AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity

Immunology | Full-Length Text

Innate phase production of IFN-γ by memory and effector T cells expressing early activation marker CD69 during infection with *Cryptococcus deneoformans* in the lungs

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ABSTRACT *Cryptococcus deneoformans* is a yeast-type fungus that causes fatal meningoencephalitis in immunocompromised patients and evades phagocytic cell elimination through an escape mechanism. Memory T (Tm) cells play a central role in preventing the reactivation of this fungal pathogen. Among these cells, tissue-resident memory T (T_{RM}) cells quickly respond to locally invaded pathogens. This study analyzes the kinetics of effector T (Teff) cells and Tm cells in the lungs after cryptococcal infection. Emphasis is placed on the kinetics and cytokine expression of T_{RM} cells in the early phase of infection. $CD4^+$ Tm cells exhibited a rapid increase by day 3, peaked at day 7, and then either maintained their levels or exhibited a slight decrease until day 56. In contrast, $CD8^+$ Tm cells reached their peak on day 3 and thereafter decreased up to day 56 post-infection. These Tm cells were predominantly composed of $CD69^+$ T_{RM} cells and $CD69^+$ $CD103^+$ T_{RM} cells. Disruption of the CARD9 gene resulted in reduced accumulation of these T_{RM} cells and diminished interferon (IFN) -γ expression in T_{RM} cells. T_{RM} cells were derived from T cells with T cell receptors non-specific to ovalbumin in OT-II mice during cryptococcal infection. In addition, T_{RM} cells exhibited varied behavior in different tissues. These results underscore the importance of T cells, which produce IFN-γ in the lungs during the early stage of infection, in providing early protection against cryptococcal infection through CARD9 signaling.

KEYWORDS *Cryptococcus deneoformans*, CLR, tissue-resident memory T cells, MPT cells, Th1 response

The two sister species, *Cryptococcus neoformans* (formerly *C. neoformans* var. *grubii*, serotype A) and *C. deneoformans* (formerly *C. neoformans* var. *neoformans*, serotype D), are yeast-type fungal pathogens characterized by thick capsules composed of polysaccharides such as glucuronoxylomannan and galactoxylomannan (1). These fungi grow in pigeon droppings and enter the lungs via an airborne route. While most healthy individuals experience asymptomatic infection, marked by granulomatous lesions in the lungs caused by these fungi, immunocompromised hosts with severely impaired cellular immunity, such as those with AIDS, frequently suffer from severe lung lesions and disseminated infections that extend to the central nervous system (2).

Host defense against cryptococcal infection is critically regulated by the balance between Th1 and Th2 cytokine responses (2–7). Th1-related cytokines, such as IFN-γ, support host defense by inducing the production of nitric oxide, which enhances macrophages' ability to eliminate *Cryptococcus* and facilitates granuloma formation at infection sites, impeding the fungi from expanding in the infected lungs (6, 8–10). In

Editor Mairi C. Noverr, Tulane University, New Orleans, Louisiana, USA

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The authors declare no conflict of interest.

See the funding table on p. 17.

Received 18 January 2024
Accepted 9 April 2024
Published 3 May 2024

contrast, Th2 immune responses, characterized by IL-4 and IL-13 production, counteract Th1-mediated responses by suppressing granulomatous responses and hindering the host's ability to fend off the fungal pathogen (6, 8, 11). A recent study conducted by our team demonstrated that IL-17A, one of the Th17-related cytokines, regulates Th1-mediated host defense against cryptococcal infection (12). Other studies have also reported the diverse functions of Th17-related cytokines in this context (13–17). Thus, the commitment of Th phenotypes critically influences host susceptibility to cryptococcal infection.

Innate immune cells, such as macrophages and dendritic cells, highly express pattern recognition receptors (PRRs). These PRRs play a crucial role in recognizing pathogen-associated molecular patterns composed of microbial components and danger-associated molecular patterns released from damaged cells (18). C-type lectin receptors (CLRs) are well-known as representative PRRs and act as sensor molecules for fungal pathogen cell wall polysaccharides (19, 20). Numerous studies have demonstrated the important roles of CLRs in recognizing various fungi and in host defense against these microorganisms, including *Candida albicans*, *Pneumocystis carinii*, *Aspergillus fumigatus*, and *C. deneoformans* (21–25). However, the roles of CLRs in host defense against cryptococcal infection remain incompletely understood, despite investigations into the impact of deficiencies in Dectin-1, Dectin-2, Dectin-3 (also known as MCL, Clec4D, and Clecsf8), and Mincle on the clinical course of the infection (7, 26–32).

In our previous study using a mouse model, we observed that deficiency of caspase recruitment domain-containing protein 9 (CARD9), a common adaptor molecule for CLRs-mediated signaling, rendered mice highly susceptible to pulmonary infection with *C. deneoformans* mediated by Th1 immune responses (25). In addition, the early-phase production of IFN-γ from memory phenotype T (MPT) cells after cryptococcal infection was significantly affected in CARD9-deficient mice. MPT cells represent a novel T cell subset characterized by a memory T cell phenotype, even in the absence of exposure to foreign antigens (33, 34). Several reports revealed that IFN-γ production from MPT cells plays an important role in infections with *Listeria monocytogenes* and *Toxoplasma gondii*, not limited to *C. deneoformans* (35–37). On the other hand, tissue-resident memory T (T_{RM}) cells, another novel memory T cell subset, are known for their unique ability to migrate to the tissue and reside there for an extended time without recirculation (38). T_{RM} cells exist in various tissues, such as the gastrointestinal tract, lungs, skin, and genital tract, playing a key role in the acute phase of infection control against invading microbes (39–41). While the role of T_{RM} cells is well understood in viral infection (41–43), it remains unclear how T_{RM} cells contribute to infections with other microorganisms and the relationship between T_{RM} cells and MPT cells.

In the present study, we aim to investigate the details of the accumulated MPT and T_{RM} cells in the lungs after cryptococcal infection, shedding light on the involvement of CLRs in these cells.

MATERIALS AND METHODS

Mice

C57BL/6 mice, purchased from CLEA (Tokyo, Japan), were used as controls [wild-type (WT)]. CARD9 gene-disrupted [knockout (KO)], Dectin-2KO, and MincleKO mice were generated and established as described previously (24, 44, 45) and backcrossed to C57BL/6 mice for more than eight generations. OT-II transgenic mice (OT-II mice) (46), which express T cell receptor (TCR) α and β chains that recognize the major histocompatibility complex (MHC) class II I^b–restricted ovalbumin (OVA) peptide (residues 323–339) in a C57BL/6 background, were kindly provided by Dr. N. Ishii (Tohoku University, Sendai, Japan). Male or female mice at 6–8 weeks of age and 16–24 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°C–26°C; humidity, 40%–60%; 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 utmost care to minimize any pain and suffering experienced by the mice. Mice were sacrificed by cervical dislocation prior to analysis.

Inoculation with *Cryptococcus deneoformans*

A serotype D 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 (Eiken, Tokyo, Japan) plates for 2–3 days before use. Mice were anesthetized by an intramuscular injection of 0.3 mg/kg of midazolam (Fuji Pharma, Tokyo, Japan) and 0.02 mg/kg of medetomidine hydrochloride (Nippon Zenyaku Kogyo, Fukushima, Japan) and an intraperitoneal injection of 15 mg/kg of pentobarbital (Abbott Laboratory, North Chicago, IL, USA). Live *C. deneoformans* (1×10^6 cells) was inoculated in a 50 µL volume into the trachea of each mouse using a 24-gauge catheter (Terumo, Tokyo, Japan).

Treatment with FTY720

FTY720 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Mice were orally administered FTY720 by *ad libitum* availability of water containing 10.5 µg/mL FTY720 every day from 2 days before infection with *C. deneoformans*.

Preparation of lung leucocytes

Pulmonary intraparenchymal leukocytes were prepared as described previously (12, 25, 27, 32, 47). 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 with stainless mesh, were incubated in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal calf serum (FCS; Biowest, Nuaillé, France), 100 U/mL penicillin G, 100 µg/mL streptomycin, 10 mM HEPES (4–2-hydroxyethyl-1-piperazineethanesulfoni acid), 50 µM 2-mercaptoethanol, 20 U/mL collagenase, and 1 µg/mL DNase I (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 60 min at 37°C with vigorous shaking, the tissue fragments and most dead cells were removed by passing through the 40 µm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). In the intracellular staining experiments, to prevent the decrease of intracellular IFN-γ by extracellular excretion during the preparation of lung leukocytes, the lungs were incubated with 2 nM monensin from the time of collection until the intrapulmonary leukocytes were separated by Percoll density gradient centrifugation. 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 20°C, the cells at the interface were collected, washed three times, and counted with a hemocytometer.

Preparation of splenocytes

Mouse spleens were collected in RPMI 1640 with 10% FCS, 100 U/mL penicillin G, and 100 µg/mL streptomycin and teased apart between two ground glass slides. After centrifugation at $320 \times g$ for 5 min at 4°C, the cell pellet was hemolyzed using a red blood cell lysis solution (155 mmol/L NH4Cl and 17 mmol/L Tris, pH 7.2) and washed twice, and the tissue fragments and most dead cells were removed by passing through a 40 µm cell strainer.

Preparation of peripheral blood leukocytes

A total of 500 µL of peripheral blood was collected from mice using heparin (AY Pharmaceuticals, Tokyo). The peripheral blood was hemolyzed using a red blood cell lysis

solution and washed twice. This hemolysis process was repeated four times to isolate peripheral blood leukocytes.

Flow cytometry

Lung leukocytes, splenocytes, and peripheral blood leukocytes were pre-incubated with anti-FcγRII/III mAb, which was prepared from the culture supernatants of hybridoma cells (clone 2.4G2) on ice for 15 min in phosphate-buffered saline (PBS) containing 1% FCS and 0.1% sodium azide. The cells were stained with PE-conjugated anti-CD3 mAb (clone 145–2C11; Biolegend, San Diego, CA, USA), APC/Cy7-conjugated anti-CD4 mAb (clone GK1.5; Biolegend) or anti-CD8 mAb (clone 53–6.7; Biolegend), Biotin-conjugated anti-CD127 mAb (clone A7R34; Biolegend), Pacific Blue–conjugated anti-CD44 mAb (clone IM7; Biolegend), APC-conjugated anti-CD69 mAb (clone H1.2F3; Biolegend), FITC-conjugated anti-CD103 mAb (clone 2E-7; Biolegend), PE-conjugated anti-CD4 mAb, APC/Cy7-conjugated anti-TCR Vα2 mAb (clone B20.1; Biolegend), APC-conjugated anti-TCR Vβ5.1/5.2 mAb (clone MR9-4; Biolegend), Biotin-conjugated anti-CD127 mAb, Pacific Blue–conjugated anti-CD44 mAb, PE/Cy7-conjugated anti-CD69 mAb (clone H1.2F3; Biolegend), and FITC-conjugated anti-CD103 mAb. After washing twice, the cells were stained with PerCP/Cy5.5-conjugated streptavidin (Biolegend). After washing twice, the cells were incubated in the presence of Cytofix/Cytoperm (BD Biosciences), washed twice in BD Perm/Wash solution (BD Biosciences), and stained with PE/Cy7-conjugated anti-IFN-γ (clone XMG1.2; Biolegend). Isotype-matched IgG was used for control staining. The stained cells were analyzed using a BD FACS Canto II flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). The gating strategy is shown in Fig. S1.

Statistical analysis

Data were analyzed using JMP Pro 11.2.0 software (SAS Institute Japan, Tokyo, Japan). Data are expressed as the mean ± 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

Kinetics in the accumulation of effector T and memory T cells in the lungs after cryptococcal infection

First, we analyzed $CD4^+$ or $CD8^+$ memory T (Tm) cells in the lungs after cryptococcal infection. WT mice were infected intratracheally with *C. deneoformans*, and then naïve T (Tnaïve) cells, effector T (Teff), and Tm cells were identified on days 0, 3, 7, 14, 28, and 56 after infection. As depicted in Fig. 1A, the number of $CD4^+$ Tm cells drastically increased as early as day 3. $CD4^+$ Tm cells reached their peak on days 7 and 14 and then gradually declined by day 56 post-infection. Similarly, $CD4^+$ Teff cells markedly increased on day 3, peaked on day 7, and then gradually decreased until day 56 (Fig. 1A). In contrast, the number of $CD8^+$ Tm cells peaked on day 3, decreased to basal levels by day 28, and then increased again on day 56 post-infection. Similarly, the number of $CD8^+$ Teff cells reached its peak on days 3 and 7, decreased to basal levels by day 28, and then increased again on day 56. These results suggest that both $CD4^+$ and $CD8^+$ Teff and Tm cells may contribute to host defense in the early phase, such as on day 3 after infection with *C. deneoformans*.

Tm cells, traditionally associated with the acquired immunity phase, exhibited a significant increase during the innate immunity phase. Therefore, we investigated whether these Tm cells were related to T_{RM} cells, which are known for their role in innate immune responses. T_{RM} cells are defined as expressing either or both CD69 and CD103 in $CD44^{bright+}$ $CD127^+$ Tm cells (48–50). To explore this, WT mice were infected with *C. deneoformans*, and the various T_{RM} cell subsets in the lungs were analyzed on days 0, 3, 7, 14, 28, and 56 post-infection. $CD69^+$ $CD103^-$ and $CD69^+$ $CD103^+$ T_{RM} (double-positive

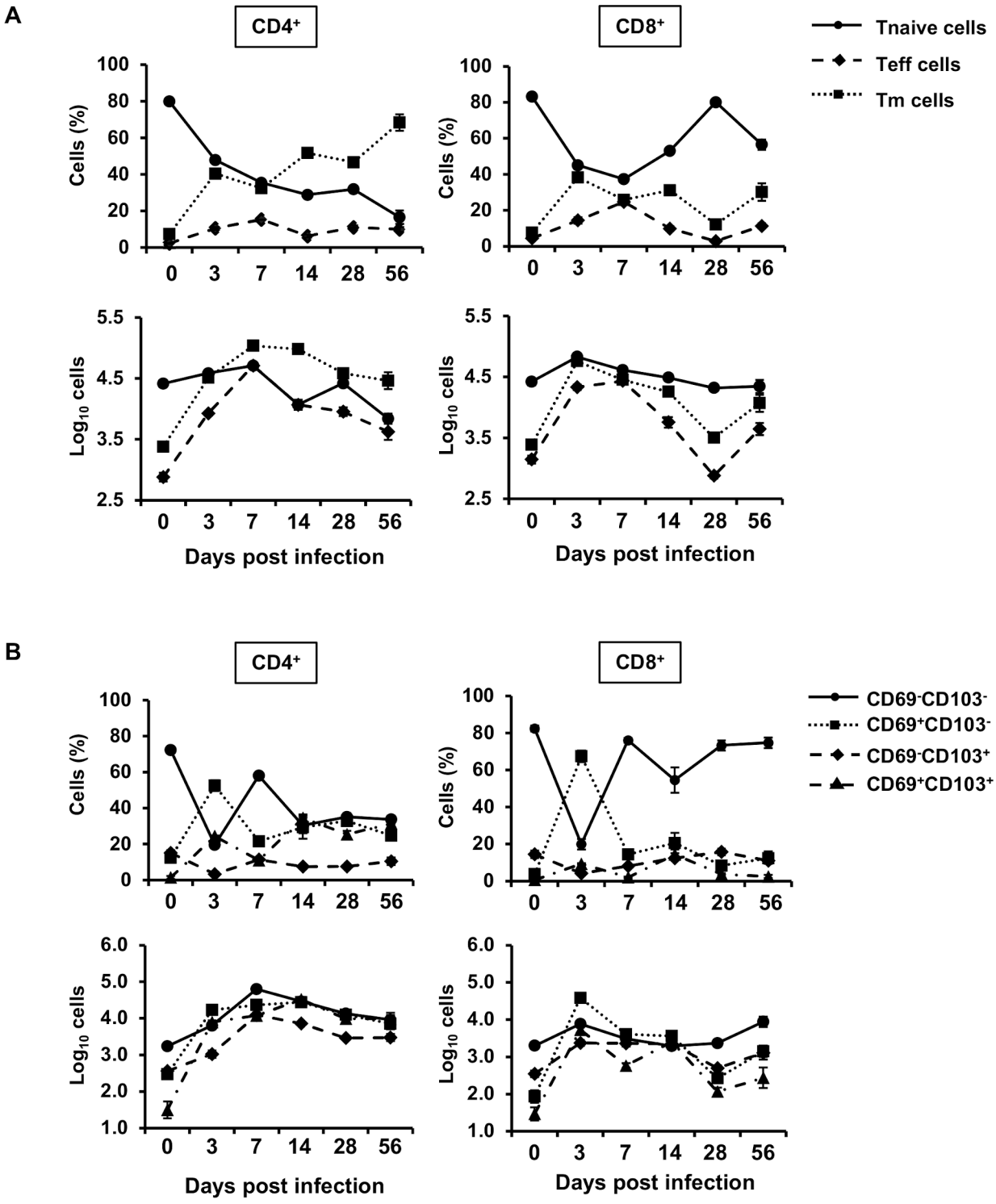

FIG 1 Kinetics of pulmonary T cells after *C. deneoformans* infection WT mice were infected intratracheally with *C. deneoformans*. The number of naïve T (T naïve: CD44$^-$ CD127$^+$), effector T (Teff: CD44$^{bright+}$ CD127$^-$), and memory T (Tm: CD44$^{bright+}$ CD127$^+$) cells (A) and CD69 and/or CD103-expressing Tm cells (B) in pulmonary CD4$^+$ or CD8$^+$ T cells were analyzed using flow cytometry on day 0 (uninfected; n = 6), 3 (n = 4), 7 (n = 3), 14 (n = 3), 28 (n = 5), and 56 (n = 5) post-infection. Each column represents the mean ± SD. Representative data demonstrating similar results from independent experiments are presented. Experiments were conducted once for day 0, three times for day 3, twice for days 7 and 14, once for day 28, and once for day 58.

T_{RM}: DP T_{RM}) cells in CD4$^+$ and CD8$^+$ Tm cells significantly increased on day 3 after infection (Fig. 1B). CD69$^+$ CD103$^-$ and DP T_{RM} cells in CD8$^+$ T_{RM} cells were increased with a

peak level on day 3 and subsequently decreased over the course of infection. However, $CD69^+$ $CD103^-$ and DP T_{RM} cells in $CD4^+$ T_{RM} cells were increased to the same level as the peak, and these cells did not exhibit a significant decrease over the course of infection. $CD69^-$ $CD103^+$ T_{RM} and $CD69^-$ $CD103^-$ Tm (double-negative Tm: DN Tm) cells reached their peak on day 7 after infection. These findings suggest that $CD69^+$ $CD103^-$ and DP T_{RM} cells, which predominantly compose the increased Tm cells, are involved in host defense against cryptococcal infection in the early phase of infection.

Effect of CARD9, Dectin-2, or Mincle deficiency on Teff and Tm cells in the lungs after cryptococcal infection

In a previous study, we demonstrated that IFN-γ-producing MPT cells were induced via CARD9 signaling in the early phase of cryptococcal infection (25). Consequently, we analyzed the effect of CARD9 deficiency on the accumulation of Teff and Tm cells in the early phase of cryptococcal infection. Three days post-infection, both Teff and Tm cells in $CD4^+$ and $CD8^+$ T cells were significantly lower in the lungs of CARD9KO mice compared to WT mice (Fig. 2A). Therefore, we next investigated which CLRs upstream of CARD9 were involved in the accumulation of these T cells. We previously reported that Dectin-2 and Mincle recognized cryptococcal chitin deacetylase 2 (Cda2) and glucosylceramide (GlcCer), respectively (7, 27–29, 32). However, the accumulation of Teff and Tm cells was not affected by Dectin2 and Mincle deficiency (Fig. 2B and C).

We next examined the effect of CLRs on IFN-γ expression in these T cells in the early phase of cryptococcal infection. Similar to our previous study, 3 days after infection, IFN-γ expression in both Teff and Tm cells in $CD4^+$ and $CD8^+$ T cells was lower in the lungs of CARD9KO mice compared to WT mice (Fig. 3A). IFN-γ-expressing $CD8^+$ Teff cells and $CD4^+$ Teff cells decreased in Dectin-2KO and MincleKO mice, respectively, compared to WT mice, although not to the same extent as observed in CARD9KO mice (Fig. 3B and C).

Effect of CARD9 deficiency on the synthesis of IFN-γ by Tm and Teff cells expressing CD69 or CD103 in the lungs after cryptococcal infection

Tm and Teff cells were induced in the lungs 3 days after cryptococcal infection, which precedes the establishment of adaptive immunity, and they produced IFN-γ through CARD9 signaling (Fig. 1to 3A). To confirm the tissue residency of these T cells and assess the effect of CARD9 deficiency on it, we analyzed the expression of CD69 and CD103 on these T cells in WT and CARD9KO mice. While many Tm and Teff cells expressed CD69, CD103-expressing cells were a small proportion in WT mice (Fig. 4A). These CD69-expressing Tm and Teff cells were significantly decreased in CARD9KO mice compared to WT mice (Fig. 4A). In addition, $CD69^+$ Tm and Teff cells, which predominantly expressed IFN-γ in WT mice, were abolished by CARD9 deficiency (Fig. 4B).

Effect of FTY720 treatment on Tm and Teff cells in the lungs after cryptococcal infection

To investigate whether Tm and Teff cells, which increased in the lungs of WT mice in the early phase of cryptococcal infection, migrated to the lungs after proliferating in response to antigen presentation in the lymph nodes, we analyzed these T cells through FTY720 treatment, which suppressed the transmigration of T cells from lymphoid tissues (51). Although $CD4^+$ and $CD8^+$ T naïve cells were significantly decreased by FTY720 treatment compared to the control, the populations of Tm, Teff, and IFN-expressing T cells did not decrease (Fig. 5).

Origin of T_{RM} cells after cryptococcal infection

To investigate the origin of T_{RM} cells, which rapidly increased in the early phase of infection and might be involved in IFN-γ production, we examined T_{RM} cells in the lungs of OT-II mice after cryptococcal infection. In OT-II mice, most T cells express high levels of

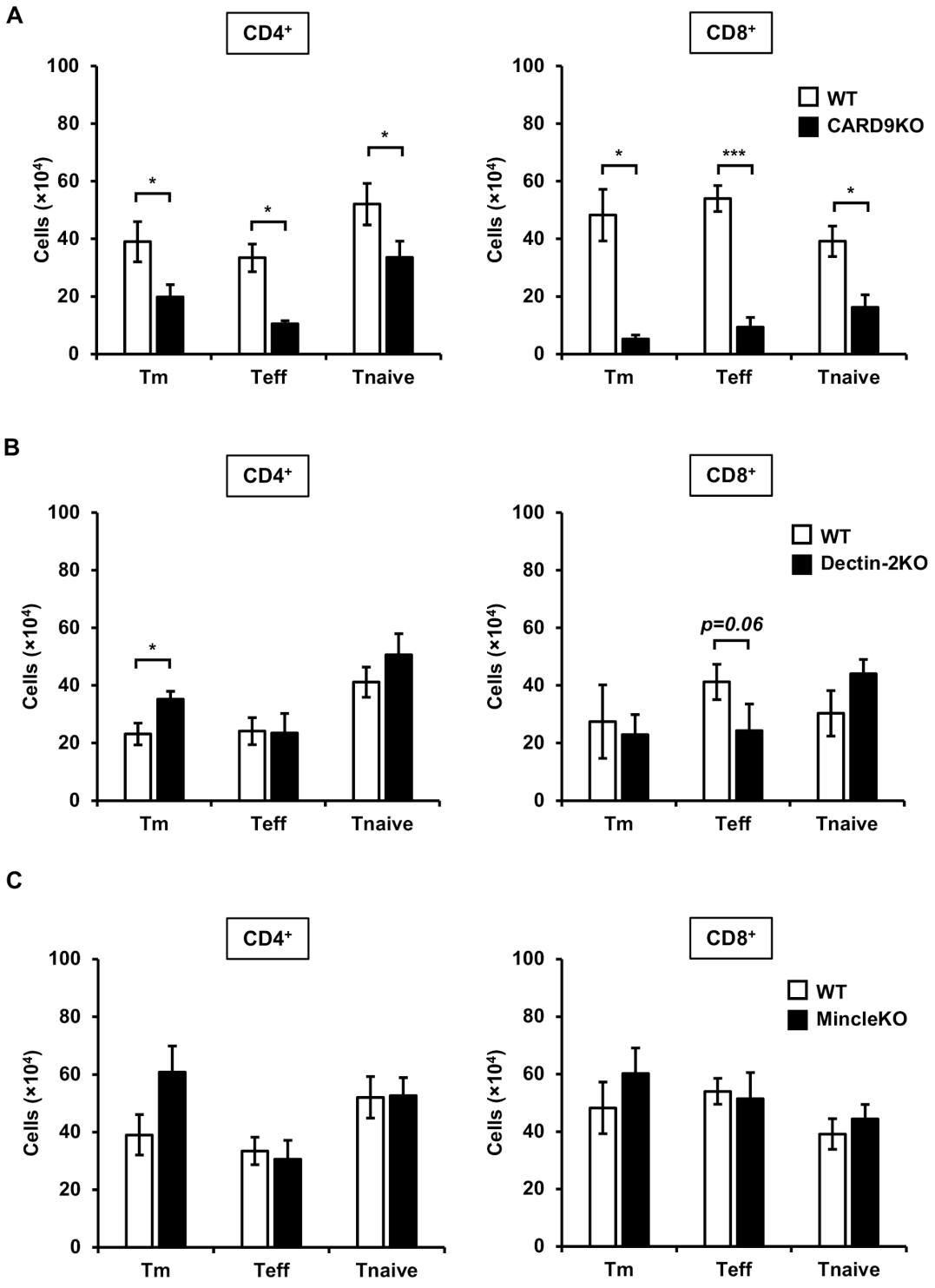

FIG 2 Effects of CLR signaling on T cell accumulation in the lungs after *C. deneoformans* infection WT (n = 3), CARD9KO (A, n = 3), Dectin-2KO (B, n = 5), and MincleKO (C, n = 4) mice were infected intratracheally with *C. deneoformans*. The number of each T cell subset was analyzed using flow cytometry on day 3 post-infection. Each column represents the mean ± SD. *, $P < 0.05$. ***, $P < 0.005$. Representative data demonstrating similar results from independent experiments are shown. Experiments were conducted three times for A and B and twice for C. The results for A and C represent experiments conducted simultaneously, and the outcomes for WT mice utilize the same materials.

TCRs specific to OVA, and these cells are considered nearly naïve T cells that have not encountered antigens. $CD4^+$ Tm cells in transgenic T (Tg-T) cells expressing TCR specific

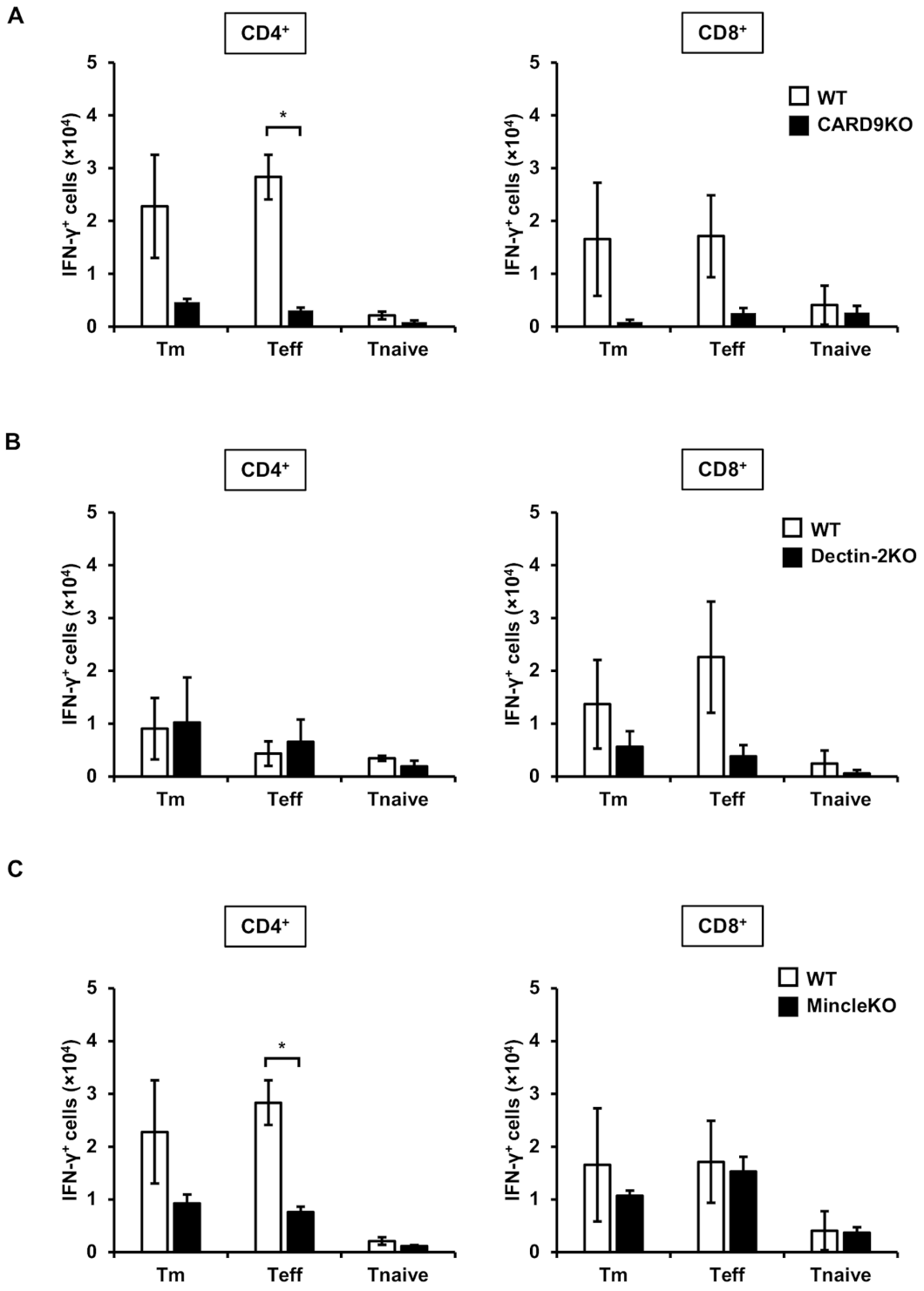

FIG 3 Effects of CLR signaling on IFN-γ expression in pulmonary T cells after *C. deneoformans* infection WT ($n = 3$), CARD9KO (A, $n = 3$), Dectin-2KO (B, $n = 5$), and MincleKO (C, $n = 4$) mice were infected intratracheally with *C. deneoformans*. The number of each IFN-γ-expressing T cell subset was analyzed using flow cytometry on day 3 post-infection. Each column represents the mean ± SD. *, $P < 0.05$. Representative data demonstrating similar results from independent experiments are shown. Experiments were conducted three times for A and B and twice for C. The results for A and C represent experiments conducted simultaneously, and the outcomes for WT mice utilize the same materials.

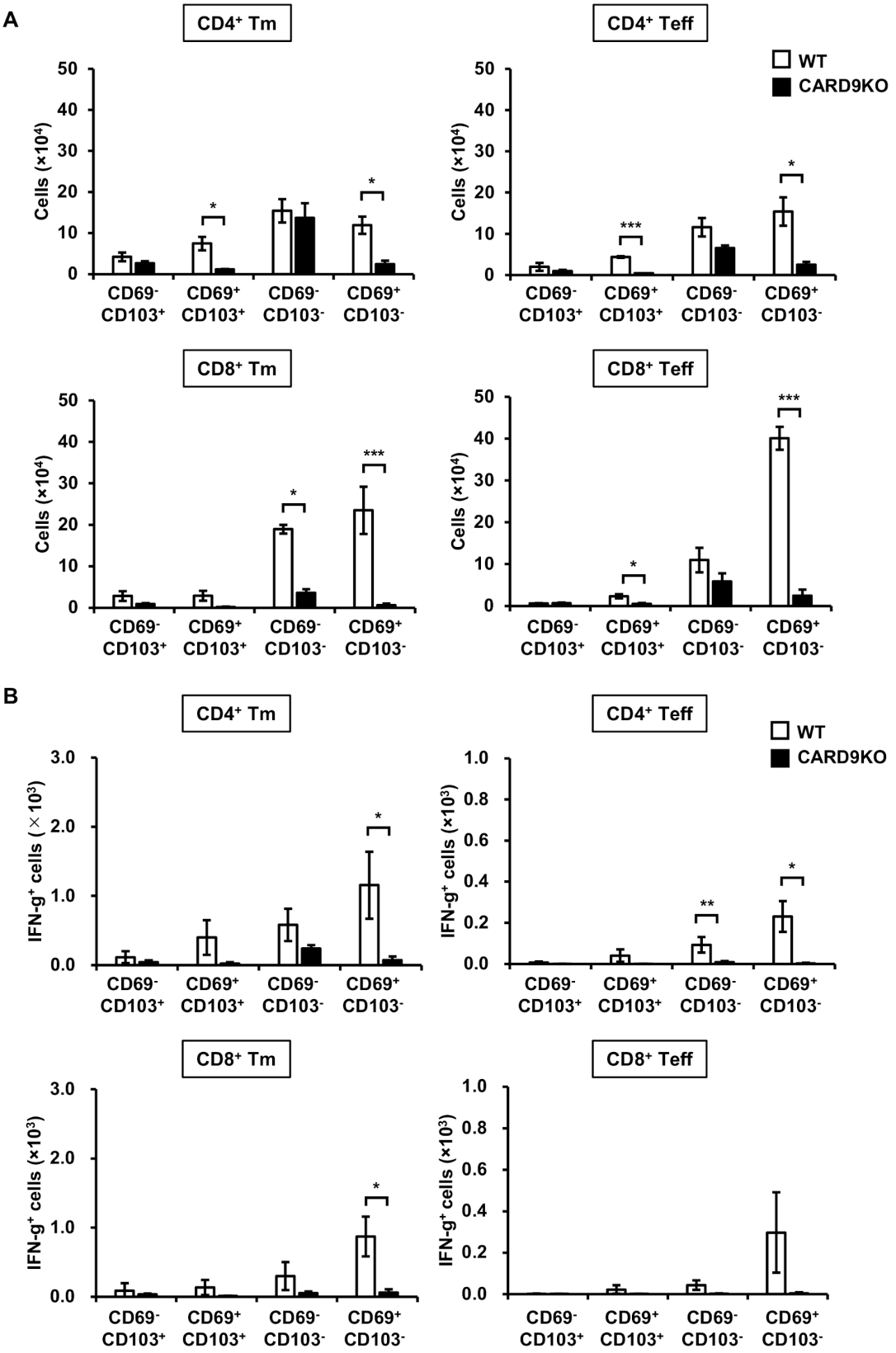

FIG 4 Effects of CARD9 deficiency on tissue-resident T cells in the lungs after *C. deneoformans* infection WT (n = 3) and CARD9KO (n = 3) mice were infected intratracheally with *C. deneoformans*. The number of CD69 and/or CD103-expressing T cells (A) and each IFN-γ-expressing T cell subset (B) in the lungs was analyzed using flow cytometry on day 3 post-infection. Each column represents the mean ± SD. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.005$. Representative data from three independent experiments with similar results are shown.

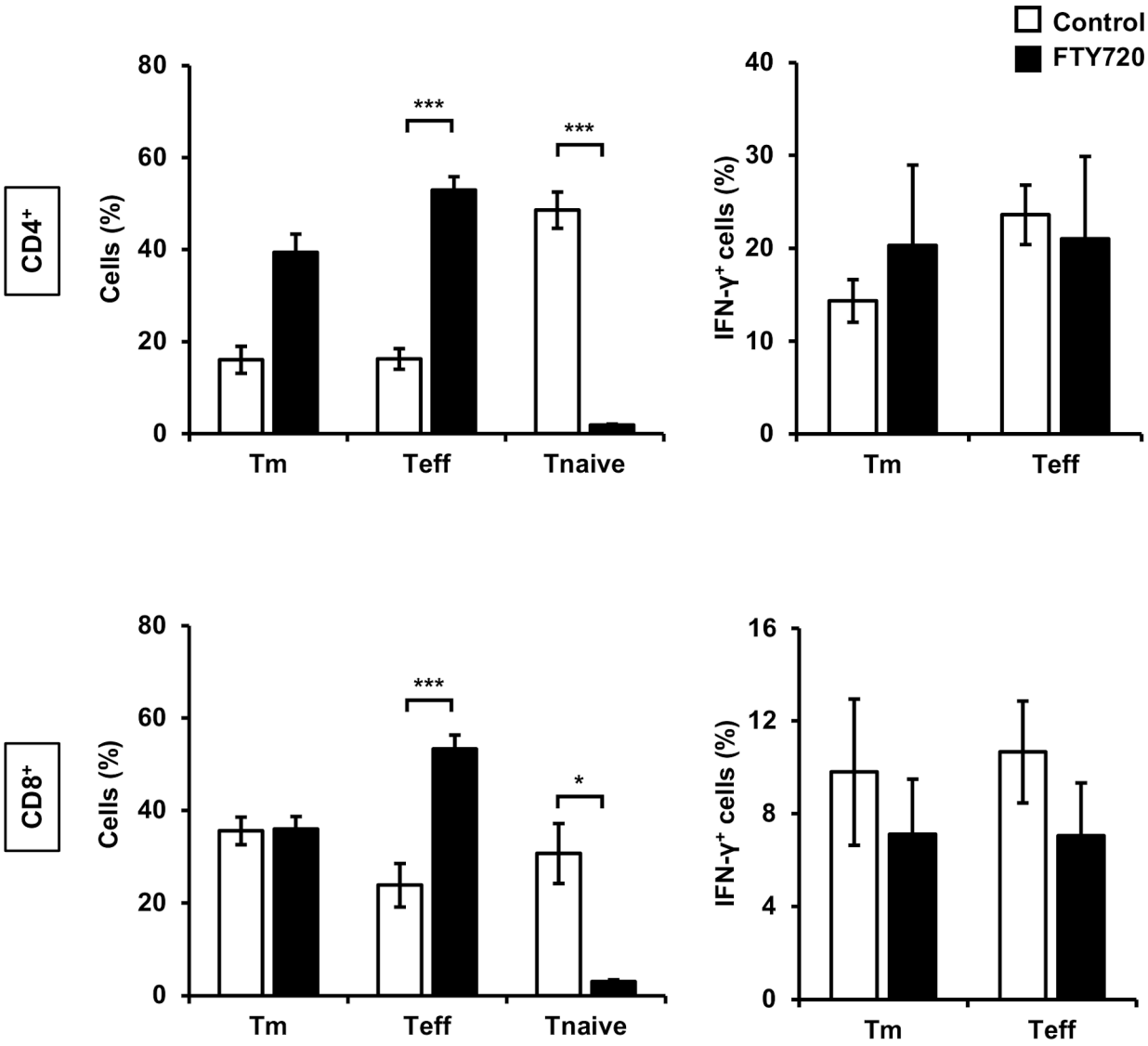

FIG 5 Effects of FTY720 on T cells in the lungs after *C. deneoformans* infection WT mice were orally administered distilled water (control, $n = 3$) or FTY720 ($n = 6$) each day starting from 2 days before infection, followed by intratracheal infection with *C. deneoformans*. The number of each T cell subset (A) and each IFN-γ-expressing T cell subset (B) in the lungs was analyzed using flow cytometry on day 3 post-infection. Each column represents the mean ± SD. *, $P < 0.05$. ***, $P < 0.005$. Representative data from two independent experiments with similar results are shown.

to OVA did not exhibit a marked change on day 3 post-infection (2.1%) compared to uninfected mice (0.3%; Fig. 6A). In addition, almost all Tg-T cells were composed of T naïve cells, and Tm cells did not exhibit a significant increased at any time point after infection compared to uninfected mice (Fig. 6B). These findings suggest that $CD4^+$ Tm cells did not differentiate from Tg-T cells, which retained their T naïve cells after infection.

Next, we examined whether $CD4^+$ T_{RM} cells originated from non-transgenic T (nonTg-T) cells expressing OVA-non-specific TCR. Tm cells in nonTg-T cells significantly increased on day 3 post-infection (45.3%) compared to uninfected mice (23.6%; Fig. 7A). In addition, we investigated T naïve and Tm cells in nonTg-T cells on days 0, 3, 7, 14, and 28 after infection. Tm cells in nonTg-T cells were significantly increased at all time points after infection compared to uninfected mice (Fig. 7B). In $CD4^+$ Tm cells in nonTg-T cells, $CD69^+$ $CD103^-$ and DP T_{RM} cells exhibited a significant increase, whereas $CD69^-$ $CD103^+$ T_{RM} cells were significantly decreased at almost every time point after infection compared to uninfected mice (Fig. 7C). These findings suggest that $CD4^+$ Tm cells, especially

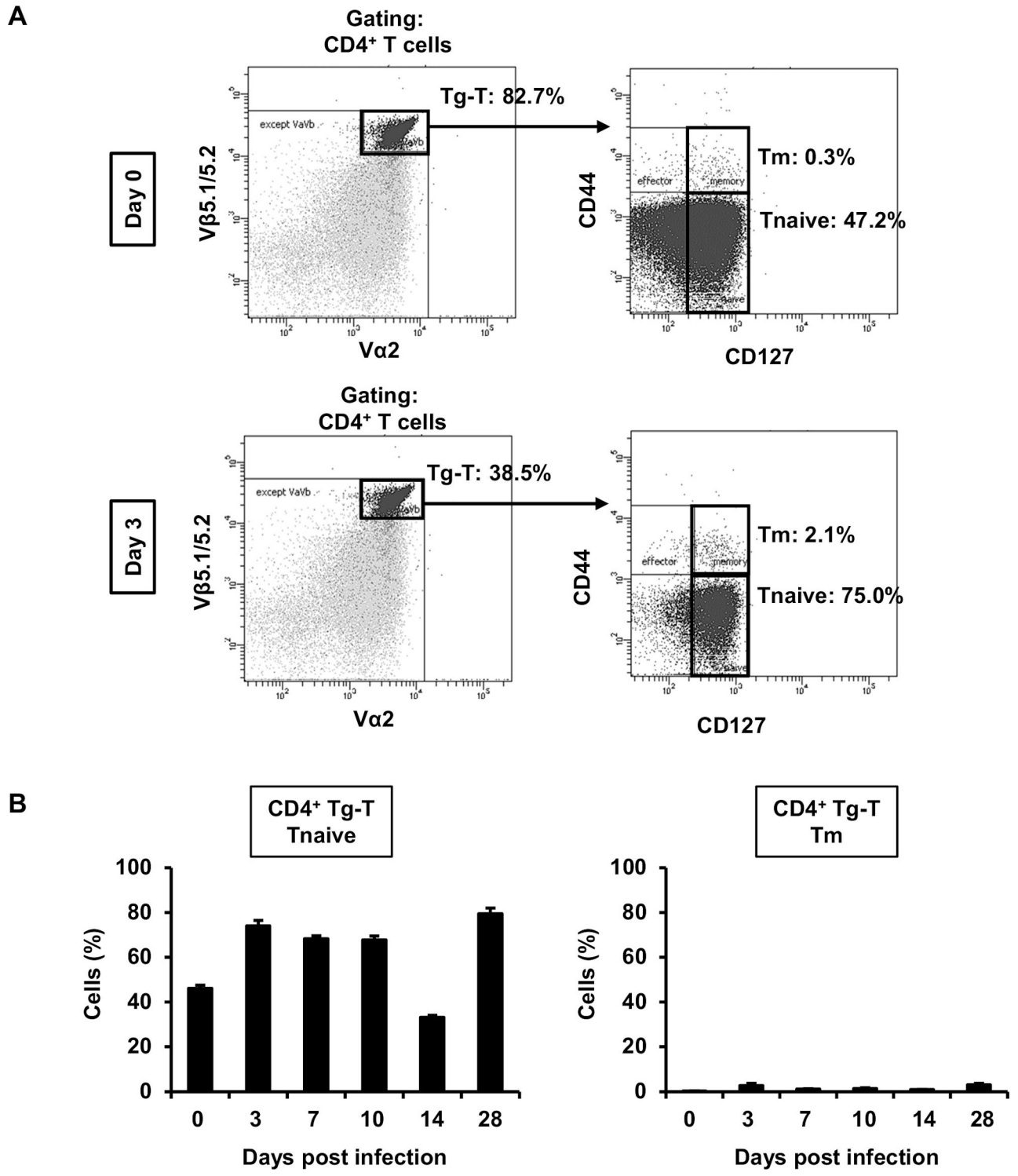

FIG 6 Analysis of T_{RM} and T naïve cells in the lungs of OT-II mice after *C. deneoformans* infection OT-II mice were infected intratracheally with *C. deneoformans*. (A) Pulmonary T naïve, Teff, and Tm cells in $CD4^+$ Tg-T cells expressing OVA-specific TCR were analyzed using flow cytometry on days 0 (uninfected, $n = 3$) and 3 ($n = 3$) post-infections. Representative dot plots are shown. (B) The number of pulmonary T naïve and Tm cells in $CD4^+$ Tg-T cells were analyzed using flow cytometry on days 0 (uninfected; $n = 3$), 3 ($n = 3$), 7 ($n = 5$), 14 ($n = 4$), and 28 ($n = 6$) post-infection. Each column represents the mean ± SD. Representative data demonstrating similar results from independent experiments are shown. Experiments were conducted twice for day 0, four times for day 3, twice for days 7 and 14, once for day 28, and once for day 58. The results for Fig. 6 and Fig. 7 represent experiments conducted simultaneously.

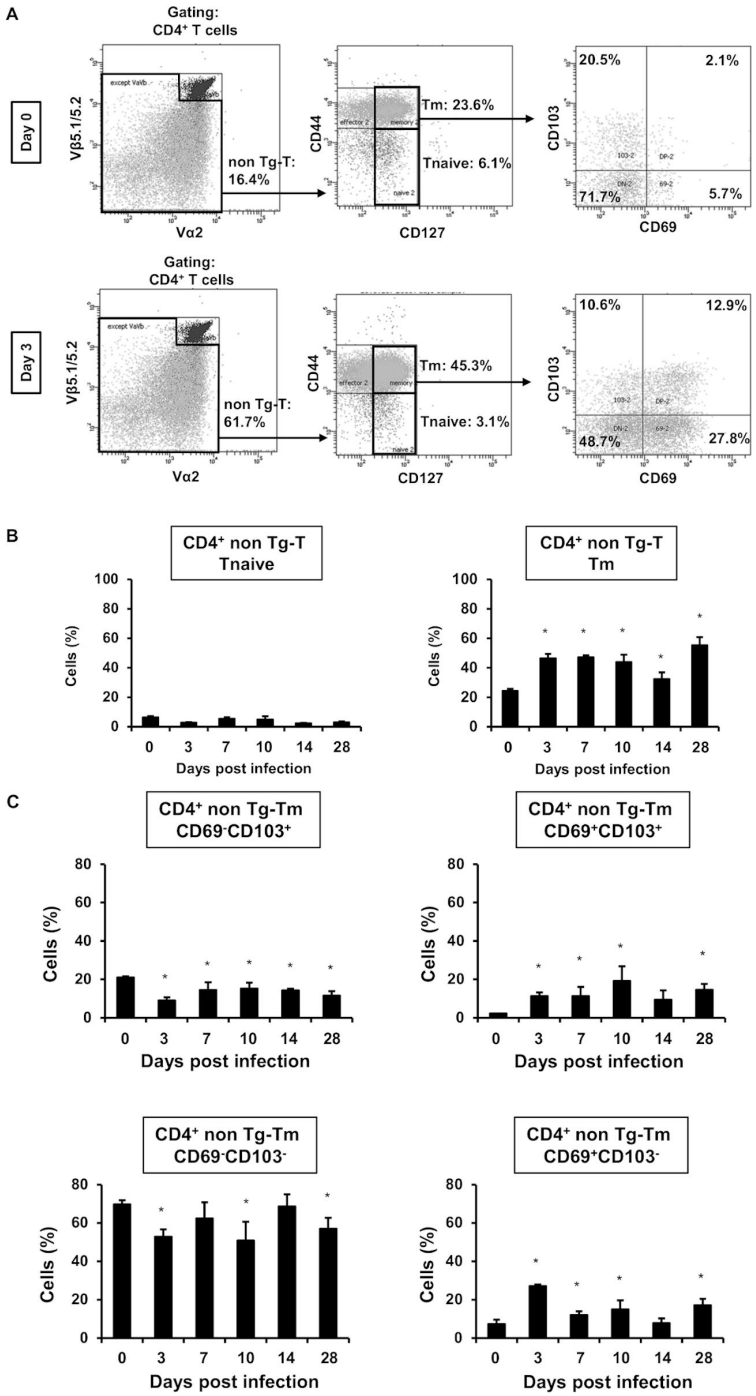

FIG 7 Analysis of T_{RM} and nonTg-T cells in the lungs after *C. deneoformans* infection OT-II mice were infected intratracheally with *C. deneoformans*. (A) Pulmonary T_{RM} cells in $CD4^+$ nonTg-T cells expressing OVA non-specific TCR were analyzed using flow cytometry on days 0 (uninfected, n = 3) and 3 (n = 3) post-infection. Representative dot plots are shown. The number of pulmonary T naïve and Tm cells (B) and CD69- and/or CD103-expressing Tm cells (C) in $CD4^+$ nonTg-T cells were analyzed using flow cytometry on days 0 (uninfected; n = 3), 3 (n = 3), 7 (n = 5), 14 (n = 4), and 28 (n = 6) post-infection. Each column represents the mean ± SD. *, $P < 0.05$ (vs day 0 post-infection). Representative data demonstrating similar results from independent experiments are shown. Experiments were conducted twice for day 0, four times for day 3, twice for days 7 and 14, once for day 28, and once for day 58. The results for Fig. 6 and 7 represent experiments conducted simultaneously.

CD69$^+$ CD103$^-$ and DP T_{RM} cells, were differentiated from nonTg-T cells due to cryptococcal infection in the early phase of infection.

Variation of T_{RM} cells in each tissue

T_{RM} cells are considered not to exist in the peripheral blood and secondary lymphoid tissues as they are primarily tissue-resident memory T cells (39, 40, 52). Therefore, we examined CD4$^+$ T_{RM} cells in peripheral blood lymphocytes and splenocytes after cryptococcal infection. Tm cells in nonTg-T cells, not Tg-T cells, increased in both peripheral blood lymphocytes and splenocytes on day 3 post-infection compared to uninfected mice (Fig. 8A). Similar to the lungs, CD69$^+$ CD103$^-$ T_{RM} cells in nonTg-T cells were significantly increased in splenocytes, not peripheral blood lymphocytes, on day 3 after infection compared to uninfected mice (Fig. 8B). On the contrary, DP T_{RM} cells were significantly increased only in the lungs on day 3 after infection compared to uninfected mice. In contrast to the lungs, CD69$^-$ CD103$^+$ T_{RM} cells were significantly increased in peripheral blood lymphocytes on day 3 after infection compared to uninfected mice. DN T_{RM} cells were significantly decreased only in the lungs on day 3 after infection compared to uninfected mice.

DISCUSSION

In this study, we analyzed the kinetics of T_{RM} cells, whose role in infection is not well understood, and MPT cells, previously reported in our research. We also assessed the production of IFN-γ from these cells using various mouse strains during infection with *C. deneoformans*. While memory T cells are generally defined as CD44$^+$CD127$^+$ cells, it is known that some MPT cells, effector, and central memory T cells exhibit low expression of CD127 (53, 54). Therefore, there is a possibility that these cells may be misidentified as effector T cells in this study.

T_{RM} cells are defined as expressing either or both CD69, an early activation marker regulating lymphocyte migration, and CD103, which binds to epithelial cells, in CD44$^{bright+}$ CD127$^+$ Tm cells (48–50). CD69$^+$ and DP T_{RM} cells significantly increased in the lungs on day 3 after cryptococcal infection, suggesting that the increased Tm cells in the early phase were predominantly composed of CD69$^+$ and DP T_{RM} cells. On the contrary, Tm and Teff cells in CD4$^+$ and CD8$^+$ T cells exhibited no increase in the lungs of CARD9KO mice after cryptococcal infection. Moreover, CD69$^+$ and DP T_{RM} cells were significantly decreased in CARD9KO mice compared to WT mice. In addition, IFN-γ expression was observed in CD4$^+$ and CD8$^+$ Tm cells and CD4$^+$ and CD8$^+$ Teff cells in the lungs of WT mice on day 3 after cryptococcal infection, and these cells were decreased in CARD9KO mice compared to WT mice. These findings are consistent with our previous report indicating a decrease in IFN-γ production in the early phase of cryptococcal infection in CARD9KO mice, suggesting that the Tm and Teff cells detected in this study are the same as the MPT cells reported in our previous studies (25). These Tm cells and Teff cells were detected in the lungs during the innate immune phase of cryptococcal infection under specific pathogen free (SPF) conditions, suggesting that these cells were responsive to various antigens encountered thus far, rather than being *C. deneoformans*-specific T cells. It has been reported that MPT cells are not only found in SPF mice but also in germ-free mice, suggesting that their production is influenced by self-antigens or dietary antigens (37).

We next investigated which CLRs upstream of CARD9 were involved in the accumulation of these T cells. Compared to WT, Dectin-2KO mice exhibited a decrease in IFN-γ-producing CD8$^+$ Teff cells, and MincleKO mice showed a decrease in IFN-γ-producing CD4$^+$ Teff cells. However, the effects observed were not as pronounced as those seen in CARD9KO mice, suggesting that other CLRs might contribute to the induction of these cells upon recognition of *C. deneoformans*. It has been reported that individual CLRs do not play major roles in phagocytosis and innate defense (55). In addition, since FcRγ and DAP12, which are not part of CLRs, require CARD9 signals (56), further research is needed

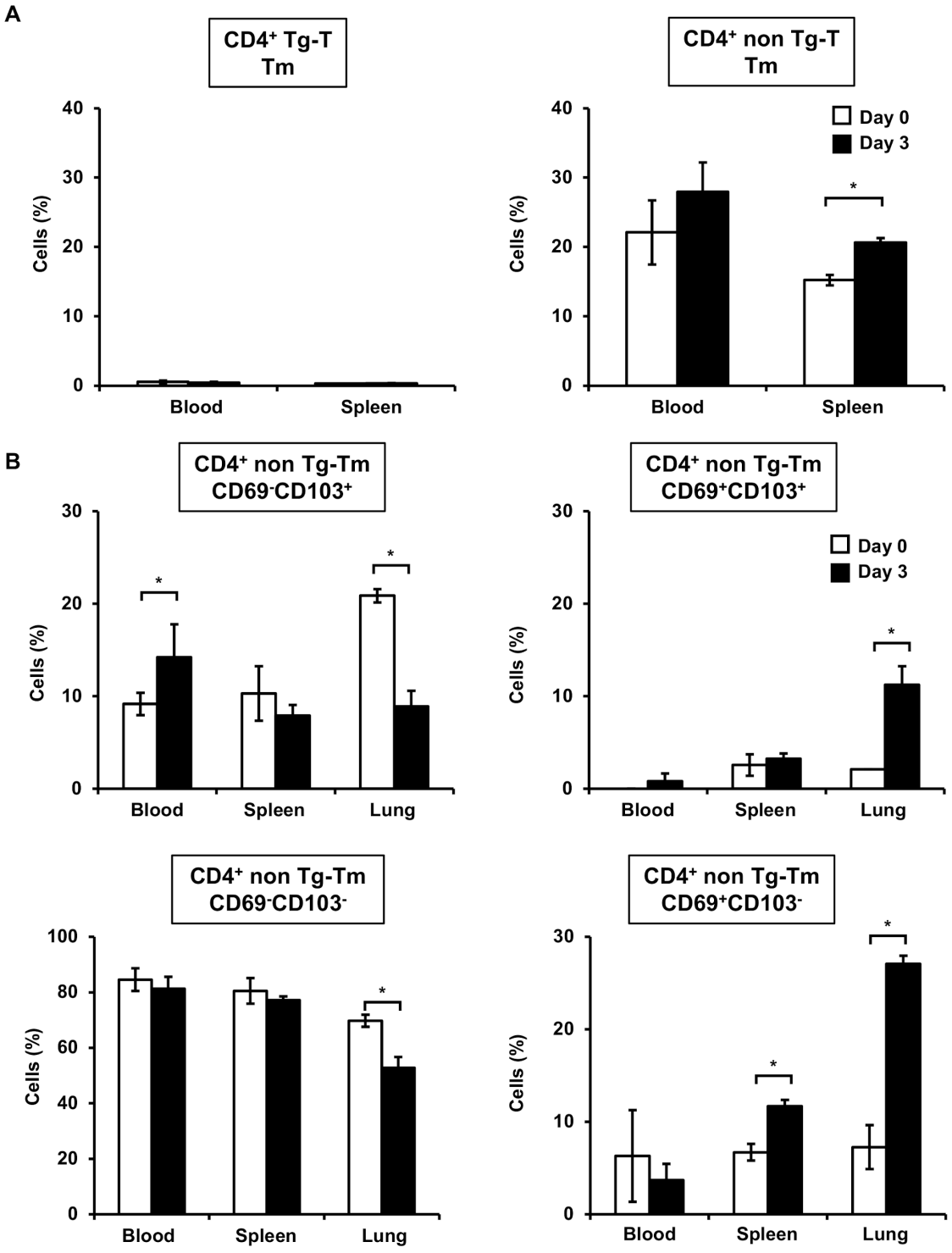

FIG 8 Analysis of T_{RM} cells in the peripheral blood and spleen after *C. deneoformans* infection OT-II mice were infected intratracheally with *C. deneoformans*. (A) Tm cells in $CD4^+$ Tg-T and nonTg-T cells in the peripheral blood and spleen were analyzed using flow cytometry on days 0 (uninfected, $n = 3$) and 3 ($n = 3$) post-infection. (B) T_{RM} cells in $CD4^+$ nonTg-T cells in the peripheral blood, spleen, and lungs were analyzed using flow cytometry on days 0 (uninfected, $n = 3$) and 3 ($n = 3$) post-infection. Each column represents the mean ± SD. *, $P < 0.05$. Representative data demonstrating similar results from independent experiments are shown. Experiments were conducted once for day 0 and twice for day 3. The results for each tissue represent results from the same experiment.

to investigate the involvement of other CLRs, such as the mannose receptor, DC-SIGN, as well as FcRγ and DAP12.

CD69$^+$ and DP T_{RM} cells, along with IFN-γ expressing cells, were reduced in CARD9KO mice compared to WT mice, suggesting that these T cells were activated within the lungs at the site of infection rather than in secondary lymphoid tissues through CARD9 signaling. CD69 is known to be an initial activating antigen expressed in T cells, B cells, and NK cells activated by anti-CD3/TCR or anti-CD2 antibodies, phorbol 12-myristate 13-acetate that activates protein kinase C (PKC), and phytohemagglutinin known as a T cells' mitogen (57, 58). In addition, it has been reported that CD69 expression is enhanced by the inflammatory cytokine tumor necrosis factor-α (TNF-α) (59), which is induced by CARD9 signaling (60). These findings suggest that the decreased antigen presentation and/or TNF-α production may lead to decreased CD69 expression in CARD9KO mice. Indeed, some reports have demonstrated that macrophages and dendritic cells derived from CARD9KO mice exhibit reduced phagocytosis of *L. monocytogenes* and *C. deneoformans* (29, 61).

To analyze the tissue residency of Tm cells and Teff cells, mice were administered FTY720. The binding of the phospholipid mediator S1P to its receptor triggers the migration of mature T cells from the thymus and secondary lymphoid tissues (48). FTY720, acting as an agonist for the S1P receptor, inhibits the migration of mature T cells from secondary lymphoid tissues by inducing the internalization of the S1P receptor from the cell surface (62). While CD4$^+$ and CD8$^+$ naïve T cells significantly decreased with FTY720 treatment compared to the control, the populations of Tm, Teff, and IFN-expressing T cells did not exhibit a decrease. The decrease in naïve T cells is likely due to the suppression of T cell migration from secondary lymphoid tissues and the decrease in the number of circulating T cells. IFN-γ-expressing Tm and Teff cells were not decreased by FTY720 treatment, suggesting that these cells were present in the lungs before infection and did not migrate to the lungs after activation and proliferation in the regional lymph nodes. Tm and Teff cells detected in the early phase of cryptococcal infection are considered to be T cells resident in the lungs, similar to T_{RM} cells. However, T_{RM} cells are involved in the rapid initial immune response at the infected site during reinfection, and it remains unclear whether these cells respond non-specifically during the initial infection. In addition, as Teff cells, not only Tm cells, were also found to be tissue resident, further investigation is needed to determine if these cells are different from T_{RM} cells.

Next, we attempted to analyze the origin of CD69$^+$ and DP T_{RM} cells using OT-II mice which highly express TCRs that are specific to OVA. T cells in OT-II mice are considered almost naïve T cells that have not encountered antigens. Tm cells were not detected in Tg-T cells before and after infection but were observed in nonTg-T cells. In addition, Tm cells and T_{RM} cells in nonTg-T cells were already present before infection, and their numbers significantly increased after 3 days of infection. CD69$^+$, CD103$^+$, and DP T_{RM} cells exhibited fluctuations over the course of the infection. Therefore, Tm cells and T_{RM} cells were already produced through encounters with various antigens, and these cells were thought to be activated in an antigen-non-specific manner upon infection with *C. deneoformans*.

Recently, a novel class of lymphocytes, distinct from T cells or B cells, has been identified as innate lymphoid cells (ILCs), involved in innate immunity. ILCs are recognized as important contributors to mucosal immunity, tissue homeostasis, and immune adjustment, categorized into three types by cytokine production profiles (63, 64). ILC1 produces Th1 cytokines such as IFN-γ, ILC2 produces Th2 cytokines such as IL-5 and IL-13, and ILC3 produces IL-17 and IL-22 (64). ILC1 produces IFN-γ and TNF-α and expresses the transcription factor T-bet upon stimulation with IL-12. It is distinguished from CD127$^-$ NK cells, CD103$^+$ ILC1, and CD127$^+$ ILC1 (63–65). These ILCs exist as either tissue-resident cells in lymphoid tissues or not, and they fulfill their function by changing the external environment or multiplying (63, 66). In this study, we consider Tm cells expressing CD69 as one subtype of T_{RM} cells, but their function is similar to ILC1, suggesting that these cells might be T_{RM}-like cells that could be classified as a new subtype of ILC. Understanding the processes by which Tm cells and T_{RM} cells, which are present

during the non-infection phase, transition from antigen-specific T cells to effector T cells, memory T cells, and ultimately become T_{RM} cells, as well as identifying the cytokines or transcription factors participating in the expression of CD69 and CD103, are potential future research topics.

MPT cells and T_{RM} cells both exhibited a rapid increase on day 3 after infection, indicating their involvement in the innate phase of infection. They were stimulated by antigen non-specifically and produced IFN-γ. However, MPT cells were mostly of the $CD8^+$ type and were found in the peripheral blood or secondary lymphoid tissues. In contrast, T_{RM} cells migrate to tissues after infection with pathogenic microorganisms and reside within the tissues (38). While MPT cells and T_{RM} cells are similar in that they both play an important role against initial infection, considering their nature and cell surface markers, it is assumed that both cells are activated by different mechanisms and perform different functions. In this study, the actual conditions and relevance of these cells were not clarified.

In this study, we demonstrated that (i) $CD69^+$ Tm cells and $CD69^+$ Teff cells in the lungs increased as early as 3 days after cryptococcal infection, (ii) these cells produced IFN-γ through non-specific signaling, and (iii) these cells were resident in tissues. In addition, it was revealed that CARD9 signaling was involved in the induction of these cells and IFN-γ production. The production of IFN-γ from these cells is considered non-specific signaling, as it occurs during the innate immune phase. However, whether specific responses exist during this phase has not been investigated in this study. These results suggest that T cells producing IFN-γ in the lungs during the early stage of infection are important for early protection against cryptococcal infection. It is essential for future research to investigate whether these cells are also detected during infection with other pathogens and whether they are related to antigen-specific Tm cells and Teff cells that are formed during acquired immunity.

ACKNOWLEDGMENTS

We thank the Biomedical Research Unit of Tohoku University Hospital and the Biomedical Research Core, Animal Pathology Platform of Tohoku University Graduate School of Medicine for providing technical support.

This work was supported in part by a Grant-in-Aid for Scientific Research (B; 18H02851 and 21H02965) and Early-Career Scientists (19K17920 and 21K16314) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the Strategic International Collaborative Research Program (SICORP), AMED (JP19jm0210073, JP20jm0210073, and JP21jm0210073); by the MSD Life Science Foundation, Public Interest Incorporated Foundation (ID-014); and by the Joint Usage/Research Program of the Medical Mycology Research Center, Chiba University (20-02, 21-04).

Conceived and designed the experiments: A.M., A.U., K.S., and Kazuyoshi K. Performed the experiments: A.M., A.U., K.S., T.M., I.M., T.Z., T.K., A.O., Kotone K., R.Y., and K.Y. Analyzed the data: A.M., A.U., K.S., H.Y., T.M., D.T., X.Y., E.K., H.T., K.I., and Kazuyoshi K. Contributed reagents/materials/analysis tools: H.H., S.Y., S.S., and Y.I. Contributed to the writing of the manuscript: A.M., A.U., K.S., and Kazuyoshi K. All authors reviewed the manuscript.

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FUNDING

Funder	Grant(s)	Author(s)
Ministry of Education, Culture, Sports, Science and Technology (MEXT)	18H02851, 21H02965	Kazuyoshi Kawakami
Ministry of Education, Culture, Sports, Science and Technology (MEXT)	19K17920, 21K16314	Ko Sato
Japan Agency for Medical Research and Development (AMED)	JP19jm0210073, JP20jm0210073, JP21jm0210073	Kazuyoshi Kawakami
MSD Life Science Foundation, Public Interest Incorporated Foundation (SD Life Science Foundation)	ID-014	Ko Sato
medical mycology research center, chiba university	20-02, 21-04	Kazuyoshi Kawakami

ETHICS APPROVAL

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: 2013 IDOU-257, 2013 IDOU-500, 2015 IDOU-061, 2016 IDOU-032, 2016 IDOU-070, 2018 IDOU-031, 2018 IDOU-056, 2019 IDOU-109, 2013 IKUMIKAE-125, 2013 IKUMIKAE-074, 2016 IKUMIKAE-141, 2018

IKUMIKAE-109, and 2018 IKUMIKAE-030). All experiments were performed under anesthesia, and all efforts were made to minimize the suffering of the animals.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1 (IAI00024-24-s0001.pdf). Gating strategy of T cells.

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