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FUNGAL AND PARASITIC INFECTIONS

# Limited Role of Mincle in the Host Defense against Infection with *Cryptococcus deneoformans*

**Yuki Sato,[a] Ko Sato,[b] Hideki Yamamoto,[a]* Jun Kasamatsu,[b] Tomomitsu Miyasaka,[c] Daiki Tanno,[a]* Anna Miyahara,[a] Takafumi Kagesawa,[a] Akiho Oniyama,[a] Kotone Kawamura,[a] Rin Yokoyama,[a]* Yuki Kitai,[a] Aya Umeki,[a] Shigenari Ishizuka,[a] Kazuki Takano,[a] Ryuhei Shiroma,[a] Nana Nakahata,[a] Kaori Kawakami,[c] Emi Kanno,[d] Hiromasa Tanno,[d] Sho Yamasaki,[e,f,g,h] Hiromitsu Hara,[i] Keiko Ishii,[a] Kazuyoshi Kawakami[a,b]**

[a]Department of Medical Microbiology, Mycology, and Immunology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[b]Department of Intelligent Network for Infection Control, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[c]Division of Pathophysiology, Department of Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan
[d]Department of Science of Nursing Practice, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[e]Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan
[f]Laboratory of Molecular Immunology, Immunology Frontier Research Center, Osaka University, Suita, Osaka, Japan
[g]Division of Molecular and Cellular Immunology, Research Center for Infectious Diseases, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan
[h]Division of Molecular Immunology, Medical Mycology Research Center, Chiba University, Chiba, Japan
[i]Department of Immunology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan

**ABSTRACT** *Cryptococcus deneoformans* is an opportunistic fungal pathogen that frequently causes fatal meningoencephalitis in patients with impaired cell-mediated immune responses such as AIDS. Caspase-associated recruitment domain 9 (CARD9) plays a critical role in the host defense against cryptococcal infection, suggesting the involvement of one or more C-type lectin receptors (CLRs). In the present study, we analyzed the role of macrophage-inducible C-type lectin (Mincle), one of the CLRs, in the host defense against *C. deneoformans* infection. Mincle expression in the lungs of wild-type (WT) mice was increased in the early stage of cryptococcal infection in a CARD9-dependent manner. In Mincle gene-disrupted (Mincle KO) mice, the clearance of this fungus, pathological findings, Th1/Th2 response, and antimicrobial peptide production in the infected lungs were nearly comparable to those in WT mice. However, the production of interleukin-22 (IL-22), tumor necrosis factor alpha (TNF-$\alpha$), and IL-6 and the expression of AhR were significantly decreased in the lungs of Mincle KO mice compared to those of WT mice. In *in vitro* experiments, TNF-$\alpha$ production by bone marrow-derived dendritic cells was significantly decreased in Mincle KO mice. In addition, the disrupted lysates of *C. deneoformans*, but not those of whole yeast cells, activated Mincle-triggered signaling in an assay with a nuclear factor of activated T cells (NFAT)-green fluorescent protein (GFP) reporter cells expressing this receptor. These results suggest that Mincle may be involved in the production of Th22-related cytokines at the early stage of cryptococcal infection, although its role may be limited in the host defense against infection with *C. deneoformans*.

**KEYWORDS** *Cryptococcus deneoformans*, Mincle, Th22, host defense

The two sister species *Cryptococcus neoformans* (formerly *C. neoformans* var. *grubii* serotype A) and *Cryptococcus deneoformans* (formerly *C. neoformans* var. *neoformans* serotype D) are yeast-type opportunistic fungal pathogens with thick polysaccharide capsules. These fungi cause life-threatening meningoencephalitis in patients with impaired immunity such as AIDS (1–4). Both of these *Cryptococcus* spp. are

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Address correspondence to Ko Sato, ko-sato@med.tohoku.ac.jp.

* Present address: Hideki Yamamoto, Center for Transdisciplinary Research, Institute of Research Promotion, Niigata University, Niigata, Japan; Daiki Tanno, Department of Clinical Laboratory, Fukushima Medical University, Fukushima, Japan; Rin Yokoyama, Department of Clinical Laboratory, University of Tokyo Hospital, Tokyo, Japan.

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intracellularly growing yeasts, and the cellular immune mechanism plays a central role in eradication of these fungi, which is critically regulated by Th1-Th2 immune balance (2, 5–8). Th1-related cytokines accelerate the killing of *Cryptococcus* via macrophages by inducing the production of nitric oxide (NO) (9–11). In contrast, the Th2 immune response disturbs the host defense against this infection by suppressing macrophage killing and granuloma formation (8, 9, 12). It remains to be clarified how Th17-related cytokines, such as interleukin-17A (IL-17A), IL-22, and IL-23, contribute to the host defense against this infection (13–18).

Pathogen-associated molecular patterns (PAMPs) are recognized by the host immune cells via pattern recognition receptors (PRRs), which trigger the inflammatory responses (19). C-type lectin receptors (CLRs) act as a sensor molecule for polysaccharides rich in the cell wall of fungal pathogens (20). Previously, we demonstrated that caspase-associated recruitment domain 9 (CARD9), a common adaptor molecule for CLR-mediated signaling, plays a critical role in the host defense to cryptococcal infection (21). The key members of CLRs, Dectin-1, Dectin-2, and Dectin-3 (also known as MCL, Clec4D, and Clecsf8) and macrophage-inducible C type lectin (Mincle), were found to participate in the immune response to fungal pathogens (22–29). In our previous studies (30–32), elimination of cryptococcal yeast cells in the infected lungs was not affected by the genetic defect of either Dectin-1 or Dectin-2 that recognizes $\beta$-1,3-glucan or high-mannose polysaccharides, respectively, although the Dectin-2 ligand is likely expressed in the cell wall of *C. deneoformans*. Huang et al. demonstrated that Dectin-3 recognizes glucuronoxylomannan of *C. neoformans* hybrid serotype AD and *Cryptococcus gattii* serotype B, but not *C. neoformans*, *C. deneoformans*, or *C. gattii* serotype C (33). In addition, Campuzano et al. revealed that Dectin-3 is not required for protection against infection with *C. neoformans* or *C. deneoformans* (34). These findings suggest that some CLR other than these molecules may be involved in the host defense against infection with *C. deneoformans*.

Mincle, a type II transmembrane protein with a short cytoplasmic tail and an extracellular ligand-binding domain, delivers activation signals through the Fc receptor $\gamma$-chain molecule containing immunoreceptor tyrosine-based activation motif (ITAM) (35, 36). This CLR recognizes a wide range of PAMPs and damage-associated molecular patterns (DAMPs), such as trehalose-6,6-dimycolate (TDM) of *Mycobacterium tuberculosis*, $\beta$-glucosyl-diacylglycerol of *Streptococcus pneumoniae*, spliceosome-associated protein 130 (SAP130), and mammalian $\beta$-glucosyl ceramide ($\beta$-GlcCer) (36–39). Wells et al. demonstrated that Mincle deficiency showed significantly increased susceptibility to systemic candidiasis (40). Yamasaki et al. analyzed more than 50 species of pathogenic fungi for their possession of Mincle-binding ligands and revealed that Mincle recognizes specific structures of $\alpha$-mannosyl residues on *Malassezia* species (41). However, the role of Mincle in the host defense against cryptococcal infection has not been clarified. Thus, in the present study, we examined the effect of Mincle deficiency on the host protection and immune response against *C. deneoformans* using a mouse model of pulmonary infection.

## RESULTS

**Kinetics in the expression of Mincle after infection with *C. deneoformans*.** Mincle expression is strongly induced in response to several inflammatory stimuli, such as infection (35). To clarify the kinetics of Mincle expression after infection with *C. deneoformans*, we first measured the expression of this mRNA in the lungs at various time points. Such expression was increased to a peak level on day 3 and then decreased to the basal level on day 7 after infection (Fig. 1a). In addition, the Mincle expression was significantly lower in CARD9 Mincle gene-disrupted (KO) mice than in wild-type (WT) mice on day 3 (Fig. 1b). To elucidate the localization of Mincle-expressing cells, we observed the immunostained tissues in the lungs of WT and Mincle KO mice on days 0 and 3 after cryptococcal infection. Although the positive cells were scarcely detected in the uninfected lungs, the Mincle-expressing cells were increased in the area surrounding this fungal pathogen in the infected lungs (Fig. 1c). Furthermore, in a flow

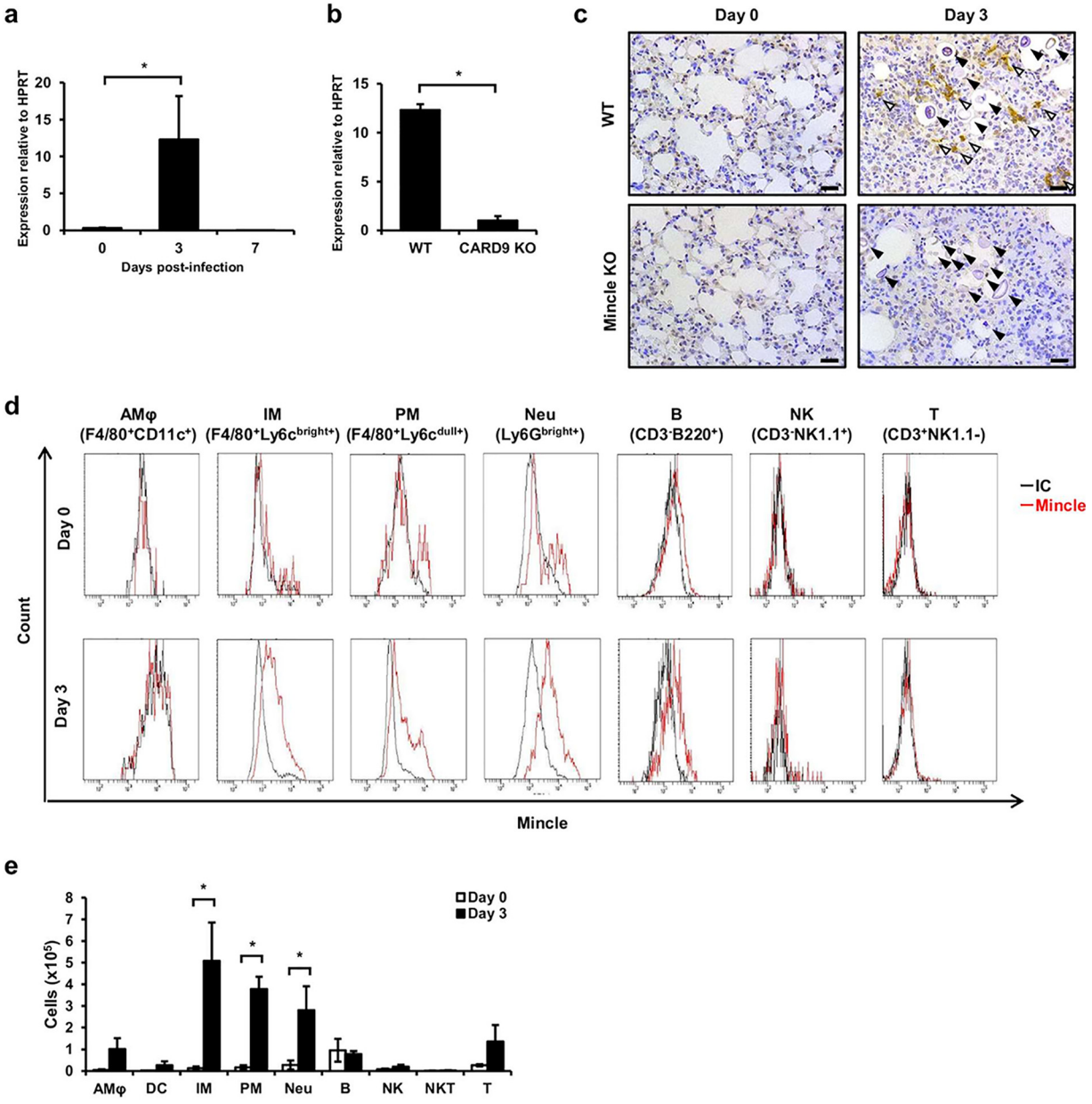


**FIG 1** Mincle expression in lungs after infection with *C. deneoformans*. (a) WT mice were infected intratracheally with *C. deneoformans*. Expression of Mincle mRNA in the lungs was measured on days 0, 3, and 7 postinfection. Each column represents the mean ± SD of three to five mice. Experiments were repeated twice with similar results, and representative results are shown. (b) WT and CARD9 KO mice were infected intratracheally with *C. deneoformans*. Expression of Mincle mRNA in the lungs was measured on day 3 postinfection. Each column represents the mean ± SD of three to five mice. Experiments were repeated twice with similar results, and representative results are shown. (c) WT and Mincle KO mice were infected intratracheally with *C. deneoformans*. Lung tissue sections of WT and Mincle KO mice uninfected and 3 days after infection were immunostained with anti-CLEC4E antibody and observed under a light microscope at ×400 magnification. Mincle KO mice were used as a negative control. Scale bars: 25 $\mu$m. Closed arrows, yeast cells; open arrows, Mincle-expressing cells. Representative pictures of four mice are shown. (d and e) WT mice were infected intratracheally with *C. deneoformans*. Lung leukocytes were prepared in uninfected mice and at 3 days postinfection, and Mincle expression in these cells was analyzed using a flow cytometer (d). AM$\varphi$, alveolar macrophages; IM, inflammatory monocytes; PM, patrolling monocytes; Neu, neutrophils; B, B cells; NK, natural killer cells; T, T cells; IC, isotype control. Representative histograms of four mice are shown. (e) The number in each cell subset was calculated. Each column represents the mean ± SD of four mice. *, $P < 0.05$.

cytometry analysis, we examined the expression of Mincle on various populations of leukocytes in the lungs. Mincle was expressed on inflammatory monocytes, patrolling monocytes, neutrophils, and B cells but not on alveolar macrophages, NK cells, and T cells in the lungs of uninfected mice, although the expression was slight (Fig. 1d). Inflammatory monocytes, patrolling monocytes, and neutrophils expressed higher

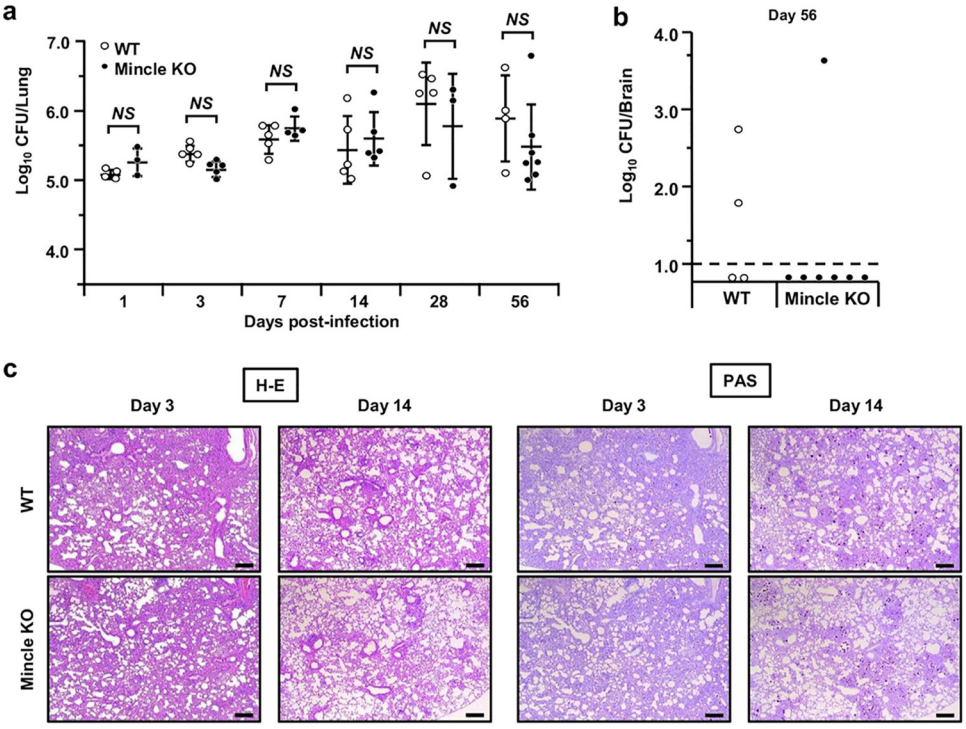


**FIG 2** Effect of Mincle deficiency on *C. deneoformans* infection. WT and Mincle KO mice were infected intratracheally with *C. deneoformans*. (a and b) The numbers of live colonies in the lungs on days 1, 3, 7, 14, 28, and 56 (a) and in the brains on day 56 postinfection (b) were counted. Each symbol represents a separate mouse, and bars indicate the mean ± SD of three to seven mice. Experiments were repeated twice with similar results, and representative results are shown. (c) Sections of the lungs on days 3 and 14 postinfection were stained with H&E or PAS and observed under a light microscope at ×40 magnification. Scale bars: 250 $\mu$m. Representative pictures of three to five mice are shown. NS, not significant.

levels of Mincle (Fig. 1d), and the number of these cells was markedly increased on day 3 after cryptococcal infection (Fig. 1e). These results suggest that the accelerated expression of Mincle may be due to an increased proportion of myeloid cells expressing this molecule and to an accumulation of these inflammatory cells in the lungs after infection with *C. deneoformans*.

**Effect of Mincle deficiency on the host protection and inflammatory response against cryptococcal infection.** Next, we examined the effect of Mincle deficiency on the host defense to cryptococcal infection. None of these mice died during the observation periods (up to day 56). The fungal burdens in the lungs were not significantly different between WT and Mincle KO mice on days 1, 3, 7, 14, 28, and 56 postinfection (Fig. 2a). Similarly, the fungal burdens in the brain were not increased in Mincle KO mice compared to those in WT mice on day 56 postinfection (Fig. 2b). In addition, we investigated how Mincle deficiency affected the inflammatory response in the lungs after cryptococcal infection. There was not an apparent difference in the infiltration of inflammatory cells or formation of granulomatous lesions in hematoxylin and eosin (H&E)-stained lung tissues between WT and Mincle KO mice on days 3 and 14 (Fig. 2c). In periodic acid-Schiff (PAS)-stained lung tissues, the fungal burdens also did not clearly differ between the two mouse strains (Fig. 2c).

**Effect of Mincle deficiency on the production of TNF-$\alpha$ and IL-12 by BM-DCs.** During infection with various microorganisms, proinflammatory cytokines play a central role in the initiation of the inflammatory response (42). Therefore, we examined how Mincle deficiency affected the *in vitro* production of proinflammatory cytokines, tumor necrosis factor alpha (TNF-$\alpha$) and IL-12p40, by bone marrow-derived dendritic cells (BM-DCs) upon stimulation with *C. deneoformans*. TNF-$\alpha$ production was slightly, but significantly, decreased in Mincle KO mice compared to in WT mice. However, IL-12p40

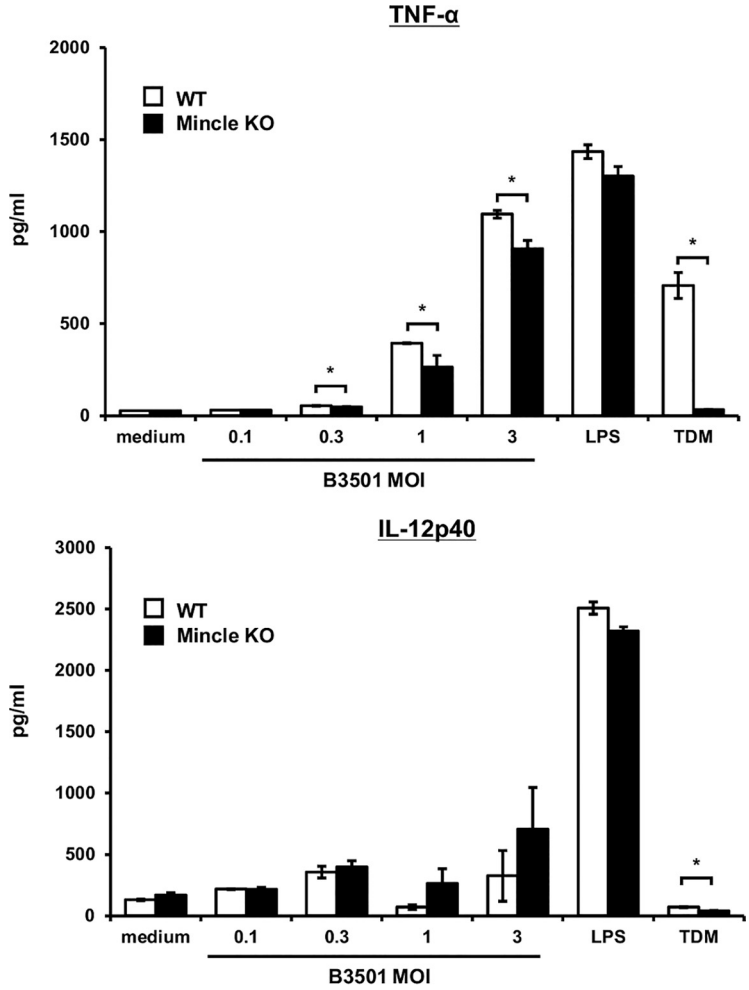


**FIG 3** Effect of Mincle deficiency on the production of proinflammatory cytokines by BM-DCs. BM-DCs were prepared from WT and Mincle KO mice and stimulated with the indicated dose of *C. deneoformans*, LPS (1 $\mu$g/ml), or TDM (1 $\mu$g/ml) for 24 h. Production of TNF-$\alpha$ and IL-12p40 in the culture supernatants was analyzed. Each column represents the mean $\pm$ SD of triplicate cultures. Experiments were repeated twice with similar results, and representative results are shown. *, $P < 0.05$. MOI, multiplicity of infection.

production was not decreased in Mincle KO mice. BM-DCs significantly produced these cytokines when stimulated with TDM, a well-known ligand for Mincle (Fig. 3).

**Suppressed IL-22 production in Mincle KO mice after infection with *C. deneoformans*.** Host defense to cryptococcal infection is largely regulated by a balance between the Th1 and Th2 immune responses (8, 9). In the next series of experiments, the effect of Mincle deficiency on the cytokine and chemokine production in responses to *C. deneoformans* was examined by measuring their concentration and mRNA expression in the lungs on days 1, 3, 7, 14, and 28 after infection. In the Th1/Th2-related cytokines, production of IL-12p40 was significantly decreased in Mincle KO mice compared to WT mice on day 7, whereas there was no significant difference in the production of interferon-$\gamma$ (IFN-$\gamma$), IL-4, IL-5, and IL-13 and the expression of IL-12p35 mRNA at any time points (Fig. 4a and b). Production of proinflammatory cytokines (IL-6 and TNF-$\alpha$), with the exception of IL-1$\beta$, was significantly reduced in Mincle KO mice compared to WT mice on day 3 (Fig. 4a). Production and expression of anti-inflammatory cytokines (IL-10 and transforming growth factor $\beta$ [TGF-$\beta$]) and inflammatory chemokines (keratinocyte-derived chemokine [KC], macrophage inflammatory protein [MIP]-1$\alpha$, MIP-2, and regulated on activation, normal T cell expressed and secreted [RANTES]) were not significantly different between the two mouse strains at any time points (Fig. 3a and b). In the Th17-related cytokines, IL-17A production and

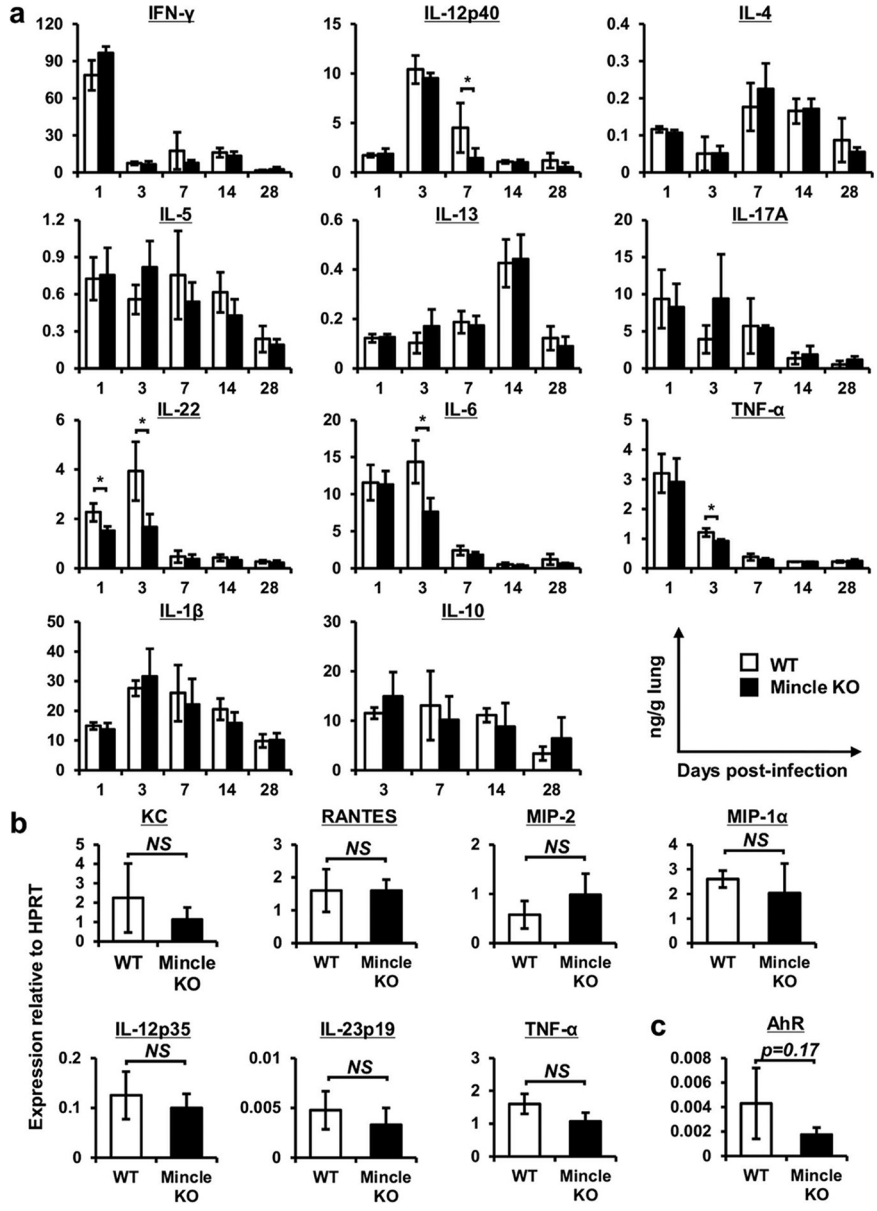


**FIG 4** Effects of Mincle deficiency on the production of cytokines and chemokines in the lungs after infection with *C. deneoformans*. WT and Mincle KO mice were infected intratracheally with *C. deneoformans*. (a) IFN-$\gamma$, IL-12p40, IL-4, IL-5, IL-13, IL-17A, IL-22, IL-6, TNF-$\alpha$, IL-1$\beta$, and IL-10 production in lung homogenates was measured on days 1, 3, 7, 14, and 28 postinfection. Each column represents the mean ± SD of three to seven mice. Experiments were repeated twice with similar results, and representative results are shown. (b) Expression of IL-12p35, IL-23p19, TGF-$\beta$, KC, MIP-1$\alpha$, MIP-2, and RANTES mRNA in the lungs was measured on day 3 postinfection. Each column represents the mean ± SD of three to five mice. Experiments were repeated twice with similar results, and representative results are shown. (c) Expression of AhR mRNA in the lungs was measured on day 3 postinfection. Each column represents the mean ± SD of three mice. Experiments were repeated twice with similar results, and representative results are shown. *, $P < 0.05$. NS, not significant.

IL-23p19 mRNA expression were not significantly different between the two mouse strains at any time points, whereas production of IL-22 was significantly decreased in Mincle KO mice on days 1 and 3 (Fig. 4a and b). The aryl hydrocarbon receptor (AhR) is a transcription factor that enhances the production of IL-22 (43). Therefore, we examined the expression of AhR mRNA in the lungs on day 3 after infection with *C. deneoformans*. AhR expression showed a tendency of suppression in Mincle KO mice compared to WT mice, although this difference was not statistically significant (Fig. 4c).

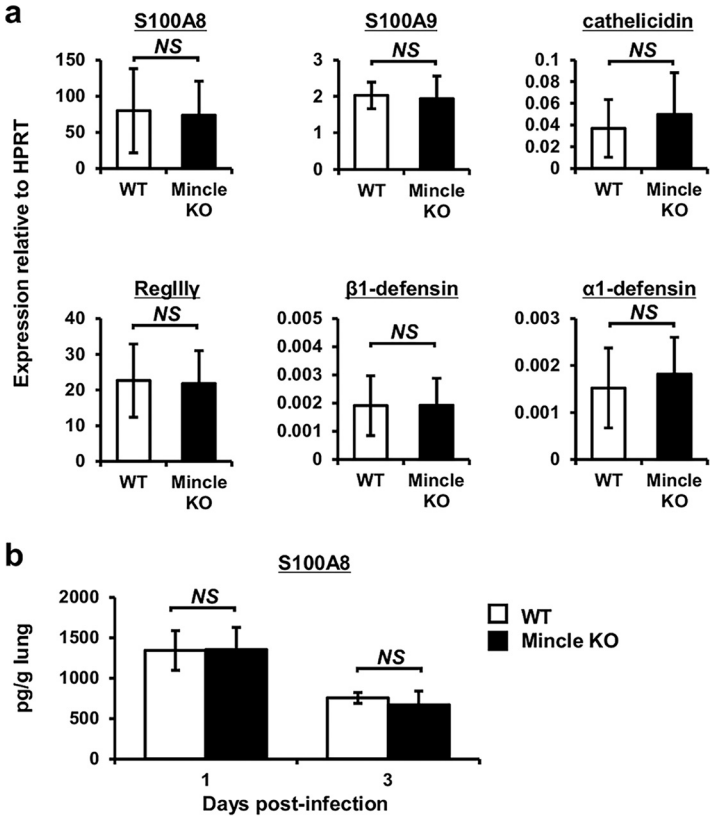


**FIG 5** Effects of Mincle deficiency on the secretion of antimicrobial peptides in the lungs after infection with *C. deneoformans*. WT and Mincle KO mice were infected intratracheally with *C. deneoformans*. (a) Expression of S100A8, S100A9, cathelicidin, RegIII$\gamma$, $\beta$1-defensin, and $\alpha$1-defensin mRNA in the lungs was measured on day 3 postinfection. Each column represents the mean $\pm$ SD of three to five mice. Experiments were repeated twice with similar results, and representative results are shown. (b) Production of S100A8 in lung homogenates was measured on days 1 and 3 postinfection. Each column represents the mean $\pm$ SD of three to five mice. NS, not significant.

**Effect of Mincle deficiency on the production of antimicrobial peptides.** IL-22 is known to induce the secretion of antimicrobial peptides, such as S100A8, S100A9, $\beta$-defensin, and RegIII$\gamma$, involved in the host defense in the skin, airway, and intestine (44, 45). To clarify how Mincle deficiency affects such production, we examined the production of S100A8, S100A9, $\beta$1-defensin, $\alpha$1-defensin, cathelicidin, and RegIII$\gamma$ in the lungs on days 1 and 3 after infection with *C. deneoformans*. As shown in Fig. 5a, expression of these antimicrobial peptide mRNAs was not significantly different between Mincle KO and WT mice on day 3 after infection (Fig. 5a). In addition, consistent with mRNA expression, there was no significant difference in production of S100A8 peptide between the two mouse strains on days 1 and 3 after infection (Fig. 5b).

**Activation of Mincle-triggered signal by *C. deneoformans*.** We examined whether *C. deneoformans* directly triggered an activation signal via Mincle using an NFAT-GFP reporter assay. As shown in Fig. 6a, whole *C. deneoformans* yeast cells did not induce GFP expression in this reporter assay (data not shown), and the disrupted lysates of these yeast cells activated the reporter cells, although such response was not marked. These results suggested that some putative Mincle ligands might exist within the yeast cells but not on their surface. Mammalian $\beta$-GlcCer, which differs in structure from that of yeasts, has been reported as one of the Mincle ligands (37, 46). Thus, we finally examined whether fungal $\beta$-GlcCer, which was derived from *Candida utilis* and showed the same structure as that from *Cryptococcus* (46, 47), affected this Mincle reporter assay. As shown in Fig. 6b, the fungal $\beta$-GlcCer apparently activated the reporter cells to induce GFP expression, whereas ethanol EtOH, a $\beta$-GlcCer solvent, did not affect the expression of GFP.

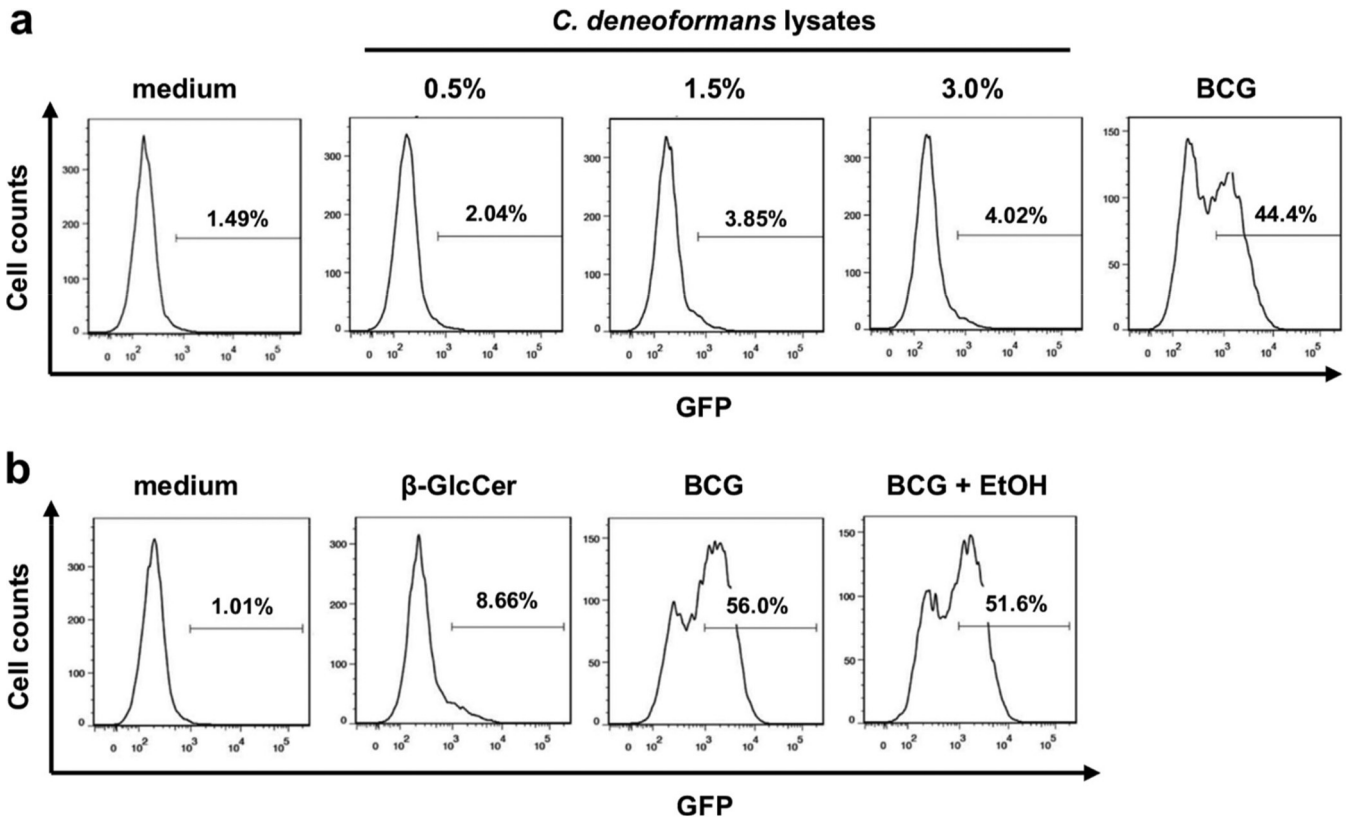


**FIG 6** Activation of Mincle reporter cells by *C. deneoformans* lysates and β-GlcCer. (a and b) The NFAT-GFP reporter cells expressing Mincle were cultured with the indicated dose of disrupted *C. deneoformans* lysates (a) or β-GlcCer (50 μg/ml) (b), and the expression of GFP was analyzed using a flow cytometer. BCG (MOI, 5) and ethanol (EtOH, 0.3%), a β-GlcCer solvent, were used as a positive control. Experiments were repeated twice with similar results, and representative results are shown. MOI, multiplicity of infection.

## DISCUSSION

In the present study, we demonstrated that Mincle was quickly expressed in the lungs in a CARD9-dependent manner during the innate immune phase after infection with *C. deneoformans*. The expression of Mincle is positively regulated by signaling through macrophage C-type lectin (MCL), one of the CLRs, which leads to enhancement of Mincle-mediated signaling (48). These reports suggest that Mincle or MCL ligand may be present in this fungal pathogen. In addition, Kerscher et al. revealed that Mincle was induced by signaling through a myeloid differentiation primary response gene 88 (MyD88), a canonical adaptor molecule delivering signals triggered by Toll-like receptors (TLRs) (49). Nakamura et al. demonstrated that cryptococcal DNA was recognized by TLR9 (50), suggesting that the expression of Mincle might be induced via a TLR9-dependent pathway. Further investigation is necessary to focus on the cross talk between Mincle and the putative counterpart signaling molecules such as TLR9. To date, Mincle has been shown to be largely expressed on myeloid cells, including macrophages, monocytes, DCs, and neutrophils (51). Similarly, in the current study, Mincle was expressed largely in inflammatory monocytes, patrolling monocytes, and neutrophils in the lungs infected with *C. deneoformans*.

Recently, Mincle has garnered a lot of attention in the host defense against infection with various microorganisms. Behler-Janbeck et al. demonstrated that Mincle is a receptor for glucosyl-diacylglycerol from *S. pneumoniae* and that deficiency of this molecule leads to increased bacterial loads and decreased survival rates after pneumococcal infection (39). Sharma et al. revealed the protective role of Mincle in the host defense against *Klebsiella pneumoniae* pneumonia by promoting the bacterial clearance mechanism of neutrophils (52). Wells et al. demonstrated that Mincle deficiency significantly increased susceptibility to systemic infection with *Candida albicans* (40). In

*Mycobacterium tuberculosis* infection, TNF-$\alpha$ and MIP-2 were produced by bone marrow-derived macrophages upon *in vitro* stimulation with TDM in a Mincle-dependent manner, whereas Mincle-deficient mice mounted an efficient granulomatous and protective immune response *in vivo* (38, 53). Among these studies, similar to *Mycobacterium tuberculosis* infection, in the current study, *in vitro* production of TNF-$\alpha$ by BM-DCs stimulated with *C. deneoformans* and several cytokine syntheses (TNF-$\alpha$, IL-6, IL-12p40, and IL-22) *in vitro* in the lungs after cryptococcal infection were decreased in Mincle KO mice, while Mincle deficiency did not affect the granuloma formation and fungal clearance. These observations suggest that some compensatory mechanism for the defect of Mincle may operate through other PRRs, such as TLR9, which may make the role of Mincle in these responses unclear.

IL-22 is produced by $CD4^+$ T, $\gamma\delta$T, NK, and NK T cells and promotes antimicrobial immunity, inflammation, and tissue repair at barrier surfaces, including the skin, pancreas, intestine, liver, lung, and kidney (42, 54–56). The expansion of IL-22-producing cells appears to be regulated by the AhR transcription factor (43, 56). In addition, the expression of AhR is regulated by TNF-$\alpha$ (57). In the current study, IL-22 and TNF-$\alpha$ production and AhR expression in the lungs were decreased in Mincle KO mice during the innate immune phase after cryptococcal infection, which suggests that the AhR/IL-22 axis may be regulated by Mincle-dependent production of TNF-$\alpha$. IL-22 and IL-17A are known to induce the secretion of antimicrobial peptides, such as S100A8, S100A9, and $\beta$-defensin, which are involved in the local host defense to microorganisms in the skin, airway, and intestine (44, 45). Mambula et al. demonstrated that calprotectin, a complex of S100A8 and S100A9, expresses anticryptococcal activity via nutritional deprivation of zinc (58). However, Wozniak et al. revealed that IL-22-deficient mice are resistant to infection with *C. neoformans*, as shown by equivalent levels of antimicrobial peptide production and survival rates compared to WT mice (17). In the current study, production of S100A8, S100A9, and $\beta$-defensin was not decreased in Mincle KO mice, although IL-22 production was reduced. These results suggest that some compensatory mechanism may operate to keep the secretion levels of antimicrobial peptides unchanged at the mucosal area in the skin, airway, and intestine. Although the precise mechanism remains unclear, other cytokines such as IL-17A may be candidates for such compensation (43, 44).

In the final series of experiments, we addressed the possibility that *C. deneoformans* may possess some ligand for Mincle to deliver activation signals in the immune cells. Although whole *C. deneoformans* did not activate the reporter cells expressing Mincle, its disrupted lysates showed small, but significant, activity in this reporter assay. These results suggest the possible existence of a Mincle ligand inside the yeast cells but not on their surface. Yamasaki et al. showed that three strains of *Cryptococcus* spp. fail to activate the reporter cell expression (41), which could be explained by the possible existence of Mincle ligands inside this fungus. On the other hand, it has been reported that mammalian $\beta$-GlcCer activates the Mincle-mediated signaling pathway by acting as DAMPs (37, 46). In addition, $\beta$-GlcCer is located at the cell membrane and masked by the capsule in this fungus (59). In the current study, we showed that $\beta$-GlcCer derived from *C. utilis* activated the reporter cells expressing Mincle, although $\beta$-GlcCer from *C. deneoformans* was not used. In previous studies, the structure of $\beta$-GlcCer was not reported to differ between *C. utilis* and *C. neoformans* (46, 47). These results suggest that $\beta$-GlcCer could be a candidate molecule to trigger the Mincle-mediated signaling pathway and induce the inflammatory response during infection with *C. deneoformans*. Further investigations are required to clarify this possibility.

In conclusion, the present study demonstrated that Mincle expression in lungs is induced in a CARD9-dependent manner at the early stage of cryptococcal infection and suggested that Mincle may be involved in the production of Th22-related cytokines, including IL-22, TNF-$\alpha$, and IL-6. *C. deneoformans* may possess some ligand such as $\beta$-GlcCer for Mincle to trigger the activation signals through CARD9, a common adapter molecule for CLRs (60). However, Mincle deficiency did not

affect the fungal elimination, Th1/Th2 cytokine balance, or secretion of antimicrobial peptides, suggesting that Mincle may not be essential and that some compensational mechanism may exist for promoting the host protective response against *C. deneoformans* infection. Thus, this study may provide important implications for better understanding the mechanism involved in the recognition of this fungal pathogen by host immune cells.

## MATERIALS AND METHODS

**Ethics statement.** This study was performed in strict accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiments 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. All experiments were performed under anesthesia, and all efforts were made to minimize the suffering of the animals.

**Mice.** C57BL/6 mice, purchased from CLEA (Tokyo, Japan), were used as controls (WT). Mincle gene-disrupted (KO) and CARD9 KO mice were generated and established as described previously (41, 61) and backcrossed to C57BL/6 mice for more than eight generations. Male or female mice at 6 to 8 weeks of age and 16 to 24 g of weight were used in the experiments. Mice were allocated to each experimental group randomly. All mice were kept under specific pathogen-free conditions at the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine. The conditions of the breeding room were as follows: room temperature, 20 to 29°C; humidity, 30 to 70%; light/dark cycle, 12 h; and *ad libitum* availability of water and food. Microbial monitoring of mice was regularly carried out by the Central Institute for Experimental Animals. We took the utmost care to alleviate any pain and suffering on the part of the mice. Mice were sacrificed by cervical dislocation prior to analysis.

***Cryptococcus deneoformans*.** A strain of *C. deneoformans*, designated B3501 (a kind gift from Kwong Chung, National Institutes of Health, Bethesda, MD, USA) was used. The yeast cells were cultured on potato dextrose agar (PDA; Eiken, Tokyo, Japan) plates for 2 to 3 days before use. Mice were anaesthetized by an intraperitoneal injection of 70 mg/kg of pentobarbital (Abbott Laboratory, North Chicago, IL, USA) and restrained on a small board. Live *C. deneoformans* ($1 \times 10^6$ cells) was inoculated at 50 $\mu$l into the trachea of each mouse using a 24-gauge catheter (Terumo, Tokyo, Japan).

**Preparation of lung leukocytes.** Pulmonary intraparenchymal leukocytes were prepared as previously described (62). Briefly, the chest of the 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-$\mu$m cell strainer (BD Falcon, Bedford, MA, USA), were incubated in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 5% fetal calf serum (FCS; BioWest, Nuaillé, France), 100 U/ml penicillin G, 100 $\mu$g/ml streptomycin, 10 mM HEPES, 50 $\mu$M 2-mercaptoethanol (ME; Sigma-Aldrich), and 2 mM L-glutamine containing 20 U/ml collagenase and 1 $\mu$g/ml DNase I (Sigma-Aldrich). After incubation for 60 min at 37°C accompanied by vigorous shaking, the tissue fragments and the majority of dead cells were removed by passing the mixture through a 40-$\mu$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.

**Histological examination.** Lung specimens obtained from mice were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stain using standard staining procedures at the Biomedical Research Core, Animal Pathology Platform of the Tohoku University Graduate School of Medicine. The stained sections were observed using a Leica DM750 microscope (Leica Microsystems, Wetzlar, Germany). The photographs were taken with a Leica ICC50 HD camera and analyzed with Leica LAS EZ software (Leica Microsystems).

**Immunohistochemical analysis.** Lung tissues were fixed in 10% neutral buffered formalin. After paraffin-embedded blocks had been cut into 5-$\mu$m sections and mounted onto slides, the specimens were deparaffinized and rehydrated. High-temperature antigen retrieval involved boiling the slides in citrate buffer (10 mM, pH 6.0) for 5 min followed by blocking with 10% rabbit serum. The samples were incubated with rabbit anti-CLEC4E polyclonal antibody (Bioss Antibody, USA) at a dilution of 1:400 overnight at 4°C. Endogenous peroxidase activity was blocked by treatment with 30% $H_2O_2$ blocking solution for 20 min. After washing, slides were incubated with Simple Stain Mouse MAX-PO (Nichirei, Tokyo, Japan) and were then incubated with horseradish peroxidase-conjugated streptavidin (Nichirei) and washed. The slides were incubated with diaminobenzidine substrate and counterstained with Carrazzi's hematoxylin solution (Wako, Osaka, Japan).

**Preparation and culture of BM-DCs.** Dendritic cells (DCs) were prepared from bone marrow (BM) cells as described by Lutz et al. (63). Briefly, BM cells from mice were cultured at $2 \times 10^5$/ml in 10 ml RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin G, 100 $\mu$g/ml streptomycin, and 50 $\mu$M 2ME containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Wako). 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 with *C. deneoformans*, lipopolysaccharide (LPS) (Sigma-Aldrich), or TDM (Sigma-

**TABLE 1** Primers for PCR

| Gene[a] | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| Mincle | CAGTGGCAATGGGTGGATGATAC | AGTCCCTTATGGTGGCACAGTC |
| IL-12p35 | GAGTTCCAGGCCATCAACGCA | GCTTCTCCCACAGGAGGTTTCTG |
| IL-23p19 | CTCAGCCAACTCCTCCAGCCAG | CTGCTCCGTGGGCAAAGACC |
| TGF-$\beta$ | TGGACCGCAACAACGCCATCTATGAGAAAACC | TGGAGCTGAAGCAATAGTTGGTATCCAGGGCT |
| KC | GACCCTGAAGCTCCCTTGGTTC | GACAGGTGCCATCAGAGCAGTC |
| RANTES | TCTTCTCTGGGTTGGCACACAC | CCTCACCATCATCCTCACTGCA |
| MIP-2 | CTGAACAAAGGCAAGGCTAACTGAC | CACATCAGGTACGATCCAGGCTTC |
| MIP-1$\alpha$ | CACCCTCTGTCACCTGCTCAACATC | GGTTCCTCGCTGCCTCCAAGACTCT |
| AhR | GTCAAATCCTTCTAAGCGACACA | AACCAGCACAAAGCCATTCA |
| S100A8 | ACAAGGAAATCACCATGCCCTCTAC | ATGCCACACCCACTTTTATCACCA |
| S100A9 | CAACATCTGTGACTCTTTAGCCTTG | ACTGTGCTTCCACCATTTGTCT |
| Cathelicidin | GACACCAATCTCTACCGTCTCCT | TGCCTTGCCACATACAGTCTCCT |
| RegIII$\gamma$ | CCTAGCCACAAGCAAGATCCCAA | AGGGAAGGGCCAGAGAAGGAGA |
| $\beta$1-defensin | CTTTTCTCCCAGATGGAGCCAG | CCTCCATGTTGAAGGCATTTGTATTG |
| $\alpha$1-defensin | CTTTGCCCTTGTCCTGCTTGGC | TCTTCTCCTGGCTGCTCCTCAG |
| HPRT | CGTTGGGCTTACCTCACTGC | ATCGCTAATCACGACGCTGG |

[a]TGF, transforming growth factor; KC, keratinocyte-derived chemokine; RANTES, regulated on activation, normal T cell expressed and secreted; MIP, macrophage inflammatory protein; AhR, aryl hydrocarbon receptor; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

Aldrich) for 24 h at 37°C in a 5% $CO_2$ incubator. The culture supernatants and cell pellets were collected and stored at −70°C before use.

**Extraction of RNA and quantitative real-time RT-PCR.** Total RNA was extracted using Isogen (Wako Pure Chemical, Osaka, Japan), and the first-strand cDNA was synthesized using a PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed in a volume of 20 $\mu$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 those 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 illustrated as relative expression compared with HPRT expression.

**Enumeration of viable *C. deneoformans*.** Mice were sacrificed 1, 2, 4, and 8 weeks after infection, and the lungs and brains were dissected carefully and excised and then homogenized separately in 5 ml and 1 ml, respectively, of distilled water by teasing with a stainless-steel mesh at room temperature. The homogenates were diluted appropriately with distilled water and inoculated at 100 $\mu$l on PDA plates and cultured for 2 to 3 days before the resulting colonies were counted.

**Cytokine assay.** Mice were sacrificed on days 1, 3, 7, 14, and 28 after infection, and the lungs were excised and then homogenized separately in 5 ml of phosphate-buffered saline (PBS) by teasing through a stainless-steel mesh. After centrifugation, the supernatants were collected and stored at −70°C before use. Concentrations of IFN-$\gamma$, IL-12p40, IL-4, IL-5, IL-13, IL-17A, IL-22, IL-6, TNF-$\alpha$, IL-1$\beta$, and IL-10 in lung homogenates and the culture supernatants were measured using each particular enzyme-linked immunosorbent assay (ELISA) kit (BioLegend for IFN-$\gamma$, IL-12p40, IL-4, IL-5, IL-17A, IL-22, IL-6, TNF-$\alpha$, and IL-1$\beta$ and eBioscience [San Diego, CA, USA] for IL-13).

**Mincle-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 (64). This cell line was transfected with Mincle and FcR$\gamma$ genes, and a Mincle-free version of the same cell line was used as a control. These cells were stimulated for 20 h at $2.5 \times 10^5$/ml with disrupted lysates of *C. neoformans*, which had been prepared using a multibead shocker (Yasui Kikai, Osaka, Japan) according to the manufacturer's instructions, $\beta$-GlcCer derived from *Candida utilis* (a kind gift from Toshiya Sato, Kojin Life Sciences, Tokyo, Japan), and Bacille de Calmette et Guérin (BCG; Japan BCG Laboratory, Tokyo, Japan), 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.

**Flow cytometry.** The cells were washed three times in PBS containing 1% FCS and 0.1% sodium azide and then stained with 7-AAD, PE-anti-F4/80 monoclonal antibody (MAb) (clone BM8; BioLegend, San Diego, CA, USA), APC-anti-CD11c MAb (clone N418; BioLegend), Pacific Blue-anti-Ly6c MAb (clone HK1.4; BioLegend), APC/Cy7-anti-Ly6G MAb (clone 1A8; BioLegend), PE-anti-CD45R/B220 MAb (clone RA3-6B2; BioLegend), PE/Cy7-anti-NK1.1 MAb (clone PK136; BioLegend), APC-anti-CD3$\varepsilon$ MAb (clone 145-2C11; BioLegend), anti-Mincle-MAb (clone 1B6; MBL Life Science, Nagoya, Japan), and fluorescein isothiocyanate (FITC)-anti-rat IgG polyclonal antibody (pAb) (MBL Life Science). Isotype-matched IgG was used for control staining. The stained cells were analyzed using a BD FACS Canto 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 populations.

**Statistical analysis.** Data were analyzed using JMP Pro 11.2.0 software (SAS Institute Japan, Tokyo, Japan). Data are expressed as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Welch's *t* test. A *P* value less than 0.05 was considered significant.

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We declare no conflict of interest.

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