

IL-33 Priming Regulates Multiple Steps of the Neutrophil-Mediated Anti-*Candida albicans* Response by Modulating TLR and Dectin-1 Signals

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IL-33 is known to play an important role in Th2 immunity. In this study, we investigated the effect of IL-33 pretreatment on anti-fungal response using an acute *Candida albicans* peritoneal infection model. IL-33 pretreatment induced a rapid fungal clearance and markedly reduced the *C. albicans* infection-associated mortality. The priming effect of IL-33 occurred during multiple steps of the neutrophil-mediated anti-fungal response. First, the anti-fungal effect occurred due to the rapid and massive recruitment of neutrophils to the site of infection as a result of the release of CXCR2 chemokines by peritoneal macrophages and by reversal of the TLR-induced reduction of CXCR2 expression in neutrophils during IL-33 priming. Second, conditioning of neutrophils by IL-33 activated the TLR and dectin-1 signaling pathways, leading to the upregulation of complement receptor 3 expression induced by *C. albicans*. Upregulated CR3 in turn increased the phagocytosis of opsonized *C. albicans* and resulted in the production of high levels of reactive oxygen species and the subsequent enhanced killing activity of neutrophils. Taken together, our results suggest that IL-33 can regulate the anti-fungal activity of neutrophils by collaborative modulation of the signaling pathways of different classes of innate immune receptors. *The Journal of Immunology*, 2012, 189: 287–295.

Interleukin-33 is a member of the IL-1 family and plays an important role in innate and adaptive immunity (1). Even though early studies focused on the role of IL-33 in the Th2 immune response, there is accumulating evidence implicating IL-33 in acute and chronic inflammation. Similar to IL-1 α , IL-33 is localized to the nucleus and is released by necrotic but not apoptotic cells, a characteristic of alarmins that can alert the immune system (1–4). IL-33 acts on a variety of cells of the innate arm of immunity, including neutrophils, macrophages, dendritic cells, mast cells, basophils, and eosinophils, and functions as an amplifier of inflammation (5–13). Recent studies have suggested that IL-33 plays a role in infection-related inflammation, as IL-33 is protective against infection by opportunistic pathogens such as *Pseudomonas aeruginosa*, *Pneumocystis murina*, *Cryptococcus neoformans*, and polymicrobes (6, 8, 9, 14). Prior to this study, there had been no publications regarding the role of IL-33 in *Candida albicans* infections.

Neutrophils represent a major group of phagocytic cells that are recruited rapidly to sites of *C. albicans* infection (15). They

moderately express TLR2, TLR4, and dectin-1 and strongly express phagocytic receptors such as complement receptor 3 (CR3; also referred to as Mac-1, CD11b/CD18, or $\alpha_M\beta_2$ integrin) and Fc γ Rs (16, 17). Except for Fc γ Rs, these receptors can directly recognize the cell wall components of *C. albicans* and deliver signals leading to a variety of biological activities. Dectin-1 and CR3 have been described as receptors for β -glucan, even though CR3 binds to other ligands (16–20). Dectin-1 signals play a critical role in controlling *C. albicans* infections by promoting the phagocytosis and fungicidal activity of macrophages and cytokine production in macrophages and dendritic cells (21–25). However, little is known about the importance of dectin-1 in defense mechanisms mediated by neutrophils against *C. albicans* infection. Even though the ligands responsible for stimulating TLRs remain largely unknown, phospholipomannan and the O-linked mannoses of *C. albicans* were identified as ligands for TLR2 and TLR4, respectively (18). Recent studies have demonstrated that TLR signals are critical for neutrophil migration through the regulation of CXCR2 expression (26) and that IL-33 reverses the downregulation of CXCR2 mediated by TLR signals (6).

CR3 and its signals are involved in neutrophil activities such as firm adhesion, migration, phagocytosis, and killing. CR3 expression is rapidly upregulated by a range of stimulators, including GM-CSF, fMLF, PMA, TNF- α , and IL-10 (27–31), and this upregulation of CR3 expression is important for neutrophil migration through venules during inflammation (32). Mice lacking C3 or CR3 are susceptible to infection with *C. albicans*, and neutrophils derived from such mice show reduced ability to kill *C. albicans* (33, 34). The CR3 of neutrophils recognizes non-opsonized and opsonized *C. albicans*, and signaling via CR3 results in the killing of these pathogens by a respiratory burst response in a spleen tyrosine kinase (Syk)-dependent manner (35–37).

In this report, we demonstrate that IL-33 priming regulates the expression of CXCR2 and CR3 induced by *C. albicans* infection on neutrophils in such a way that neutrophils are rapidly recruited to the infection site, where their phagocytic and killing activities

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Abbreviations used in this article: CR, complement receptor; CR3, complement receptor 3; MFI, mean fluorescence intensity; MOI, multiplicity of infection; ROS, reactive oxygen species; Syk, spleen tyrosine kinase.

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are elevated. Modulation of the TLR and/or dectin-1 signaling pathways is critical for IL-33 to enhance the responsiveness of neutrophils to *C. albicans*. In sum, we identify IL-33 as a novel regulator of neutrophil activities.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice, 6–8 wk of age, were purchased from Orient. MyD88^{-/-} mice with a C57BL/6 background were purchased from The Jackson Laboratory and bred under pathogen-free conditions at the University of Ulsan animal facility. The University of Ulsan Animal Care Committee approved the study protocol. Most experiments were performed using BALB/c mice, and C57BL/6 mice were used as a control for MyD88^{-/-} mice. We observed similar effects of IL-33 in both strains.

Production of recombinant IL-33 protein

Recombinant IL-33 protein was produced as previously described (38). LPS was removed using the Thermo Scientific Detoxi-Gel endotoxin Removing Gel (Pierce Biotechnology). The *Limulus* Amebocyte Lysate test (Lonza) confirmed that LPS contamination was low (less than 0.05 endotoxin unit per microgram). IL-33 (1 µg per mouse) was i.p. injected 1 h before infection, unless otherwise noted.

C. albicans infection

C. albicans (ATCC 26555) were i.p. injected into mice at a lethal dose of 5×10^7 CFU. In some experiments, i.v. infection of 5×10^5 CFU was conducted. Survival was monitored every 12 h for 7 d. To determine candidal burdens, mice were infected with a sublethal dose of 2×10^7 CFU *C. albicans*. Organs were harvested 24 h postinfection and homogenized by mechanical disruption in PBS. Serial dilutions were plated on Sabouraud agar and incubated at 37°C for 24 h. Colonies were counted and expressed as the log₁₀ (CFU/ml) or log₁₀ (CFU/organ). To deplete neutrophils, mice were i.p. injected with anti-Gr-1 (RB6-8C5) mAbs (300 µg per mouse) 2 d before undergoing i.p. challenge with *C. albicans*.

Flow cytometry

The following FITC-, PE-, and PE-Cy5-conjugated mAbs to mouse surface proteins were purchased from BD Biosciences and used for cell staining: CD11b, CD11c, CXCR2, and Ly6G. Peritoneal lavage was performed with 3 ml PBS. Cells were blocked with 2.4G2 mAbs in FACS buffer and then incubated with the relevant mAbs at 4°C for 30 min. Finally, the cells were washed twice with FACS buffer. Flow cytometric analysis was performed with a FACSCanto II (BD Biosciences), and data were analyzed using FACSDiva (BD Biosciences) and FlowJo software (Tree Star). In some experiments, intracellular staining was performed by treating with anti-GRK2 mAb, followed with Texas red-conjugated anti-rabbit IgG.

ELISA

Levels of CXCL1 and CXCL2 in the serum and from the peritoneal cavity lavage were determined using ELISA, following the manufacturer's instructions (R&D Systems). In some experiments, peritoneal macrophages or bone marrow neutrophils (4×10^5 cells/well) were pretreated with IL-33 (150 µg/ml) 2 h before addition of heat-killed *C. albicans* [multiplicity of infection (MOI) = 10]. Supernatant was harvested 1 h after heat-killed *C. albicans* challenge and used for ELISA.

Immunohistochemistry

Neutrophils were isolated as described later or cells harvested from the peritoneal cavity (3×10^5) were cytopsin onto slides and fixed in 4% paraformaldehyde for 20 min after permeabilization with 0.1% Triton X-100 for 10 min. The cells were blocked with 2% BSA in PBS for 30 min and then incubated with biotinylated rabbit anti-CXCL1, anti-CXCL2 (10 µg/ml; R&D Systems), or anti-GRK2 (2 µg/ml; Santa Cruz Biotechnology) at 4°C overnight. After washing with PBS, the cells were incubated with streptavidin-FITC or Texas red-conjugated anti-rabbit IgG for 1 h at room temperature. Images were obtained using an Olympus Fluoview FV500 confocal microscope with $\times 40$ objectives.

In vitro migration

Neutrophils were purified from the bone marrow using an Anti-Ly6G MicroBead kit (Miltenyi Biotec), in which purities of >90% were routinely achieved. The isolated neutrophils were preincubated with IL-33 (150 ng/ml) for 2–3 h before mixing with heat-killed *C. albicans*

(MOI = 10) or curdlan (50 µg/ml) (Sigma) for 2 additional hours. In some experiments, laminarin (500 µg/ml; Sigma) and GRK2 inhibitor (1 µM; Calbiochem) were added 30 min before stimulation with heat-killed *C. albicans* or curdlan. IL-33-primed or unprimed neutrophils (8×10^5 in 0.1 ml assay medium) were seeded in the upper chamber, and 0.6 ml of medium containing 100 ng/ml CXCL1 was added to the lower chamber. The cells were then allowed to migrate to the lower chamber for 1 h at 37°C. Migrated cells were enumerated using a hemocytometer.

Phagocytosis assay

In vivo phagocytosis was analyzed 1 h after i.p. infection with 5×10^8 CFU FITC-labeled heat-killed *C. albicans*. Peritoneal lavage was performed with 3 ml cold PBS, and cells were stained with the indicated Abs on ice. After being washed twice with cold FACS buffer, stained cells were quenched in a quenching solution containing 0.04% trypan blue and 1% formaldehyde to exclude extracellular fluorescence. Cells phagocytosing FITC-labeled *C. albicans* were analyzed using gating settings that were specific to CD11b⁺Ly6G^{hi} neutrophils using a FACSCanto II. The extent of phagocytosis was expressed as the mean fluorescence intensity (MFI). To assess in vitro phagocytosis, purified neutrophils were resuspended in RPMI 1640 medium and adjusted to a concentration of 2×10^6 cells/ml. The cells were preincubated with IL-33 (150 ng/ml) at 37°C for 2–3 h. After the addition of either non-opsonized or serum-opsonized FITC-labeled heat-killed *C. albicans* (MOI = 10), the mixtures were incubated with slow rotation at 37°C for the indicated times. In some experiments, cells were treated with inhibitors, curdlan, or purified Abs 30 min before exposure to *C. albicans*: piceatannol (Calbiochem) for Syk inhibition; anti-CD16/CD32 mAbs (clone 2.4G2) for blocking FcRs; anti-CD11b and anti-CD18 mAbs for blocking CR3; anti-CD11c and anti-CD18 mAbs for blocking CR4; anti-CD11b, anti-CD11c, and anti-CD18 mAbs for blocking CRs; and anti-CD16/CD32, anti-CD11b, anti-CD11c, and anti-CD18 mAbs for blocking FcRs and complement receptors (CRs). Phagocytosis was stopped by the immediate transfer of cells onto ice, and the cells were washed thoroughly with cold FACS buffer. Extracellular fluorescence was quenched as described earlier, and cells containing fungi were analyzed using flow cytometry. Phagocytosis was expressed as the percentage of neutrophils phagocytosing FITC-labeled *C. albicans* or as the MFI.

Depletion of cell surface CD11b

Purified neutrophils were incubated with biotinylated anti-CD11b mAb on ice for 20 min. The cells were washed twice with RPMI 1640 and incubated at 37°C. Cells were harvested at various time points, stained with FITC-conjugated streptavidin, and analyzed using a FACSCanto II to examine levels of residual cell surface CD11b. In some experiments, neutrophils were stained with FITC-conjugated anti-CD11b mAb to observe the internalization of CD11b-anti-CD11b complexes using an Olympus confocal microscope. Phagocytosis assays were performed in neutrophils that were depleted of cell surface CD11b, as described earlier.

Killing assay

Live *C. albicans* were opsonized with mouse serum and added to neutrophil suspensions (MOI = 1). The mixture was incubated at 37°C with shaking for 20 min to allow for the phagocytosis of live *C. albicans*. The cells were washed thoroughly in cold PBS, resuspended in warm medium, and further incubated at 37°C. At the indicated time, a 200-µl sample was taken, the cells were lysed in PBS containing 0.1% Triton X-100, and the CFUs were enumerated by plating on agar. The percentage killing of fungi was calculated as $[1 - (\text{CFUs after incubation/phagocytized CFUs at the start of incubation})] \times 100$. To inhibit reactive oxygen species (ROS), DPI (10 µM; Sigma) was added 30 min before exposure to *C. albicans*.

ROS production

ROS were detected using the fluorescent probe DCF-DA (Invitrogen). Isolated neutrophils (3×10^5) were seeded into a 96-well plate and primed with IL-33 (150 ng/ml) for 2 h prior to being challenged by opsonized heat-killed *C. albicans* (MOI = 10). After a 1-h incubation, DCF-DA (10 µM) was added and fluorescence was measured by a fluorescent plate reader at an excitation/emission = 485/530 nm at an interval of 10 min for 1 h. The fluorescence intensity was defined as the relative fluorescence units. In some experiments, DPI (10 µM), piceatannol (50 µM), or anti-CD11b/CD18 mAbs were added 30 min before exposure to *C. albicans*.

Statistical analysis

All data were analyzed using GraphPad Prism Software version 4. Unpaired Student *t* tests were used to compare differences between the groups. The

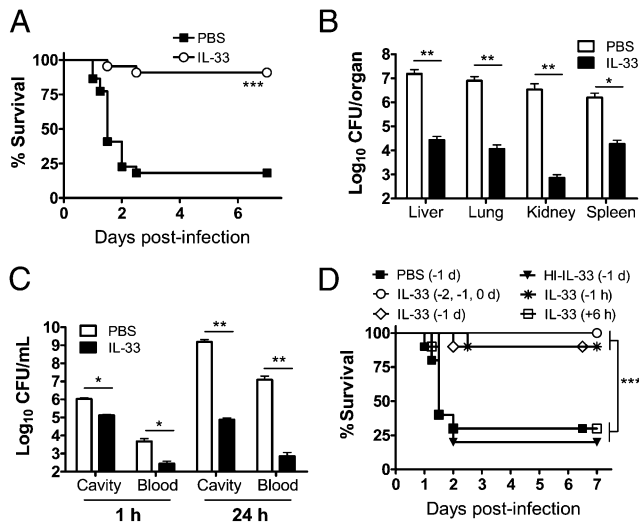


FIGURE 1. IL-33 protects mice from lethal infection with *C. albicans*. IL-33 (1 μ g per mouse) was i.p. injected daily for 3 d, unless otherwise noted, before inoculation with 5×10^7 CFU *C. albicans*. PBS was used as a control. Results presented are representative of at least two independent experiments. (A) Survival curve of mice after infection ($n = 22$ mice per group). $***p < 0.001$. (B) Fungal growth in organs at 24 h postinfection ($n = 5$ to 6 mice per group). $*p < 0.05$, $**p < 0.01$ (between the two indicated groups). (C) Fungal growth in peritoneal cavity and blood at 1 and 24 h postinfection ($n = 5$ to 6 mice per group). $*p < 0.05$, $**p < 0.01$ (between the two indicated groups). (D) Survival curve for mice injected with IL-33 at various times ($n = 10$ mice per group). $***p < 0.001$ (between the indicated two groups of cohorts). HI-IL-33, Heat-inactivated IL-33.

log-rank test and the Mann–Whitney *U* test were used to analyze survival curves and fungal counts, respectively. Error bars represent the SEM of the mean. A *p* value < 0.05 was considered statistically significant.

Results

IL-33-injected mice are resistant to C. albicans infection

We assessed the role of IL-33 in an acute peritoneal sepsis that was induced by the i.p. injection of 5×10^7 CFU *C. albicans* into

BALB/c mice. Mice that received three consecutive daily injections of IL-33 showed a significantly increased survival rate compared with PBS-injected mice (Fig. 1A). The higher survival rate was correlated with a lower number of *C. albicans* in various organs at 24 h postinfection (Fig. 1B). The number of fungi in the peritoneal cavity and blood did not change between 1 and 24 h postinfection in the IL-33–treated mice, whereas there was a 1000-fold increase over the same time interval in PBS-treated mice (Fig. 1C). This result suggested that IL-33–treated mice controlled fungal growth efficiently beginning at the early stages of infection. IL-33 given before infection was critical for protection (Fig. 1D). Heat-inactivated IL-33 failed to protect mice from mortality caused by *C. albicans* infection (Fig. 1D), confirming that the protection was due to the sp. act. of IL-33.

IL-33 rapidly induces neutrophil migration to the site of infection through peritoneal macrophage production of CXCL1 and CXCL2

The rapid recruitment of neutrophils to infection sites is critical for protection from sepsis induced by cecal ligation and puncture or *C. albicans* infections (6, 15). At 1 h postinfection, the number of CD11b⁺Ly6G^{hi} neutrophils was more than 5-fold higher in IL-33–treated mice than that in PBS-treated mice (Fig. 2A). The massive recruitment of neutrophils in IL-33–treated mice was associated with a significantly higher expression of CXCR2 in peritoneal neutrophils (Fig. 2B) as well as increased levels of CXCR2 ligands CXCL1 and CXCL2 in serum and the peritoneal cavity (Fig. 2C). Immunohistochemical analysis showed that F4/80⁺ peritoneal macrophages were the major sources of these chemokines (Fig. 2D). IL-33–primed isolated peritoneal macrophages, but not purified neutrophils, secreted higher levels of CXCL1 and CXCL2 in response to *C. albicans* compared with unprimed counterparts (Fig. 2E, 2F). IL-33 increased levels of serum CXCL1 and CXCL2 by stimulating their release from vessel endothelial cells and smooth muscle cells (W. Kim, H.T. Le, J. Kim, V.G. Tran, H.J. Kim, Q.-T. Nguyen, B.-S. Kim, J.-B. Jun, H.R. Cho, and B. Kwon, submitted for publication). Taken together, our results suggested that IL-33 treatment created an in vivo envi-

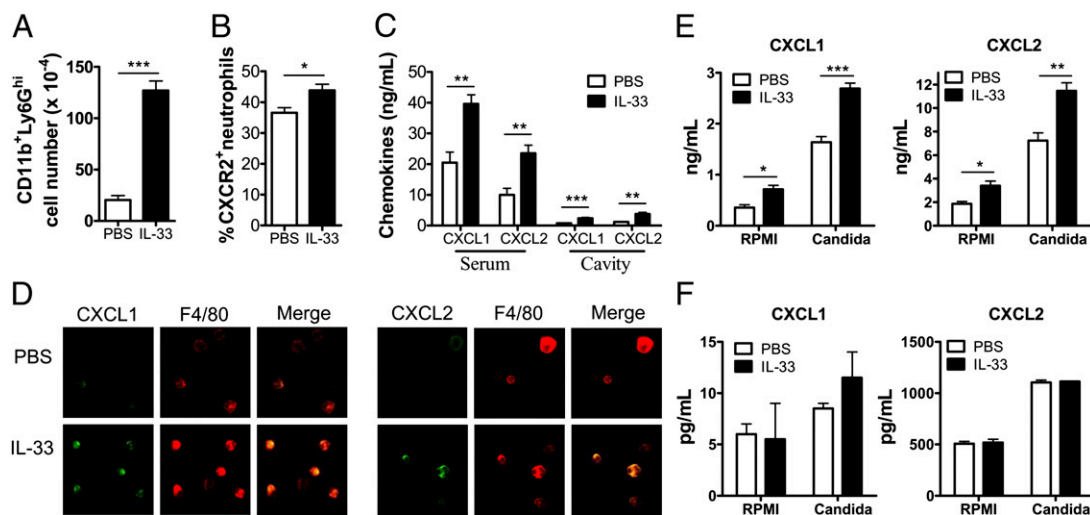


FIGURE 2. IL-33 increases neutrophil migration. (A–D) Mice were i.p. injected with IL-33 (1 μ g per mouse) 1 h before infection with 5×10^7 CFU *C. albicans*. Cells were harvested from the peritoneal cavity 1 h postinfection. Experiments were repeated two to four times, and similar results were obtained. (A) The number of CD11b⁺Ly6G^{hi} neutrophils recruited into the peritoneal cavity ($n = 5$ mice per group). $***p < 0.001$. (B) Percentage of CXCR2-positive neutrophils among the total peritoneal cells ($n = 5$ mice per group). $*p < 0.05$. (C) Concentrations of CXCL1 and CXCL2 in serum and from the peritoneal cavity ($n = 5$ mice per group). $**p < 0.01$, $***p < 0.001$ (between the indicated two groups). (D) Intracellular staining for CXCL1 and CXCL2 in peritoneal cells. F4/80 was used as a marker of peritoneal macrophages. Original magnification $\times 40$. (E and F) Levels of CXCL1 and CXCL2 secreted by peritoneal macrophages or neutrophils. Cells were stimulated by *C. albicans* after 2-h preincubation with IL-33. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (between the indicated two groups).

ronment that promotes the rapid and robust recruitment of neutrophils to infection sites in response to *C. albicans*.

Pretreatment with IL-33 blocks the downregulation of CXCR2 expression induced by C. albicans in neutrophils

We investigated whether IL-33 would affect the expression of CXCR2 in neutrophils upon exposure to *C. albicans*, as seen in cases of sepsis (6). Heat-killed *C. albicans* markedly lowered the percentage of CXCR2-expressing neutrophils, and IL-33 pretreatment significantly blocked the *C. albicans*-induced downregulation of CXCR2 expression (Fig. 3A). Changes in CXCR2 expression in neutrophils were correlated with their chemotactic responses to CXCL1 (Fig. 3B). A deficiency of MyD88 prevented the downregulation of CXCR2 expression induced by heat-killed *C. albicans* in neutrophils (Fig. 3A), and MyD88-deficient neutrophils were intact in their ability to migrate to CXCL1 (Fig. 3B), suggesting that *C. albicans* decreased CXCR2 expression through TLR signals. Even though stimulation of dectin-1 using curdlan

marginally downregulated the percentage of CXCR2-expressing neutrophils compared with that of control neutrophils (Fig. 3A), curdlan-primed neutrophils had a significantly decreased migratory activity to CXCL1 (Fig. 3C). However, IL-33 pretreatment had no effect on CXCR2 expression and migration of curdlan-treated neutrophils (Fig. 3A, 3C). Consistent with this observation, IL-33 priming increased the migratory capacity of heat-killed *C. albicans*-exposed neutrophils to CXCL1 under the condition of dectin-1 blockade (Fig. 3D). Finally, IL-33 blocked the induction of GRK2—which is known to suppress the expression of CXCR2 in neutrophils (6, 26)—by heat-killed *C. albicans* (Fig. 3E). Stimulation of dectin-1 with curdlan, however, did not induce GRK2 expression in neutrophils (Fig. 3E). In the same context, GRK2 inhibitor significantly restored the decreased migratory capacity of neutrophils after exposure to heat-killed *C. albicans* while only having a milder effect on the migration of curdlan-treated neutrophils (Fig. 3F). Taken together, our results clearly demonstrated that IL-33 specifically suppressed the TLR-mediated,

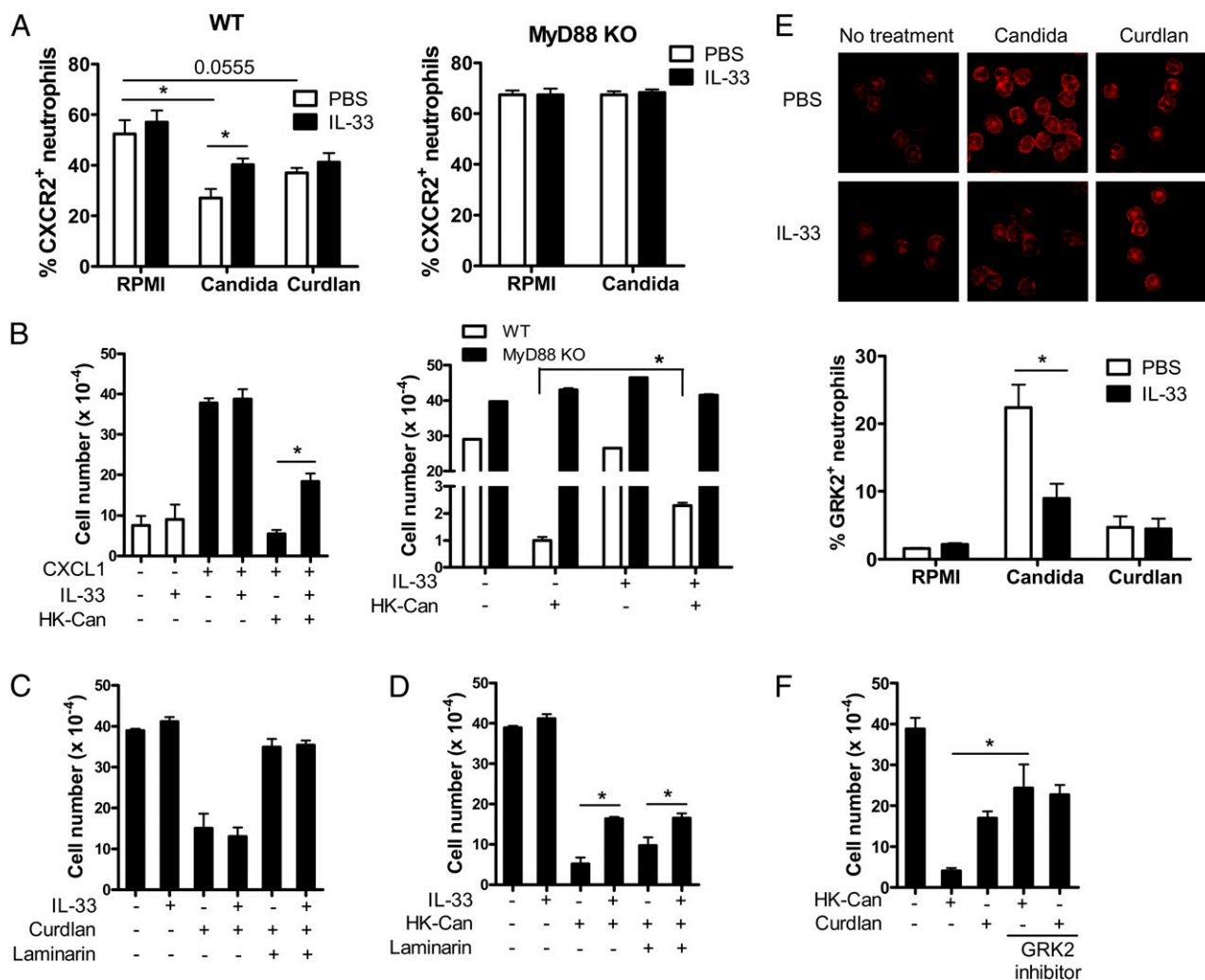


FIGURE 3. IL-33 inhibits the downregulation of CXCR2 induced by *C. albicans* in neutrophils. Purified neutrophils were preincubated with IL-33 (150 ng/ml) for 2 h and then stimulated with heat-killed *C. albicans* (MOI = 10) or curdlan (50 μ g/ml). Laminarin (500 μ g/ml) or GRK2 inhibitor (1 μ M) was added 30 min before stimulation with *C. albicans* or curdlan. (A) CXCR2 expression was analyzed after the 2-h stimulation period, and the percent of CXCR2⁺ cells was presented. Results are representative of three independent experiments. (B–D and F) Isolated neutrophils were cultured under the indicated conditions and allowed 1-h migration to CXCL1 (100 ng/ml). Chemotaxis was measured as the number of migrated cells. Data were pooled from two to five independent experiments. **p* < 0.05 (between the indicated groups). (E) Intracellular staining for GRK2 expression in neutrophils 1 h after stimulation with heat-killed *C. albicans* or curdlan. Upper panel, Confocal microscopic analysis. Cells were stained with anti-GRK2 followed by Texas red-conjugated anti-rabbit IgG. Original magnification $\times 40$. Lower panel, The percentage of cells positively stained for GRK2 was assessed by FACS (*n* = 3 to 4 per group). Data are representative of two independent experiments. **p* < 0.05 (between the indicated groups).

but not the dectin-1–mediated, expression of GRK2, a serine–threonine protein kinase that induces internalization of CXCR2 (6).

IL-33 enhances the phagocytic activity of neutrophils

Next, we examined whether IL-33 could directly act on neutrophils and enhance their phagocytic activity. After a 2-h preincubation with IL-33, isolated neutrophils were challenged with FITC-labeled, opsonized, heat-killed *C. albicans*. Neutrophils preincubated with IL-33 displayed increases in the percentage of FITC-positive neutrophils and the MFI of FITC-positive neutrophils at 30 and 60 min after incubation with *C. albicans* (Fig. 4A, 4B). These results suggest that IL-33 not only broadened the pool of neutrophils with phagocytic capacity but also increased the phagocytic activity of individual neutrophils. By contrast, IL-33 priming had no effect on the phagocytic activity of neutrophils for non-opsonized heat-killed *C. albicans* (Fig. 4C). We also performed in vivo phagocytic assays by challenging FITC-labeled heat-killed *C. albicans* in mice that were pretreated with IL-33 for 1 h. IL-33–primed neutrophils had a significantly higher MFI than PBS-primed neutrophils (Fig. 4D). Taken together, our results demonstrated that IL-33 directly acted on neutrophils and increased their phagocytic activity for opsonized *C. albicans*.

IL-33 priming increases the phagocytic activity of neutrophils through upregulation of their cell surface CR3 expression

Because FcRs and CRs are important targets of opsonic phagocytosis, we investigated which receptors would be responsible for the IL-33–enhanced phagocytic activity of neutrophils on exposure to *C. albicans*. Blockage of FcRs using anti-CD16/CD32 (FcγRIII and FcγRII) mAbs slightly, but not significantly, increased the phagocytic activity of neutrophils preincubated with

either PBS or IL-33 (Fig. 5A). In contrast, the phagocytic activity of neutrophils was markedly diminished by blocking of CRs using anti-CD11b, anti-CD11c, and anti-CD18 mAbs or by blocking both FcR and CRs (Fig. 5A). This result suggested that CRs, but not FcRs, were primarily responsible for the phagocytosis of opsonized *C. albicans* by neutrophils. Specifically blocking CR3 (CD11b/CD18) markedly inhibited the phagocytic activity of neutrophils, regardless of preincubation with IL-33, whereas CR4 (CD11c/CD18) blockage slightly increased this activity (Fig. 5B). Finally, inhibition of Syk, an important signaling molecule that is involved in the CR3 signaling pathway, decreased the phagocytic activity of neutrophils that were previously increased by preincubation with IL-33 to the basal level observed in neutrophils preincubated with PBS (Fig. 5C), confirming that IL-33 priming enhanced the CR3-mediated phagocytosis of neutrophils for opsonized *C. albicans*.

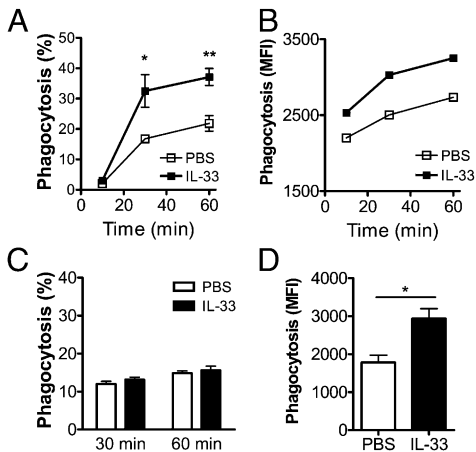


FIGURE 4. IL-33 enhances the phagocytic activity of neutrophils. (A and B) Purified neutrophils were preincubated with IL-33 (150 ng/ml) for 2 h and challenged with opsonized, FITC-labeled, heat-killed *C. albicans* (MOI = 10) for the indicated times. Shown are the percentage of FITC-positive cells (A) and the MFI of FITC-positive cells (B). Data are presented as the mean ± SEM of at least three independent experiments. **p* < 0.05, ***p* < 0.01 (between the two groups at the indicated time point). (C) Neutrophils preincubated with IL-33 were challenged with non-opsonized, FITC-labeled, heat-killed *C. albicans* (MOI = 10) for the indicated times. Data were pooled from three independent experiments. (D) Mice were pretreated with IL-33 before being i.p. challenged with 5 × 10⁸ CFU FITC-labeled heat-killed *C. albicans*. One hour later, peritoneal cells were stained and analyzed by flow cytometry with gating settings for CD11b⁺ Ly6G^{hi} neutrophils. The extent of phagocytosis was expressed as the MFI (*n* = 6 mice per group). **p* < 0.05.

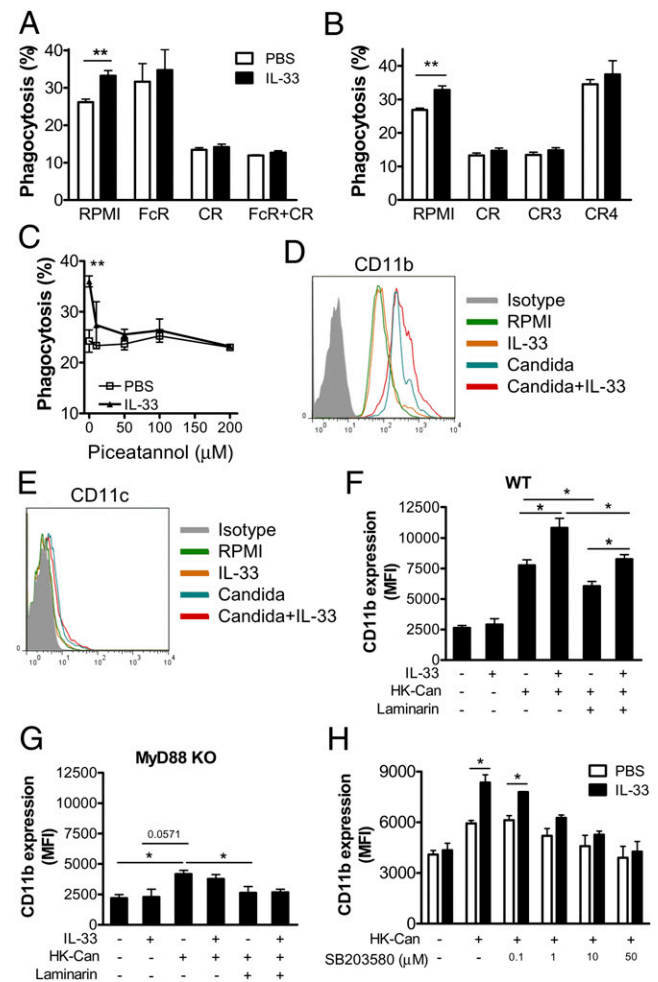


FIGURE 5. CR3 is critical for the IL-33–mediated phagocytic activity of neutrophils. Purified neutrophils were primed with IL-33 (150 ng/ml) or PBS for 2 h. (A–C) Phagocytosis was assayed for opsonized, FITC-labeled, heat-killed *C. albicans* in the presence of various blocking Abs or inhibitors. Blocking Abs were used as described in *Materials and Methods*. Piceatannol was added 30 min before the challenge with *C. albicans*. Data were pooled from at least three independent experiments. ***p* < 0.01 (between the two indicated groups). (D–H) Expression of CD11b (D, F–H) or CD11c (E) was determined 30 min after the addition of heat-killed *C. albicans*. Laminarin (500 μg/ml) and SB203580 were added 30 min before the challenge with *C. albicans*. Data are representative of two to three independent experiments. **p* < 0.05 (between the indicated groups).

FACS analysis showed that the surface expression of CD11b in neutrophils was correlated with their phagocytic activity for *C. albicans*: preincubation with IL-33 further upregulated the CD11b expression induced by *C. albicans* (Fig. 5D, 5F). CD11c expression in neutrophils was very low, and incubation with *C. albicans* did not change its expression in either unprimed or IL-33-primed neutrophils (Fig. 5E). A deficiency of MyD88 signaling resulted in a marked decrement in CD11b expression (Fig. 5G), indicating that TLR signals were essential for the upregulation of CD11b expression induced by *C. albicans*. This view was supported by the observation that blockage of dectin-1 using laminarin slightly decreased the levels of CD11b expression previously increased by *C. albicans* (Fig. 5F). Notably, IL-33 priming did not completely recover the decreased CD11b expression in the absence of dectin-1 signals (Fig. 5F), suggesting that TLR and dectin-1 signals had to work cooperatively for IL-33 priming to upregulate CD11b expression induced by *C. albicans*. Consistent with this interpretation, the increment of CD11b expression induced by *C. albicans* was significantly impaired under the condition of a dectin-1 blockade in MyD88-deficient neutrophils (Fig. 5G). Finally, inhibition of p38 MAPK, a molecule involved in both TLR and dectin-1 signaling, abrogated the priming effect of IL-33 on CD11b expression induced by *C. albicans* (Fig. 5H). Taken together, our findings suggested that CR3 was the major phagocytic receptor of neutrophils for opsonized *C. albicans* and that IL-33 pretreatment increased the phagocytic activity of neutrophils through the upregulation of CR3 expression upon exposure to *C. albicans* that was mediated by the synergistic action of TLR and dectin-1 signals.

It was expected that a deficiency of CD11b in neutrophils would abrogate the priming effect of IL-33 on the phagocytic activity of neutrophils for opsonized *C. albicans*. We depleted cell surface CD11b molecules of neutrophils by inducing the internalization of CD11b into the cell using anti-CD11b mAb. As seen in Fig. 6A and 6B, anti-CD11b mAb was rapidly internalized after its binding to CD11b, and basal levels of cell surface CD11b remained in neutrophils by 2 h after anti-CD11b treatment and thereafter. CD11b-depleted neutrophils had no priming effect of IL-33 on their phagocytic activity upon exposure to *C. albicans* (Fig. 6C).

Recent studies have shown that dectin-1 signaling is critical for the activation of CR3 to promote the phagocytic activity of neutrophils and *C. albicans* clearance (39). The previous results

shown in Fig. 5F and 5G, however, demonstrated that *C. albicans* could upregulate CD11b expression in neutrophils through dectin-1 signaling in a TLR-independent manner. To confirm further this observation, we stimulated isolated MyD88-deficient neutrophils with curdlan and examined the effect of dectin-1 signaling on CD11b expression and phagocytosis in neutrophils. As expected, 30-min prestimulation of dectin-1 resulted in significantly increased CD11b expression and phagocytosis in MyD88-deficient neutrophils upon exposure to *C. albicans* (Fig. 7A). The effect of IL-33 priming was apparent in curdlan-prestimulated wild-type neutrophils and control counterparts (Fig. 7B), validating our experiments. These results suggest that dectin-1 signaling contributes to the phagocytic activity of neutrophils by upregulating CD11b expression and that IL-33 priming can increase dectin-1-mediated upregulation of CD11b expression.

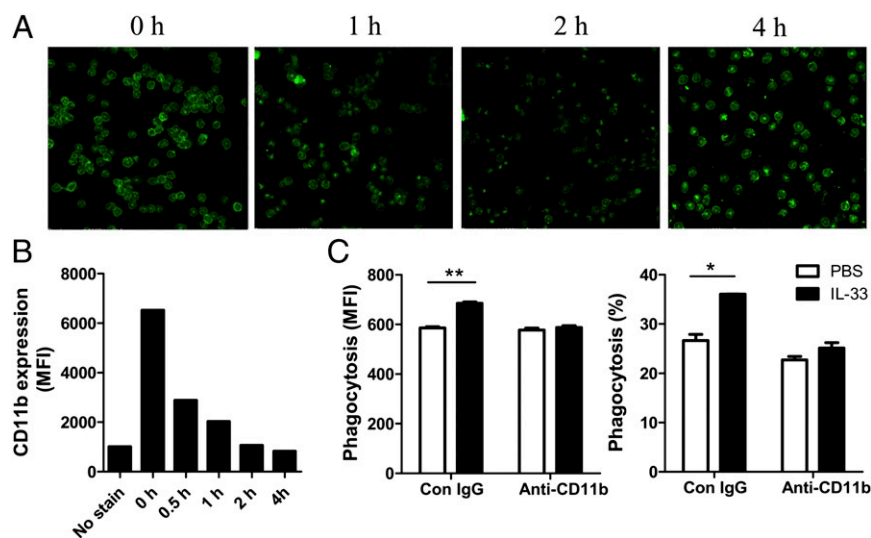
IL-33 priming accelerates the fungicidal activity of neutrophils

Killing assays demonstrated that phagocytosed *C. albicans* were killed more rapidly inside neutrophils primed by IL-33 (Fig. 8A), suggesting that IL-33 priming elevated the killing activity of neutrophils. *C. albicans* induced the production of higher levels of ROS in IL-33-primed neutrophils than those found in PBS-primed neutrophils (Fig. 8B). Blockade of the CR3 signaling pathway using either anti-CD11b/CD18 mAbs or Syk inhibitor markedly decreased ROS production induced by *C. albicans* in neutrophils primed by IL-33 or PBS (Fig. 8C), suggesting that CR3 signals were involved in the enhanced killing activity of neutrophils primed by IL-33. Indeed, inhibition of ROS production completely abrogated the effect of IL-33 priming on the fungicidal activity of neutrophils (Fig. 8D).

Neutrophils are essential for IL-33-mediated fungal clearance

The data presented so far have suggested that the priming effect of IL-33 on neutrophil activities might result in the rapid clearance of *C. albicans*. To address this issue, we assessed the fungal clearance in mice that were depleted of neutrophils. A single injection of anti-Gr-1 mAb 2 d before infection completely depleted the neutrophil population in the peritoneal cavity (Fig. 9A). Depletion of neutrophils accelerated the fungal burden in the peritoneal cavity (Fig. 9B) and blood (Fig. 9C) at 4 h post-infection in both IL-33-injected and PBS-injected mice to a similar extent, confirming that neutrophils were a major *in vivo* target of IL-33.

FIGURE 6. Depletion of cell surface CD11b abrogates IL-33-induced increase of neutrophil phagocytosis. (A) Purified neutrophils were treated with FITC-conjugated anti-CD11b mAb (10 μ g/ml), and its internalization was observed using a confocal microscope. Original magnification $\times 40$. (B) Purified neutrophils were treated with biotinylated anti-CD11b mAb, and CD11b remaining on the cell surface was analyzed by staining with streptavidin-conjugated FITC. The MFI was obtained using FACS. (C) Phagocytosis was assayed for opsonized, FITC-labeled, heat-killed *C. albicans* (MOI = 10) in neutrophils that were depleted of cell surface CD11b by 4-h incubation with biotinylated anti-CD11b mAb. Data are representative of two to three independent experiments. * $p < 0.05$, ** $p < 0.01$ (between the indicated groups, $n = 3$ per group).



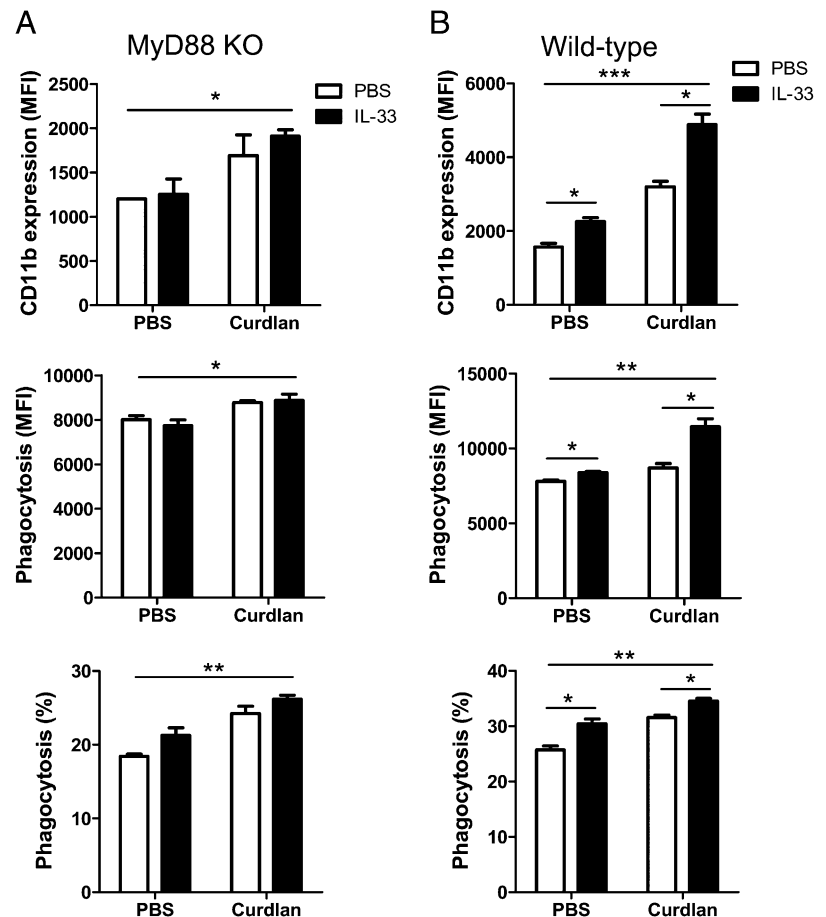


FIGURE 7. Dectin-1 signaling increases the phagocytic activity of neutrophils through upregulation of CD11b expression. Purified neutrophils of MyD88 KO (A) and wild-type (B) mice were primed with IL-33 (150 ng/ml) or PBS for 2 h. Curdlan (50 μ g/ml) was added 30 min before the challenge with *C. albicans* (MOI = 10). CD11b expression and phagocytosis was analyzed 30 min postinfection with *C. albicans*. CD11b expression was shown as the MFI (top panels). Phagocytosis was expressed as the MFI (middle panels) or the percent of cells containing *C. albicans* (lower panels). Data are representative of three to four independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 (between the indicated groups, n = 3).

IL-33 is protective against i.v. infection of *C. albicans*

We examined the effect of IL-33 on i.v. infection of *C. albicans*, which is an accepted model of disseminated candidiasis in humans. Injection of IL-33 at 2-d intervals starting from 1 d before infection with 5×10^5 CFU (total four times) significantly inhibited mortality compared with PBS-treated counterparts (Fig. 10A). At 4-d postinfection, 2 to 3 log-fold decrease in CFUs was recovered from kidneys, lungs, and blood of IL-33-treated compared with PBS-treated mice (Fig. 10B). Splens and livers of both groups of mice had a very low number of CFUs at this time point. Together, these results suggest that IL-33 has a protective effect on i.v. infection of *C. albicans*.

Discussion

Control of fungal dissemination and effective fungal clearance are key strategies for survival during infection. In this study, we demonstrated that IL-33 pretreatment effectively protected mice from mortality caused by *C. albicans* (Figs. 1, 10). This protective effect occurred because IL-33-primed neutrophils effectively limited the fungal burden at the early phase of infection in a coordinated fashion. First, IL-33 promoted the recruitment of neutrophils to the site of infection by adopting two means: 1) through priming peritoneal macrophages in such a way that they actively produced CXCL1/CXCL2 in response to *C. albicans* (Fig. 2); and 2) through reversing the downregulation of CXCR2 induced by *C. albicans* in neutrophils (Fig. 3). Second, IL-33 facilitated the upregulation of CR3 expression, which resulted in the enhancement of two neutrophil activities critical for fungal clearance: phagocytosis and killing. We believe that neutrophils were the

main in vivo target of IL-33 for fungal clearance, as early fungal growth was not controllable in normal mice infected with lethal doses of *C. albicans* (Fig. 1C), and the effect of IL-33 on fungal clearance was completely abolished as a result of neutrophil depletion (Fig. 9).

TLRs and dectin-1 were previously reported to be necessary for protection from *C. albicans* infection (15–18, 39–41). Collaborative recognition of distinct microbial components by TLRs and dectin-1 synergistically induced the production of proinflammatory cytokines (42, 43). In this respect, it is not a surprise that the simultaneous modulation of TLRs and dectin-1 pathways by IL-33 was critical for enhancing anti-fungal responses. In our model, IL-33 conditioned the signaling pathways leading to the expression of CR3 in neutrophils (Figs. 5–7) that was responsible for their opsonic phagocytic and killing activities for *C. albicans* (Figs. 4, 8). It seems that one signaling molecular candidate implicated in involvement in this phenomenon is p38 MAPK, whose activation is known to be mediated commonly by signaling through TLRs, dectin-1, and ST2 (44–46). This interpretation is strongly supported by our observation that the inhibition of p38 MAPK abrogated the effect of IL-33 priming on the upregulation of CR3 expression induced by *C. albicans* (Fig. 5H). Recent studies by Mayadas's group (39) have provided a novel mechanism of anti-fungal activities of neutrophils involving the dectin-1–CR3 axis: dectin-1 signaling results in the activation of CR3 and its subsequent binding to *C. albicans*, and coengagement of dectin-1 and CR3 by *C. albicans*, in turn, delivers coordinated signals that lead to phagocytosis and killing of neutrophils. Taken together, activation and upregulation of CR3 by signaling through

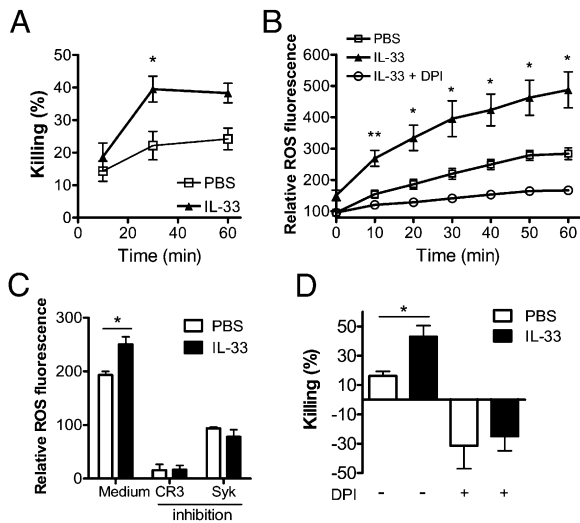


FIGURE 8. IL-33 increases the killing activity of neutrophils. **(A)** IL-33-primed neutrophils were exposed to opsonized *C. albicans* for 20 min for uptake. The killing activity was measured at the indicated times. Data were pooled from three independent experiments. $*p < 0.05$ (between the two groups at the indicated time point). **(B)** IL-33-primed neutrophils were challenged with opsonized heat-killed *C. albicans* after treatment with DCF-DA. Fluorescence was measured at a 10-min time interval for 1 h. DPI (10 μ M) was added for ROS inhibition 30 min before the challenge with *C. albicans*. Data were pooled from at least three independent experiments. $*p < 0.05$, $**p < 0.01$ (between the PBS-treated and IL-33-treated groups at the indicated time point). **(C)** ROS production was measured in the presence of anti-CD11b and anti-CD18 mAb (10 μ g/ml) or piceatannol (50 μ M). Data were pooled from two to three independent experiments. $*p < 0.05$ (between the two groups). **(D)** Killing assays were performed in the presence or absence of DPI (10 μ M). Data were pooled from two to three independent experiments. $*p < 0.05$ (between the two groups at the indicated time point).

dectin-1 and TLRs seem to serve as an early auto-amplification loop to restrict *C. albicans*.

C. albicans downregulated CXCR2 expression in neutrophils by activating TLRs, and IL-33 reversed the TLR-induced reduction of CXCR2 expression (Fig. 3). The mechanism behind this was that IL-33 blocked the TLR-mediated upregulation of GRK2, a key promoter for CXCR2 internalization (Fig. 3E). However, inhibition of neutrophil migration after dectin-1 stimulation was not dependent upon GRK2 upregulation (Fig. 3C, 3F), and IL-33 priming was not effective in blocking this process (Fig. 3C–F). It

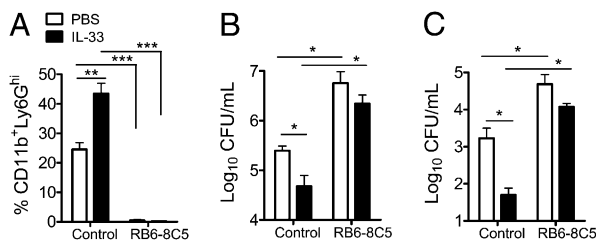


FIGURE 9. Neutrophils are the major effector cells involved in the removal of *C. albicans*. For depletion of neutrophils, mice were i.p. injected with anti-Gr-1 mAb (300 μ g) 2 d before being i.p. challenged with *C. albicans*. Mice were sacrificed 4 h postinfection. **(A)** The percentage of neutrophils among the total peritoneal cells ($n = 5$ mice per groups). $**p < 0.01$, $***p < 0.001$ (between the indicated two groups). **(B)** and **(C)** Fungal growth was determined in the peritoneal cavity (B) and blood (C). $*p < 0.05$ (between the indicated two groups).

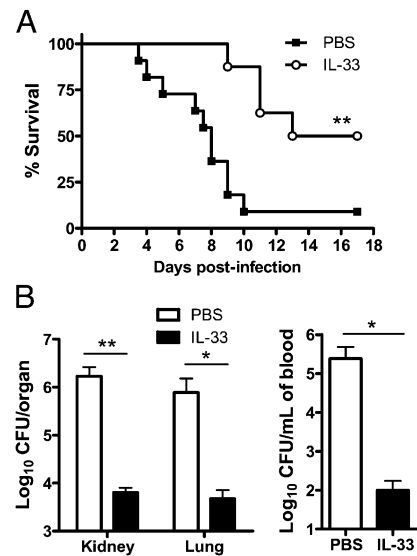


FIGURE 10. IL-33 is protective against lethal i.v. infection with *C. albicans*. IL-33 (1 μ g per mouse) was i.v. injected four times at 2-d intervals, starting from 1 d before inoculation with 5×10^5 CFU *C. albicans*. PBS was used as a control. **(A)** Survival curve of mice after infection. Data were pooled from two independent experiments ($n = 8$ to 12 mice per group). $**p < 0.01$. **(B)** *Left panel*, Fungal growth in organs at 4 d postinfection ($n = 5$ to 6 mice per group). *Right panel*, Fungal growth in blood at 4 d postinfection ($n = 5$ mice per group). Similar experiments were repeated at least two times. $*p < 0.05$, $**p < 0.01$ (between the two indicated groups).

is necessary to clarify how dectin-1 signaling regulates neutrophil migration in a CXCR2-independent manner.

In our *C. albicans* infection model, IL-33 priming increased the phagocytic activity of neutrophils for opsonized but not non-opsonized *C. albicans* (Fig. 4). Blocking experiments identified CR3 as the main phagocytic receptor for opsonized *C. albicans* (Fig. 5A, 5B). The conditioning of neutrophils by IL-33 may have potentially activated collaborative signaling by TLRs and dectin-1 to upregulate CR3 expression induced by *C. albicans* (Fig. 5F, 5G). It will be necessary to determine how the upregulation of CR3 expression preferentially increased the phagocytosis of opsonized versus non-opsonized *C. albicans*, considering that CR3 is important for both opsonic and non-opsonic phagocytosis (19, 20, 33, 34, 47). One possibility is that opsonized *C. albicans* has a higher affinity for CR3 because two different binding sites are available: one for iC3b and the other for β -glucans (16, 48).

C. albicans infection is associated with high mortality in immunocompromised patients receiving anti-cancer therapies or immunosuppressants (49, 50). New clinical approaches must be developed to complement current anti-fungal agents to decrease mortality resulting from systemic fungal infections (16). Combining of chemotherapy and immunotherapy may be one possible strategy (51). Our results suggest a new potential treatment against *C. albicans* infection. IL-33 may have merits for systemic anti-fungal defense: 1) IL-33 promotes myelopoiesis in the bone marrow (W. Kim et al., submitted for publication); 2) IL-33 rapidly induces emigration of neutrophils from the bone marrow to the periphery through increased production of CXCL1/2 by vessel cells (W. Kim et al., submitted for publication); 3) IL-33 stimulates tissue macrophages to release CXCL1/2 in response to fungal infection; and 4) IL-33 directly acts on neutrophils and elevates their migratory, phagocytic, and killing activities. These properties of IL-33 may be advantageous for the purpose of enhancing innate immunity in immunocompromised patients.

