Dectin-2 Deficiency Promotes Th2 Response and Mucin Production in the Lungs after Pulmonary Infection with *Cryptococcus neoformans*

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Dectin-2 is a C-type lectin receptor that recognizes high mannose polysaccharides. *Cryptococcus neoformans*, a yeast-form fungal pathogen, is rich in polysaccharides in its cell wall and capsule. In the present study, we analyzed the role of Dectin-2 in the host defense against *C. neoformans* infection. In Dectin-2 gene-disrupted (knockout) (Dectin-2KO) mice, the clearance of this fungus and the inflammatory response, as shown by histological analysis and accumulation of leukocytes in infected lungs, were comparable to those in wild-type (WT) mice. The production of type 2 helper T (Th2) cytokines in lungs was higher in Dectin-2KO mice than in WT mice after infection, whereas there was no difference in the levels of production of Th1, Th17, and proinflammatory cytokines between these mice. Mucin production was significantly increased in Dectin-2KO mice, and this increase was reversed by administration of anti-interleukin 4 (IL-4) monoclonal antibody (MAb). The levels of expression of β 1-defensin, cathelicidin, surfactant protein A (Sp-A), and Sp-D in infected lungs were comparable between these mice. In *in vitro* experiments, IL-12p40 and tumor necrosis factor alpha (TNF- α) production and expression of CD86 and major histocompatibility complex (MHC) class II by bone marrow-derived dendritic cells and alveolar macrophages were completely abrogated in Dectin-2KO mice. Finally, the disrupted lysates of *C. neoformans*, but not of whole yeast cells, activated Dectin-2-triggered signaling in an assay with nuclear factor of activated T cells (NFAT)-green fluorescent protein (GFP) reporter cells expressing this receptor. These results suggest that Dectin-2 may oppose the Th2 response and IL-4-dependent mucin production in the lungs after infection with *C. neoformans*, and it may not be required for the production of Th1, Th17, and proinflammatory cytokines or for clearance of this fungal pathogen.

Cryptococcus neoformans, an opportunistic yeast-form fungal pathogen, infects the host via an airborne route (1). This fungus resists killing induced by macrophages, which enables its intracellular growth within these cells (2). The host defense against cryptococcal infection is mediated by the cellular immune response (3), and the type 1 helper T (Th1) response plays a critical role in eradicating this infection, whereas Th2 cytokines counter-regulate this response (4). Mice with a genetic disruption of Th1-related cytokines, such as gamma interferon (IFN- γ), interleukin 12 (IL-12), and IL-18, are highly susceptible to cryptococcal infection compared to wild-type (WT) control mice (5–7). In contrast, mice lacking Th2 cytokines, such as IL-4, IL-10, and IL-13, are more resistant to this infection than WT mice (8–10). For this reason, individuals with an impaired cellular immune response, such as hematological malignancy and AIDS, suffer from a severe *C. neoformans* infection that leads to disseminated infection in the central nervous system (11, 12).

When microorganisms infect the host, pathogen-associated molecular patterns (PAMPs) are recognized by the host immune cells via their pattern recognition receptors (PRRs), which initiates the host defense response (13). C-type lectin receptors (CLRs), a PRR-recognizing PAMP composed of polysaccharides, have garnered the attention of many investigators in the study of host defense against fungal infection (14). Recently, we demonstrated that caspase-associated recruitment domain 9 (CARD9), a common adaptor molecule delivering signals triggered by CLRs, plays a critical role in the host defense against infection with *C. neoformans* (15), suggesting the possible involvement of CLRs. In

our earlier study (16), the genetic defect of Dectin-1, a representative CLR recognizing β 1,3-glucan, did not influence the clearance of *C. neoformans*, and the inflammatory response during infection with this fungal pathogen suggests that other CLRs may be involved in this response.

Dectin-2 is expressed by a variety of myeloid cells, such as macrophages and dendritic cells (DCs), and its expression is enhanced during the inflammatory response (17, 18). Dectin-2 is

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TABLE 1 Primers for real-time PCR

Target ^a	Sense primer (5'–3')	Antisense primer (5'–3')
IFN- γ	ACTGCCACGGCAGATCATT	TCACCATCCTTTTGCCAGTTTCCT
IL-4	ACGAGGTCACAGGAGAAGGGA	TTGGAAGCCCTACAGACGAGC
IL-5	GCAATGGAAGGCTGAGGCTG	GGGTATGTGATCCTCCTGCGTC
IL-13	CTCTTGCTTGCCTTGGTGGTCT	ACTCCATACCATGCTGCCGTG
IL-17A	AACCGTTCCACGTCACCCTG	GTCCAGCTTTCCCTCCGCAT
MUC5AC	ACACCGCTCTGATGTTCTCACC	ATGTCCTGGGTTGAAGGCTCGT
Cathelicidin	GACACCAATCTCTACCGTCTCCT	TGCCTTGCCACATACAGTCTCCT
β 1-Defensin	GAGCATAAAGGACGAGCGA	CATTACTCAGGACCAGGCAGA
Sp-A	TCGGAGGCAGACATCCACA	GCCAGCAACAACAGTCAAGAAAG
Sp-D	TCAGTACCCAACACCTGCACCC	TCTCACCCCGTGGACCTTCTCT
HPRT	GCTTCCTCCTCAGACCGCTT	TCGCTAATCACGACGCTGGG

^a HPRT, hypoxanthine-guanine phosphoribosyltransferase; Sp-, surfactant protein.

involved in the recognition of high mannose polysaccharides, and its triggering leads to the production of various cytokines and chemokines, including proinflammatory Th1, Th17, and also Th2 cytokines (17, 18). Dectin-2 is known to recognize a variety of fungal pathogens, including *Candida albicans*, *Aspergillus fumigatus*, and noncapsular *C. neoformans* (19, 20), and indeed to play a critical role in the host defense against infection with *C. albicans* through inducing the Th17-mediated immune response (21). Like other fungal microorganisms, *C. neoformans* is rich in high-mannose polysaccharides, such as glucuronoxylomannan, galactoxylomannan, and mannoprotein, in its cell wall (22), which suggests that Dectin-2 may contribute to the recognition of this fungus by host immune cells. Thus, in the present study, we addressed the question of how dectin-2 is involved in the host defense immune response to infection with *C. neoformans*.

MATERIALS AND METHODS

Ethics statement. This study was performed in strict accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan, 2006. All experimental procedures involving animals followed the Regulations for Animal Experiments and Related Activities at Tohoku University, Sendai, Japan, and were approved by the Institutional Animal Care and Use Committee at Tohoku University (approval numbers 22 IDOU-108, 2011 IDOU-86, 2012 IDOU-124, and 2013 IDOU-257). All experiments were performed with the animals under anesthesia, and all efforts were made to minimize the suffering of the animals.

Mice. Dectin-2 gene-disrupted (knockout [KO]) mice were generated and established as described previously (21), and they were backcrossed to C57BL/6 mice for 7 generations. Littermate mice were used as wild-type (WT) control mice. Male or female mice at 6 to 8 weeks of age were used in the experiments. All mice were kept under specific-pathogen-free conditions at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

Microorganisms. A serotype D strain of *C. neoformans*, designated B3501 (provided by Kwong Chung, National Institutes of Health, Bethesda, MD, USA), was used. In some experiments, *C. albicans* (ATCC 18804) was used. The fungal microorganisms were cultured on potato dextrose agar (PDA; Eiken, Tokyo, Japan) plates for 2 to 3 days before use. Mice were anesthetized using an intraperitoneal injection of 70 mg pentobarbital/kg of body weight (Abbott Laboratory, North Chicago, IL, USA) and restrained on a small board. Live *C. neoformans* (1×10^6 cells or 1×10^5 cells in some experiments) was inoculated at 50 μ l into the trachea of each mouse using a 24-gauge catheter (Terumo, Tokyo, Japan).

Enumeration of viable *C. neoformans* cells. Mice were sacrificed on days 7, 14, and 28 after infection. The lungs were dissected carefully,

excised, and then homogenized separately in 5 ml of distilled water by teasing them with a stainless-steel mesh at room temperature. The homogenates, diluted appropriately with distilled water, were inoculated at 100 μ l on PDA plates and cultured for 2 to 3 days before the resulting colonies were counted.

Histological examination. The lung specimens obtained from mice were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin (H-E) or periodic acid-Schiff (PAS) stain using standard staining procedures at the Biomedical Research Core, Animal Pathology Platform, of Tohoku University Graduate School of Medicine. Mucin production was estimated on the PAS-stained sections as the proportion of mucin-producing bronchi relative to total bronchi. The mucin-producing bronchi were classified into three categories: –, mucin-negative bronchi; +, bronchi with mucin production in 0 to 50% of bronchoepithelial cells; and ++, bronchi with mucin production in 50 to 100% of bronchoepithelial cells.

Cytokine assay. Concentrations of IL-1 β , IL-6, tumor necrosis factor alpha (TNF- α), IL-12p40, gamma interferon (IFN- γ), IL-17A, IL-4, IL-5, IL-13, IL-10, and transforming growth factor β (TGF- β) in the lung homogenates and culture supernatants were measured using the appropriate enzyme-linked immunosorbent assay (ELISA) kits (BioLegend for IL-1 β , IL-6, IFN- γ , IL-17A, IL-4, IL-5, and IL-10; eBioscience [San Diego, CA, USA] for IL-13 and TGF- β ; BD Bioscience [Franklin Lakes, NJ, USA] for TNF- α and IL-12p40).

Extraction of RNA and quantitative real-time RT-PCR. Total RNA was extracted from the infected lungs using Isogen (Wako Pure Chemical, Osaka, Japan), and the first-strand cDNA was synthesized using a PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time reverse transcription (RT)-PCR was performed in a volume of 20 μ l using gene-specific primers and FastStart Essential DNA Green Master (Roche Applied Science, Branford, CT, USA) in a LightCycler Nano system (Roche Applied Science). The primer sequences for amplification are shown in Table 1. Reaction efficiency with each primer set was determined using standard amplifications. Target gene expression levels and that of hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a reference gene were calculated for each sample using the reaction efficiency. The results were analyzed using a relative quantification procedure and are illustrated as expression relative to HPRT expression.

Preparation of lung leukocytes. Pulmonary intraparenchymal leukocytes were prepared as previously described (23). Briefly, the chest of each mouse was opened and the lung vascular bed was flushed by injecting 3 ml of chilled physiological saline into the right ventricle. The lungs were then excised and washed in physiological saline. The lungs, teased apart with a 40- μ m cell strainer (BD Falcon, Bedford, MA, USA), were incubated in RPMI 1640 medium (Nipro, Osaka, Japan) with 5% fetal calf serum (FCS; BioWest, Nuaille, France), 100 U/ml penicillin G, 100 μ g/ml streptomycin, 10 mM HEPES, 50 μ M 2-mercaptoethanol, and 2 mM L-glutamine

FIG 1 *C. neoformans* infection in Dectin-2KO mice. WT and Dectin-2KO mice were infected intratracheally with *C. neoformans*. The numbers of live colonies in the lungs were counted on days 7, 14, and 28 after infection. Each bar represents the mean $\pm$ SD of results for five mice. Experiments were repeated twice, with similar results. NS, not significant.

containing 20 U/ml collagenase and 1 μ g/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 60 min at 37°C with vigorous shaking, the tissue fragments and the majority of dead cells were removed by passing the cells through the 40- μ m cell strainer. After centrifugation, the cell pellet was resuspended in 4 ml of 40% (vol/vol) Percoll (Pharmacia, Uppsala, Sweden) and layered onto 4 ml of 80% (vol/vol) Percoll. After centrifugation at 600 $\times$ g for 20 min at 15°C, the cells at the interface were collected, washed three times, and counted using a hemocytometer. The obtained cells were centrifuged onto a glass slide at 110 $\times$ g for 3 min using Cytospin-2 (StatSpin Inc., Norwood, MA, USA), stained with Diff-Quick (Sysmex, Kobe, Japan), and observed under a microscope. The number of leukocyte fractions was estimated by multiplying the total leukocyte number by the proportion of each fraction in 200 cells.

Flow cytometry. The lung leukocytes were cultured at 1×10^6 /ml with 5 ng/ml phorbol 12-myristate 13-acetate (PMA), 500 ng/ml ionomycin, and 2 nM monensin (Sigma-Aldrich) in RPMI 1640 medium supplemented with 10% FCS (BioWest, Nuaillé, France) for 4 h. The cells were

washed three times in phosphate-buffered saline (PBS) containing 1% FCS and 0.1% sodium azide and then stained with allophycocyanin (APC)-anti-CD3 ϵ monoclonal antibody (MAb) (clone 145-2C11; BioLegend), phycoerythrin (PE)-anti-CD4 MAb (clone GK1.5; BioLegend), and allophycocyanin cyanine 7 (APC/Cy7)-anti-CD8a MAb (clone 53-6.7; BioLegend). After being washed twice, the cells were incubated in the presence of Cytofix/Cytoperm (BD Bioscience), washed twice in BD Perm/Wash solution (BD Bioscience), and stained with fluorescein isothiocyanate (FITC)-anti-IFN- γ or IL-17A MAb (clone XMG1.2 for IFN- γ [BioLegend] and clone TC11-18H10.1 for IL-17A [BioLegend]) and Alexa Fluor 488-anti-IL-4 (clone 11B11; BioLegend). Isotype-matched IgG was used for control staining. The stained cells were analyzed using a BD FACSCanto II flow cytometer (BD Bioscience). Data were collected from 20,000 to 30,000 individual cells using forward-scatter and side-scatter parameters to set a gate on the lymphocyte or myeloid cell population. The number of particular cells was estimated by multiplying the lymphocyte or myeloid cell number, calculated as mentioned above, by the proportion of each subset.

Administration of anti-IL-4 MAb. Anti-IL-4 MAb was purified from culture supernatants of hybridoma (clones 11B11) using a protein G column kit (Kierkegaard & Perry Laboratories, Gaithersburg, MD, USA). To neutralize the biological activity of IL-4, mice were injected intraperitoneally with 200 μ g of MAb against this cytokine on day 1 before and days 0, 3, and 7 after infection.

Preparation and culture of DCs. Dendritic cells (DCs) were prepared from bone marrow (BM) cells as described by Lutz and coworkers (24). Briefly, BM cells from mice were cultured at 2×10^5 /ml in 10 ml RPMI 1640 medium (Nipro, Osaka, Japan) supplemented with 10% FCS, 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF; Wako, Osaka, Japan). 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 or 9, nonadherent cells were collected and used as BM DCs. The obtained cells were cultured with *C. neoformans* or lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 37°C in a 5% CO₂ incubator. The culture supernatants were measured for synthesis of IL-12p40 and TNF- α by ELISA. The cells were stained with FITC-anti-CD11c (clone N418; eBioscience), Pacific Blue-anti-CD86 (clone GL1; BioLegend), and PE-anti-I-A/I-E MABs (clone M5/114.15.2; BioLegend), and the expression

FIG 2 Effect of Dectin-2 deficiency on the inflammatory response in lungs. WT and Dectin-2KO mice were infected intratracheally with *C. neoformans*. (A) Sections of the lungs on day 14 postinfection were stained with H-E and observed under a light microscope at $\times 50$ or $\times 200$ magnification. Representative pictures from the five to six mice are shown. Experiments were repeated twice, with similar results. (B) The lung leukocytes prepared on day 7 postinfection were stained with Diff-Quick and observed under a light microscope. The amount of cells in each leukocyte fraction was counted. Each bar represents the mean $\pm$ SD of results for five mice. NS, not significant.

of CD86 and MHC class II on CD11c⁺ cells was analyzed using a flow cytometer.

Preparation of alveolar macrophages. The chests of WT and Dectin-2KO mice were opened, and their tracheae were cannulated (22-gauge intravenous [i.v.] catheter). PBS (1 ml) was infused intratracheally and withdrawn. This procedure was performed three times. Bronchoalveolar lavage (BAL) fluids were centrifuged at $450 \times g$ for 10 min at 4°C, and the pellets were collected and suspended in the culture medium. The cells contained more than 95% alveolar macrophages. The obtained cells were cultured at 1×10^6 /ml with *C. neoformans* or LPS for 24 h at 37°C in a 5% CO₂ incubator.

Dectin-2-NFAT-GFP reporter assay. T cell hybridoma 2B4 was transfected with the NFAT-GFP construct prepared by fusing three tandem NFAT-binding sites with enhanced GFP cDNA (25). This cell line was transfected with Dectin-2 and FcRγ genes, and the same cell line but lacking Dectin-2 was used as a control. These cells were stimulated for 20 h at 2.5×10^5 /ml with *C. neoformans* or its disrupted lysates, which had been prepared using Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) according to the manufacturer's instructions, and the expression of GFP was analyzed on the CD3⁺ cells, but not on dead 7-aminoactinomycin D (7-AAD)-stained cells, by flow cytometry.

Statistical analysis. Data were analyzed using JMP Pro 10.0.2 software (SAS Institute Japan, Tokyo, Japan). Data are expressed as means $\pm$ standard deviations (SD). Differences between groups were examined for statistical significance using Welch's *t* test. A *P* value of less than 0.05 was considered significant.

RESULTS

Effect of Dectin-2 deficiency on the clearance of *C. neoformans* and the inflammatory response in lungs. To elucidate the effect of Dectin-2 deficiency on the host defense against cryptococcal infection, the clinical courses between WT and Dectin-2KO mice after infection with *C. neoformans* were compared. None of these mice died during the observation period (up to day 56). Next, the numbers of live colonies in lungs between these mice on days 7, 14, and 28 postinfection were compared. As shown in Fig. 1, there was no significant difference in the numbers of live *C. neoformans* cells at any time point between WT and Dectin-2KO mice. The live colonies of *C. neoformans* were not detected in the brains of both mouse strains on day 28 postinfection (data not shown). In addition, similar results were obtained when mice were infected with a lower dose of *C. neoformans*, 1×10^5 CFU/mouse (see Fig. S1 in the supplemental material). We then investigated how Dectin-2 deficiency affected the inflammatory response in the lungs after cryptococcal infection. Histological analysis did not find any apparent difference in the HE-stained lung sections on day 14 after infection (Fig. 2A). In addition, we examined the leukocyte fractions obtained from the lungs on day 14 after infection. The total numbers of leukocytes were not significantly different between WT and Dectin-2KO mice ($3.7 \times 10^5 \pm 2.3 \times 10^5$ versus $5.1 \times 10^5 \pm 2.2 \times 10^5$ [5 mice each]), and the lymphocytes, macrophages, neutrophils, and eosinophils were found in comparable proportions and counts in these mice (Fig. 2B and C). These results indicated that Dectin-2 was not required for the clearance of *C. neoformans* and the inflammatory response caused by this infection in the lungs.

Increased production of Th2 cytokines in Dectin-2KO mice after infection with *C. neoformans*. In the next series of experiments, the effect of Dectin-2 deficiency on the cytokine response to *C. neoformans* was examined by measuring the concentrations of various cytokines in the lung homogenates on days 0, 1, 3, 7, 14, and 21 postinfection. As shown in Table 2, proinflamma-

TABLE 2 Effect of Dectin-2 deficiency on cytokine production in lungs

Cytokine	Production (pg/g lung) in indicated mice on ^a :											
	Day 0			Day 1			Day 3			Day 7		
	WT	KO	WT	WT	KO	WT	WT	KO	WT	WT	KO	KO
IL-1β	9,262 $\pm$ 1,605	9,007 $\pm$ 1,270	8,849 $\pm$ 3,009	9,939 $\pm$ 3,027	11,313 $\pm$ 1,032	8,411 $\pm$ 2,192*	5,051 $\pm$ 577	5,218 $\pm$ 727	6,003 $\pm$ 1,933	6,715 $\pm$ 1,349	9,256 $\pm$ 1,456	8,092 $\pm$ 1,357
IL-6	2,714 $\pm$ 694	2,606 $\pm$ 302	2,939 $\pm$ 1,277	2,826 $\pm$ 1,429	4,243 $\pm$ 2,004	2,574 $\pm$ 1,083	663 $\pm$ 213	867 $\pm$ 174	1,163 $\pm$ 255	1,695 $\pm$ 402*	2,468 $\pm$ 665	2,711 $\pm$ 360
TNF-α	570 $\pm$ 79	568 $\pm$ 73	559 $\pm$ 100	724 $\pm$ 269	1,624 $\pm$ 1,023	1,366 $\pm$ 547	277 $\pm$ 67	362 $\pm$ 115	334 $\pm$ 83	358 $\pm$ 79	338 $\pm$ 100	318 $\pm$ 93
IL-12p40	2,088 $\pm$ 226	1,829 $\pm$ 202	3,400 $\pm$ 574	3,126 $\pm$ 308	23,193 $\pm$ 2,822	25,590 $\pm$ 3,577	4,530 $\pm$ 713	3,632 $\pm$ 1,216	2,334 $\pm$ 196	2,127 $\pm$ 136	6,198 $\pm$ 2,230	6,415 $\pm$ 2,914
IFN-γ	1,168 $\pm$ 342	1,121 $\pm$ 252	10,389 $\pm$ 2,303	9,539 $\pm$ 691	14,323 $\pm$ 4,695	15,319 $\pm$ 3,378	5,214 $\pm$ 538	6,234 $\pm$ 674	7,715 $\pm$ 1,611	7,132 $\pm$ 534	2,853 $\pm$ 954	3,182 $\pm$ 377
IL-17A	2,522 $\pm$ 437	2,617 $\pm$ 294	2,303 $\pm$ 616	2,831 $\pm$ 993	1,965 $\pm$ 399	1,582 $\pm$ 384	1,391 $\pm$ 536	1,166 $\pm$ 287	941 $\pm$ 151	1,232 $\pm$ 213*	1,749 $\pm$ 548	1,754 $\pm$ 327
IL-4	106 $\pm$ 20	86 $\pm$ 21	176 $\pm$ 89	139 $\pm$ 45	170 $\pm$ 79	226 $\pm$ 69	287 $\pm$ 49	1,307 $\pm$ 534*	631 $\pm$ 488	848 $\pm$ 328	182 $\pm$ 80	312 $\pm$ 49*
IL-5	215 $\pm$ 25	215 $\pm$ 19	3,011 $\pm$ 482	3,050 $\pm$ 280	1,731 $\pm$ 163	2,644 $\pm$ 782*	1,295 $\pm$ 99	1,542 $\pm$ 195*	2,465 $\pm$ 366	3,593 $\pm$ 637*	130 $\pm$ 40	126 $\pm$ 17
IL-13	486 $\pm$ 77	449 $\pm$ 44	417 $\pm$ 96	439 $\pm$ 132	198 $\pm$ 22	276 $\pm$ 73*	257 $\pm$ 54	547 $\pm$ 208*	396 $\pm$ 81	748 $\pm$ 178*	595 $\pm$ 168	882 $\pm$ 113*
IL-10	4,423 $\pm$ 756	4,593 $\pm$ 684	3,423 $\pm$ 1,187	4,554 $\pm$ 1,376	737 $\pm$ 116	1,724 $\pm$ 820*	641 $\pm$ 100	1,137 $\pm$ 356*	1,222 $\pm$ 286	2,071 $\pm$ 540*	1,966 $\pm$ 908	1,829 $\pm$ 214
TGF-β	8,519 $\pm$ 2,058	7,512 $\pm$ 2,060	8,597 $\pm$ 2,343	10,242 $\pm$ 3,376	9,287 $\pm$ 2,391	10,007 $\pm$ 3,611	5,192 $\pm$ 2,407	4,584 $\pm$ 2,997	7,784 $\pm$ 2,296	6,272 $\pm$ 3,362	4,889 $\pm$ 1,375	4,432 $\pm$ 2,355

^a Cytokine production (pg/g lung) at the indicated time intervals after *C. neoformans* infection. Data shown are means $\pm$ SD of results for four to seven mice. Experiments were repeated twice with similar results. *, *P* < 0.05, compared to value for WT mice.

FIG 3 Effect of Dectin-2 deficiency on the T cell immune response. WT and Dectin-2KO mice were infected intratracheally with *C. neoformans*. (A) IFN- γ , IL-4, IL-5, IL-13, and IL-17A mRNA expression in the lungs was measured on day 7 after infection. Each bar represents the mean $\pm$ SD of results for three to four mice. Experiments were repeated twice, with similar results. NS, not significant; *, $P < 0.05$. (B) The lung leukocytes were prepared on day 7 postinfection. IFN- γ , IL-4, and IL-17A expression in CD3⁺ CD4⁺ or CD8⁺ T cells was analyzed using flow cytometry. Each bar represents the mean $\pm$ SD of results for three to five mice. Experiments were repeated twice, with similar results. NS, not significant; *, $P < 0.05$.

tory cytokines (such as IL-1 β , IL-6, TNF- α , and IL-12p40) and Th1 and Th17 cytokines (such as IFN- γ and IL-17A, respectively) were not significantly influenced by Dectin-2 deficiency compared to those in WT mice, except for IL-1 β on day 3, IL-6 on day 14, and IL-17A on day 14; IL-1 β was reduced, and IL-6 and IL-17A were increased. In contrast, the production of Th2 cytokines, such as IL-4, IL-5, and IL-13, was significantly increased on days 7 and 21, days 3, 7, and 14, and days 3, 7, 14, and 21, respectively. Similarly, production of anti-inflammatory cytokines, such as IL-10, was significantly increased on days 3, 7, and 14. We also examined IFN- γ , IL-4, and IL-17A mRNA expression on day 7 postinfection. As shown in Fig. 3A, the expression of Th2 cytokines, such as IL-4, IL-5, and IL-13, was significantly higher in Dectin-2KO mice than that in WT mice, whereas IFN- γ and IL-17A were expressed at equivalent levels in these mice. Additionally, we examined the intracellular expression of these cytokines in lung T cells on day 7, and expression of IL-4, but not of IFN- γ and IL-17A, was significantly increased in both CD4⁺ and CD8⁺ T cells (Fig. 3B).

Increased production of mucin in Dectin-2KO mice after infection with *C. neoformans*. In previous studies, Zuyderduyn and coworkers reported that mucin production was enhanced by IL-4 under conditions of *Pseudomonas aeruginosa* infection (26). In addition, recently, Grahnert et al. demonstrated that mucin production was ameliorated in the lungs of IL-4RKO mice compared to that in WT mice after infection with *C. neoformans* (27). We next examined the expression of mucin detected by PAS staining

in the lung sections on day 14 after cryptococcal infection. As shown in Fig. 4A to C, numbers of bronchi expressing mucin were significantly increased and numbers of mucin-negative bronchi were significantly decreased in Dectin-2KO mice compared with those detected in WT mice. Mucin is composed of various core proteins, including MUC5AC, a major one secreted from bronchial epithelial cells (28). Therefore, we examined the effect of Dectin-2 deficiency on MUC5AC expression in the lungs. As shown in Fig. 4D, this expression was significantly increased in the lungs of Dectin-2KO mice compared with that in WT mice. In addition, we addressed whether IL-4 was involved in the increased production of mucin in Dectin-2 KO mice by testing the effect of neutralizing anti-IL-4 MAb. Administration of this MAb led to significant reduction in the mucin production observed in Dectin-2KO mice (Fig. 5A and B). However, the same treatment did not show any influence on the clearance of *C. neoformans* in lungs on day 14 postinfection (data not shown). These results indicated that Dectin-2 deficiency led to the increase of mucin production in the lungs after cryptococcal infection, which suggested that Dectin-2 may be involved in the regulation of the Th2-mediated response and mucosal surface barrier in the lungs.

Effect of Dectin-2 deficiency on the humoral host defense factors. Mucous layers on the bronchoepithelial cells contain various humoral host defense factors, such as β -defensin, cathelicidin, and collectins (collagen-containing C-type lectins), which show antimicrobial and proinflammatory activities (29–31). Sur-

FIG 4 Mucin production after infection with *C. neoformans*. WT and Dectin-2KO mice were infected intratracheally with *C. neoformans*. (A) Sections of the lungs on day 14 postinfection were stained with PAS and observed under a light microscope at $\times 200$ magnification. Arrows indicate the mucin secreted by bronchoepithelial cells. Representative pictures from the five to six mice are shown. The proportion of mucin-producing bronchi (B) and the classification of mucin-producing bronchi (C) were determined. Each bar represents the mean $\pm$ SD of results for five to six mice. Experiments were repeated twice, with similar results. *, $P < 0.05$. (D) MUC5AC mRNA expression in the lungs was measured on day 7 after infection. Each bar represents the mean $\pm$ SD of results for three to four mice. Experiments were repeated twice, with similar results. *, $P < 0.05$.

factant protein A (Sp-A) and Sp-D host factors belonging to collectins are reported to bind to *C. neoformans*, and Sp-D promotes the phagocytosis of this fungus by macrophages (32, 33). We examined whether these host defense factors were affected by Dectin-2 deficiency during infection with *C. neoformans*. As shown in Fig. 6, there was no significant difference in the levels of expression of $\beta 1$ -defensin, cathelicidin, and Sp-A in the lungs of WT and Dectin-2KO mice 7 days after infection, although the Sp-D level was significantly lower in Dectin-2KO mice.

Reduced production of TNF- α and IL-12p40 by BM DCs and alveolar macrophages in Dectin-2KO mice. We examined how Dectin-2 deficiency affected *in vitro* proinflammatory cytokine production by BM DCs and alveolar macrophages and expression of the maturation markers CD86 and MHC class II by BM DCs upon stimulation with *C. neoformans*. As shown in Fig. 7A and B, TNF- α and IL-12p40 production by BM DCs and TNF- α production by alveolar macrophages were strongly suppressed in Dectin-2KO mice compared to production levels in WT mice when these

FIG 5 Effect of anti-IL-4 MAb on mucin production. Dectin-2KO mice were infected intratracheally with *C. neoformans*. Mice were injected intraperitoneally with anti-IL-4 MAb and control rat IgG. Sections of the lungs on day 14 postinfection were stained with H-E or PAS and observed under a light microscope. The proportion of mucin-producing bronchi (A) and the classification of mucin-producing bronchi (B) were determined. Each column represents the mean $\pm$ SD of results for five mice. *, $P < 0.05$.

FIG 6 Effect of Dectin-2 deficiency on antimicrobial peptides and surfactant proteins. Cathelicidin, β 1-defensin, Sp-A, and Sp-D mRNA expression in the lungs was measured on day 7 after infection. Each bar represents the mean $\pm$ SD of results for three to four mice. Experiments were repeated twice, with similar results. NS, not significant; *, $P < 0.05$.

cells were stimulated with *C. neoformans*. IL-12p40 was not detected in the culture supernatants of alveolar cells stimulated with *C. neoformans*. Similar results were obtained in the expression of CD86 and MHC class II (Fig. 7C). These results suggested that Dectin-2 may be involved in the recognition of this fungal pathogen by DCs and macrophages.

Activation of a Dectin-2-triggered signal by *C. neoformans*.

We examined whether *C. neoformans* directly triggered an activation signal via Dectin-2 using an NFAT-GFP reporter assay. As shown in Fig. 8A, whole *C. neoformans* yeast cells did not induce GFP expression by the reporter cells, whereas heat-killed *C. albicans* (HKCA) caused this activation. To address the possibility that Dectin-2 ligand may not be expressed on their surfaces, we examined whether disrupted *C. neoformans* lysates activated the reporter cells. As shown in Fig. 8B, the disrupted lysates induced GFP expression by the reporter cells, although this activity was not as high as that of HKCA. In contrast, the control cells lacking Dectin-2 did not show activation of the GFP gene by any stimulation.

DISCUSSION

Major findings in the present study are the following: (i) levels of clearance of *C. neoformans* in the lungs were almost equivalent in Dectin-2KO mice and WT mice; (ii) there was no apparent difference in the inflammatory responses in the infected lungs of these mice; (iii) production of Th2 cytokines, but not Th1, Th17, and proinflammatory cytokines, after infection was significantly increased in Dectin-2KO mice compared to that in WT mice; (iv) mucin and MUC5AC production in infected lungs was higher in Dectin-2KO mice than in WT mice; (v) increased mucin production was reversed by administration of anti-IL-4 MAb to Dectin-2KO mice; (vi) the levels of expression of β 1-defensin, cathelicidin, Sp-A, and Sp-D in infected lungs were comparable between

these mice; (vii) TNF- α and IL-12p40 production by BM DCs and alveolar macrophages and CD86 and MHC class II expression by BM DCs were mostly abrogated in Dectin-2KO mice compared to those in WT mice; and (viii) disrupted *C. neoformans* lysates, but not whole yeast cells, induced the activation signal triggered by Dectin-2 in an NFAT-GFP reporter assay. These results suggest that Dectin-2 may play a role in the host response during infection with *C. neoformans*.

In *in vivo* experiments, *C. neoformans* clearances and the inflammatory responses, as shown by the histological analysis and analysis of the accumulated leukocytes in infected lungs, were almost comparable between WT and Dectin-2KO mice, which suggests that Dectin-2 may not be required for host defense against this infection. In contrast, Dectin-2KO mice were more susceptible to infection with *C. albicans* than WT mice, as shown by the shortened survival and attenuated clearance of fungal microorganisms in the kidney, and this reduced host resistance was due to impairment in the appropriate host immune response (21). Similar results were reported for *C. glabrata* infection (34). In host defense against candida infection, neutrophils play a central role (35), and IL-17A is an important cytokine for the appropriate immune response (36). Consistently with this notion, in these studies with *Candida* spp., the Th17 response was ameliorated under conditions lacking Dectin-2. In the present study, Th1 cytokine production and inducible NO synthase expression (see Fig. S2 in the supplemental material) were not hampered in Dectin-2KO mice during infection with *C. neoformans*. These results were consistent with the data showing no impairment in the clearance of *C. neoformans* from these mice, because the Th1 response and NO production are required for the host defense against this fungus (4, 37). In addition, levels of IL-17A production were equivalent

FIG 7 Production of proinflammatory cytokines by BM DCs and alveolar macrophages in Dectin-2KO mice. (A, C) BM DCs were prepared from WT and Dectin-2KO mice and stimulated with the indicated doses of *C. neoformans* or LPS for 24 h. IL-12p40 and TNF- α production in the culture supernatants (A) and cell surface expression of CD86 and MHC class II (C) were analyzed. MHC class II expression was expressed as mean fluorescence intensity (Log₁₀MFI). (B) Alveolar macrophages were prepared from WT and Dectin-2KO mice and stimulated with the indicated doses of *C. neoformans* or LPS for 24 h, and production of TNF- α was measured. Each bar represents the mean $\pm$ SD of results for triplicate cultures. Experiments were repeated twice, with similar results. MOI, multiplicity of infection. *, $P < 0.05$.

lent in WT and Dectin-2KO mice during this infection, which is in contrast to what occurred during infection with *Candida* spp., although the role of the Th17 response in the host defense against *C. neoformans* seems limited (38, 39).

Dectin-2KO mice showed increases in their production of Th2 cytokines, such as IL-4, IL-5, and IL-13, but not Th1 and Th17 cytokines, after infection with *C. neoformans*. Previously, Barrett and coworkers demonstrated that Dectin-2 is an important recognition receptor of house dust mites for inducing the Th2-type immune response through the production of cysteinyl leukotriene (40, 41). These previous findings were obtained in an allergic-disease model, and a similar mechanism may exist during cryptococcal infection. Although it remains to be elucidated how the Th2 response was promoted under Dectin-2-deficient conditions in our model, the production of Th2 cytokines was increased in the adaptive immune phase on days 7 and 14, and both CD4⁺ and CD8⁺ T cells expressed IL-4 within their cytoplasm on day 7 after infection with *C. neoformans*. This suggests that Th2 differentiation is promoted in Dectin-2KO mice. For Th2 cell differentiation, IL-4 is required during the innate immune phase (42), and innate immune cells, such as NKT cells, are thought to be the source of IL-4 production (43). In our unpublished data, there was no significant difference between WT and Dectin-2KO mice in IL-4 expression by NKT cells, a potent lymphocyte subset that

synthesizes IL-4 (44). Other innate immune cells may contribute to the early production of this cytokine.

C. neoformans resists macrophage killing, which enables it to grow within these phagocytes (2). Therefore, the cell-mediated immune response promoted by Th1 cytokines is essential for the host defense against this fungus, and Th2 cytokines suppress the protective Th1 response (3–10). In Dectin-2KO mice, Th2 cytokine production was enhanced after infection with *C. neoformans*, but the Th1 response and clearance of this fungal pathogen were not affected, suggesting that the increased Th2 response may not be enough to ameliorate the host defense against this infection. On the other hand, mucin expression in infected lungs was promoted under conditions lacking Dectin-2, which was reversed by administration of neutralizing anti-IL-4 MAb. In agreement with these results, Zuyderduyn and coworkers previously demonstrated that Th2 cytokines were involved in the elimination of *Pseudomonas aeruginosa* by bronchoepithelial cells through inducing the production of mucin and antimicrobial peptides (26). In addition, Grahner et al. recently reported that IL-4R α KO mice showed an attenuated host defense to *C. neoformans* infection through reduced mucin production in the lungs (27). Taken together with these findings, Dectin-2 may have an impact on host defense against cryptococcal infection through regulating IL-4-dependent mucin production by bronchoepithelial cells, although an effect

FIG 8 Dectin-2-NFAT-GFP reporter assay. The NFAT-GFP reporter cells expressing Dectin-2 or control cells were cultured with *C. neoformans* (A) or the disrupted lysates (B), and the expression of GFP was analyzed using a flow cytometer. Heat-killed *C. albicans* (HKCA) was used as a positive control. Representative data from three independent experiments are shown. MOI, multiplicity of infection.

on the production of antimicrobial peptides was not found in our study.

In *in vitro* experiments, alveolar macrophages and BM DCs almost completely lost their ability to produce TNF- α and IL-12p40 and to increase the expression of CD86 and MHC class II upon stimulation with *C. neoformans*, suggesting the existence of a certain ligand for Dectin-2 in this fungus. However, unexpectedly, whole *C. neoformans* yeast cells did not induce the activation of Dectin-2-expressing NFAT-GFP reporter cells. We addressed another possibility, namely, that the putative Dectin-2 ligand may not be expressed on the surfaces of, but rather inside, yeast cells. In agreement with this possibility, disrupted *C. neoformans* lysates triggered the activation signal via Dectin-2, and Dectin-2 did not bind to whole *C. neoformans* yeast cells in a binding assay using Dectin-2-Fc fusion protein (see Fig. S2 in the supplemental material). In an earlier study by McGreal and coworkers (20), Dectin-2-Fc fusion protein bound to an acapsular strain of *C. neoformans*, Cap67, but not to the capsular strain, B3501, used in the current study, suggesting that this acapsular strain may trigger the activation signal by Dectin-2. However, whole yeast cells of the Cap67 strain did not induce GFP expression by the reporter cells, although its lysates showed this activation (data not shown). In addition, Dectin-2 fused with the Fc portion of human immunoglobulin (Dectin-2-Fc) did not bind to the acapsular strain (Cap67), which is a result similar to that for the capsular strain (B3501) (see Fig. S3 in the supplemental material). It is currently

not clear why the results in these two studies showed a difference in levels of binding of Dectin-2-Fc fusion protein to the acapsular *C. neoformans* strain.

Thus, the current results suggest that a certain ligand that is not expressed on the surface of *C. neoformans* may be detected by Dectin-2 and may play a role in the host immune response to this fungal pathogen. However, even though this possibility exists, it is not known how the internal ligand accesses Dectin-2 through the capsule. To address this issue, it is necessary to define the Dectin-2 ligand in this fungal pathogen. An explanation for the discrepant results between the *in vitro* and *in vivo* experiments remains unclear. A compensatory mechanism may operate to activate the critical Th1-mediated immune response in place of the Dectin-2-mediated pathway. Recently, Viriyakosol and coworkers reported a similar discrepancy between *in vitro* and *in vivo* experiments in a fungal infection, in which *in vitro* production of proinflammatory cytokines by macrophages stimulated with *Coccidioides immitis* was reduced in Dectin-2KO mice, whereas the clearance of this fungal pathogen in the lungs and spleen was comparable to that in WT mice (45).

In our recent study, mice lacking CARD9, a common adapter molecule in signaling via CLRs, including Dectin-2, were highly susceptible to infection with *C. neoformans*, which was associated with the amelioration of early IFN- γ production (15). This was consistent with the possibility that Dectin-2 is involved in the host defense against this fungus. However, in contrast to CARD9KO

mice, Dectin-2KO mice were resistant to this infection, with no impairment of IFN- γ production, but the Th2 response and IL-4-dependent mucin production were promoted. These distinct results between CARD9KO and Dectin-2KO mice suggest that the immune response during *C. neoformans* infection may be modified by signals via other CLR receptors besides Dectin-2. There are CLR receptors, including Dectin-1 and Mincle, besides Dectin-2 that use CARD9 as a common adaptor molecule to transduce the activation signal (46). Our previous study showed that, among these receptors, Dectin-1 was not likely to be involved in this response because its deficiency disturbed neither the Th1-mediated immune response nor *C. neoformans* clearance (16). There is currently no information on the role of Mincle in host defense to this fungal pathogen.

In conclusion, we demonstrated that the absence of Dectin-2 led to an acceleration of the Th2 response and IL-4-dependent mucin production but did not affect the proinflammatory response, Th1 and Th17 cytokine production, or *C. neoformans* clearance in infected lungs. These results suggested that Dectin-2 may be involved in triggering a negative signal for the production of Th2 cytokines and mucin and that it may be involved in the recognition of *C. neoformans* for this response. Thus, the current study has important implications for understanding the precise mechanism of the host defense against this infection. However, there are many issues to be solved. For example, *C. neoformans* used in this study was serotype D, not serotype A, the latter of which is more common in AIDS patients (47). Because the carbohydrates on serotypes A and D are different, the current findings may not apply to serotype A. Thus, further investigation is necessary for a better understanding of the innate immune response during infection with *C. neoformans*.

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