agar plates (Eiken) for 2–3 days before use.

Pulmonary infection and enumeration of viable Cn
To induce pulmonary infection, mice were anesthetized by i.p. injection of 70 mg/kg pentobarbital (Abbott Laboratories) and restrained on a small board. Live Cn, Cap67 strain (5 × 10⁷ cells), were inoculated at 50 μl/mouse by insertion of a 25-gauge blunt needle into and parallel to the trachea. Mice were sacrificed 2 wk after infection, and lungs and brains were dissected carefully and excised, then separately homogenized in 5 and 2 ml of distilled water, respectively, by teasing with a stainless mesh at 100 μl on potato dextrose agar plates (Eiken) for 2–3 days before use.

Preparation of Cn lysates and DNA
Cn yeast cells (1 × 10¹¹) were treated with 70% ethanol for 30 min. washed three times with PBS, and then received five cycles of disruption with 0.3 mm of zirconia beads by use of Multibeads shocker (Yasuiki Kikai). For preparation of Cn DNA, the yeast cells were lysed with 100 mM Tris–hydrochloride (pH 7.5), 0.5% SDS, and 30 mM EDTA at 100°C for 15 min. The DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and isopropanol precipitation. The pellet was washed with 70% ethanol, dried, and resolved with distilled water. The obtained DNA was kept at −20°C until use. The OD₂₆₀/OD₂₈₀ ratio was usually between 1.5 and 2.0. The endotoxin content in the DNA preparations measured by Limulus amebocyte lysate assay was <10 pg/ml.

Culture medium and reagents
RPMI 1640 medium was obtained from Nippon and FCS from Cansera. Bafilomycin A, DNase, RNase, peptidoglycan (PG), and LPS were purchased from Sigma-Aldrich, polymixin B from MP Biotechnology, and chlo-roquine from Wako Biochemicals. NaCl-oxidized Candida albicans (OX-CA; a particle form β-1,6-branched β-1,3-glucan was prepared as described previously (30)). Cpg-ODN (Cpg1826: TCC ATG ACG TTC and Cap67 DNA (10 μg/ml), OX-CA (1 μg/ml), or Cap67 DNA (10 μg/ml). e and f, BM-DCs were cultured with DNA from Cap67, B3501, YC-11, and YC-13 at the indicated doses. Data are the mean ± SD of triplicate cultures. Flow cytometry data are representative of three independent experiments. *p < 0.05.

Preparation of Cn culture supernatants
Cn yeast cells (YC-11) were cultured at 1 × 10⁸/ml in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin G, and 100 μg/ml streptomycin for 48–72 h in a 5% CO₂ incubator. The culture supernatants were...
collected and passed through 0.2-μm Millipore filter and kept at −70°C before use.

**Treatment of DNA with DNase, RNase, and methylase**

Cap67 DNA was incubated with 10 μg/ml DNase or RNase in water bath at 37°C for 4 h. After incubation, the DNAs were heated at 68°C for 20 min to inactivate DNase and again purified by isopropanol precipitation. Methylated dsCpG1826 or Cap67 DNA was synthesized by incubating each DNA with Sss CpG methylase (10 U/μg DNA) for 24 h at 37°C in a water bath. Then, DNAs were heated at 68°C for 20 min to inactivate Sss CpG methylase and centrifuged by adding isopropanol for purification.

**Preparation and culture of DCs**

DC were prepared from BM cells as described by Lutz et al. (31), with some modifications. Briefly, BM cells from WT, TLR9KO, MyD88KO, TRIFKO, and dectin-1KO mice were cultured at 2 × 10^5/ml in 10 ml RPMI 1640 medium supplemented with 10% FCS (Cansera), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 50 μM 2-ME containing 20 ng/ml murine GM-CSF (WakoCytomation). On day 3, 10 ml of the same medium was added, followed by a half change with the GM-CSF-containing culture medium on day 6. On day 8, nonadherent cells were collected and used as BM-DCs. The obtained cells were cultured at 1 × 10^5/ml with various stimulants for 24 h at 37°C in 5% CO₂ incubator.

**Cytokine assay**

The concentration of IL-12p40 in the culture supernatants was measured by ELISA using capture and biotinylated developing Abs (BD Biosciences). The detection limit was 15 pg/ml.

**Flow cytometric analysis**

Cells were preincubated with anti-FcγRII and III mAb, prepared by a protein G column kit (Kirkegaard & Perry Laboratories) from the culture supernatants of hybridoma cells (clone 2.4G2), on ice for 15 min in PBS containing 1% FCS and 0.1% sodium azide, stained with FITC-conjugated anti-CD11c mAb (clone HL3; BD Biosciences), and PE-conjugated anti-CD40 mAb (clone 1C10; eBioscience) for 25 min and then washed three times in the same buffer. Isotype-matched irrelevant Abs were used for control staining. The propidium iodide-stained population was excluded as dead cells. The stained cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter). Data were collected from 15,000 to 20,000 individual live cells using parameters of forward scatter/side scatter and FL1 to set a gate on the CD11c⁺ lymphocyte population.

**TLR9 cloning and NF-κB reporter assay**

The full-length tlr9 was PCR amplified from a cDNA of mouse macrophage cell line RAW264.7 cells (RIKEN Cell Bank). Specific primers for amplification of tlr9 were designed based on the GenBank accession number AF314224 (32). The sequences of primers were 5'-CAC CAT GGT TCT CCG TCG AAG G-3' (forward) and 5'-CTA TTC TGC TGT AGG TCC CCG GC-3' (reverse). The amplified full-length cDNA was cloned

**FIGURE 2.** Effects of chloroquine and baflomycin A. BM-DCs pre-treated or not with chloroquine (3 μg/ml) or baflomycin A (60 nM) were cultured with LPS (1 μg/ml), CpG1826 (150 nM), or Cap67 DNA (10 μg/ml). IL-12p40 levels in the culture supernatants (a) and expression of CD40 on BM-DCs (b) were measured. Data are mean ± SD of triplicate cultures. Flow cytometry data are representative of three independent experiments.

**FIGURE 3.** Effect of inhibitory ODN. BM-DCs were cultured with CpG1826 (150 nM) or Cap67 DNA (10 μg/ml) in the presence or absence of the indicated doses of ODN2088 or ODN2114. IL-12p40 levels in the culture supernatants (a) and expression of CD40 on BM-DCs (b) were measured. Data are mean ± SD of triplicate cultures. * and **, p < 0.05, compared with CpG1826 and Cn-DNA, respectively. Flow cytometry data are representative of three independent experiments.
into pcDNA6.2/TOPO (Invitrogen Life Technologies) and sequenced. Endothelial leukocyte adhesion molecule 1 luciferase reporter plasmids were provided by Dr. M. Mitsuyama (Kyoto University, Kyoto, Japan). For the NF-κB luciferase reporter assay, HEK293T cells were transiently transfected with 100 ng of reporter plasmid along with 100 ng of tlr9 expression plasmids or empty control plasmid. At 24 h after transfection, the cells were treated with PG, CpG-ODN, or cryptococcal DNA for 6 h. Luciferase activity in the total cell lysate was measured with the dual-luciferase reporter assay system (Promega). The Renilla-luciferase reporter gene was simultaneously transfected as an internal control. Relative luciferase activities were calculated as folds of induction compared with unstimulated vector control.

Florescent labeling and confocal imaging
DNA was labeled with Alexa Fluor 647 using ULYSIS Nucleic Acid Labeling kits (Molecular Probes) according to the instructions provided by the manufacturer. The relative efficiency of a labeling reaction was evaluated by calculating the approximate ratio of bases to dye molecules. Rhodamine-labeled CpG-ODN was purchased from Hokkaido System Science. FITC-conjugated TLR9 mAb (clone 26C593.2) was purchased from IMGENEX. Cells were incubated in microtubes, cytospun to glass slides, and coverslips were mounted on the glass slides with a ProLong Antifade Kit (Molecular Probes). Confocal studies were performed with an oil immersion objective (×60 Plan Apo; numerical aperture 1.4) and a Nikon TIRF-C1 confocal microscope. The software, Nikon EZ-C1 version 2.00, were used to acquire and process the confocal images. Dual-color images were acquired using a sequential acquisition mode to avoid cross-excitation.

Statistical analysis
Analysis was conducted using Statview II software (Abacus Concept) on a Macintosh computer. Data are expressed as mean ± SD. Differences between groups were examined for statistical significance using one-way ANOVA with a post hoc analysis (Fisher’s least significant difference) test. A value of p < 0.05 was considered significant.

Results
DNA from Cn activates BM-DCs
We measured the activity of Cn to induce the synthesis of IL-12p40 by BM-DCs. For this purpose, we used the acapsular mutant strain Cap67 to avoid the influence of capsular polysaccharides, which were reported to suppress various immune responses (33). The lysates of Cap67 prepared using a multibeads shocker generated IL-12p40 by BM-DCs. We tested the effect of DNase on the cytokine-inducing capability of Cap67 lysates. As shown in Fig. 4a, DNase significantly reduced the production of both cytokines.
These results suggest the involvement of Cap67 DNA in this response. Next, we tested whether Cap67-derived DNA can activate BM-DCs by measuring the synthesis of IL-12p40 and expression of CD40 on these cells. IL-12p40 was produced by this stimulation in a dose-dependent manner and such production was completely abolished by the addition of DNase, but not of RNase (Fig. 1b). Similarly, the expression of CD40 on BM-DCs was accelerated by coculture with Cap67 DNA, and this effect was inhibited by DNase, but not by RNase (Fig. 1c). These responses were not affected by the addition of polymyxin B, which completely abolished cytokine production induced by LPS (data not shown), indicating that the activity of Cap67 DNA was not mediated by LPS contamination. Since fungal secrete β-glucan that activates BM-DCs (34), it is possible that β-glucan contaminating the DNA preparation could have mediated these responses. However, this was not the case, because IL-12p40 produced by Cap67 DNA-stimulated BM-DCs was not decreased in mice lacking dectin-1, the sole receptor of β-glucan (Fig. 1d). Furthermore, we tested also whether these findings were specific for this particular mutant strain of Cn. IL-12p40 synthesis by BM-DCs was induced by DNA derived from the parent strain of Cap67, B3501, and two clinically isolated strains, YC-13 and YC-11, in a dose-dependent fashion (Fig. 1e). Similarly, the expression of CD40 on BM-DCs was enhanced by DNA from these strains (Fig. 1f). These results indicate that DNA from Cn directly activated BM-DCs to synthesize the cytokine and to express the surface activation Ag. In contrast, DNA derived from mouse splenocytes and human PBMC did not induce these responses by BM-DCs (data not shown).

To address this possibility, we investigated the role of TLR9 in Cap67 DNA-induced BM-DC activation. As shown in Fig. 4, a and b, production of IL-12p40 and expression of CD40 by Cap67 DNA- and CpG1826-stimulated cells were completely diminished in TLR9−/− mice, whereas such effect was not detected when the cells were stimulated with LPS. In this study, it should be noted that DNA was not released from Cn outside the DCs, but rather likely generated from the killed yeast cells in the endosomal compartments. Therefore, our current observations indicating the activation of BM-DCs by cryptococcal DNA have a more physiological significance, when defective expression of TLR9 interferes with activation induced by whole Cn. As shown in Fig. 4c, the amount of IL-12p40 synthesized by BM-DCs in response to whole yeast cells of Cap67, an acapsular strain, was significantly reduced in TLR9−/− mice compared with WT control mice, whereas YC-11, a highly encapsulated strain, failed to induce the production of DNA of Cn activates BM-DCs in a manner similar to CpG-ODN

Because Cn is a eukaryote, we questioned whether the activity of DNA from this fungal pathogen is different from that of a synthetic ODN containing the CpG motif, which prokaryote DNA usually carries in an unmethylated form (17). Previous studies reported that CpG-ODN-induced activation of BM-DCs was strongly inhibited by chloroquine and bafilomycin A, both of which interfere with endosomal maturation (35). Therefore, to address this issue, we compared the effects of these inhibitors on the synthesis of IL-12p40 by BM-DCs and expression of CD40 caused by a stimulatory CpG-ODN (CpG1826) and Cap67 DNA. As shown in Fig. 2a, the synthesis of IL-12p40 by Cap67 DNA-stimulated cells was strongly suppressed by addition of either chloroquine or bafilomycin A; such an effect was similar to the responses induced by CpG1826, but not by LPS. Similarly, the addition of chloroquine or bafilomycin A suppressed CD40 expression induced by Cap67 DNA and CpG1826, but not by LPS (Fig. 2b).

Previous studies identified DNA sequences that neutralized the CpG-ODN-induced activation of B cells (36). In the next experiments, therefore, we tested the effect of such ODN with an inhibitory DNA motif on the induction of IL-12p40 synthesis and CD40 expression by BM-DCs. As shown in Fig. 3, ODN2088 and ODN2114 strongly inhibited these responses induced by both CpG1826 and Cap67 DNA, whereas such inhibition was not detected on the responses by LPS. These results indicate that Cn DNA exerted its activity in a manner similar to CpG1826 and suggest that the fungal DNA may activate BM-DCs via interaction with TLR9, a recognition receptor of the unmethylated CpG motif (17, 18).
In the indicated time periods.

The case of OX-CA stimulation (Fig. 5). RAW264.1 cells were incubated with 10 μg/ml Alexa Fluor 647 (green) and 3 μM CpG-rhodamine (red) for 6 h. The luciferase activity in each sample was determined as described in Materials and Methods. Data are expressed as relative values to those of control vector and presented are mean ± SD of triplicate cultures. CNT-ODN, control ODN of CpG1826.

IL-12p40 by BM-DCs from both mice, which may raise a possibility that capsular polysaccharides affect the activation of BM-DCs caused by cryptococcal DNA. We tested the effect of the culture supernatants of YC-11 on the IL-12p40 production by BM-DCs. As shown in Fig. 4d, the production of this cytokine caused by cryptococcal DNA was strongly inhibited by the YC-11 culture supernatants in a dose-dependent fashion, whereas such inhibition was not found when BM-DCs were stimulated with LPS. In addition, we compared the clinical course of infection with Cap67 in lungs between TLR9−/− and WT mice. As shown in Fig. 4e, the number of live colonies in lungs was significantly higher in the former mice than in the latter ones 2 wk after infection, although dissemination of yeast cells to brains was not detected in both groups (data not shown).

In additional experiments, we tested how Cap67 DNA-induced activation of BM-DCs was affected in mice genetically lacking MyD88, a downstream signaling molecule of TLR9 and TLR4, or TRIF, that of TLR4, but not of TLR9 (37). BM-DCs from TRIF−/− mice failed to produce IL-12p40 induced by Cap67 DNA and LPS, but not by OX-CA (Fig. 5a). In contrast, LPS, but not Cap67 DNA and OX-CA, inhibited such production by BM-DCs from TRIF−/− mice (Fig. 5a). Similarly, CD40 expression induced by Cap67 DNA and CpG1826 was completely abrogated in MyD88−/− mice, whereas such expression was not affected in the case of OX-CA stimulation (Fig. 5b). Similar results were obtained for the synthesis of IL-12p40, when BM-DCs were stimulated with whole yeast cells (data not shown). These results indicate that TLR9 and its downstream signaling molecule are essential for Cap67 DNA-induced activation of BM-DCs.

Methylation of the CpG motif results in the loss of its capability to activate DCs via a TLR9-dependent signaling pathway (16, 24). Therefore, we tested the effect of methylation on cytokine synthesis by Cap67 DNA-stimulated BM-DCs. As shown in Fig. 6, BM-DCs stimulated by CpG1826 and Cap67 DNA pretreated with methylase did not synthesize IL-12p40. These results were consistent with the notion that Cn DNA activates BM-DCs by interacting with TLR9.

Cn DNA activates the signaling pathway via TLR9

CpG-ODN triggers the signaling pathway via TLR9, which results in the activation of NF-κB (37). In the next step, we tested the effect of Cn DNA on NF-κB. For this purpose, we performed a luciferase reporter assay using HEK293T cells transfected with the gene for TLR9 and luciferase gene linked to the promoter sequence containing an NF-κB binding site. As shown in Fig. 7, both Cap67 DNA and CpG1826 induced luciferase activity in TLR9-expressing HEK293T cells, whereas PG and control ODN of CpG1826 did not show such activity. These results indicate that Cn DNA triggered the signaling pathway for activation of NF-κB by directly interacting with TLR9.

Colocalization of Cn DNA with CpG-ODN in BM-DCs

In DCs and macrophages, CpG-ODN is internalized into early endosomes, followed by subsequent transportation to a lysosomal compartment and direct interaction with TLR9, which is redistributed from the endoplasmic reticulum (19, 20). Using confocal microscopic analysis, we compared the intracellular trafficking of Cap67 DNA and CpG1826 to further define the relationship of both agents during the activation of BM-DCs. As shown in Fig. 8a, Cap67 DNA and CpG1826 were mostly colocalized in the intracellular area of BM-DCs at 5, 30, 60, and 90 min after incubation. Thus, the intracellular trafficking of Cn DNA in BM-DCs is similar to that of CpG1826, suggesting the interaction of the fungal DNA with TLR9. In the next step, we analyzed the distribution of TLR9 in these cells after incubation with Cap67 DNA and CpG1826. Isotype-matched irrelevant Ab did not show positive staining in resting and Cap67 DNA-stimulated BM-DCs (data not shown). TLR9 was detected in the intracellular area of resting BM-DCs and redistributed in part to the area where these nucleic acids were localized after stimulation, and similar results were observed using RAW264.1 cells, a macrophage lineage cell line (Fig. 8b). These results indicate that Cn DNA interacts with TLR9 in the endosomal pathway similar to CpG1826.
Discussion

In the present study, we hypothesized that DNA from Cn can activate BM-DCs based on the finding that lysates of this fungal microorganism showed such activity, which was significantly reduced after DNase treatment. In agreement with this hypothesis, cryptococcal DNA induced the release of IL-12p40 and expression of CD40 by BM-DCs. Such activity was not mediated by contaminated LPS or β-glucan due to the following reasons: 1) the activity was abrogated by DNase treatment, 2) polymyxin B did not affect the activity, and 3) the level of activity was similar in BM-DCs from control mice, C3H/HeJ, and TLR4−/− mice (data not shown) and from dectin-1−/− mice lacking a specific receptor for β-glucan. In addition, activation of BM-DCs did not involve capsular polysaccharides because DNA from an acapsular strain of Cn was highly active. In earlier studies (24, 25), similar to our findings, DNA from two yeast-form fungi, S. pombe and P. brasiliensis, also induced the B cell proliferation and the development of Th1 immune responses and host protection, respectively, although the precise mechanism was not investigated. Thus, Cn seems to possess immunostimulatory DNA that promotes Th1-mediated host responses against this infection.

In previous studies (12, 13), MyD88, which acts as an adaptor molecule in signaling via TLRs (37), was reported to play an important role in host defense against cryptococcal infection. These findings suggested the possible involvement of certain TLRs. However, involvement of TLR2 and TLR4 in the host defense responses against this infection remains to be substantiated (12–14). Recent studies have identified the involvement of TLRs in the recognition of nucleic acids; including TLR3 for dsRNA, TLR7/8 for ssRNA, and TLR9 for unmethylated CpG motif-containing DNA (18, 38–40). Thus, we addressed the possible contribution of TLR9 to the mechanism underlying cryptococcal DNA-induced BM-DC activation, although it was unclear whether Cn possess such motifs in its DNA structure. CpG-ODN is internalized via an endocytic pathway and trafficked into late endosomal compartments, which may provide a benefit for them to reach the alveolar space and multiply in alveolar spaces. It has recently been proposed that this fungal pathogen grows inside the endosomes of macrophages based on its escape mechanism against killing effector molecules (1). Macrophages acquire the capacity to kill the fungus after their activation by IFN-γ secreted from Th1 cells (5). Therefore, it is unlikely that the DNA is released from Cn outside the macrophages and DCs, but rather generated from the killed yeast cells in the endosomal compartment, which could be moved into the TLR9-triggered signaling pathway for the initiation of host protective immune responses. Consistent with this notion, activation of BM-DCs by whole cryptococcal cells was significantly, although not completely, reduced in TLR9−/− mice, and these mice were more susceptible to pulmonary infection with Cn than WT control mice, as shown by the increased live colonies in lungs, when Cap67, an acapsular strain, was used. In this regard, it was recently demonstrated that DCs could incorporate and kill this fungal microorganism (42, 43). In our data, the culture supernatants of YC-11, a highly encapsulated Cn, containing much capsular polysaccharides suppressed the production of IL-12p40 by cryptococcal DNA and, in addition, BM-DCs failed to produce IL-12p40 upon stimulation with whole yeast cells of YC-11 in contrast to the effect of this cytokine synthesis by BM-DCs, in contrast to the effect of this treatment on CpG-ODN. Although a possibility that cryptococcal DNA was not completely methylated is not excluded, these data suggest a TLR9-mediated, but CpG-independent activation mechanism for cryptococcal DNA, which may be consistent with the recent observations that TLR9 recognizes DNA not only by the CpG motif but also by different nucleic acid sequences (41).

In the present study, BM-DCs were stimulated by adding cryptococcal DNA to the cell cultures, suggesting that the DNA was internalized through the cell membranes and moved into the endosomal pathway as described previously for CpG-ODN (19, 20). This possibility was confirmed using confocal microscopy performed by comparing the intracellular trafficking of fluorescence-labeled cryptococcal DNA and CpG-ODN. The results showed colocalization of these two nucleic acids throughout the course of incubation. Thus, the intracellular trafficking of cryptococcal ODN to the endosomal pathway is similar to that of the CpG-ODN. Latz et al. (19) demonstrated the redistribution of TLR9 toward the sites of CpG-ODN accumulation. Compatible with this observation, in the present study, redistribution of TLR9 to the sites of cryptococcal DNA accumulation was noted in BM-DCs. These results add further support to our conclusion that cryptococcal DNA activated BM-DCs for cytokine synthesis and expression of costimulatory cell surface molecules by triggering the TLR9 signaling pathway, although we could not confirm that cryptococcal DNA directly binds TLR9.

Based on our findings, we propose here a novel mechanism for recognition of fungal pathogen by the host immune system: the DCs sense Cn DNA in a TLR9-dependent fashion. Cn infects lung tissues and multiplies in alveolar spaces. It has recently been proposed that this fungal pathogen grows inside the endosomes of macrophages based on its escape mechanism against killing effector molecules (1). Macrophages acquire the capacity to kill the fungus after their activation by IFN-γ secreted from Th1 cells (5). Therefore, it is unlikely that the DNA is released from Cn outside the macrophages and DCs, but rather generated from the killed yeast cells in the endosomal compartment, which could be moved into the TLR9-triggered signaling pathway for the initiation of host protective immune responses. Consistent with this notion, activation of BM-DCs by whole cryptococcal cells was significantly, although not completely, reduced in TLR9−/− mice, and these mice were more susceptible to pulmonary infection with Cn than WT control mice, as shown by the increased live colonies in lungs, when Cap67, an acapsular strain, was used. In this regard, it was recently demonstrated that DCs could incorporate and kill this fungal microorganism (42, 43). In our data, the culture supernatants of YC-11, a highly encapsulated Cn, containing much capsular polysaccharides suppressed the production of IL-12p40 by cryptococcal DNA and, in addition, BM-DCs failed to produce IL-12p40 upon stimulation with whole yeast cells of YC-11 in contrast to Cap67 (Fig. 4, c and d), although DNA from the former strain was as potent as the latter ones (Fig. 1e). These observations raise a possibility that capsular polysaccharides interfere with the BM-DC production of IL-12p40 in response to cryptococcal DNA, which may limit the significance of our findings. However, Cn is known to have no capsule or a thin capsule when infected via the airborne route, which may provide a benefit for them to reach the alveolar space (44). This notion suggests that DCs may encounter acapsular
or possibly capsulated Cn and may be activated by DNA released from engulfed yeast cells under little influence of capsular polysaccharides at the early stage of infection. Further investigations are necessary to address this important issue. Thus, the present findings enhance our understanding of the pathogenic mechanism of intracellular cryptococcal infection in immunocompromised patients and help in the design of novel immunotherapies to combat such clinically difficult infection.

**Disclosures**

The authors have no financial conflict of interest.

**References**


