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ORIGINAL INVESTIGATION

# A comparative study of IL-33 and its receptor ST2 in a C57BL/6 J mouse model of pulmonary *Cryptococcus neoformans* infection

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## Abstract

It has been reported that IL-33 receptor ST2 deficiency mitigates *Cryptococcus neoformans* (*C. neoformans*) pulmonary infection in BALB/c mice. IL-33 may modulate immune responses in ST2-dependent and ST2-independent manners. The host genetic background (i.e., BALB/c, C57BL/6 J) influences immune responses against *C. neoformans*. In the present study, we aimed to explore the roles of IL-33 and ST2 in pulmonary *C. neoformans*-infected mice on a C57BL/6 J genetic background. *C. neoformans* infection increased IL-33 expression in lung tissues. IL-33 deficiency but not ST2 deficiency significantly extended the survival time of *C. neoformans*-infected mice. In contrast, either IL-33 or ST2 deficiency reduced fungal burdens in lung, spleen and brain tissues from the mice following *C. neoformans* intratracheal inoculation. Similarly, inflammatory responses in the lung tissues were more pronounced in both the IL-33$^{-/-}$ and ST2$^{-/-}$ infected mice. However, mucus production was decreased in IL-33$^{-/-}$ infected mice alone, and the level of IL-5 in bronchoalveolar lavage fluid (BALF) was substantially decreased in the IL-33$^{-/-}$ infected mice but not ST2$^{-/-}$ infected mice. Moreover, IL-33 deficiency but not ST2 deficiency increased iNOS-positive macrophages. At the early stage of infection, the reduced pulmonary fungal burden in the IL-33$^{-/-}$ and ST2$^{-/-}$ mice was accompanied by increased neutrophil infiltration. Collectively, IL-33 regulated pulmonary *C. neoformans* infection in an ST2-dependent and ST2-independent manner in C57BL/6 J mice.

**Keywords** *Cryptococcus neoformans* · IL-33 · ST2 · Macrophage · Neutrophil · Lung · C57BL/6 J

## Introduction

*Cryptococcus neoformans* (*C. neoformans*) is a facultative intracellular pathogen that can be inhaled into the lungs directly and results in pneumonia in immunocompromised individuals, especially AIDS patients. Genetic backgrounds, from either the fungus strains or the infected hosts, may

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influence the immune responses. H99, a highly virulent *C. neoformans* clinical isolate, potentially evokes type 2 immune responses in BALB/c mice [1]. In contrast, 52D, a moderately virulent strain, initiates type 1 immune responses in BALB/c mice [2]. In response to *C. neoformans* 52D infection, BALB/c mice develop a Th1-skewed immunity that is propitious to the clearance of infection in the lungs, while C57BL/6 mice initiate a Th2-skewed immunity that is permissive to *C. neoformans* infections [3]. The mechanisms of host defenses against *C. neoformans* pulmonary infection still need to be further explored.

IL-33 is an alarmin released by lung epithelial cells upon exposure to pathogens, including *C. neoformans* [4]. IL-33 is a dual-function cytokine. The full-length IL-33 protein (flIL-33) serves as an intranuclear gene regulator, and mature IL-33 (mIL-33) is an extracellular cytokine from damaged cells [5]. The IL-33 receptor ST2 plays a crucial role in host immune defense against *C. neoformans* infections in BALB/c mice by regulating innate type 2 immunity as well as subsequent adaptive Th2-skewed lung immunopathology [6, 7]. In particular, ST2 was required for IL-5 and IL-13 production in a pulmonary infection model of *C. neoformans* [7, 8]. Abrogation of ST2 signaling in $Foxp3^+$ Treg cells compromised their suppressive function in *C. neoformans*-infected BALB/c mice [9]. Therefore, ST2 signaling may be a key mediator in host defense against *C. neoformans* infection. However, IL-33, especially flIL-33, is fully capable of activating ST2-dependent or ST2-independent signaling [10, 11]. The roles of IL-33 and its receptor ST2 in pulmonary *C. neoformans* infection warrant further study.

To date, all of the above related studies only applied ST2-deficient BALB/c mice [6–9]. Therefore, very little is known about the precise roles of IL-33/ST2 in pulmonary *C. neoformans* infection in C57BL/6 J mice. In the present study, we explored the roles of IL-33/ST2 signaling pathways in an experimental pulmonary *Cryptococcus neoformans* infection model in wild-type (WT), IL-33-deficient ($IL\text{-}33^{-/-}$) and ST2-deficient ($ST2^{-/-}$) C57BL/6 J mice.

## Materials and methods

### *Cryptococcus neoformans* strains and culture

*Cryptococcus neoformans* H99 (ATCC, 208821) was cultivated in Sabouraud Dextrose Broth (Becton Dickinson, 238230) for 24 h at 30 °C and 200 rpm. The fungal cells were washed three times with sterile phosphate-buffered saline (PBS) and counted using a hemocytometer. Subsequently, strains were resuspended to the desired concentration in saline for in vivo infection or in PBS for in vitro stimulation.

### Mice and Intratracheal inoculation of *C. neoformans*

Wild-type (WT) female C57BL/6 mice, aged 6 to 8 weeks, were purchased from the Laboratory Animal Center, Nanjing Medical University (Nanjing, China). Age- and sex-matched IL-33-deficient ($IL\text{-}33^{-/-}$) or ST2-deficient ($ST2^{-/-}$) mice on the C57BL/6 J background were obtained from Dr. Hong Zhou's laboratory (Department of Immunology, Nanjing Medical University). All mice were maintained under specific pathogen-free conditions at the Animal Core Facility of Nanjing Medical University. All experimental protocols were reviewed and approved by the Animal Care and Use Committee of Nanjing Medical University (IACUC-1708004).

For intratracheal inoculation of *C. neoformans*, mice were anesthetized with pentobarbital sodium by intraperitoneal (i.p.) injection. A total of $1 \times 10^4$ CFU of H99 in a volume of 30 µl of sterile saline was injected into the airway by using a 1 ml syringe, followed quickly by a 200 µl volume of air to help H99 diffuse into the airways. For the survival study, mice were monitored daily for survival following inoculation. For the cryptococcal organ burden assay, cytokine determination, inflammatory cell analysis, and histology, mice were sacrificed at 14 days postinfection. For neutrophil infiltration analysis during the innate immune response at an early stage, mice were infected with $1 \times 10^5$ CFU of H99 and sacrificed 4 h after inoculation.

### Bronchoalveolar lavage fluid (BALF) harvest

Mice were sacrificed at 14 days or 4 h postinfection, and BALF was collected. Briefly, a 22-gauge catheter was inserted into the airway after the mouse was anesthetized. A volume of 0.5 ml ice-cold PBS containing 1 mM EDTA was injected into the airway via the catheter, followed by gentle aspiration. The lavage procession was then repeated twice (recovery rate > 80%). The BALF was then centrifuged at 500 × g for 5 min, and the supernatants were stored at − 80 °C for cytokine and chemokine analysis, while the cell pellet was resuspended in PBS for inflammatory cell analysis by flow cytometry.

### Cryptococcal organ burden quantification

After BALF harvest, mice were perfused with PBS, and the lung, spleen, and brain were collected into separate tubes on ice. Subsequently, the left lobe of the lung and whole spleen and whole brain were homogenized in a volume of 1 ml sterile PBS. Tenfold serial dilutions of homogenates were plated on Sabouraud Dextrose Agar (Becton Dickinson, 210950)

and incubated at 32 °C for 36 h. Subsequently, CFUs were counted and expressed as log10 (CFU/ml).

### Flow cytometry

BALF cells were washed twice with protein-free PBS and incubated with a zombie NIR™ Fixable Viability Kit (Biolegend 423105) at room temperature for 30 min in the dark. After washing twice with 1% BSA, Fc receptors were blocked with anti-CD16/32 antibody (eBioscience, 14-0161-85, Clone: 93) for 10 min. Subsequently, the BALF cells were incubated with anti-CD45-PE-Cy7 (Biolegend, 103114, Clone: 30-F11), anti-CD11b-BV510 (Biolegend, 101245, Clone: M1/70) and anti-ly6G-Alexa Fluor647 (Biolegend, 127,610, Clone: 1A8) in the dark at 4 °C for 40 min. The counting beads (eBioscience, 01-1234-42) were used to count the cell number. Data were acquired with a BD FACSCalibur and analyzed using FlowJo software (Treestar, Woodburn, OR, USA).

### Quantitative real-time PCR

Total RNA was extracted from fresh lung tissue with a TRIzol reagent kit (Life Technologies) according to the manufacturer's instructions. Briefly, 10 mg lung tissue was homogenized in 1 ml TRIzol reagent using a tissue homogenizer (Jingxin, Tiss-24), and subsequently, 200 µl chloroform was added to homogenates. After centrifugation at 12,000 × g for 15 min, the aqueous phase was collected into new tubes, and equal volumes of isopropanol were added. After centrifugation again, the pellets were washed with 1 ml of 75% ethanol, centrifuged and resuspended in nuclease-free water. Subsequently, RNA was reverse-transcribed into cDNA with a reverse-transcription kit (Abm, G490). Finally, cDNA was quantified with SYBR Green-based detection using a StepOnePlus Real-Time PCR System (ABI, USA), and each sample was run in triplicate. The relative expression of targets is shown as $-\Delta CT = CT_{Targets} - CT_{\beta\text{-actin}}$. The primers designed by referring to PrimerBank (https://pga.mgh.harvard.edu/primerbank) are listed in Table 1.

**Table 1** Primers used in the study

| Target | Primer |
|---|---|
| β-actin-F | 5′- GAGAAGCTGTGCTATGTTGCT-3′ |
| β-actin-R | 5′- CTCCAGGGAGGAAGAGGATG-3′ |
| Arg1-F | 5′- CTCCAAGCCAAAGTCCTTAGAG-3′ |
| Arg1-R | 5′- AGGAGCTGTCATTAGGGACATC-3′ |
| iNOS-F | 5′- GTTCTCAGCCCAACAATACAAGA-3′ |
| iNOS-R | 5′- GTGGACGGGTCGATGTCAC-3′ |

### Enzyme-linked immunosorbent assay (ELISA)

Cytokine and chemokine contents in BALF, lung homogenates or cell culture supernatants were measured by using commercially available ELISA kits: interleukin-4 (IL-4) (Biolegend, 431104), interleukin-5 (IL-5) (Biolegend, 431204), interleukin-13 (IL-13) (Peprotech, 900-K207), interferon gamma (IFN-γ) (Biolegend, 430804) and chemokine (C-X-C motif) ligand 1 (KC) (R&D, Y453-05). All assays were performed according to the manufacturer's instructions provided by commercially available ELISA kits.

### Histopathological analysis

Following euthanasia, the pulmonary vasculature was perfused with ice-cold PBS via the right ventricle of the heart to remove blood from the lungs. Subsequently, the right lower lobes of the lungs were embedded in paraffin and cut into 5-µm-thick tissue sections. Furthermore, sections were stained with H&E, periodic acid-Schiff (PAS) or mucicarmine reagents. Images of the slides were acquired with an Olympus BX51 light microscope (Olympus Canada) at a magnification of 400×. The severity of peribronchial inflammation was semiquantitatively graded for the following features: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1-cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; and 4, a ring of inflammatory cells 4 cells deep. The numerical scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, < 0.5% PAS-positive cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, > 75% [12].

### Immunofluorescence

For immunofluorescence, slides were fixed with 4% paraformaldehyde, rinsed twice with PBS, and permeabilized in PBS containing 0.5% Triton X-100. Samples were incubated with 5% normal goat serum for 1 h at room temperature and stained in blocking buffer with anti-F4/80 (Servicebio, GB113373, 1:500), anti-iNOS (Servicebio, GB11119, 1:3000) and anti-Arg1 (Cell Signaling Technology, #93668, Clone: D4E3 M, 1:1000) overnight at 4 °C. After the samples were washed with PBS, the corresponding secondary antibodies were added to the samples and incubated for 1 h in the dark at room temperature. The samples were finally mounted with DAPI (Yeasen, 36308) to stain the nucleus. Images were collected with a slice scanner (Pannoramic MIDI, 3Dhistech).

### Western blot analysis

Total cell or tissue protein was lysed in RIPA (Pierce, 89900) with PMSF (Beyotime Biotech, ST506) on ice and

centrifuged for 10 min at 12,000 rpm at 4 °C. The supernatant was then transferred to a new tube and denatured in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Yeasen, 20315) with heating at 100 °C for 10 min. The supernatant was then stored at − 80 °C. The proteins were separated by 15% SDS-PAGE and transferred at 300 mA for 1 h at 4 °C. The blots were then blocked with 5% nonfat dry milk in TBST and incubated with 1:1,000 primary antibodies against β-actin (Cell Signaling Technology, 4970 L, 1:1000) and IL-33 (Abcam, ab187060, Clone: EPR17831, 1:1000) overnight before adding HRP-linked anti-rabbit IgG (Cell Signaling Technology, 7074, 1:5000). After the membranes were treated with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, WBKLS0500), the binding of specific antibodies was visualized using a Syngene G:BOX Imaging System and was analyzed with ImageJ.

### Bone marrow-derived macrophage culture

Tibias and femurs were excised, and marrow cells were flushed, lysed with Red Blood Cell Lysis Buffer and resuspended at $3\times10^5$ cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin, streptomycin and 20 ng/ml recombinant mouse GM-CSF (Biolegend, 576308). The medium was changed every three days. After 7 days, the resultant nonadherent cell populations were discarded, and the remaining adherent cells were collected for further use.

*C. neoformans* H99 was opsonized in 10% normal mouse serum at 37 °C for 45 min. For stimulation with *C. neoformans*, BMDMs were plated in 24-well culture plates infected with MOI = 2 for 24 h, and then supernatants were collected for the detection of KC (CXCL1) [13]. The experiments were repeated 4 times.

### Neutrophil isolation

Mouse neutrophils were isolated from bone marrow by density gradient centrifugation as previously described [14]. Briefly, the mice were euthanized and immersed in 70% ethanol for 15 min. The bone marrow from the femur and tibia was flushed using a 1 ml sterile syringe filled with PBS and filtered through a 70 µm cell strainer (Falcon). The collected cell suspension was washed and resuspended in 1 ml sterile PBS. Then, separating medium and cell suspension were gently added into 15-ml centrifuge tubes in accordance with the following order: 3 ml of Histopaque 1119 (Sigma–Aldrich, 111191), 3 ml of Histopaque 1077 (Sigma–Aldrich 10771) and 1 ml of bone marrow cell suspension. Finally, neutrophils were obtained at the interface of the Histopaque 1119 and Histopaque 1077 layers by density gradient centrifugation.

### In vitro killing assay

Murine neutrophils were incubated with *C. neoformans* cells (MOI = 0.01) in 96-well flat-bottomed plates containing 200 µl RPMI 1640 supplemented with 40% fresh mouse serum. Control wells contained *C. neoformans* cells alone with 40% mouse serum. Cocultures were harvested and washed with PBS after 6 h by centrifugation. The pellet was released by lysing the neutrophils in distilled water. The number of *C. neoformans* CFUs was determined by plating tenfold serial dilutions for each sample onto Sabouraud dextrose agar plates after 48 h of incubation at 30 °C. The percent killing was calculated by dividing the CFU for each well by the average CFU in wells without neutrophils and then subtracting this value from 1 [15].

### Statistical analysis

All data are expressed as the mean ± SEM, and all statistical analyses were performed using GraphPad Prism 7. A Q-Q (for quartile-quartile) plot was used to visually compare the sample distribution against a Gaussian ideal. A single comparison was conducted by an unpaired *t* test. Multiple comparisons were tested by using one-way ANOVA with Tukey's adjustment. Survival study comparison was performed using Kaplan–Meier analysis. For all analyses, statistical significance was set as $p < 0.05$. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant.

## Results

### IL-33 deficiency but not ST2 deficiency extended the survival time of pulmonary *Cryptococcus neoformans*-infected C57BL/6 J mice

To explore whether IL-33 expression changed during the course of pulmonary *C. neoformans* infection, we quantified IL-33 at different times. As shown in Fig. 1A, the level of flIL-33 in lung tissues was significantly increased from infected mice 3 weeks following intratracheal injection with *C. neoformans,* while mIL-33 reached the peak 2 weeks postinfection, suggesting that IL-33 may be involved in *C. neoformans* pulmonary infection. ST2 is the putative receptor of IL-33. To determine if the protective function of ST2 deficiency was not uniquely restricted to the BALB/c mouse infection model, we evaluated the survival of infected mice and organ dissemination of fungi in C57BL/6 J mice (WT, IL-33$^{-/-}$, ST2$^{-/-}$) by intratracheal inoculation with *C. neoformans*. As shown in Fig. 1B, IL-33 deficiency markedly prolonged the survival time of infected mice (median survival time postinfection in WT: 27 days; IL-33$^{-/-}$, 35 days). IL-33 could exert its

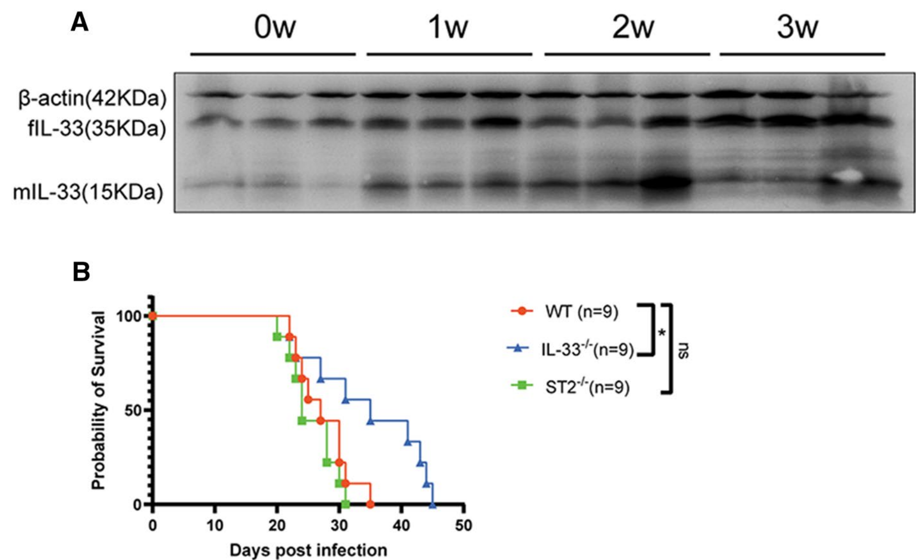


**Fig. 1** IL-33 deficiency but not ST2 deficiency prolonged the survival time of mice with pulmonary *C. neoformans* infection. **A** IL-33 in lung tissues was measured by Western blotting at 0~3 weeks postinfection**. B** WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1 \times 10^4$ *C. neoformans* strain H99 and monitored daily for survival. Data represent the mean ± SEM. ($n = 9$ per group). $^*p < 0.05$; ns, not significant

function not only in an ST2-dependent way but also in an ST2-independent way [11]. In contrast with the previous observation in BALB/c mice that ST2 deficiency significantly extended survival time [6], the survival curves of pulmonary infected WT and ST2 deficiency in C57BL6/J genetic background mice were comparable (median survival time postinfection in WT: 27 days; ST2$^{-/-}$: 24 days). Collectively, IL-33 deficiency but not ST2 deficiency was protective in pulmonary *Cryptococcus neoformans*-infected C57BL/6 J mice, suggesting that IL-33 may regulate pulmonary *Cryptococcus neoformans* infection in C57BL/6 J mice at least partially independent of ST2.

## Both IL-33 deficiency and ST2 deficiency limited fungal burdens in C57BL/6 J mice

To decipher the discrepancy between IL-33$^{-/-}$ and its receptor ST2$^{-/-}$ mice with pulmonary *C. neoformans* infection, we first quantified fungi in the lung. As shown in Fig. 2A, compared with WT mice infected with pulmonary *C.*

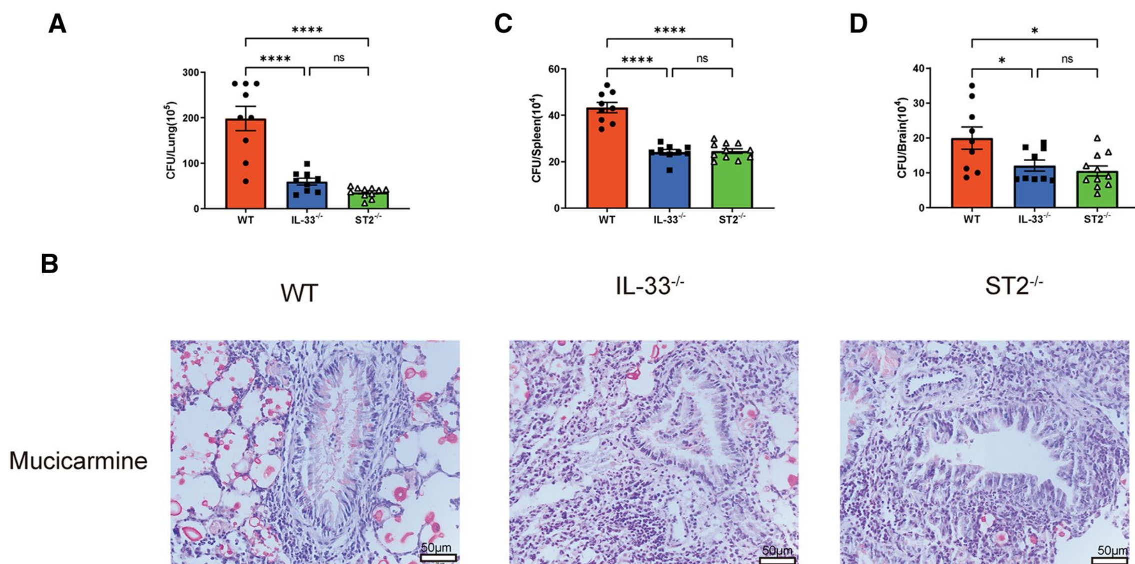


**Fig. 2** Deficiency in IL-33 or ST2 restricted *C. neoformans* fungal burdens. WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1 \times 10^4$ CFU *C. neoformans* strain H99 and sacrificed 14 days postinfection. **A** CFU in the lung tissues. **B** Representative lung sections were stained with mucicarmine, identifying *C. neoformans* as a red-stained cell surrounded by a clear capsular halo. Scale bar, 50 µm. **C** CFU in the spleen tissues. **D** CFU in the brain tissues. Data represent the mean ± SEM. ($n = 9$–11 per group). $^*p < 0.05$; $^{****}p < 0.0001$; ns, not significant

*neoformans*, either IL-33$^{-/-}$ or ST2$^{-/-}$ mice had reduced CFU in the lung tissues. Pulmonary CFUs in the IL-33$^{-/-}$ and ST2$^{-/-}$ mice following intratracheal infection were similar. To directly observe the fungi in the lung tissues, we stained the fungi with mucicarmine, which again validated the significant reduction in pulmonary fungal burden in IL-33$^{-/-}$ and ST2$^{-/-}$ mice following *C. neoformans* infection (Fig. 2B). Fungal local growth and fungal dissemination into other organs are associated with progressive infection. Therefore, we measured the fungal burdens in the spleen and brain tissues 14 days postinfection. Similar to the lung tissues, compared with WT C57BL/6 J mice, fungal burdens in the spleen and brain tissues from IL-33$^{-/-}$ or ST2$^{-/-}$ mice were significantly reduced; again, CFUs in the spleens or brains were similar in IL-33$^{-/-}$ or ST2$^{-/-}$ mice (Fig. 2C, D). The pattern of fungal burdens in the WT, IL-33$^{-/-}$ or ST2$^{-/-}$ mice was similar among the lung, spleen and brain. Collectively, these data suggested that IL-33 and ST2 may mediate the growth of *C. neoformans* in lung tissues and dissemination into extrapulmonary organs, i.e., the spleen and brain.

### IL-33 deficiency but not ST2 deficiency reduced mucus section

The discrepancy in survival curves and fungal burdens in the IL-33$^{-/-}$ and ST2$^{-/-}$ mice following *C. neoformans* infection implied that IL-33 deficiency or ST2 deficiency might result in the development of distinct lung tissue pathology. To test this hypothesis, we stained lung sections from WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice with H&E and PAS at 14 days postinfection. Consistent with previous reports [6], H&E staining revealed that inflammatory infiltration was significantly increased in the lungs of IL-33$^{-/-}$ and ST2$^{-/-}$ infected mice compared with WT C57BL/6 J mice (Fig. 3A). In contrast, PAS staining demonstrated that mucus secretion was decreased in the airways of IL-33$^{-/-}$ C57BL/6 J mice only; compared with WT, ST2 deficiency only slightly decreased mucus secretion (Fig. 3B). Taken together, our data revealed that IL-33/ST2 deficiency promoted pulmonary inflammation, and IL-33 deficiency but not ST2 deficiency reduced mucus sections in C57BL/6 J mice following *C. neoformans* infection.

### IL-33 deficiency reduced pulmonary type 2 responses at least partially independent of its receptor ST2

It has been previously shown that a proinflammatory immune response (type 1) is protective against Cryptococcus [16–18]. Conversely, an anti-inflammatory immune response (type 2) is permissive for cryptococcal infection [16, 19]. IL-33 is capable of initiating type 2 immune responses with ST2 dependency [20, 21]. To investigate whether IL-33/ST2 deficiency could alter the pattern of immune responses in C57BL/6 J mice following cryptococcal infection, we further

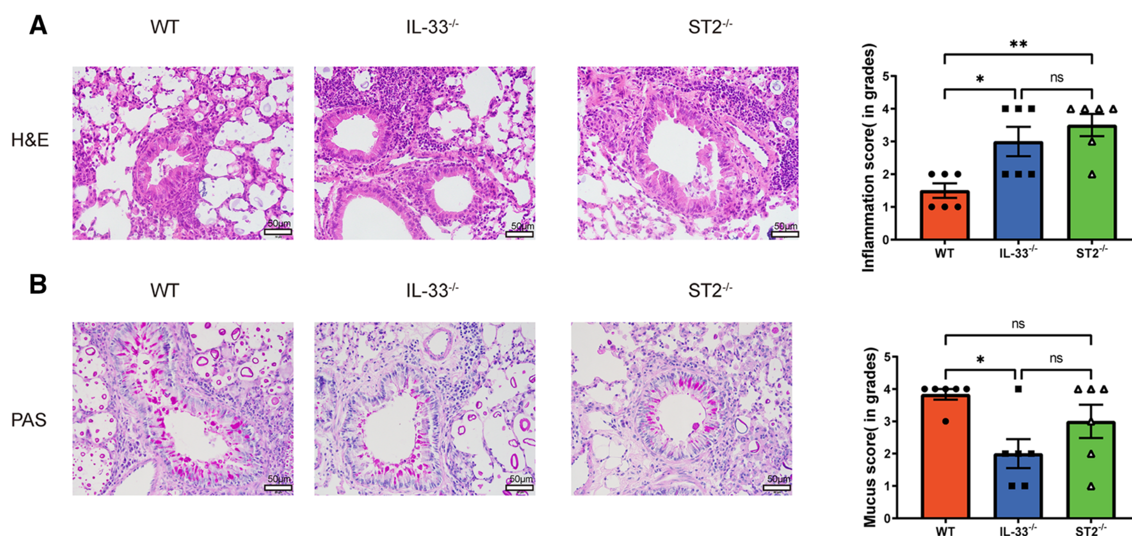


**Fig. 3** Increased inflammatory response in the lung tissues of IL-33$^{-/-}$ or ST2$^{-/-}$ mice following cryptococcal infection. WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1\times10^4$ *C. neoformans* strain H99 and sacrificed 14 days postinfection. **A** Representative lung sections were stained with hematoxylin and eosin to analyze the infiltration of inflammatory cells, and the severity of peribronchial inflammation was graded semi-quantitatively by analyzing HPF (high power fields) per sample. **B** Representative lung sections were stained with PAS to assess goblet cell hyperplasia, and the percentage of PAS-positive cells per bronchiole was calculated by analyzing HPF (high power fields) per sample. Magnification, ×400, Scale bar, 50 μm. Data represent the mean ± SEM. ($n$ = 6 per group). $*p<0.05$, $**p<0.01$; ns, not significant

assessed the contents of type 1 cytokines (IFN-γ) and type 2 cytokines (IL-4, IL-5 and IL-13) in the lung homogenates at 14 days postinfection (Fig. 4). IFN-γ in the lung tissues was increased in the IL-33$^{-/-}$ mice following pulmonary cryptococcal infection. In contrast, IL-4, IL-5 and IL-13 in the lung tissues were decreased in IL-33$^{-/-}$ mice. IL-4 in the ST2$^{-/-}$ infected mice was significantly decreased. Similarly, IL-13 in the ST2$^{-/-}$ infected mice was decreased, although it did not reach significance. In the ST2$^{-/-}$ infected mice, however, IFN-γ and IL-5 were comparable with the WT controls. In summary, IL-33 deficiency reduced pulmonary type 2 responses in C57BL/6 J mice following *C. neoformans* infection, which was at least partially in an ST2-independent manner (i.e., IL-5).

## IL-33 deficiency but not ST2 deficiency induced M1 polarization in pulmonary cryptococcal infection

It has been well established that the fungicidal activity of macrophages depends on their polarization status. Classically activated macrophages (M1) are protective, and alternatively activated macrophages (M2) are permissive for cryptococcal infection. IL-33 has recently been described to influence macrophage polarization [5, 22, 23]. Thus, we next addressed whether the improved outcomes of pulmonary *C. neoformans* infection in IL-33$^{-/-}$ or ST2$^{-/-}$ mice were associated with differential M1/M2 polarization. First, we measured the mRNA levels of iNOS (a putative M1 marker) and Arg1 (a putative M2 marker). IL-33 deficiency skewed toward M1, as evidenced by increased iNOS and decreased Arg1 in IL-33$^{-/-}$ mice following pulmonary *C. neoformans* infection. However, iNOS and Arg1 were similar in WT and ST2$^{-/-}$ mice infected with *C. neoformans* (Fig. 5A, B). Furthermore, immunofluorescence staining was used to validate the above qRT–PCR observations. As presented in Fig. 5C, macrophages expressing iNOS were increased in IL-33$^{-/-}$ but not ST2$^{-/-}$ mice infected with *C. neoformans*; meanwhile, Arg1-positive macrophages were decreased in the lungs of IL-33$^{-/-}$ mice but not in ST2$^{-/-}$ infected mice. Taken together, our results suggested that IL-33 deficiency promoted macrophages to acquire the M1 phenotype in an ST2-independent manner.

## Deficiency in IL-33 or ST2 restricted fungal growth and increased neutrophil infiltration at the early stage of pulmonary *C. neoformans* infection

Several recent studies have implicated neutrophils in cryptococcal clearance at the early stage of infection [24], and IL-33 has been demonstrated to influence the migration of neutrophils in infections such as sepsis [25, 26]. Therefore, we further investigated the clearance of *C. neoformans* at 4 h postinfection. To test this hypothesis, we analyzed the lung CFU among WT, IL-33$^{-/-}$ and

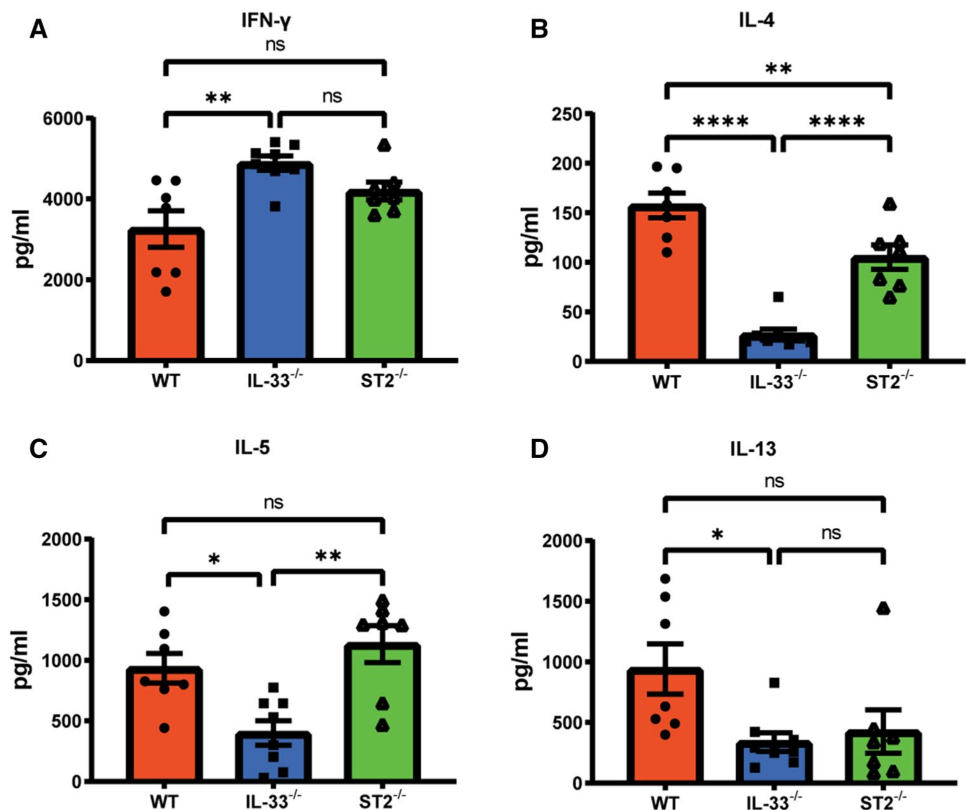


**Fig. 4** Reduced type 2 response in the lungs following cryptococcal infection in IL-33$^{-/-}$ and ST2$^{-/-}$ mice. WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1 \times 10^4$ *C. neoformans* strain H99 and sacrificed 14 days *postinfection.* IFN-γ (**A**) and IL-4 (**B**), IL-5 (**C**), and IL-13 (**D**) in the lung homogenates were measured with ELISA. Data represent the mean ± SEM. ($n = 7$–8 per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant

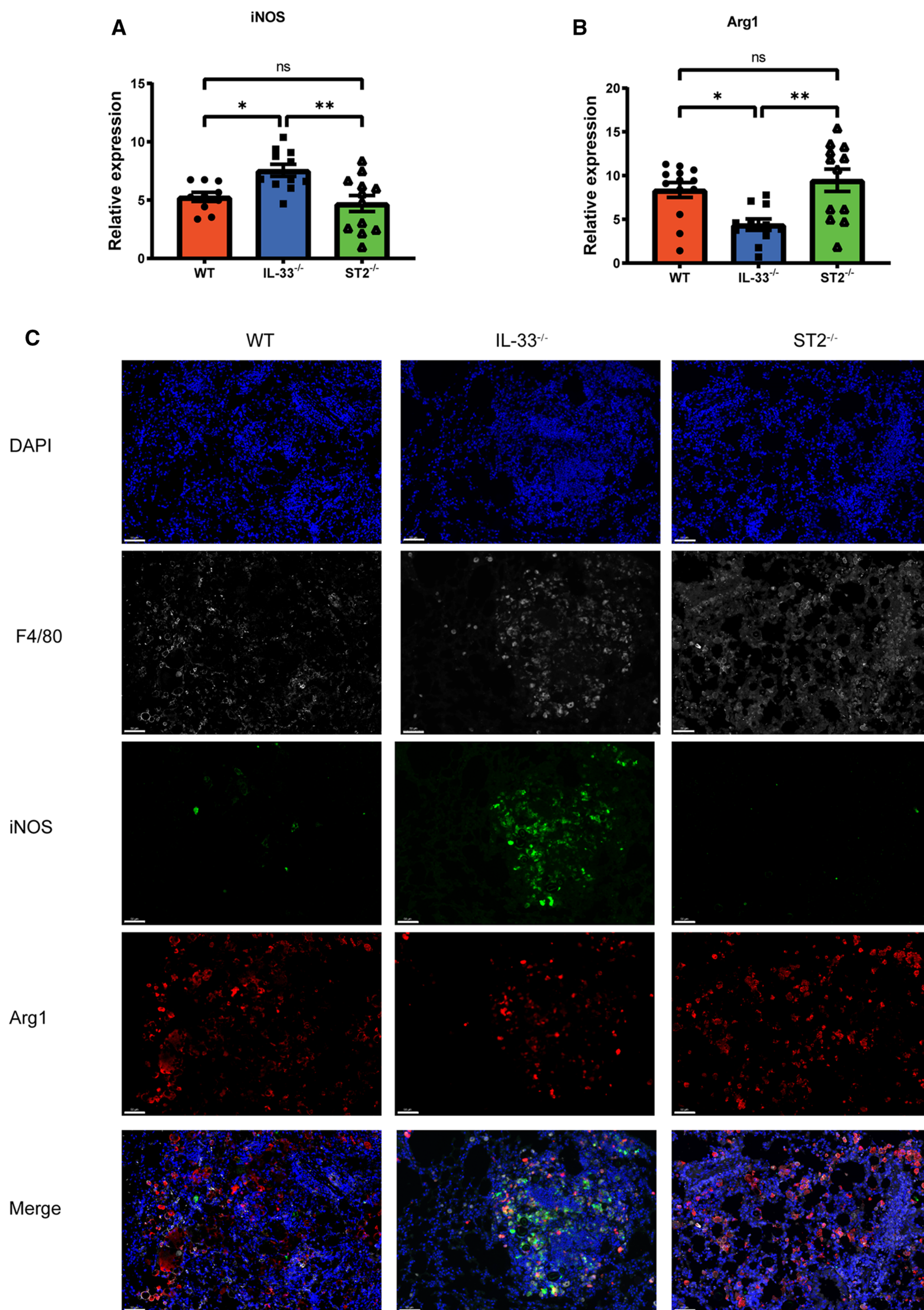


**Fig. 5** Pulmonary macrophage polarization following cryptococcal infection in IL-33$^{-/-}$ and ST2$^{-/-}$ mice. WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1 \times 10^4$ CFU *C. neoformans* strain H99 and sacrificed 14 days postinfection. **A**, **B** Lung mRNA expression levels of M1 macrophage markers (iNOS) and M2 macrophage markers (Arg1). **C** Immunofluorescence imaging of macrophages (F4/80$^+$, pseudogray) expressing iNOS (pseudogreen) and Arg1 (pseudored) in lung tissues. Scale bar, 50 µm. Data represent the mean ± SEM. ($n$ = 10–13 per group). *$p$ < 0.05; **$p$ < 0.01; ns, not significant

ST2$^{-/-}$ mice at 4 h postinfection and found that the lung fungal burden was obviously decreased in IL-33$^{-/-}$ and ST2$^{-/-}$ mice (Fig. 6A). To determine whether the clearance of *C. neoformans* was linked to an early innate immune response, we performed flow cytometry analysis (gate strategy, Figure S1) of immune cells in BALF. At 4 h postinfection, the numbers of total CD45$^{+}$ immune cells and CD45$^{+}$Ly6G$^{+}$CD11b$^{+}$ neutrophils were significantly increased in IL-33-deficient and ST2-deficient mice (Fig. 6B, C, Figure S1). In line with the increased neutrophil infiltration, the chemokine KC, which mediates neutrophil migration, was upregulated in BALF (Fig. 6D). Following *C. neoformans* infection in vitro, BMDMs defective in IL-33 or ST2 produced more KCs (Fig. 6E). Meanwhile, the fungicidal effects of WT and IL-33$^{-/-}$ neutrophils were similar; however, the cytotoxicity of ST2$^{-/-}$ neutrophils against *C. neoformans* was significantly increased (Fig. 6F). Thus, deficiency in IL-33 or ST2 restricted pulmonary *C. neoformans* growth and increased neutrophil recruitment at the early stage of pulmonary cryptococcal infection.

## Discussion

The outcome of infectious diseases is determined by the host genetic background, the virulence of microbes and the complicated interplay between the host and microbes. In the present study, we demonstrated that abrogation of IL-33 in C57BL6/J mice was protective against *C. neoformans* pneumonia, as evidenced by the extended survival time and reduced fungal burdens in the lung, spleen and brain tissues. Unexpectedly, although IL-33 receptor ST2 deficiency similarly reduced the fungal burdens, ST2 deficiency was dispensable for survival in C57BL6/J-infected mice. The protective effect of IL-33 deficiency was associated with altered immune response patterns. Specifically, in response to the established *C. neoformans* pneumonia, mice defective in IL-33 but not ST2 decreased IL-5 and mucus production and increased M1 differentiation. In the early stage of *C. neoformans* pneumonia, either IL-33 or ST2 deficiency promoted neutrophil infiltration and diminished fungal survival in the lung tissues. These observations may shed light on how IL-33/ST2 regulates *C. neoformans* pneumonia in C57BL6/J mice.

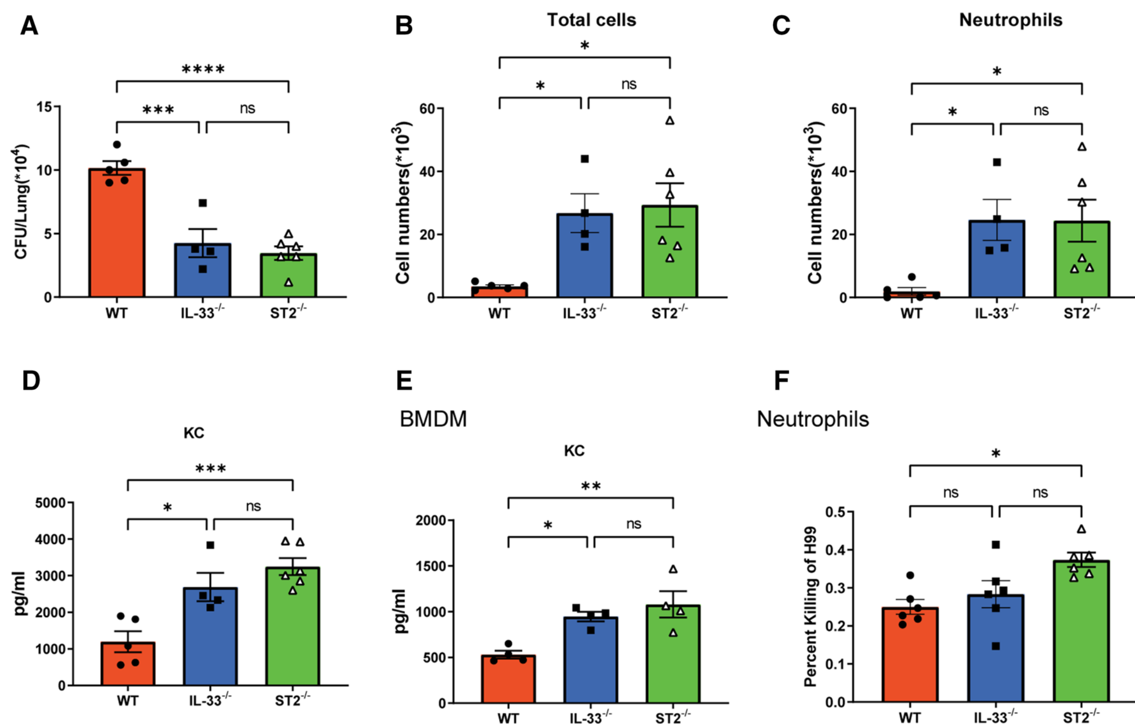


**Fig. 6** IL-33 or ST2 deficiency restricted pulmonary *C. neoformans* growth and increased neutrophil infiltration. WT, IL-33$^{-/-}$ and ST2$^{-/-}$ mice were intratracheally inoculated with $1\times10^{5}$ *C. neoformans* strain H99 and sacrificed 4 h postinfection. **A** Lung CFU. **B**, **C** Quantification of total immune cells and neutrophils in the BALF. **D** The chemokine KC in BALF was determined using ELISA. **E** Levels of KCs in the supernatant from WT, IL-33$^{-/-}$ and ST2$^{-/-}$ BMDMs infected with *C. neoformans* strain H99. **F** The fungicidal activity of WT, IL-33$^{-/-}$ and ST2$^{-/-}$ neutrophils against *C. neoformans*. Data represent the mean ± SEM. (n = 4–6 per group). *$p<0.05$; ***$p<0.001$; ****$p<0.0001$; ns, not significant

The fungal burdens in IL-33$^{-/-}$ and ST2$^{-/-}$ infected mice were similar. However, IL-33 deficiency, but not ST2 deficiency, prolonged the survival time of C57BL6/J mice, suggesting that fungal burden was not the direct cause of death in mice with fungal pneumonia. The overproduction and hypersecretion of mucus may predispose to fungal infection by disturbing the clearance of inhaled fungal spores and by causing their sustained colonization [27]. In the present study, the reduced mucus production in the IL-33$^{-/-}$ infected mice was in line with prolonged survival. In contrast, ST2 deficiency was negligible for mucus production and survival extension. Of note, goblet cell hyperplasia and increased mucus production are regulated by IL-4, IL-5, IL-9 and IL-13 [28, 29]. IL-5, which was reduced in IL-33$^{-/-}$ but not ST2$^{-/-}$ *C. neoformans*-infected mice, may be responsible for the mitigation of mucus production in IL-33$^{-/-}$ but not ST2$^{-/-}$-infected mice. Of note, in *C. neoformans*-infected mice with a Balb/c genetic background, ST2 deficiency decreased IL-5 production [7], suggesting that the host genetic background may influence the immune response.

Macrophages are one of the first lines of defense for the innate immune system against *C. neoformans*. In the present study, deficiency of IL-33 but not ST2 in mice with *C. neoformans* pneumonia promoted the differentiation of M1 macrophages (increased iNOS) and suppressed M2 macrophages (decreased Arg1). IL-33, especially mature IL-33 bound to its receptor ST2, is a potent inducer of M2 [30]. In addition to favoring M2 polarization, IL-33 also contributes to M1 chemokine production [31]. Myeloid differentiation factor 88 (MyD88) was required for the production of proinflammatory cytokines and restriction of pulmonary infection in experimental murine cryptococcosis models [32]. Interestingly, IL-33 may function in a MyD88-dependent manner, which is independent of ST2 [33].

Similar to macrophages, neutrophils are also the first responders in pulmonary cryptococcosis. In the present study, neutrophil infiltration in the airway from IL-33$^{-/}$ or ST2$^{-/-}$ mice was increased at 4 h postinfection, which was consistent with the increased KC from macrophages infected with *C. neoformans*. Fungcidal ability in IL-33$^{-/-}$ or ST2$^{-/-}$ neutrophils was not impaired; in contrast, ST2 deficiency increased the ability of neutrophils to kill *C. neoformans*. Therefore, the reduced fungal burdens may be due to the increased recruitment and fungicidal activity of neutrophils in either IL-33$^{-/}$ or ST2$^{-/-}$ infected mice.

This preliminary study has some limitations. First, the cell sources of various cytokines, i.e., IL-4, IL-5, IL-13 and IL-33, were not investigated. IL-4 may be secreted by basophils, mast cells, CD4$^{+}$NK1.1$^{+}$ T cells, ILC2 cells, and Th2 cells [34]. Similarly, Th2 cells, NK cells, and eosinophils secrete IL-5. However, IL-5-producing cells in the lung may be IL-4 negative [35], i.e., airway epithelial cells may release IL-5 but not IL-4 [36]. Interestingly, IL-5 production in the lung is strongly regulated by IL-33 [35]. In the present study, we revealed that IL-5 production was impaired in IL-33$^{-/-}$ infected mice but not ST2$^{-/-}$ infected mice, indicating that IL-4-producing cells and IL-5-producing cells in response to *C. neoformans* infection may be different. Second, mechanistic analysis in IL-33$^{-/-}$ and ST2$^{-/-}$ infected mice warrants further research, especially the ST2-independent roles of IL-33 in pulmonary infection and immune response.

In summary, we demonstrated that IL-33 and its receptor ST2 played crucial roles in *C. neoformans* pulmonary infection by regulating inflammatory responses. In the model of *C. neoformans* pulmonary infection with the C57BL6/J genetic background, IL-33 deficiency decreased IL-5, increased M1 polarization and extended survival time in an ST2-independent manner. Meanwhile, either IL-33 deficiency or ST2 deficiency promoted neutrophil infiltration in the airway and reduced fungal burdens. Our findings provide more clues that will help us better understand the mechanisms of host defenses against *C. neoformans* pulmonary infection.

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**Author contributions** ZW performed animal experiments and drafted the paper. QM analyzed the data. JJ performed the in vitro experiments. XY, EZ, YT and HH helped with improving the methodology. MH contributed to the paper revision. NJ and MZ designed and supervised the project.

**Data availability** The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors have no conflicts of interest to declare.

## References

1. Zhang Y, Wang F, Tompkins KC, McNamara A, Jain AV, Moore BB et al (2009) Robust Th1 and Th17 immunity supports pulmonary clearance but cannot prevent systemic dissemination of highly virulent *Cryptococcus neoformans* H99. Am J Pathol 175(6):2489–2500
2. Jain AV, Zhang Y, Fields WB, McNamara DA, Choe MY, Chen GH et al (2009) Th2 but not Th1 immune bias results in altered lung functions in a murine model of pulmonary *Cryptococcus neoformans* infection. Infect Immun 77(12):5389–5399

3. Chen GH, McNamara DA, Hernandez Y, Huffnagle GB, Toews GB, Olszewski MA (2008) Inheritance of immune polarization patterns is linked to resistance versus susceptibility to *Cryptococcus neoformans* in a mouse model. Infect Immun 76(6):2379–2391
4. Heyen L, Muller U, Siegemund S, Schulze B, Protschka M, Alber G et al (2016) Lung epithelium is the major source of IL-33 and is regulated by IL-33-dependent and IL-33-independent mechanisms in pulmonary cryptococcosis. Pathog Dis 74(7):ftw086
5. Li D, Guabiraba R, Besnard AG, Komai-Koma M, Jabir MS, Zhang L et al (2014) IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. J Allergy Clin Immunol 134(6):1422–32.e11
6. Flaczyk A, Duerr CU, Shourian M, Lafferty EI, Fritz JH, Qureshi ST (2013) IL-33 signaling regulates innate and adaptive immunity to *Cryptococcus neoformans*. J Immunol 191(5):2503–2513
7. Piehler D, Grahnert A, Eschke M, Richter T, Kohler G, Stenzel W et al (2013) T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. Mucosal Immunol 6(2):405–414
8. Piehler D, Eschke M, Schulze B, Protschka M, Muller U, Grahnert A et al (2016) The IL-33 receptor (ST2) regulates early IL-13 production in fungus-induced allergic airway inflammation. Mucosal Immunol 9(4):937–949
9. Alvarez F, Istomine R, Shourian M, Pavey N, Al-Aubodah TA, Qureshi S et al (2019) The alarmins IL-1 and IL-33 differentially regulate the functional specialisation of Foxp3(+) regulatory T cells during mucosal inflammation. Mucosal Immunol 12(3):746–760
10. Lefrançais E, Duval A, Mirey E, Roga S, Espinosa E, Cayrol C et al (2014) Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. Proc Natl Acad Sci USA 111(43):15502–15507
11. Luzina IG, Pickering EM, Kopach P, Kang PH, Lockatell V, Todd NW et al (2012) Full-length IL-33 promotes inflammation but not Th2 response in vivo in an ST2-independent fashion. J Immunol (Baltimore, Md: 1950). 189(1):403–410
12. Wang Z, Ji N, Chen Z, Sun Z, Wu C, Yu W et al (2020) MiR-1165-3p suppresses Th2 differentiation via targeting IL-13 and PPM1A in a mouse model of allergic airway inflammation. Allergy, Asthma Immunol Res 12(5):859–876
13. Osterholzer JJ, Chen GH, Olszewski MA, Zhang YM, Curtis JL, Huffnagle GB et al (2011) Chemokine receptor 2-mediated accumulation of fungicidal exudate macrophages in mice that clear cryptococcal lung infection. Am J Pathol 178(1):198–211
14. Ubags NDJ, Suratt BT (2018) Isolation and characterization of mouse neutrophils. Methods Mol Biol (Clifton, NJ) 1809:45–57
15. Sun D, Zhang M, Liu G, Wu H, Zhu X, Zhou H et al (2016) Real-time imaging of interactions of neutrophils with *Cryptococcus neoformans* demonstrates a crucial role of complement C5a–C5aR signaling. Infect Immun 84(1):216–229
16. Leopold Wager CM, Hole CR, Wozniak KL, Wormley FL Jr (2016) Cryptococcus and phagocytes: complex interactions that influence disease outcome. Front Microbiol 7:105
17. Olszewski MA, Zhang Y, Huffnagle GB (2010) Mechanisms of cryptococcal virulence and persistence. Future Microbiol 5(8):1269–1288
18. Zheng CF, Ma LL, Jones GJ, Gill MJ, Krensky AM, Kubes P et al (2007) Cytotoxic CD4+ T cells use granulysin to kill *Cryptococcus neoformans*, and activation of this pathway is defective in HIV patients. Blood 109(5):2049–2057
19. Osterholzer JJ, Surana R, Milam JE, Montano GT, Chen GH, Sonstein J et al (2009) Cryptococcal urease promotes the accumulation of immature dendritic cells and a non-protective T2 immune response within the lung. Am J Pathol 174(3):932–943
20. Griesenauer B, Paczesny S (2017) The ST2/IL-33 axis in immune cells during inflammatory diseases. Front Immunol 8:475
21. Cayrol C, Girard JP (2018) Interleukin-33 (IL-33): a nuclear cytokine from the IL-1 family. Immunol Rev 281(1):154–168
22. Fanny M, Nascimento M, Baron L, Schricke C, Maillet I, Akbal M et al (2018) The IL-33 receptor ST2 regulates pulmonary inflammation and fibrosis to bleomycin. Front Immunol 9:1476
23. Nelson MP, Christmann BS, Werner JL, Metz AE, Trevor JL, Lowell CA et al (2011) IL-33 and M2a alveolar macrophages promote lung defense against the atypical fungal pathogen *Pneumocystis murina*. J Immunol (Baltimore, Md: 1950). 186(4):2372–2381
24. Sun D, Zhang M, Liu G, Wu H, Li C, Zhou H et al (2016) Intravascular clearance of disseminating *Cryptococcus neoformans* in the brain can be improved by enhancing neutrophil recruitment in mice. Eur J Immunol 46(7):1704–1714
25. Alves-Filho JC, Sônego F, Souto FO, Freitas A, Verri WA Jr, Auxiliadora-Martins M et al (2010) Interleukin-33 attenuates sepsis by enhancing neutrophil influx to the site of infection. Nat Med 16(6):708–712
26. Lan F, Yuan B, Liu T, Luo X, Huang P, Liu Y et al (2016) Interleukin-33 facilitates neutrophil recruitment and bacterial clearance in *S. aureus*-caused peritonitis. Mol Immunol 72:74–80
27. Oguma T, Asano K, Tomomatsu K, Kodama M, Fukunaga K, Shiomi T et al (2011) Induction of mucin and MUC5AC expression by the protease activity of Aspergillus fumigatus in airway epithelial cells. J Immunol (Baltimore, Md: 1950). 187(2):999–1005
28. Southam DS, Ellis R, Wattie J, Glass W, Inman MD (2008) Goblet cell rebound and airway dysfunction with corticosteroid withdrawal in a mouse model of asthma. Am J Respir Crit Care Med 178(11):1115–1122
29. Nath P, Leung SY, Williams AS, Noble A, Xie S, McKenzie AN et al (2007) Complete inhibition of allergic airway inflammation and remodelling in quadruple IL-4/5/9/13-/- mice. Clin Exp Allergy 37(10):1427–1435
30. Saradna A, Do DC, Kumar S, Fu QL, Gao P (2018) Macrophage polarization and allergic asthma. Transl Res 191:1–14
31. Joshi AD, Oak SR, Hartigan AJ, Finn WG, Kunkel SL, Duffy KE et al (2010) Interleukin-33 contributes to both M1 and M2 chemokine marker expression in human macrophages. BMC Immunol 11:52
32. Biondo C, Midiri A, Messina L, Tomasello F, Garufi G, Catania MR et al (2005) MyD88 and TLR2, but not TLR4, are required for host defense against *Cryptococcus neoformans*. Eur J Immunol 35(3):870–878
33. Nishizaki T (2018) IL-33 suppresses GSK-3β activation through an ST2-independent MyD88/TRAF6/RIP/PI3K/Akt pathway. Heliyon 4(11):e00971
34. Yoshimoto T (2018) The hunt for the source of primary interleukin-4: how we discovered that natural killer T cells and basophils determine T helper type 2 cell differentiation in vivo. Front Immunol 9:716
35. Ikutani M, Yanagibashi T, Ogasawara M, Tsuneyama K, Yamamoto S, Hattori Y et al (2012) Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. J Immunol 188(2):703–713
36. Wu CA, Peluso JJ, Zhu L, Lingenheld EG, Walker ST, Puddington L (2010) Bronchial epithelial cells produce IL-5: implications for local immune responses in the airways. Cell Immunol 264(1):32–41


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