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Dendritic Cell-Based Immunization Ameliorates Pulmonary Infection with Highly Virulent *Cryptococcus gattii*

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Cryptococcosis due to a highly virulent fungus, *Cryptococcus gattii*, emerged as an infectious disease on Vancouver Island in Canada and surrounding areas in 1999, causing deaths among immunocompetent individuals. Previous studies indicated that *C. gattii* strain R265 isolated from the Canadian outbreak had immune avoidance or immune suppression capabilities. However, protective immunity against *C. gattii* has not been identified. In this study, we used a gain-of-function approach to investigate the protective immunity against *C. gattii* infection using a dendritic cell (DC)-based vaccine. Bone marrow-derived dendritic cells (BMDCs) efficiently engulfed acapsular *C. gattii* (Δ*cap60* strain), which resulted in their expression of costimulatory molecules and inflammatory cytokines. This was not observed for BMDCs that were cultured with encapsulated strains. When Δ*cap60* strain-pulsed BMDCs were transferred to mice prior to intratracheal R265 infection, significant amelioration of pathology, fungal burden, and the survival rate resulted compared with those in controls. Multinucleated giant cells (MGCs) that engulfed fungal cells were significantly increased in the lungs of immunized mice. Interleukin 17A (IL-17A)-, gamma interferon (IFN-γ)-, and tumor necrosis factor alpha (TNF-α)-producing lymphocytes were significantly increased in the spleens and lungs of immunized mice. The protective effect of this DC vaccine was significantly reduced in IFN-γ knockout mice. These results demonstrated that an increase in cytokine-producing lymphocytes and the development of MGCs that engulfed fungal cells were associated with the protection against pulmonary infection with highly virulent *C. gattii* and suggested that IFN-γ may have been an important mediator for this vaccine-induced protection.

Inhalation of the airborne fungal pathogens *Cryptococcus neoformans* and *Cryptococcus gattii* causes life-threatening infectious diseases despite treatment with antifungal drugs. These two species are genetically close, although they have some distinct features. *C. neoformans* typically causes fatal infections, such as meningitis, in immunocompromised hosts, whereas *C. gattii* causes similar infections in immunocompetent hosts. Although cryptococcosis caused by *C. gattii* is endemic in tropical areas, such as Australia and Papua New Guinea, outbreaks of *C. gattii*, including fatalities among healthy individuals, were reported on Vancouver Island and surrounding areas beginning in 1999 (1, 2). In response to this, the Centers for Disease Control and Prevention (CDC) of the United States and British Columbia organized a public health working group to promote awareness of this outbreak (3–5).

Using mouse pulmonary infection models, two groups independently showed that *C. gattii* strain R265, which was clinically isolated during the Canadian outbreak, was more virulent than *C. neoformans* strain H99, which is frequently studied (6, 7). Although the mechanisms for its hypervirulence remain unknown, there is evidence that *C. gattii* induces a less severe inflammatory response than that induced by *C. neoformans* infection. Histological and flow cytometry analyses showed reduced migration of inflammatory cells into the lungs of mice infected with R265 compared with those infected with H99 (7–9). Additionally, a smaller amount of inflammatory cytokines was found in the lungs of mice infected with *C. gattii* (9) and in the cerebrospinal fluid of humans infected with *C. gattii* (10, 11). These findings suggest that *C. gattii* has a superior ability to suppress or evade the inflammatory response.

Previous studies indicated that one of the capsular components of *C. gattii* may have been involved in immune avoidance or immune suppression and was required for the complete virulence of *C. gattii* (12, 13). Because *C. gattii* can induce immune avoidance or suppression, an analysis of loss-of-function using gene knockout (KO) mice is not applicable for studying any protective immune responses against *C. gattii*, and the protective immunity

Received 30 December 2014 **Returned for modification** 19 January 2015
Accepted 27 January 2015

Accepted manuscript posted online 2 February 2015

Citation Ueno K, Kinjo Y, Okubo Y, Aki K, Urai M, Kaneko Y, Shimizu K, Wang D-N, Okawara A, Nara T, Ohkouchi K, Mizuguchi Y, Kawamoto S, Kamei K, Ohno H, Niki Y, Shibuya K, Miyazaki Y. 2015. Dendritic cell-based immunization ameliorates pulmonary infection with highly virulent *Cryptococcus gattii*. Infect Immun 83:1577–1586. doi:10.1128/IAI.02827-14.

Editor: G. S. Deepe, Jr.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.02827-14.

doi:10.1128/IAI.02827-14

against highly virulent *C. gattii* has not been well characterized. Thus, it is important to unravel any protective immunity against *C. gattii* to garner insights for the prevention, diagnosis, and treatment of cryptococcosis with highly virulent *C. gattii*.

Dendritic cells (DCs) play a central role in inducing effector T cells (14) and can also be utilized as antigen delivery systems for vaccinations for cancers or infections (15, 16). Adoptive transfer of DCs pulsed with fungal cells or with fungal RNA has also been utilized as a means to assess T cell-mediated immunity against pathogenic fungi (17–19). In this study, we implemented a gain-of-function approach to investigate protective immunity against *C. gattii*. We tested whether a DC-based vaccine could augment protective immunity against the highly virulent *C. gattii* strain R265 by evaluating the pathology, fungal burden, and survival rate after pulmonary infection in mice. Further, we examined cytokine-producing cells in the mouse spleen and lung. Moreover, we assessed the role of gamma interferon (IFN-γ) in the protective effect exerted by this DC-based vaccine using IFN-γ knockout mice.

MATERIALS AND METHODS

Ethics. All our animal experiments were in compliance with the guidelines and policies of the Principles of Morality for Animal Experiments of the National Institute of Infectious Disease, Japan (approval numbers 213072, 21608, 214044, 114004, and 114029).

Mice. C57BL/6J mice were purchased from Japan SLC, Inc. IFN-γ knockout (KO) mice (C57BL/6 background) were purchased from the Jackson Laboratory. Mice used in experiments were 6 to 8 weeks old and were maintained under specific-pathogen-free conditions at the National Institute of Infectious Diseases of Japan.

C. gattii. *C. gattii* strain R265 (genotype VGII, mating type α, and serotype B) was kindly provided by Kyung J. Kwon-Chung (National Institutes of Health, Bethesda, MD); in 2001, this strain was clinically isolated from bronchial washings of infected patients during the Vancouver Island outbreak (20). To construct the acapsular *C. gattii* Δ*cap60* strain, *CAP60* (GenBank accession number CGB_A6290C) of *C. gattii* strain PNG18 (genotype VGI, mating type α, and serotype B) was disrupted by gene replacement using the nourseothricin resistance gene *NAT1*, which was also clinically isolated from an infected person in Papua New Guinea (21). The disruption strategy used is shown in Fig. S1 in the supplemental material. The primers and sequences that were used for strain construction are listed in Table S1 in the supplemental material.

To prepare a gene replacement cassette, DNA fragments, such as for a marker gene and homologous regions, were amplified by PCR. The marker fragment *NAT*, including the *ACT1* promoter, *NAT1*, and the *TRP1* terminator, were amplified using the primers NAT-F and NAT-R derived from plasmid pCH233 used as a template (22). Homologous upstream and downstream regions (≈1 kb) of *CAP60* were amplified with the primers CAP60up-F and CAP60up-R for the upstream region and CAP60down-F and CAP60down-R for the downstream region using *C. gattii* genomic DNA as a template. The primer pairs CAP60up-R and NAT-R and CAP60down-F and NAT-F contained complementary sequences that allowed them to anneal with the marker fragment *NAT*. Three fragments, (i) 5′ upstream region, (ii) 3′ downstream region, and (iii) *NAT* fragment, were equally mixed and used as a template for PCR with the primers CAP60up-F and CAP60down-R, which harbored a single gene disruption cassette. This cassette was introduced into *C. gattii* strain PNG18 using a helium-driven biolistic system (Bio-Rad) as described previously (23). The transformants were screened on a yeast extract-peptone-dextrose (YPD) agar plate (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto peptone, and 2% [wt/vol] dextrose) that contained 100 μg/ml of nourseothricin. Homologous integration events were confirmed by PCR using the primers shown in Fig. S1. The *CAP60* open reading frame (ORF), the region from the ATG codon to the TAG stop codon, was completely deleted in the Δ*cap60* strain.

Plasmid pJAF12-CAP60 was constructed to integrate the *CAP60* ORF with a 1-kb flanking region into the genomic DNA of the Δ*cap60* strain. Primers CAP60up-NotI-F and CAP60down-SacII-R were used to amplify the *CAP60* locus, and the amplified fragment was digested with NotI and SacII. The digested fragment was cloned into the NotI and SacII sites of pJAF12 (24). Plasmid pJAF12-CAP60 was introduced in the Δ*cap60* strain as described above, and the transformants were screened on a YPD agar plate that contained 100 μg/ml of nourseothricin and 200 μg/ml of Geneticin (G418). Homologous integration events were confirmed by PCR with the primers shown in Fig. S1 in the supplemental material.

To prepare heat-killed fungal cells, *C. gattii* was cultured in YPD medium with shaking overnight at 30°C. Cells were harvested and washed with sterile Dulbecco's phosphate-buffered saline (DPBS; Invitrogen), resuspended in DPBS, and boiled for 1 h. Heat-killed *C. gattii* cells were not washed further. The morphologies of heat-killed *C. gattii* were not altered based on microscopic observations. A suspension of heat-killed *C. gattii* cells was spread onto YPD agar and cultured at 30°C for 7 days to check whether all *C. gattii* cells were dead.

A conventional India ink preparation was used to observe capsule formation. *C. gattii* cells were grown in YPD medium at 30°C with shaking overnight. Then, 100 μl of a culture suspension was centrifuged and the harvested cells were resuspended in 20 μl of an ink solution, which included equal amounts of 4% (wt/vol) paraformaldehyde and India ink. Then, 5 μl of an ink-cell suspension was placed on a slide glass with a coverslip. Cells were observed by differential interference contrast microscopy (Inverted microscope IX81; Olympus).

BMDCs. Bone marrow (BM) cells were harvested from the femurs and tibias of female C57BL/6J mice. Red blood cells were lysed with lysis buffer (9 volumes of 0.83% [wt/vol] NH_4Cl and 1 volume of 200 mM Tris-HCl, pH 7.6). BM cells (3×10^6 cells/10 ml per dish) were cultured in RPMI 1640 (complete) medium (Sigma) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) streptomycin-penicillin solution (Sigma; 10,000 U of penicillin and 10 mg/ml of streptomycin), 44 μM 2-mercaptoethanol, and 10 ng/ml of mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF; PeproTech, Inc.) at 37°C under 5% CO_2. Sterile petri dishes (not treated for cell culture) were used for cell culture. On day 3, 6 ml of the culture medium was removed, and 7 ml of fresh complete medium was added to the dishes. On day 5, 5 ml of fresh complete medium was added to the dishes. On day 6, nonadherent cells were collected and used as BM-derived dendritic cells (BMDCs).

Lung leukocytes. Mouse lungs were perfused and rinsed with saline prior to being minced with scissors. Lung pieces were enzymatically digested at 37°C for 60 min with 5 ml/lung of a digestion solution (RPMI 1640 medium, 5% FBS, 2 mg/ml of collagenase D [Roche], and 10 μg/ml of DNase I [Sigma]) in 15-ml conical tubes and a tube rotator. After digestion, 250 μl of 100 mM EDTA solution was added to the tubes to stop digestion (final concentration, 5 mM EDTA). The lung pieces were then homogenized with a 70-μm cell strainer (BD-Falcon), and cell suspensions were harvested. A cell pellet was resuspended in 2 ml of 30% Percoll (GE Healthcare) and layered onto 4 ml of 44% and 70% Percoll. All Percoll solutions contained 2 mM EDTA to prevent cell aggregation. After centrifugation at 1,000 × *g* without acceleration and deceleration for 20 min at room temperature, cells at the 44-70% interface were collected and washed twice with complete RPMI 1640 medium. Cells (2×10^5) were attached to a glass slide using a Cytofuge-12 (Statspin, Inc.) and stained with Diff-Quik (Sysmex Corporation) for Wright-Giemsa staining, followed by determination of the proportion of each cell type under a microscope. The numbers of lymphocytes, macrophages, and polymorphonuclear cells (PMNs) were determined by multiplying the total leukocyte count by the proportion of each cell type.

Phagocytosis assay. Prior to doing a phagocytosis assay, heat-killed *C. gattii* cells (1×10^9 cells/ml) were stained with acridine orange (stock concentration, 0.1 mg/ml; working concentration, 0.01 mg/ml) at room

temperature for 1 h in the dark. Stained cells were washed with DPBS and resuspended in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated FBS. We confirmed that capsular strains as well as the wild-type strain were stained. A suspension of stained cells was stored at 4°C overnight. Harvested BMDCs (1×10^6 cells/ml) were incubated with prestained *C. gattii* cells (5×10^6 cells/ml; multiplicity of infection [MOI] = 5) in complete RPMI 1640 medium that contained mGM-CSF in 24-well culture plates. After incubation, 500 μl of 4% (vol/vol) paraformaldehyde (Fixation Buffer; Biolegend) was added and cells were fixed at room temperature for 5 min, followed by a washing with DPBS. Fungal cells that were not engulfed by BMDCs were stained with calcofluor white (Sigma; 1/10 dilution) at room temperature for 5 min. Culture wells were rinsed with DPBS before fluorescence microscopic analysis (inverted microscope IX81; Olympus). A mercury apo lamp, Olympus filter cube WU (BP330–385, DM400, BA420), and Olympus filter cube GFP (BP460–480, DM485, BA495–540) were used for the fluorescence imaging. At least five fields of view with ≈100 BMDCs/field were randomly selected. At least three fields were used to determine a phagocytosis rate, defined as the number of BMDCs engulfing fungal cells/total BMDCs × 100%. ImageJ software (National Institutes of Health, USA) was used for cell counting and color image merging.

Activation marker expression on BMDCs and cytokine-producing T cells. BMDCs (2×10^6 cells/ml) were incubated in complete RPMI 1640 medium that contained mGM-CSF and heat-killed *C. gattii* cells (2×10^4 cells/ml; MOI = 0.1) or 100 ng/ml of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma) for 24 h. Cells were then collected and stained for flow cytometry analysis. All antibodies and buffer used for cell staining were from Biolegend. Fc receptors were blocked with an anti-CD16/32 antibody (clone 93), after which cells were stained with the following antibodies: anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD86 (clone GL-1), anti-CD40 (clone MR1), and anti-I-Ab (clone AF6-120.1).

To assess cytokine-producing T cells, spleen cells (2×10^6 cells/ml) were cultured in complete RPMI 1640 medium with 100 ng/ml of anti-CD28 monoclonal antibody (MAb) (clone 37.51) and 100 ng/ml of anti-CD49d MAb (clone 9C10) in the presence of heat-killed Δ*cap60* cells (MOI = 0.1) for 5 to 6 days. Lung leukocytes were incubated in complete RPMI 1640 medium with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 3 h. To stop cytokine release, brefeldin A (final concentration, 5 μg/ml; Biolegend) and monensin (final concentration, 2 μM; Sigma; stock concentration, 72 mM in methanol) were added for the last 1.5 to 4 h of culture. After blocking of Fc receptors, cell surface molecules on harvested cells were stained with the following antibodies: anti-CD4 (clone GK1.5), anti-CD3 (clone 145-2C11), and anti-Thy1.2 (clone 30-H12). Intracellular cytokines were stained according to the manufacturer's instructions (Biolegend) with the following antibodies: anti-IFN-γ (clone XMG1.2), anti-interleukin 17A (anti-IL-17A) (clone TC11-18H10.1), and anti-tumor necrosis factor alpha (anti-TNF-α) (clone MP6-XT22). For RORγt staining, a FOXP3/transcription factor staining buffer set and anti-human/mouse RORγt (clone AFKJS-9) were used according to the manufacturer's instructions (eBioscience Inc.). Isotype-matched IgG was used for control staining for IFN-γ, IL-17A, TNF-α, and RORγt. Data were acquired with a BD FACSCaliburor BD FACSCantoII flow cytometer (BD Bioscience) and analyzed using FlowJo software (TreeStar Inc.).

Cytokine determinations. BMDCs (1×10^6 cells/ml) were incubated with heat-killed *C. gattii* cells for 24 h. After centrifugation, culture supernatants were collected and cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA). Cytokines in lung homogenates prepared from *C. gattii*-infected mice were also measured by ELISA. A MaxiSorp plate and a DuoSet ELISA kit (R&D Systems) or BD OptEIA ELISA sets (BD Bioscience) were used according to the manufacturers' instructions.

Vaccination. BMDCs (1×10^6 cells/ml) were incubated in complete RPMI 1640 medium that contained mGM-CSF and heat-killed *C. gattii* cells (1×10^7 cells/ml; MOI = 10) for 24 h in petri dishes. After incubation, nonadherent cells were collected and washed twice with PBS. BMDCs that were pulsed with heat-killed *C. gattii* cells (5×10^5 cells/mouse) were injected via a tail vein, both at 14 days and 1 day before intratracheal infection with *C. gattii* R265.

Infection study. *C. gattii* R265 was cultured in YPD at 30°C with shaking overnight. Cells were then washed and resuspended in PBS. A mouse was anesthetized with isoflurane, after which 50 μl of a fungal suspension (3×10^3 CFU) was intratracheally injected using a 24-gauge indwelling needle (TOP Corporation, Japan). Mice were euthanized by carbon dioxide inhalation, and their lungs were harvested to determine their weight and fungal burden. Lungs were homogenized using a stainless steel mesh in 5 ml of PBS. Homogenates were diluted and spread onto YPD plates. These plates were incubated at 30°C for 24 h, after which colonies were counted.

Histological analysis. Histological analyses were as previously described (8). To prepare specimens, three isolated lungs were fixed in 10% formalin, dehydrated, and embedded in paraffin. Paraffin blocks were cut into 4-μm sections and stained with hematoxylin and eosin, alcian blue, or Elastica van Gieson stain for light microscopy analysis. The cross point intervals of alveoli were measured to assess alveolar spaces and the levels of alveolar destruction as described previously (25). Multinucleated giant cells (MGCs) were examined to assess macrophage recruitment into lungs after infection. The numbers of MGCs per unit area (square millimeters), the numbers of nuclei within each MGC, and the nuclear density in MGCs were determined as described previously (26).

Statistical analysis. GraphPad Prism5 (GraphPad Software, Inc.) was used for statistical analyses. *P* values of <0.05 were considered significant.

RESULTS

BMDCs are activated by acapsular *C. gattii*. To design an effective DC-based vaccine, we first evaluated the phagocytosis efficiency, costimulatory molecule expression, and cytokine production by BMDCs that were cultured with *C. gattii* cells. Because the capsular component of *C. neoformans* is known to suppress several immune responses by BMDCs (27, 28), we constructed an acapsular Δ*cap60* strain (see Fig. S1 in the supplemental material) and compared this with encapsulated strains (Fig. 1). In a phagocytosis assay, 80% of BMDCs engulfed several Δ*cap60* cells within 24 h, while only 10% of BMDCs engulfed one or two cells of the encapsulated strains (Fig. 1B; see also Fig. S2 in the supplemental material). Major histocompatibility complex class II (MHC-II) molecules (I-Ab) are essential for antigen presentation to CD4 T cells, and CD86 and CD40 are important costimulatory molecules for T cell stimulation. The percentages of BMDCs that expressed these molecules were significantly increased when these cells were stimulated with the acapsular Δ*cap60* strain (Fig. 1C; see also Fig. S3 in the supplemental material). Additionally, BMDCs that were stimulated with the Δ*cap60* strain produced more inflammatory cytokines, including IL-12p40 and TNF-α, than BMDCs that were stimulated with encapsulated strains (Fig. 1D). These data showed that BMDCs could be activated by the acapsular Δ*cap60* strain and suggested that BMDCs pulsed with this strain could be used for a DC-based vaccine to induce T cell responses against *C. gattii*.

Transferring CAP60Δ/DCs ameliorates pulmonary infection with highly virulent *C. gattii*. We tested for a protective effect after vaccination with Δ*cap60* strain-pulsed DCs (CAP60Δ/DCs) in a mouse model of pulmonary *C. gattii* infection. Transferring CAP60Δ/DCs significantly suppressed fungal growth in mouse lungs and improved mouse survival rates after pulmonary infection with the highly virulent strain R265 (Fig. 2). Although *C.*

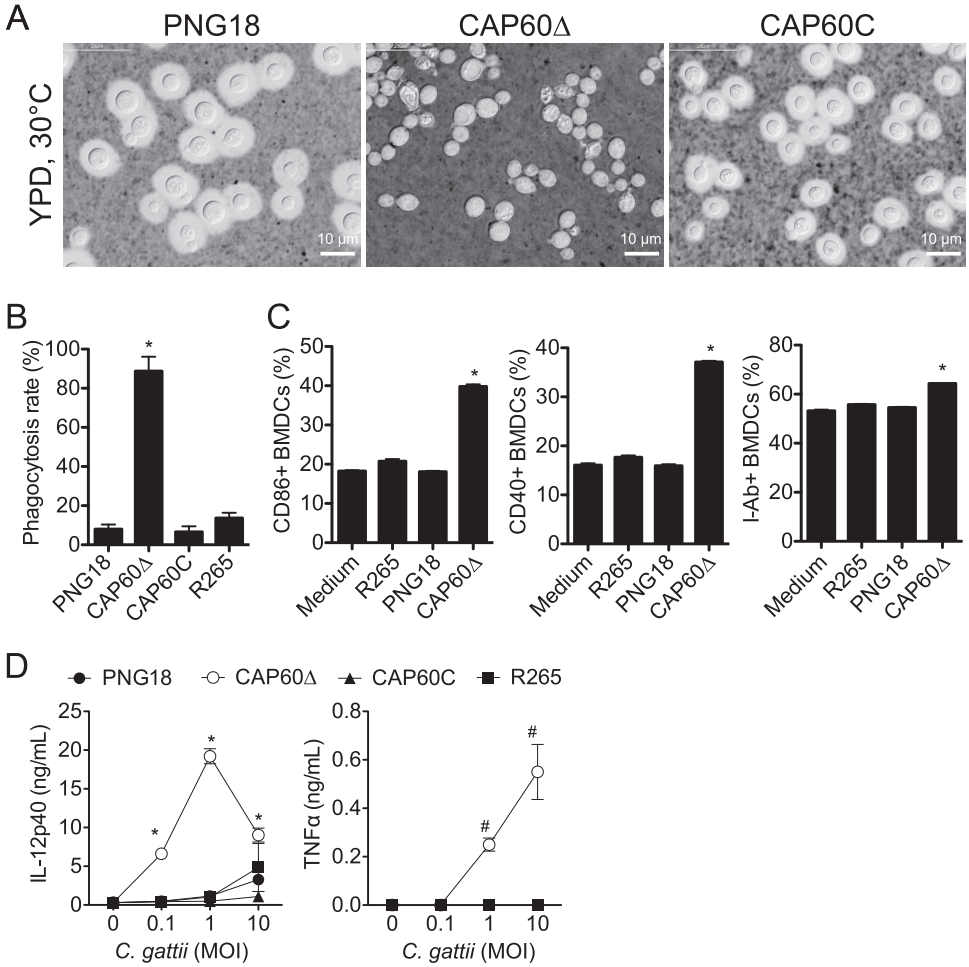

FIG 1 The *C. gattii* acapsular Δ*cap60* strain efficiently activates BMDCs. (A) Capsule formation was assessed using the conventional India ink method. Parental strain PNG18, the Δ*cap60* strain, and the revertant (CAP60C) were grown in YPD medium at 30°C overnight. BMDCs were incubated with heat-killed *C. gattii* cells for 24 h, and the phagocytosis rate (B), costimulatory molecule expression (C), and cytokine production (D) were evaluated by fluorescence microscopy, flow cytometry, and ELISA, respectively. For flow cytometry analysis, gates were set for $CD11c^+$ $CD11b^+$ cells. Representative data (means ± SDs) from 2 or 3 independent experiments are shown. *, $P < 0.05$ versus PNG18 by unpaired *t* test; #, $P < 0.05$ versus PNG18 by Mann-Whitney U test.

neoformans acapsular mutants have been successfully used as vaccines (29), vaccination using heat-killed R265 or heat-killed Δ*cap60* strain without DCs had no protective effect (see Fig. S4 in the supplemental material). In these experiments, we could not evaluate fungal burdens in the brain or spleen because of the limited number of fungal cells that had disseminated to the brain and spleen (data not shown). A previous study also showed that *C. gattii* R265 only minimally disseminated from the lungs to other organs in a murine pulmonary infection model (7).

To determine how our CAP60Δ/DC vaccine suppressed fungal growth, we histologically assessed mouse lung sections after pulmonary infection with highly virulent *C. gattii*. Lungs were significantly lighter in those mice immunized with our CAP60Δ/DC vaccine than in unvaccinated mice at day 13 postinfection (see Fig. S5 in the supplemental material). In unvaccinated mice, the increased numbers of fungal cells destroyed alveolar structures by enlarging alveolar spaces. In contrast, fewer fungal cells, increased numbers of leukocytes, and reduced alveolar destruction were observed in the lungs of mice that had been immunized with Δ*cap60* strain-loaded DCs (Fig. 3; see also Fig. S5 and Table S2 in the supplemental material). The majority of these leukocytes were mononuclear cells, including lymphocytes and macrophages, which developed into multinucleated giant cells (MGCs) that engulfed the fungal cells in the lungs of mice immunized with our CAP60Δ/DC vaccine. Large MGCs with a number of nuclei and an eosinophilic cytoplasm that represented the accumulated macrophages and well-matured cytoplasmic organelles, respectively, were significantly increased in immunized mice (Fig. 3C). Although transferring antigen-unloaded DCs also induced leukocyte accumulation and the development of MGCs in the lungs to some extent, MGCs with lesser eosinophilic cytoplasm and engulfing fewer fungal cells were observed in those mice that received antigen-unloaded DCs compared with those in immunized mice (Fig. 3). Increased numbers of leukocytes and multinucleated cells were also observed in the lungs of vaccinated mice at day 7 posttransfer (see Fig. S5). Taken together, these data suggested that the development of MGCs that engulfed fungal cells had suppressed the fungal growth in the lungs of immunized mice.

Transferring CAP60Δ/DCs induces cytokine-producing lymphocytes in spleen and lungs. The development of MGCs that

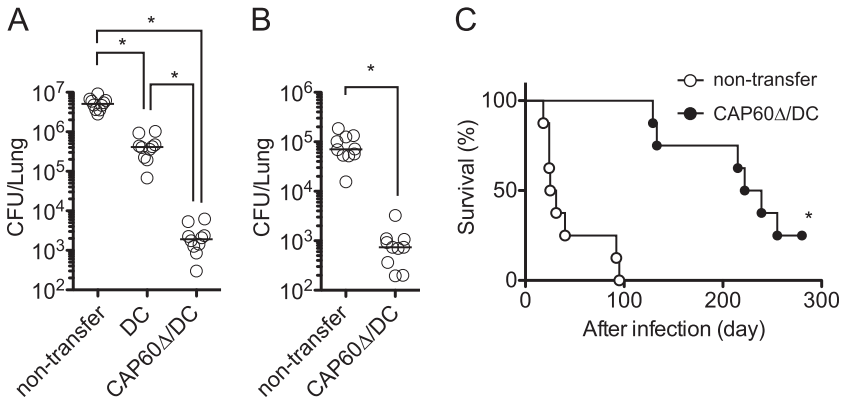

FIG 2 Transferring CAP60Δ/DCs ameliorates pulmonary infection with highly virulent *C. gattii* strain R265. CAP60Δ/DCs or unloaded DCs were injected into tail veins of mice at 14 days and 1 day prior to intratracheal infection with 3×10^3 CFU of strain R265. (A and B) Fungal burdens ($n = 5$) in the lungs were determined at day 14 (A) and day 3 (B). Pooled data from two independent experiments are plotted, and median values are shown by short horizontal lines. *, $P < 0.05$ by analysis of variance with Dunn's *post hoc* test (A) or $P < 0.05$ by Mann-Whitney U test (B). (C) Survival curves ($n = 8$) were compared by log rank test. Representative data from two independent experiments are shown. *, $P < 0.01$. Median survival times were 28 days in the case of nontransfer and 238 days in the case of CAP60Δ/DC transfer.

engulf fungal cells in granulomas is associated with protection from cryptococcosis due to *C. neoformans*, which is also associated with an increase in cytokine-producing T cells, such as Th1 and Th17 cells (30–33). Because DC-based vaccines reportedly augmented cytokine-producing effector T cells (17, 34), we assessed cytokine-producing T cells in the spleen and lungs after vaccination. IFN-γ^+ and TNF-α^+ CD4 T cells that responded to antigen restimulation were significantly increased in the spleens of vaccinated mice at day 14 after infection (Fig. 4). However, we could not detect any IL-17A-producing T cells (data not shown). Because the numbers of total splenocytes and proportions of $CD4^+$ cells among splenocytes were equal between unvaccinated and vaccinated mice at day 14 postinfection, the increased proportion of these cells indicated an increase in the numbers of this cell population.

Also, we utilized short-pulse stimulation using phorbol 12-myristate 13-acetate (PMA) and ionomycin to determine the numbers of cytokine-producing T cells in the lungs of vaccinated mice. IL-$17A^+$ RORγt^+ T cells and innate lymphoid cells (ILCs) were significantly increased in the lungs of vaccinated mice at day 1 postinfection (Fig. 5). CD4 T cells that produced IL-17A, IFN-γ, or TNF-α were also increased in the lungs of vaccinated mice at day 7 after transfer of CAP60Δ/DCs (see Fig. S6 in the supplemental material). At day 14 after infection, the amounts of IFN-γ, IL-17A, TNF-α, and CXCL1 (KC) were significantly increased in the lungs of immunized mice, and comparable amounts of IL-1β and smaller amounts of CCL2 (MCP-1) were detected (see Fig. S6). Because numerous lymphocytes and macrophages had already accumulated in the lungs of immunized mice, the amounts of MCP-1 may have decreased by day 14 after infection. These data suggested that increased T cell cytokine responses in vaccinated mice may have contributed to the development of MGCs that engulfed fungal cells after pulmonary infection with *C. gattii*.

IFN-γ may be an important mediator for vaccine-induced protection against pulmonary infection with highly virulent *C. gattii*. Previous studies showed that IFN-γ-producing T cells had a protective role in pulmonary infection with *C. neoformans* (35, 36). However, surprisingly, the fungal burden was significantly reduced in the lungs of IFN-γ knockout (KO) mice after the pulmonary infection with *C. gattii* compared with that in C57BL/6J wild-type (WT) mice (Fig. 6). Thus, we investigated a role for IFN-γ in DC vaccine-induced protection by determining the reduction rate of the fungal burden in the lungs of vaccinated mice. The DC-based vaccine induced an 8,900-fold reduction and a 2,700-fold reduction in the fungal burdens in the lungs of WT mice and IFN-γ KO mice, respectively. The protective effect of our DC vaccine was significantly reduced in IFN-γ knockout mice. This suggested that IFN-γ may be an important mediator for DC vaccine-induced protection against pulmonary infection with highly virulent *C. gattii*.

DISCUSSION

In this study, we developed a new DC-based vaccine to investigate protective immunity against highly virulent *C. gattii*. Our results showed that an increase in cytokine-producing CD4 T cells and the development of MGCs that engulfed fungal cells were associated with protection against pulmonary infection with highly virulent *C. gattii*.

Recently, Chaturvedi et al. reported a protein-based vaccine against highly virulent *C. gattii*. They identified several protein antigen candidates using two-dimensional PAGE (2D-PAGE) and immunoblotting with sera from vaccinated mice after infection (37). They immunized mice three times at 4-week intervals without any adjuvant and then challenged mice intranasally with 1×10^4 CFU of *C. gattii* R265 at 10 days after the final immunization. Although the protein vaccine significantly ameliorated the fungal burden in lungs and improved the survival rate after this challenge, this protein-based vaccine induced no more than about a 5-fold reduction in the fungal burden in lungs on day 14 postinfection (37). In our study, Δ*cap60* strain-loaded DCs were transferred at day 14 and day 1 prior to infection, and this DC-based vaccine provided for an 8,900-fold reduction in the fungal burden in mouse lungs at day 14 postinfection (Fig. 6). This DC-based vaccine still effectively suppressed fungal growth even when mice were infected with *C. gattii* at 2 months after the final transfer of CAP60Δ/DCs (unpublished data). Interestingly, vaccination with DCs loaded with the Δ*cap60* strain (VGI) effectively suppressed the growth of R265 (VGII). Thus, our data suggested that our

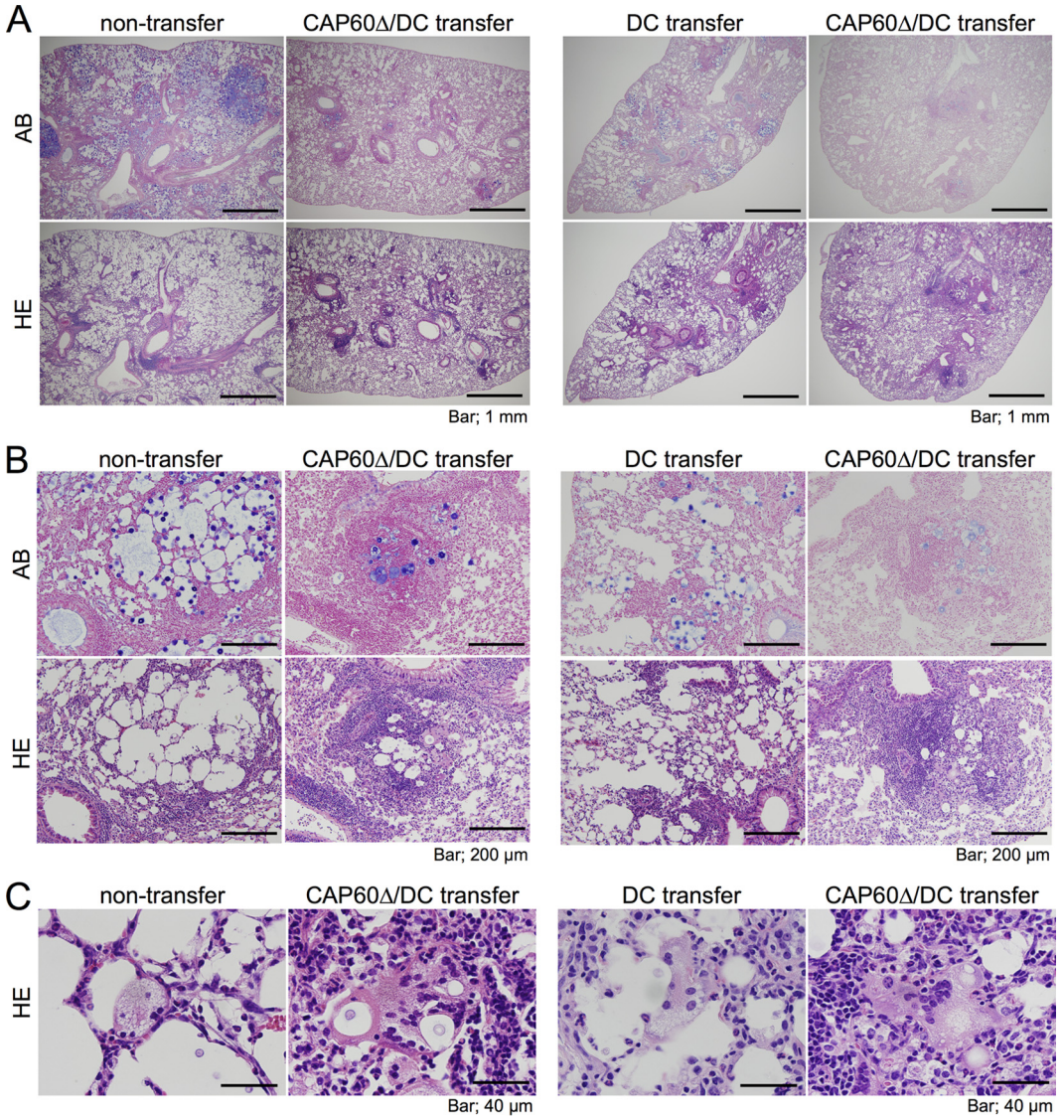

FIG 3 Multinucleated giant cells (MGCs) engulf fungal cells in the lungs of immunized mice. Lung sections obtained from three mice, at 13 days after infection with strain R265, were stained with hematoxylin-eosin (HE) or alcian blue (AB). *C. gattii* cells were stained with alcian blue. Each section was observed at magnifications of ×40 (A), ×200 (B), and ×1,000 (C).

CAP60Δ/DC vaccine could induce protection against *C. gattii* in a cross-strain manner and that this DC-based vaccine was useful for studying protective immunity against highly virulent *C. gattii*.

Huston et al. showed that human DCs derived from $CD14^+$ peripheral blood mononuclear cells (PBMCs) could engulf and kill *C. gattii* strain R265 but that DCs failed to mature in the presence of *C. gattii* (38). They also showed that TNF-α could restore DC maturation to induce T cell responses in the presence of *C. gattii*, which suggested the potential of DC-based therapies to improve the outcomes of patients with *C. gattii* infections. Another study showed that encapsulated *C. neoformans* strain B3501 (serotype D) could induce human DC maturation based on DC expression of CD40, CD86, and MHC-II (28). In our study, murine BMDCs did not mature in the presence of encapsulated *C. gattii* strains, because they did not efficiently engulf encapsulated *C. gattii* strains (Fig. 1). One study showed that murine BMDCs could mature after upregulated CD86 and MHC-II expression and cytokine production in the presence of encapsulated *C. neoformans* strain 1841 (serotype D) (27). Thus, both human DCs and murine BMDCs can become mature in the presence of encapsulated *C. neoformans*, but they did not mature in the presence of encapsulated *C. gattii*, as the phagocytosis rate for *C. gattii* cells seemed to be different between human DCs and murine BMDCs.

This failure of DC maturation may have negatively affected protection against pulmonary infection with *C. gattii*. In fact, transferring antigen-unloaded DCs prior to infection induced about a 10-fold reduction in the fungal burden in the lungs at day 14 postinfection (Fig. 2). Because BMDCs were cultured in the presence of GM-CSF, even antigen-unloaded DCs secreted a small amount (~300 pg/ml) of IL-12p40 (Fig. 1). Thus, transferring unloaded DCs seemed to induce a small number of IFN-γ-producing CD4 T cells in the spleen (Fig. 4). However, the protective

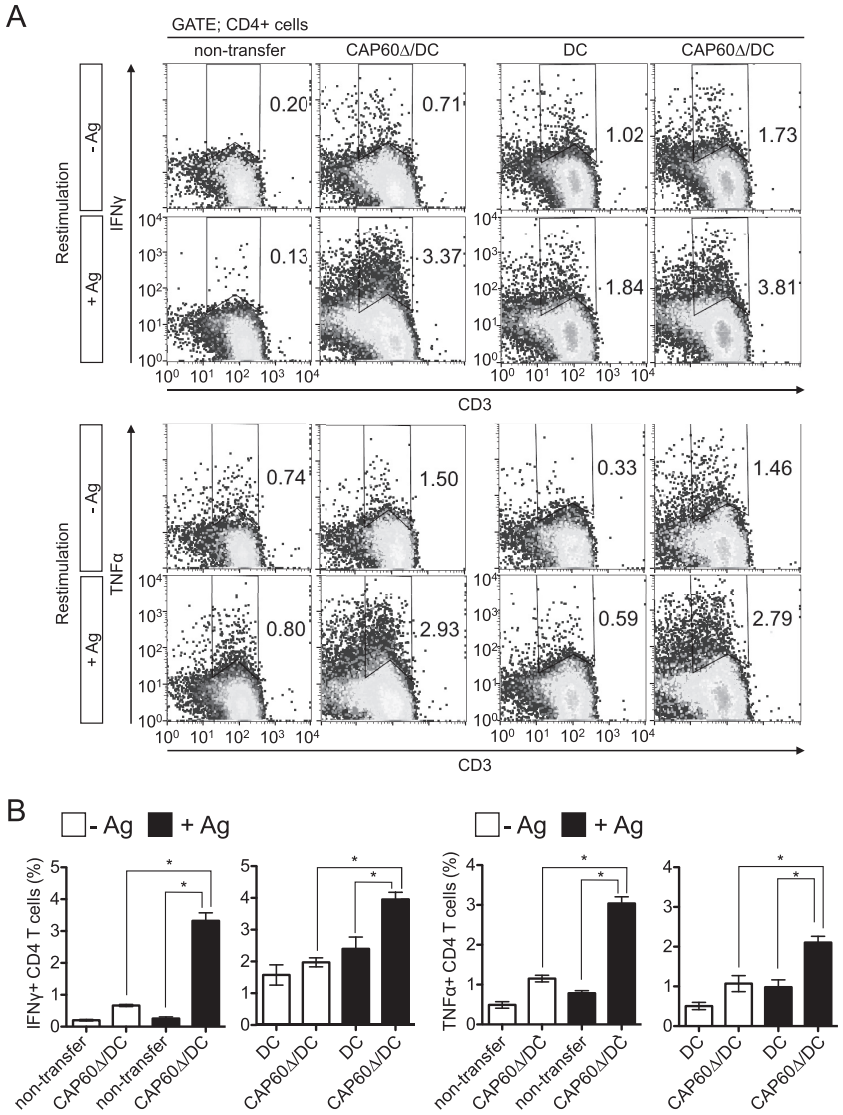

FIG 4 Transferring CAP60Δ/DCs induces cytokine-producing CD4 T cells in spleen. Splenocytes were obtained from three mice at 14 days after infection and cultured with (+Ag) or without (−Ag) antigen and with or without heat-killed Δ*cap60* cells (MOI = 0.1) for 5 to 6 days. For flow cytometry analysis, gates were set for $CD4^+$ cells. Representative flow cytometry profiles (A) and histograms for statistical analysis (B) are shown. Pooled data from two separate experiments were used to prepare histograms (means ± SEMs). *, $P < 0.05$ by analysis of variance with Dunnett's *post hoc* test.

effect was limited in those mice that received unloaded DCs compared with the effect in those that received Δ*cap60* strain-pulsed DCs, as transferring unloaded DCs induced fewer antigen-specific T cells. These findings suggested that the complete activation of DC and T cell responses was required for protection against pulmonary infection with highly virulent *C. gattii*.

In a strict sense, vaccination using CAP60Δ/DCs only delayed the progression of infection, as the fungal burden in the lungs at day 14 after infection was not lower than the inoculated burden (Fig. 2). In fact, even though mice were immunized with CAP60Δ/DCs, most of these mice ultimately died (Fig. 2C). This implied that highly virulent *C. gattii* could cause a persistent infection or could change to a latent state during a protective immune response. Persistent infection with *C. neoformans* was shown in a previous report, as *C. neoformans* could be detected in the lungs of immunocompetent rats at 18 months after an intratracheal infection, along with reduced nitric oxide synthase in pulmonary granulomas that harbored this pathogen (39). *C. gattii* may also cause a persistent infection by similar mechanisms. To overcome persistent *C. gattii* infection, the DC-based vaccine used in this study needs to be refined. Previous studies showed that treating antigen-loaded DCs with rapamycin, an mTOR inhibitor, enhanced T cell responses and augmented the vaccine's efficacy against tuberculosis or tumors. Because rapamycin can enhance DC autophagy, which can enhance antigen processing and presentation and also improve DC life span (34, 40), it may be useful to augment the protective effect of our DC-based vaccine against *C. gattii* infection.

Previous studies showed that T cells, particularly IFN-γ-producing T cells, were essential for protection against *C. neoformans* infection in mice (35, 36). IFN-γ production induced by *C. neoformans* infection enhanced the migration and killing capacity of

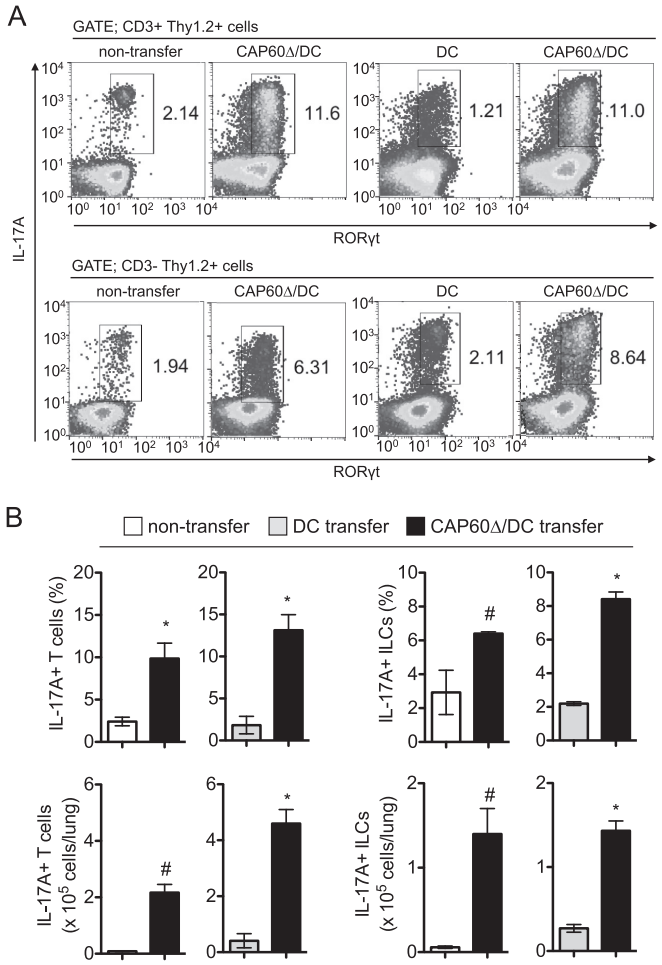

FIG 5 Transferring CAP60Δ/DCs induces IL-17A-producing cells in lungs. Lung leukocytes were obtained from three mice at 1 day after infection and were stimulated with PMA and ionomycin for 3 h. For flow cytometry analysis, gates were set for $CD3^+$ $Thy1.2^+$ cells or $CD3^-$ $Thy1.2^+$ cells. Representative flow cytometry profiles (A) and histograms for statistical analysis (B) from two independent experiments are shown. Results in histograms are means ± SDs. *, $P < 0.05$ versus control by unpaired *t* test; #, $P < 0.05$ versus control by unpaired *t* test with Welch's correction.

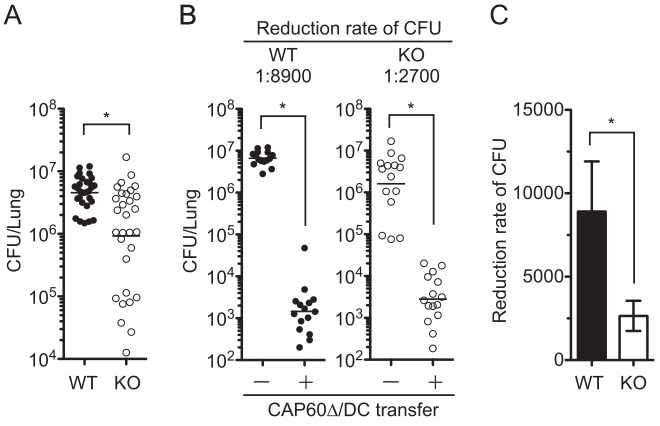

FIG 6 DC-based vaccine amelioration of the fungal burden is partially reduced in IFN-γ KO mice. (A) Fungal burdens in the lungs of C57BL/6J (WT) mice and IFN-γ KO mice were determined on days 9 to 14 postinfection. Data from six independent experiments were combined. (B and C) Vaccination and infection were performed as described in the legend to Fig. 2, and the fungal burden in lungs was determined on day 14 postinfection. Data from three independent experiments were combined. Vaccine effects between WT and KO mice were compared based on the reduced fungal burden rate (C). Panels A and B show dot plots with geometric means, and panel C shows means ± SEMs. *, $P < 0.05$ by Mann-Whitney U test.

macrophages (32, 41). Although transferring CAP60Δ/DCs strongly induced an IFN-γ response that was partially required for protection against *C. gattii*, this DC-based vaccine was still effective for inhibiting fungal growth in IFN-γ KO mice (Fig. 6). This suggested that both IFN-γ-dependent and IFN-γ-independent responses were involved in vaccine-induced protection against pulmonary infection with *C. gattii*.

The role of IL-17A in cryptococcosis is controversial. Several reports showed that IL-17A-producing CD4 T cells were increased in the lungs after pulmonary infection with *C. neoformans* (33, 42). Two reports indicated that IL-17A was dispensable for protection against pulmonary infection with *C. neoformans* (42, 43), while another report showed that IL-17A enhanced host defenses against pulmonary *C. neoformans* infection (33). It has also been shown that IL-17A was required for leukocyte accumulation, including numerous granulocytes, at the early phase of infection and for the development of MGCs that engulfed fungal cells at the late phase of *C. neoformans* infection. Thus, fungal clearance was impaired in IL-17A KO mice (33).

In our study, $IL\text{-}17A^+$ T cells and $IL\text{-}17A^+$ innate lymphoid cells (ILCs) increased in the lungs of immunized mice at day 1 postinfection (Fig. 5), and the fungal growth in mouse lungs was significantly suppressed at day 3 postinfection (Fig. 2). Additionally, the amount of IL-17A was also increased in the lungs of immunized mice at day 14 after infection (see Fig. S6 in the supplemental material). In agreement with previous findings that an IL-17A response induced neutrophil recruitment, the number of neutrophils increased in the lungs of immunized mice (see Fig. S5). Collectively, these results implied that IL-17A might contribute to vaccine-induced protection against pulmonary infection with highly virulent *C. gattii*. Further studies will be needed to determine the main factors of DC-based vaccine-induced immunity for protection against pulmonary infection with highly virulent *C. gattii*.

In summary, we demonstrated that transferring CAP60Δ/DCs strongly induced cytokine-producing CD4 T cells and MGCs that engulfed fungal cells and that this was associated with protection against pulmonary infection with highly virulent *C. gattii*. We propose that this DC-based vaccine is useful for (i) determining which antigens can induce cytokine-producing T cells and (ii) assessing those memory T cell responses that contribute to protection against highly virulent *C. gattii*. This may lead to the development of new means to control lethal *C. gattii* infections.

ACKNOWLEDGMENTS

This work was supported by Health Science Research Grants for Research on Emerging and Re-emerging Infectious Diseases (H25-Shinkou-Shitei-001, H25-Shinkou-Shitei-002, H25-shinkou-Wakate-005, H25-Shinkou-Ippan-006, and H26-Shinkoujitsuyouka-Ippan-010) from the Ministry of Health, Labor and Welfare of Japan, by a grant from the Strategic Research Foundation Grant-aided Project for Private Schools at Heisei 23rd, KAKENHI (26860774), from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and by a grant from the Life Science Foundation of Japan.

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