DOCTOR OF PHILOSOPHY

The Renal Immune Network Created By CCR5 and IL-33 Regulates Sequential Events of Innate Immune Defense Against Systemic *Candida albicans* Infection

The Graduate School of the University of Ulsan

Department of Biological Science

Nguyen Nu Zen Na

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Supervisor : ByungSuk Kwon, Ph.D.

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Nguyen Nu Zen Na

Department of Biological Sciences

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This certifies that the dissertation of Nguyen Nu Zen Na is approved by

Committee Member Dr. 최혜선

Committee Member Dr. 박정우

Committee Member Dr. 이종수

Committee Member Dr. 서수길

Committee Member Dr. 박성호

Committee Member Dr. 권병석

Department of Biological Sciences

Ulsan, Korea

November 2021

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Abstract

Candida albicans (*C. albicans*) is a crucial pathogen that cause a serious health problem, inducing high mortality rates in immunocompromised patients. Understanding the innate immune pathway that controls antifungal defense in kidney, a major target organs, remains to be clarified. In this thesis, using in vitro and in vivo experiments systems, I demonstrated that C-C chemokine receptor type 5 (CCR5) and Interleukin- 33 (IL-33) played a critical role in innate defense to systemic *C.albicans* infection.

In the first part of study, I showed that using CCR5-deficient (*Ccr5*$^{-/-}$) mice, CCR5 contributes to an effective defense mechanism against systemic *C. albicans* infection. CCR5 was required for recruitment of NK cells to the kidney after systemic *C. albicans* infection. In *Ccr5*$^{-/-}$ mice, there were lower levels of GM-CSF in the kidney, which resulted in impaired neutrophils' fungal clearance. Taken together with previous results showing that *C. albicans* activated the dendritic cell (DC) → IL-23 → NK cell → GM-CSF → neutrophil defense axis, the results obtained form this study indicates that CCR5 play a main role in recruiting NK cells to the kdiney during systemic *C. albicans* infection.

In the second part, I investigated in vivo function of endogenous Il-33 in systemic *C. albicans* infection using *Il33*$^{-/-}$ mice. IL-33 initiated an innate defense mechanism by co-stimulating DCs to produce IL-23 after systemic *C. albicans* infection. As a result, NK cells could not secrete amounts of GM-CSF suffieint to stimulate nuetrophils to phagocytize proliferating *C. albicans*. The susceptibility of *Il33*$^{-/-}$ mice was also associated with increased levels of IL-10 and neutralization of

IL-10 resulted in enhanced fungal clearance in these mice. However, depletion of IL-10 overrided the effect of IL-33 on fungal clearance. There were abnormally differentiationed $MHCII^{+}F4/80^{+}$ macrophages in *Il10*$^{-/-}$ mouse kidnneys, which were superior to $MHCII^{-}F4/80^{+}$ macrophage that were presnt in WT mouse kidneys, in killing of extracellular hyphal form of *C. albicans*.

Taken together, our results identify IL-33 and CCR5 as critical regulator creating an innate immune network cirtical for defense during systemic *C. albicans* infection.

Content

List of Figures

Chapter 1: CCR5-MEDIATED RECRUITMENT OF NK CELLS TO THE KIDNEY IS A CRITICAL STEP FOR HOST DEFENSE TO SYSTEMIC *CANDIDA ALBICANS* INFECTION.

1.1 ABSTRACT

C-C chemokine receptor type 5 (CCR5) regulates the trafficking of various immune cells to sites of infection. In this study, we showed that expression of CCR5 and its ligands was rapidly increased in the kidney after systemic *Candida albicans* infection, and infected *Ccr5*$^{-/-}$ mice exhibited increased mortality and morbidity, indicating that CCR5 contributes to an effective defense mechanism against systemic *C. albicans* infection. The susceptibility of *Ccr5*$^{-/-}$ mice to *C. albicans* infection was due to impaired fungal clearance, which in turn resulted in exacerbated renal inflammation and damage. CCR5-mediated recruitment of NK cells to the kidney in response to *C. albicans* infection was necessary for the anti-microbial activity of neutrophils, the main fungicidal effector cells. Mechanistically, *C. albicans* induced expression of IL-23 by CD11c^{+} DCs. IL-23 in turn augmented the fungicidal activity of neutrophils through GM-CSF production by NK cells. As GM-CSF potentiated production of IL-23 in response to *C. albicans*, a positive feedback loop formed between Natural Killer (NK) cells and DCs seemed to function as an amplification point for host defense. Taken together, our results suggest that CCR5-mediated recruitment of NK cells to the site of fungal infection is an important step that underlies innate resistance to systemic *C. albicans* infection.

1.2 INTRODUCTION

C. albicans is the most prevalent human fungal pathogen and causes invasive candidiasis [1]. This infectious disease presents a serious clinical challenge to immunocompromised patients and has a high mortality rate in these individuals, despite treatment with antifungal drugs [2, 3]. In particular, neutropenia is a major risk factor for invasive candidiasis [4, 5]. Systemic infections occur when colonizing or gut-residing *C. albicans* penetrates the mucocutaneous barriers. The kidney acts as the major site of fungal replication [6]. Recognition of *C. albicans* by a variety of pattern recognition receptors (PPRs) initiates innate immunity [2, 3]. The C-type lectin receptor family is particularly important in anti-*Candida* defense [7, 8]. Signal transduction through these receptors orchestrates a complex series of molecular and cellular events that underlies innate resistance to *C. albicans* infection, including production of inflammatory mediators, inflammasome activation, and phagocytic and fungicidal activities [7, 8]. Failure of innate defense mechanisms frequently results in life-threatening *Candida* sepsis due to hyperactivation of the immune system [9, 10].

Recent studies have demonstrated that NK cells occupy a central position in an important defense pathway linked to the *Candida*-killing activity of neutrophils [11–13]. During acute *Candida* infection, DCs and Ly6C^{+} monocytes produce IL-

23 and IL-15, respectively, both of which stimulate NK cells to produce GM-CSF [12–14]. Considering that NK cells are one of the cell types recruited most rapidly to sites of infection, it is surprising that Ly6C$^+$ monocytes act upstream of NK cells. To understand the sequential events determining host defense, therefore, it is a prerequisite to defining the regulatory immune networks that are created during *C. albicans* infection. One approach to this type of study is to dissect the chemotactic system that regulates the recruitment of immune cells to the infection site. Although trafficking of Ly6C$^+$ monocytes to the kidney is known to depend upon CCR2 after systemic *C. albicans* infection [15], it has yet to be clarified how other types of immune cells are recruited to the infected kidney. We previously showed that injury of tubular epithelial cells by ischemia and reperfusion induces recruitment of NK cells in a CCR5-dependent manner [16]. In addition, host defense to some infections relies upon CCR5-mediated NK cell recruitment to infection sites [17]. Based upon these observations, we hypothesized that ablation of CCR5 in NK cells would compromise innate resistance to systemic *C. albicans* infection and leads to death from fulminant candidiasis. Indeed, we showed that CCR5 was critical in NK cell recruitment and neutrophil activation during acute *C. albicans* infection. Unexpectedly, production of IL-23 was impaired in *Candida*-infected *Ccr5*$^{-/-}$ mouse kidneys. GM-CSF secreted by NK cells was shown to promote IL-23 production,

thereby forming a positive feedback loop for mutual regulation of these factors during acute *C. albicans* infection.

1.3 MATERIALS AND METHODS

1.3.1 Mice

C57BL/6 mice were purchased from Orient Bio-Charles River (Seoul, Korea). *Ccr5*$^{-/-}$ mice with a C57BL/6 background were maintained in a specific pathogen-free facility and used between 7-8 wk of age. All experiments were conducted according to the regulations of the Animal Committee of the University of Ulsan.

1.3.2 Fungal strains and growth conditions

C. albicans (ATCC26555) was grown in peptone dextrose extract at 30°C overnight, and aliquots were frozen at -80°C. To obtain hyphal forms, yeasts were suspended in Rosewell Park Memorial Institute (RPMI)-1640 medium (Welgene) at a final concentration of 5 x 10^6 cells/mL and were further cultured on a rotary shaker at 37 ºC for 120 min. To kill *C. albicans* hyphae, organisms were harvested by centrifugation, and pellets were washed twice in sterile PBS and resuspended at a density of 1 x 10^8 cells/mL before heat killing at 90°C for 30 min.

1.3.3 Experimental systemic Candidiasis

C. albicans were inoculated intravenously into the lateral caudal tail veins with 3 × 10^5 CFUs (lethal dose).

1.3.4 Counting CFUs

Mice were euthanized, and kidneys were removed aseptically to determine fungal burdens. Harvested kidneys were homogenized in 2 mL PBS, and serial dilutions of homogenates were plated on Sabouraud agar and incubated at 37 ℃ for 24 h. Colonies were counted, and results were expressed as $\log_{10}$(CFUs/organ).

1.3.5 Phagocytosis assay

Bone marrow cells were collected from femurs and tibias, suspended in RPMI-1640 medium (Welgene) supplemented with 10% FBS, penicillin/streptomycin (100 U/mL), 2 mM L-glutamine (GIBCO), and 50 μM 2-mercaptoethanol. To purify neutrophils from bone marrow cells, anti-Ly6G magnetic activated cell sorting (MACS) beads were used according to the manufacturer's instructions (Miiltenyi Biotech). Purified neutrophils were resuspended in RPMI 1640 medium and adjusted to a concentration of 2 x 10^6 cells/mL. The cells were preincubated with GM-CSF (10 ng/ml) at 37 ˚C for 4 h. After the addition of serum-opsonized FITC-labeled heat-killed *C. albicans* hyphae [multiplicity of infection (MOI) = 10], the mixtures were incubated with slow rotation at 37 ˚C for 30 min. 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 in a quenching solution containing 0.04% trypan blue and 1% formaldehyde, and cells

containing fungi were analyzed using flow cytometry. Phagocytosis was expressed as the percentage of neutrophils phagocytosing FITC-labeled *C. albicans*.

1.3.6 Diff-Quik staining of neutrophils

Purified neutrophils were resuspended in PBS. Specimens were prepared using the Cytospin™ 4 Cytocentrifuge (Thermo Fisher Scientific). The centrifuged specimens were then stained with a Diff-Quik™ staining set (Siemens Healthcare Diagnostics) and examined under microscope.

1.3.7 Histology

Kidneys were fixed in 10% (v/v) formalin, embedded in paraffin, sectioned (5 μm), stained with H&E or periodic acid–Schiff (PAS), and analyzed.

1.3.8 Depletion of neutrophils and NK cells

Neutrophil depletion was achieved by injecting 200 μg anti-Gr-1 (RB6-8C5) mAb intraperitoneally into mice 2 d before *C. albicans* infection. For NK cell depletion, anti-NK1.1 (PK136; eBioscience) mAb was injected 1 d before and 2 d after infection.

1.3.9 Real-time RT-PCR

Total RNA was extracted from kidneys or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Whole tissues were

homogenized with a TissueLyzer tissue homogenizer (QUAGEN), and cDNA was prepared with SuperScript reverse transcription (Invitrogen). Real-Time PCR was performed using SYBR Green PCR Master Mix (Qiagen) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The primers used in the experiments were as follows. *Ccr5*: 5'-AGATCTCTGCAGCTGCCCTCA-3' (forward) and 5'-GGAGCACCTGCTGCTGGTGTAG-3' (reverse); *Il23a* : 5'-CCAGCAGCTCTCTCGGAATC-3' (forward) and 5'-TCATA GTCCCGCTGGTGC-3'(reverse); *Il-12b*: 5'-CCTGGTTTGCCATCGTTTTG-3' (forward) and 5'-TCAGAGTCTCGCCTCCTTTGTG-3'(reverse); *β-actin*: 5'-CATTACTGCTCTGGCTCCTACC-3' (forward) and 5'-GACTCATCGTACTCCTGCTTGC-3' (reverse). All PCRs were performed in triplicate and normalized to internal control *β-actin* mRNA. Relative expression was presented using the $2^{-\triangle\triangle CT}$ method.

1.3.10 Measurement of cytokines, chemokines, and myeloid peroxidase (MPO)

Cytokines and chemokines present in total kidney homogenates and cell culture supernatants were measured using a Cytometric Bead Array kit (BD Biosciences) or by ELISA (eBioscience), respectively, according to the manufacturers' protocols.

1.3.11 Preparation of kidney cells

Kidneys were perfused, minced, and placed in DMEM (Life Technologies) containing 1 mg/mL collagenase IA and 100 ng/mL DNAse I (Sigma-Aldrich) at 37°C for 30 min. Digested kidney tissues were passed through a 40-μm cell strainer (BD Falcon), and the cell suspension obtained was centrifuged at 300 x g for 10 min. Cells were washed in PBS containing 2% BSA, suspended in 36% Percoll (Amersham Pharmacia Biotech), and gently overlaid onto 72% Percoll. After centrifugation at 900 x g for 30 min at RT, cells were retrieved from the Percoll interface and washed twice in DMEM and once with staining buffer (PBS containing 2% BSA and 0.1% sodium azide).

1.3.12 Flow cytometry

The following FITC-, PE-, PE-Cy5 (PE-cytochrome 5), PerCP- or APC (allophycocyanin)-conjugated monoclonal antibodies (mAbs) to mouse proteins were purchased from BD Biosciences or eBioscience and used for cell staining: CD45, CD11b, Ly6G, CD3, NK1.1, CD11c, CCR5, IL-23p19, GM-CSF, TCRβ chain, and Rat IgG2a κ chain. Prepared cells were blocked with 2.4G2 monoclonal antibody in a staining buffer at 4 ^{0}C for 20 min. Before washing twice with a staining buffer, cells were incubated with the relevant antibody at 4^{0}C for 30 min. For intracellular cytokine staining, after staining of surface markers, cells were fixed and

permeabilized using Cytofix/Cytoperm and Perm/wash buffer (BD Biosciences), followed by staining with monoclonal antibody to mouse GM-CSF and IL-23p19 (eBioscience). Flow cytometric analysis was performed using a FACS Canto II (BD Biosciences) cytometer and data were analyzed using FACS Diva software (BD Biosciences) and FlowJo software (Tree Star).

1.3.13 Analysis of renal function

To determine kidney function, concentrations of creatinine and blood urea nitrogen (BUN) in sera were measured colorimetrically using the Quantichrom Urea Assay and the Quantichrom Creatine Assay kits (Bioassay Systems).

1.3.14 Culture of bone marrow-derived DCs (BMDCs)

Bone marrow cells were collected from femurs and tibias, suspended in RPMI-1640 medium supplemented with 10% FBS, penicillin/streptomycin (100 U/mL), 2 mM L-glutamine (GIBCO), 50 μM 2-mercaptoethanol and Flt3L (150 ng/mL), were distributed into 6-well plates at a density of 1×10^6 cells/well for 7 d. At day 7, GM-CSF (10 ng/mL) was added, and cells were harvested at day 9. Cells was stimulated with GM-CSF (10 ng/ml) and heat-killed *Candida albicans* hyphae (MOI = 10).

1.3.15 Statistical analysis

All data were analyzed using GraphPad Prism Software version 4. Unpaired Student t-test or one- or two-way ANOVA with posthoc analysis were used to compare differences between the groups. The 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 below 0.05 was considered statistically significant.

1.4 RESULTS

1.4.1 Increased expression of CCR5 and its ligands in the kidney during *C. albicans* infection.

To investigate the role of CCR5 and its ligands in systemic *C. albicans* infection, we first investigated changes in their expression in the kidney with candidiasis. Expression of *Ccr5* transcripts was significantly increased at days 1 and 3 after infection (Fig. 1-1A). FACS analysis showed that a higher percent of CCR5-expressing neutrophils was infiltrated into the kidney at day 1 after infection than before infection (Fig. 1-1B). The percentages of $CCR5^+$ macrophages were significantly greater at either day 1 or day 3 after infection (Fig. 1-1B). In a similar context, production of CCR5 ligands, CCL3, CCL4, and CCL5, by infected kidneys was higher at days 1 and 3 after infection (Fig. 1-1C).

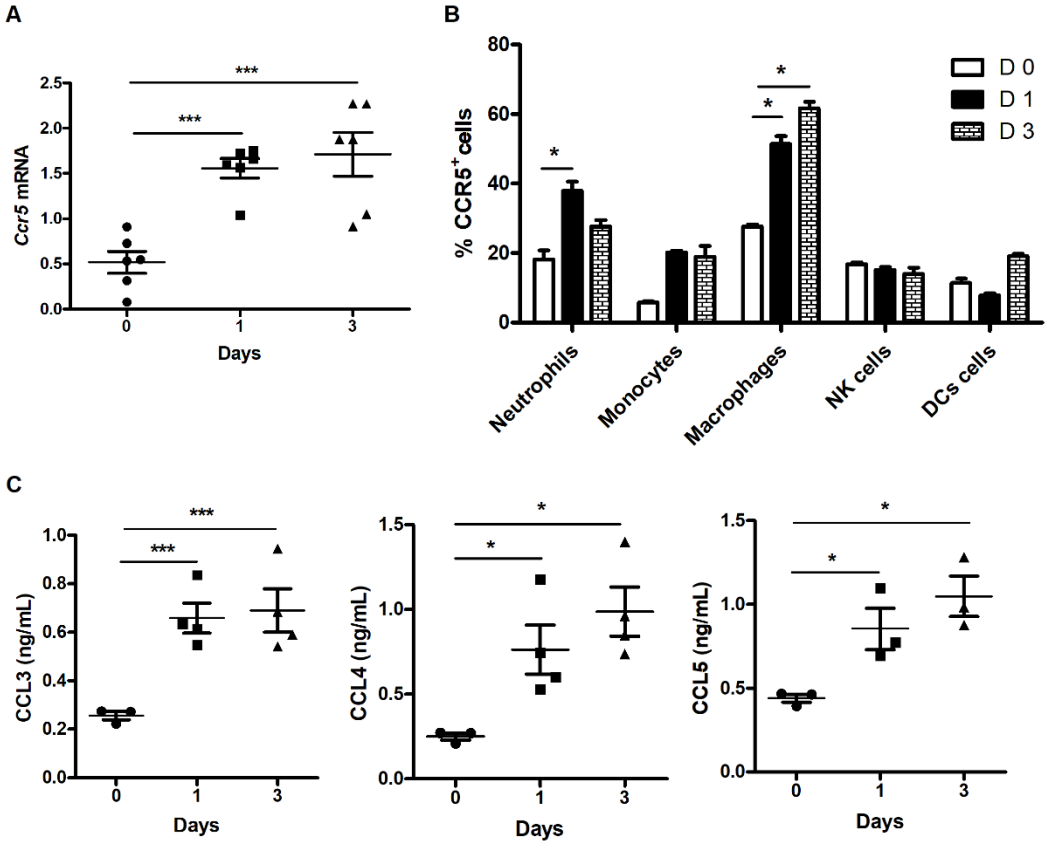

Figure 1-1. Upregulation of CCR5 and its ligands in the kidney during systemic candidiasis.

C57BL/6 (WT) mice were intravenously injected with 3 x 10^5 CFUs of *C. albicans* and the kidneys were harvested at the indicated time points. (A) Levels of *Ccr5* mRNA in the kidneys were determined by real-time RT-PCR (n = 6 kidneys/group). (B) Percentages of CCR5-expressing kidney cells were determined using FACS at the indicated time points (n = 3-5 kidneys/group). (C) Levels of

CCR5 ligands (CCL3, CCL4, and CCL5) were measured from kidney lysates using ELISA (n = 3-4 kidneys/group). Results are representative of 2-3 experiments and are presented as the mean ± SEM. One-way ANOVA test was used. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

1.4.2 *Ccr5*$^{-/-}$ mice are susceptible to systemic *C. albicans* infection

To define the role of CCR5 during systemic candidiasis, *Ccr5*$^{-/-}$ mice were challenged with a lethal dose of *C. albicans* and monitored for survival. *Ccr5*$^{-/-}$ mice succumbed to *C. albicans* infection more rapidly than WT mice (Fig. 1-2A). *Ccr5*$^{-/-}$ mice also experienced more severe body weight loss (Fig. 1-2B), and their kidneys appeared less pink, more swollen (Fig. 1-2C), and were significantly heavier than WT kidneys at day 3 post-infection (Fig. 1-2D). Gross observations also indicated that *Ccr5*$^{-/-}$ kidneys had many more distinguishable nodules than WT kidneys (Fig. 1-2C), indicating a more rapid proliferation of fungi and extensive abscess formation. Indeed, *Ccr5*$^{-/-}$ mice had a significantly increased fungal burden in the kidney at day 3 post-infection compared with WT mice (Fig. 1-2E). Histopathological analysis showed that *Ccr5*$^{-/-}$ kidneys had numerous multifocal areas of abscess formation as compared to WT kidneys (Fig. 1-2F). In particular, PAS staining revealed more prominent hyphae within abscesses of *Ccr5*$^{-/-}$ kidneys compared with those of WT mice (Fig. 1-2F).

The data presented in Fig. 1-2 suggests that impaired suppression of fungal proliferation might result in uncontrolled inflammatory responses and subsequent tissue damage in *Ccr5*$^{-/-}$ mice. To test this hypothesis, we looked into the severity of renal inflammation. As expected, we detected higher levels of inflammatory

cytokines and chemokines (IL-6, TNF-α, CXCL1, and CXCL2) in *Ccr5*$^{-/-}$ kidneys at day 3 post-infection than in those of WT mice (Figs. 1-3, A-D). Consistent with these results, there was greater infiltration of CD45^{+} leukocytes, predominantly CD11b^{+}Ly6G^{+} neutrophils, into infected *Ccr5*$^{-/-}$ kidneys (Figs. 1-3, E and F). In addition, higher levels of serum creatinine and BUN indicated that *Ccr5*$^{-/-}$ mice had more severe impairment of kidney function at day 3 post-infection (Figs. 1-3, G and H). Taken together, our results suggest that uncontrolled fungal proliferation in *Ccr5*$^{-/-}$ mice might cause severe renal inflammation linked to fatal immunopathology.

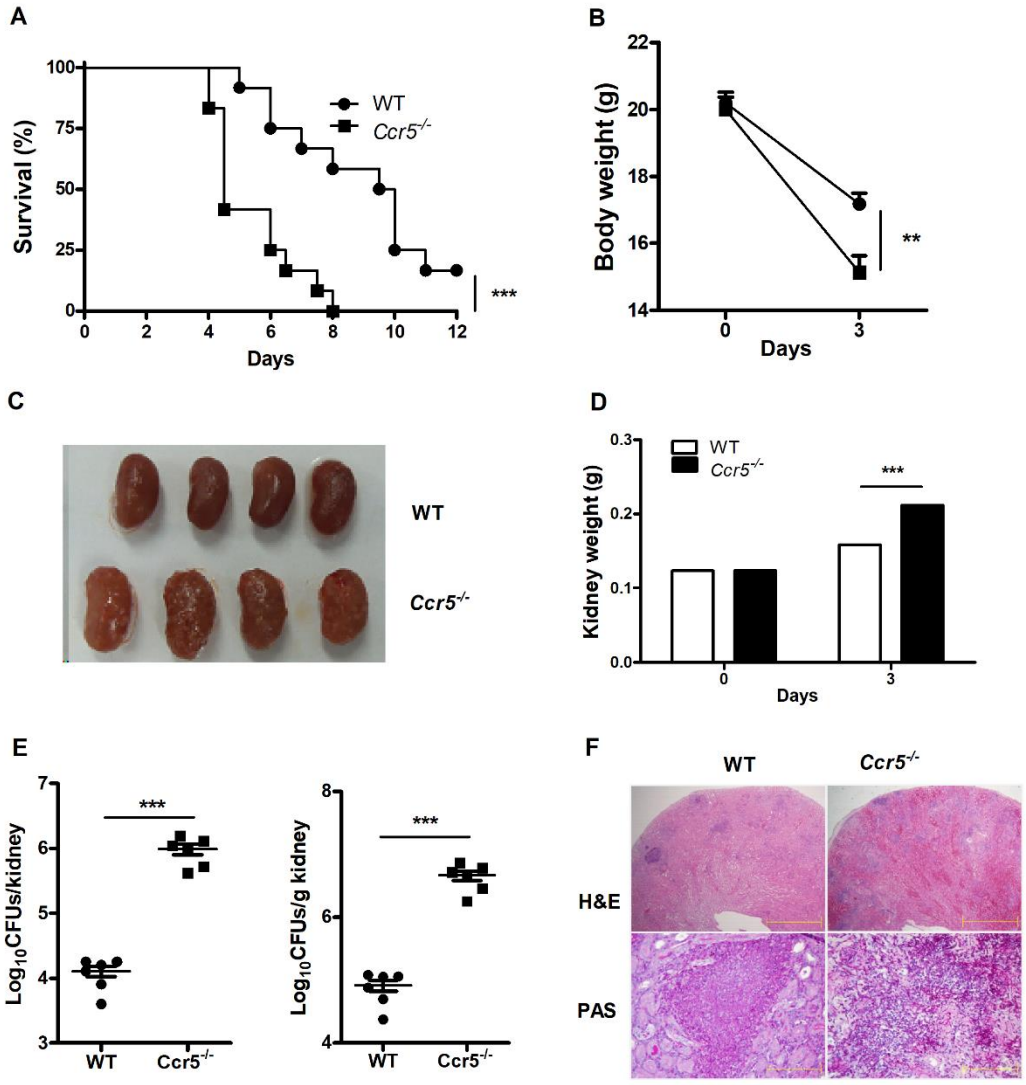

Figure 1-2. *Ccr5*$^{-/-}$ mice are susceptible to systemic candidiasis

WT and *Ccr5*$^{-/-}$ mice were intravenously injected with 3 x 10^5 CFUs of *C. albicans*. (A) Survival curves (n = 12 mice/group). The log-rank test was used. (B) Changes in body weight (n = 4 or 5 mice/group). Unpaired Student t-test was used. (C) Gross morphology of kidneys at day 3 post-infection. (D) Changes in kidney weight (n = 8 or 10 kidneys /group). Two-way ANOVA test was used. (E) Fungal

burdens at day 3 post-infection were presented per kidney or gram basis (n = 6 kidneys/group). The Mann-Whitney U test was used. (F) Histopathologic findings: H&E staining (*upper panels*); PAS staining, (*lower panels*). Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001

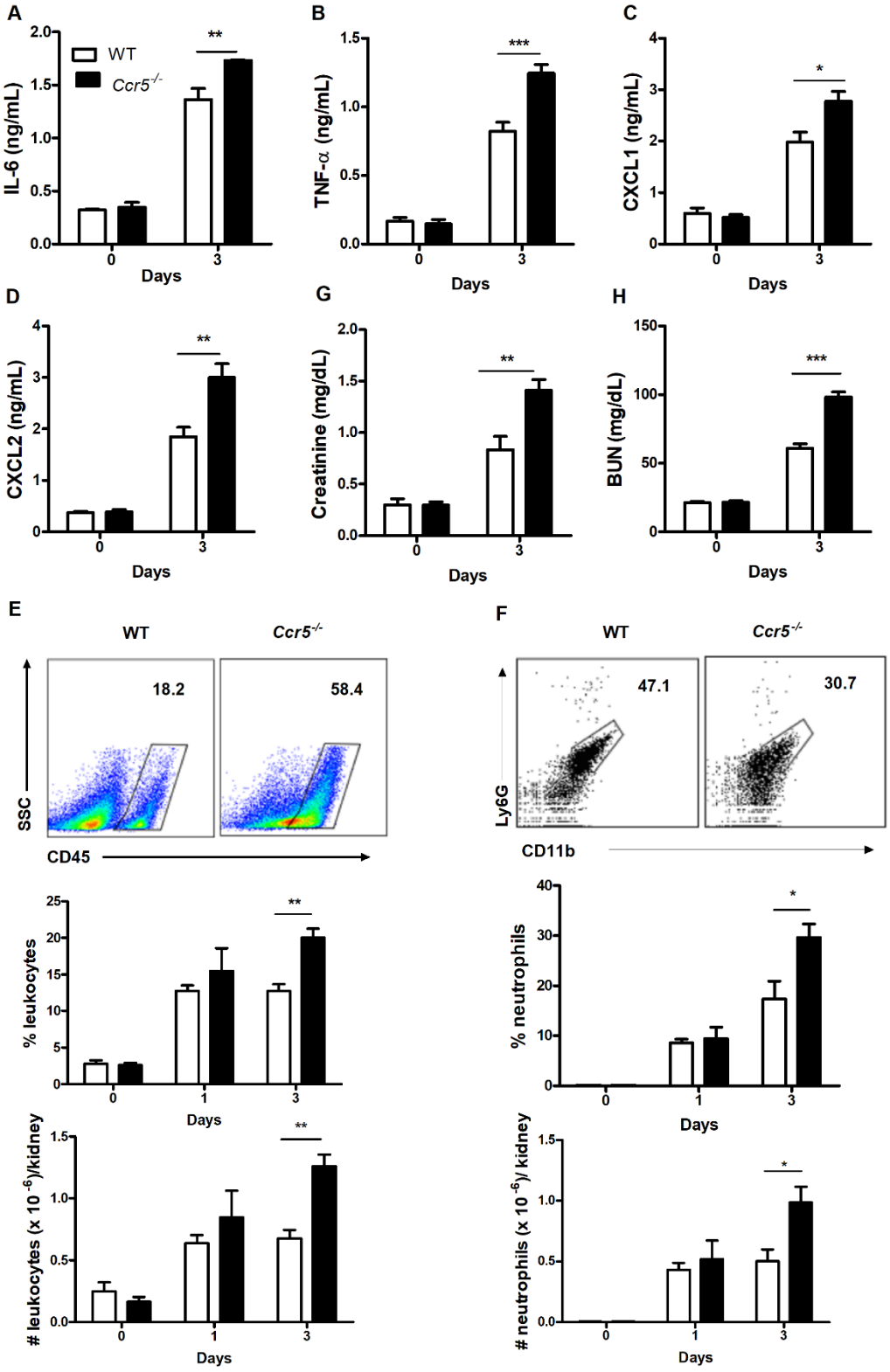
A
IL-6 (ng/mL)
WT
Ccr5-/-
**
Days
B
TNF-α (ng/mL)
***
Days
C
CXCL1 (ng/mL)
*
Days
D
CXCL2 (ng/mL)
**
Days
G
Creatinine (mg/dL)
**
Days
H
BUN (mg/dL)
***
Days
E
WT
Ccr5-/-
18.2
58.4
SSC
CD45
% leukocytes
**
Days
leukocytes (x 10-6)/kidney
**
Days
F
WT
Ccr5-/-
47.1
30.7
Ly6G
CD11b
% neutrophils
*
Days
neutrophils (x 10-6)/ kidney
*
Days

Figure 1-3. *Ccr5*$^{-/-}$ mice have increased inflammatory responses to systemic *C. albicans* infection.

WT and *Ccr5*$^{-/-}$ mice were intravenously injected with 3 x 10^5 CFUs of *C. albicans*. (A-D) Levels of IL-6 (A), TNF-α (B), CXCL1 (C), and CXCL2 (D) in kidney lysates at day 3 post-infection (n = 4 or 6 kidneys/group). (E and F) Kidney cells were isolated, and stained with anti-CD45, anti-CD11b, and anti-Ly6G antibodies. (E) Representative FACS plots for $CD45^+$ leukocytes and their percentages and absolute numbers were presented (n = 4 or 6 kidneys/group). (F) $CD45^+$ leukocytes were gated and FACS plots for $CD11b^+$ and $Ly6G^+$ neutrophils and their percentages in leukocytes were presented (n = 4 or 6 kidneys/group). (G and H) Serum creatinine and BUN levels (n = 3 or 4 mice/group). Two-way ANOVA test was used. Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

1.4.3 Impaired recruitment of NK cells to the kidney is linked to the susceptibility of *Ccr5*$^{-/-}$ mice to *C. albicans* infection.

The increased presence of renal inflammation of *Ccr5*$^{-/-}$ mice after *C. albicans* infections seemed to suggest that although neutrophils had impaired function for fungal clearance, they induced severe immunopathology, presumably as a result of their increased presence in the kidney. Indeed, kidney neutrophils from *Ccr5*$^{-/-}$ mice had decreased levels of myeloperoxidase (MPO), a major constituent of azurophil granules necessary for generation of ROS, a key component of the neutrophils' killing arsenal [13, 18] (Fig. 1-4A). Taking their numbers into account, the concentration of MPO per cell was approximately 2-fold lower compared to WT kidney neutrophils (Fig. 1-4B). Depletion of neutrophils using anti-Gr-1 Ab completely abrogated differences in survival and fungal clearance between *Ccr5*$^{-/-}$ and WT mice (Figs. 1-4, C and D), confirming that neutrophils were important effector cells for fungal clearance. As anti-Gr-1 Ab depletes other types of Gr-1-expressing cells, however, we cannot exclude their contribution to fungal clearance. We next investigated the possibility that *Ccr5*$^{-/-}$ neutrophils have intrinsic functional defects which might cause the susceptible phenotype of *Ccr5*$^{-/-}$ mice to *C. ablicans* infection. Either WT or *Ccr5*$^{-/-}$ neutrophils isolated from the bone marrow had a similar premature neutrophil's nuclear morphology (Fig. 1-4E). Treatment with

GM-CSF induced nuclear segmentation of either type of neutrophils to a similar extent (Fig. 1-4E). Consistent with this result, there was no difference in their phagocytic activity and MPO production after treatment with GM-CSF (Fig. 1-4, F and G). However, it seems that *Ccr5*$^{-/-}$ bone marrow neutrophils have a lower phagocytic capacity in steady state (Fig. 1-4F).

As NK cells have been shown to be indispensable for neutrophil activation [12, 13], we hypothesized that CCR5 mediates recruitment of NK cells to the kidney during *C. albicans* infection. In accordance to our hypothesis, a significantly lower number of NK cells were present in *Ccr5*$^{-/-}$ kidneys at days 1 and 3 post-infection (Fig. 1-5, A and B). Consistent with previous studies showing that GM-CSF is produced by NK cells for neutrophil activation [12, 13], there were decreased levels of GM-CSF in *Ccr5*$^{-/-}$ kidneys at day 3 post-infection (Fig. 1-5C). We further showed that depletion of NK cells in WT mice reduced levels of renal GM-CSF (Fig. 1-5D) and fungal clearance to some extent (Fig. 1-5E), confirming that NK cell numbers are associated with levels of GM-CSF and fungal clearance. Unexpectedly, individual kidney NK cells produced less GM-CSF in *Ccr5*$^{-/-}$ mice (Fig. 1-5F), indicating that signaling upstream of NK cells may be defective in *Ccr5*$^{-/-}$ mice during *C. albicans* infection.

We next examined whether GM-CSF can displace NK cells in activating neutrophils and promoting their subsequent fungal clearance. Indeed, injection of GM-CSF significantly recovered the anti-fungal immunity of *Ccr5*$^{-/-}$ mice (Fig 1-6A) but in our hands, exogenous GM-CSF did not reduce the fungal clearance to the extent observed in WT mice. Concomitantly, infiltrating neutrophil numbers were significantly reduced by injection of GM-CSF in *Ccr5*$^{-/-}$ mice (Fig. 1-6, B and C). This result seems to support our previous observations that neutrophil activation is directly associated with effective fungal clearance and less severe renal inflammation. In sum, our data demonstrate that impaired recruitment of NK cells to the kidney in *Ccr5*$^{-/-}$ mice leads to defects in fungal clearance by neutrophils.

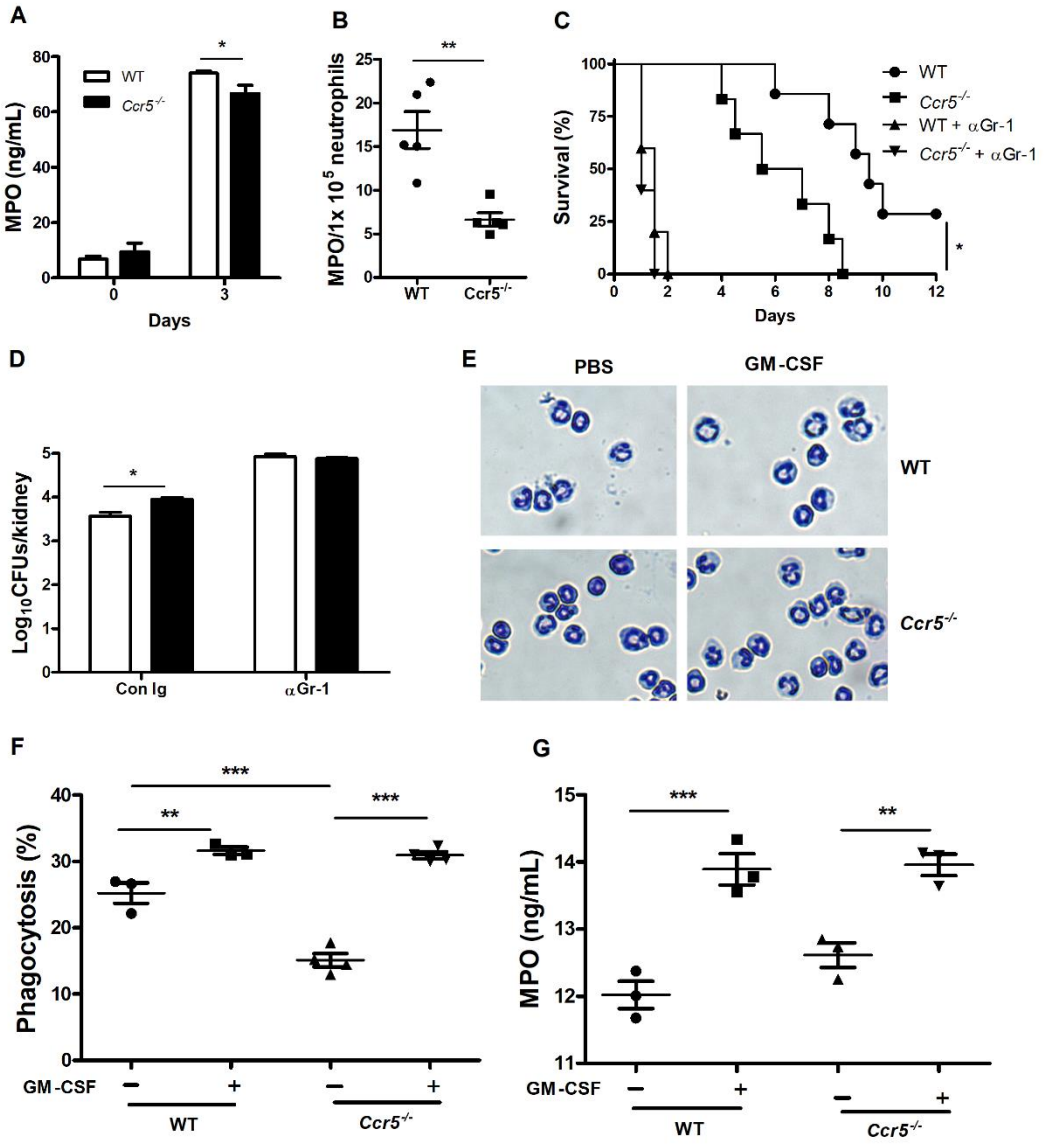

Figure 1-4. Impaired recruitment of NK cells to the kidney results in characteristic phenotypic changes of *Ccr5*$^{-/-}$ mice after *C. albicans* infection.

(A and B) Levels of MPO in kidney lysates obtained at day 3 post-infection. Data are presented as a concentration per kidney or normalized to 1 x 10^5 renal neutrophils (n = 4 or 5 kidneys/group). (C and D) WT mice were administered anti-

Gr-1 mAb (200 μg/mouse) or control IgG antibody 2 d before *C. albicans* infection. (C) Survival curves (n = 5-7 mice/group). (D) Fungal CFUs at day 3 post-infection (n = 4 or 5 mice/group). (E-G) Bone marrow neutrophils were isolated from WT and *Ccr5*$^{-/-}$ mice and were treated with GM-CSF for 4 h. (E) Diff-Quik staining. (F) Phagocytosis assay. (G) Measurement of MPO (n = 3 kidneys/group). Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

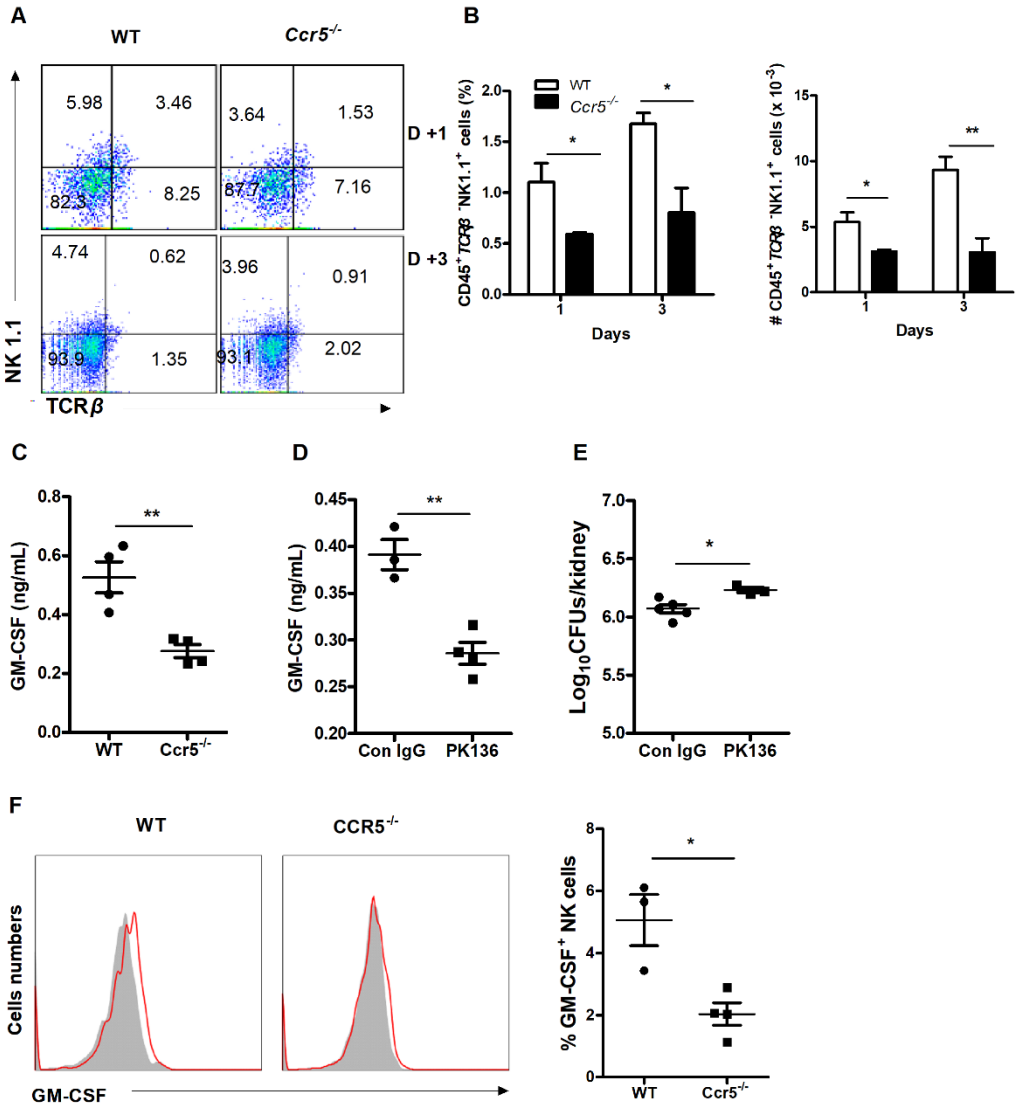

Figure 1-5. Impaired recruitment of NK cells and their GM-CSF production in *Ccr5*$^{-/-}$ mice.

(A and B) Renal cells were stained with anti-CD45, anti-TCRβ, and anti-NK1.1 antibodies. (A) CD45^{+} leukocytes were gated and analyzed for expression of NK1.1 and TCRβ. Representative FACS dot plots and percentages of CD45^{+}TCRβ^{-}NK1.1^{+}

NK cells in leukocytes were presented. (B) Absolute numbers of $CD45^{+}TCR\beta^{-}$ $NK1.1^{+}$ NK cells (n = 4 or 6 kidneys/group). (C) Levels of GM-CSF in kidney lysates at day 3 post-infection (n = 4 mice/group). (D and E) Mice were depleted of NK cells using anti-NK1.1 antibody (PK136). Kindeys were harvested at day 3 post-infection. (D) Levels of renal GM-CSF (n = 3 or 4 kidneys/group). (E) Fungal CFUs (n = 4 or 5 kidneys/group). (F) Intracellular staining was performed in kidney $CD45^{+}CD3^{-}NK1.1^{+}$ NK cells at day 3 post-infection. Shown are FACS histograms and percentages of $GM\text{-}CSF^{+}$ cells (n = 3 or 4 mice/group). Results are representative of 2-4 experiments and are presented as the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

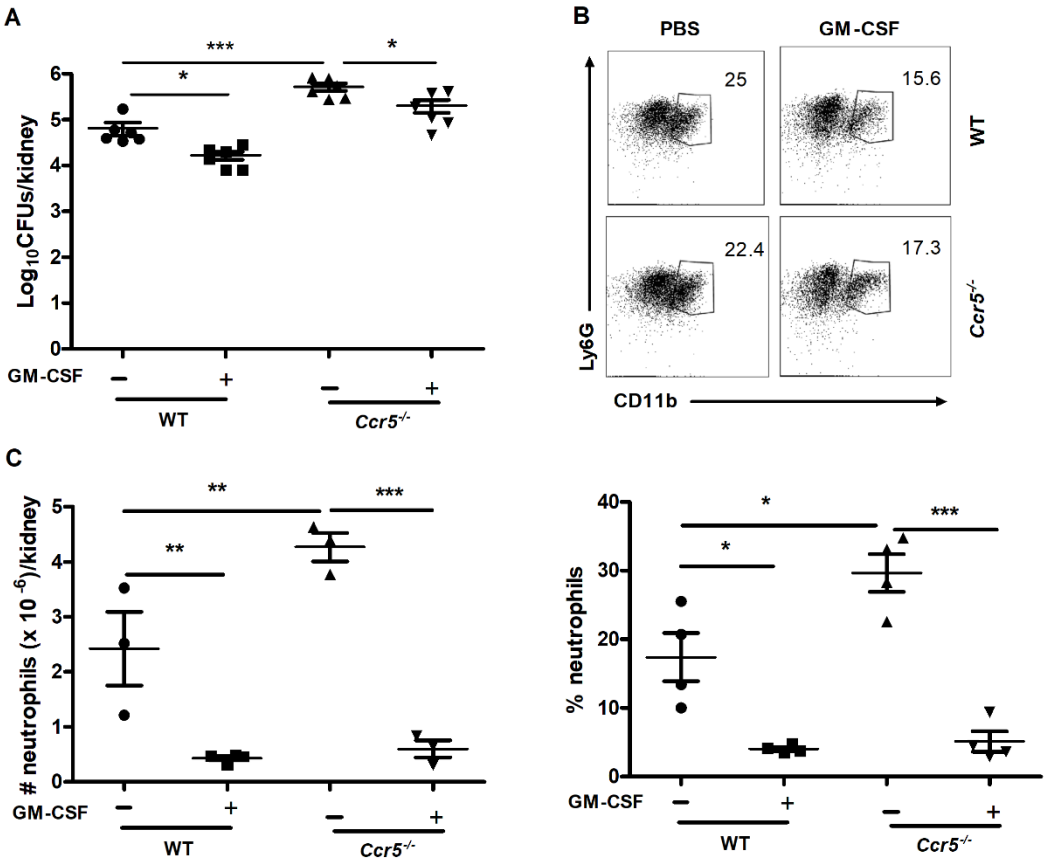

Figure 1-6. GM-CSF augments fungal clearance but reduces renal inflammation.

Mice were intraperitoneally injected with GM-CSF (5 μg/mouse) 24 h and 36 h after infection. (A) Fungal CFUs were counted in kidneys at day 3 post-infection (n = 6 kidneys/group). (B) Representative FACS dot plots for $CD45^+CD11b^+Ly6G^+$ renal neutrophils and their percentages in leukocytes were presented (n = 4 or 5). (C) Absolute numbers of renal neutrophils (n = 3 or 4 kidneys/group). Results are

representative of 3 experiments and are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

1.4.4 DCs and NK cells reciprocally regulate production of IL-23 and GM-CSF during systemic *C. albicans* infection.

We next investigated why individual NK cells of *Ccr5*$^{-/-}$ mice have reduced levels of GM-CSF during systemic *C. albicans* infection. *Ccr5*$^{-/-}$ mice had decreased expression of IL-23 at the mRNA and protein levels in 3-d post-infection kidneys (Figs. 1-7, A-C). We noticed that expression of *Il12b* (*IL-12p40*) was reduced in *Ccr5*$^{-/-}$ mouse kidneys to larger extent than that of *Il23a* (*IL-23p19*) (Fig. 1-7, A and B), suggesting that this common subunit may be a better control target for inflammation regulation. Intracellular levels of IL-23 in CD11c^{+}MHCII^{+} kidney DCs were significantly lower in *Ccr5*$^{-/-}$ mice at day 3 post-infection compared to WT mice (Fig. 1-7D). To verify that GM-CSF can affect *C. albicans*-induced production of IL-23 by DCs, we stimulated BMDCs with combination of GM-CSF and heat-killed (HK) *C. albicans* hyphae. Combined treatment had a synergistic effect on IL-23 production compared to GM-CSF or HK *C. albicans* alone (Fig. 1-7E). There was no difference in renal DC numbers between naïve WT and *Ccr5*$^{-/-}$ mice (Fig. 1-7F). Thus, GM-CSF can increase its own production indirectly through DC stimulation of IL-23 production during systemic *C. albicans* infection.

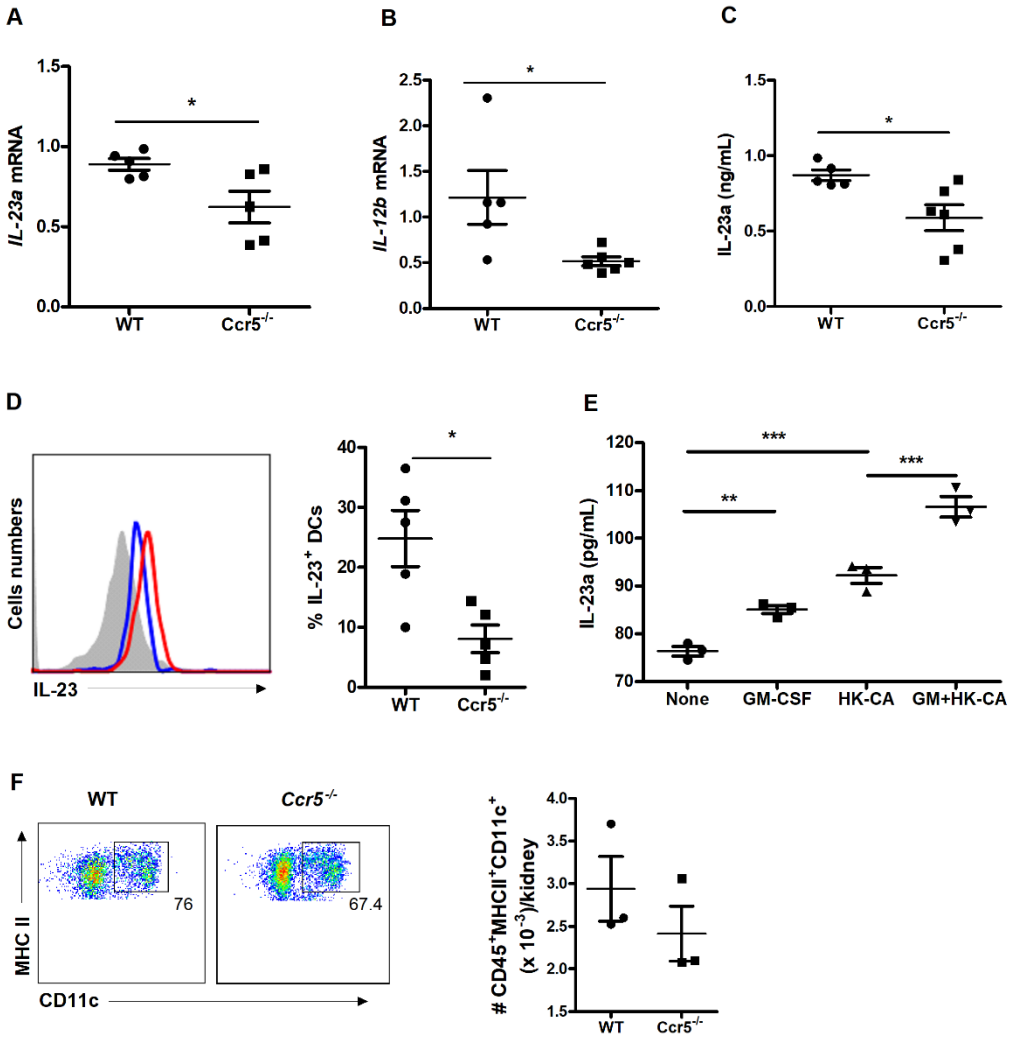

Figure 1-7. GM-CSF enhances IL-23 production by DCs.

(A-D) Kidneys were harvested at day 3 post-infection and their expression of IL-23 was investigated. (A and B) Transcriptional levels of *IL23a* and *IL12b* (n = 4 or 5 kidneys/group). (C) Concentrations of renal IL-23 protein (n = 5 or 6 kidneys/group). (D) FACS hitograms for IL-23p19 intracellular staining in renal $CD45^+MHCII^+CD11c^+$ DCs and percentages of $IL\text{-}23^+$ cells. Red line: WT; blue line:

Ccr5$^{-/-}$ (n = 5 kidneys/group). (E) BMDCs were cultured with combination of GM-CSF and hyphae forms of heat-kille (HK) *C. albicans* for 24 h. Concentrations of IL-23 in culture supernantants were determined using ELISA (n = 3/group). (F) The percentage of $CD45^+MHCII^+CD11c^+$ DCs in naïve mice (n = 3 kidneys/group). Results are representative of 2-3 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

1.5 DISCUSSION

Innate immune cells play critical roles in host defense mechanisms against invasive candidiasis [18]. Previous studies have reported chemotactic roles for CCR2 and CXCR2 in recruiting inflammatory monocytes and neutrophils, respectively, to infection sites during candidiasis [14, 19], but little has been known regarding the chemokine/chemokine receptor systems involved in chemotaxis of other types of innate immune cells. In this study, we first showed that CCR5-mediated recruitment of NK cells to the kidney is important for innate resistance to systemic *C. albicans*. Although the percent of CCR5-expressing renal NK cells was lower compared to neutrophils and macrophages (Fig. 1-1B), CCR5 was highly specific for recruiting NK cells to the kidney (Fig. 1-5, A and B). Secondly, our results support the previous findings that NK cells maintain a central position in an innate defense line established early after *Candida* infection [11–14]. Finally, we demonstrated that there is a positive feedback loop between NK cells and DCs that amplifies fungal clearance by neutrophils. If this defense mechanism fails, uncontrolled fungal proliferation may result in uncontrolled renal inflammation and a life-threatening renal immunopathology (Fig. 1-7).

NK cells are a particularly important regulator for acute renal inflammation, regardless of its causes. During ischemia-reperfusion kidney injury, tubular

epithelial cells (TECs) that undergo hypoxic damage initiate acute inflammation by recruiting NK cells through secretion of CCR5 ligands [16]. NK cells, in turn, stimulate TECs to produce CXCR2 ligands to recruit neutrophils [20]. During systemic candidiasis, however, tissue-resident macrophages seem to rapidly respond to invading *C. albicans* and release CCR5 ligands to recruit NK cells (our unpublished data). Thus, NK cells contribute to acute renal inflammation by two mechanisms: recruitment of neutrophils (in ischemia-reperfusion kidney injury and candidiasis) and enhancement of their effector functions (in candidiasis). As effector cells, however, NK cells can kill damaged TECs during ischemia-reperfusion kidney injury [20, 21]. Interestingly, human NK cells recognize β-1,3-glucan, a component of the fungal cell wall, through the activating receptor NKp30 [22, 23]. This recognition triggers signaling that sequentially leads to degranulation of NK cells, delivers effector molecules, such as perforin, to the fungal wall, and finally kills *C. albicans*. This process requires phagocytosis of a pathogen [24]. Therefore, defects in the fungicidal activity of NK cells in *Ccr5*$^{-/-}$ mice could contribute to their susceptibility to systemic *C. albicans* infection.

In the current study, our results suggest that the higher mortality rate of *Ccr5*$^{-/-}$ mice may be caused primarily by early neutrophil-mediated immunopathology, which occurs as a result of uncontrollable fungal proliferation. However, there is a

second wave of neutrophil recruitment to the kidney in WT mice, whose peak time is around day 9 after infection [25]. In this case, CCR1 but not CXCR2 is responsible for recruitment of neutrophils, whose main functions are to amplify late renal immunopathology without affecting fungal clearance [25]. Thus, this phenotypic dichotomy of neutrophils, based on chemokine receptors, reflects their functional differences during invasive candidiasis.

Mutations in *CX$_3$CR1* and *CXCR1* genes are associated with the susceptibility of humans to *Candida* infections [26–28]. Loss of function mutations in these genes is related to neutrophil degranulation and survival of macrophages and monocytes, respectively. Considering that these defects commonly cause impaired fungal clearance, screening of human cohorts for *Ccr5* gene mutations may reveal a relation between this gene and invasive candidiasis in humans.

GM-CSF is a pleiotropic cytokine produced by a variety of cell types, including lymphoid cells such as NK cells, Th17 cells, invariant natural killer T (iNKT) cells, type 2 innate lymphoid cells (ILC2s), and ILC3s [12–14, 29–33]. IL-1, IL-2, and IL-23 trigger GM-CSF production in Th17 cells, whereas IL-23 and IL-15 do so in NK cells. GM-CSF targets granulocytes, monocytes, macrophages, DCs, and glial cells, promoting tissue inflammation and host defense. During systemic *C. albicans* infection, GM-CSF secreted by NK cells is critical for fungal clearance for

neutrophils [11–13] but additional targets are not known. Our observation showing that impairment of NK cell recruitment in *Ccr5*$^{-/-}$ mice decreased production of IL-23 (Fig. 1-7) indicates that there is a mutual regulation between IL-23 and GM-CSF. Indeed, we showed that GM-CSF and *C. albicans* synergistically promote production of IL-23 by DCs (Fig. 1-7E). Although a mechanism underlying the crosstalk between PRRs and GM-CSF in DCs has yet to be explored, it is likely that GM-CSF enforces or co-stimulates PRR signaling. Thus, GM-CSF may be an important amplification point in the axis of resistance comprised of DCs, inflammatory monocytes, and neutrophils in the kidney and this resistance mechanism seems to override immunopathology incurred by GM-CSF-activated neutrophils, contributing to early survival. However, as mentioned previously, persistent fungal proliferation can induce later mortality in the survived host by recruitment of neutrophils that cause fatal renal immunopathology. Considering the diversity of GM-CSF source and its target, the immune network involving GM-CSF may be more complex than what has been known so far.

Overall, our results indicate that GM-CSF is a cytokine that plays a central role in activation of neutrophils to increase their phagocytic activity (Fig. 1-8). Nonetherless, injection of GM-CSF in *Ccr5*$^{-/-}$ mice did not induce fungal clearance as effectively as in WT mice (Fig. 1-6A). This observation suggests that there may

be an impairment in GM-CSF target cells other than NK cells and neutrophils in *Ccr5*$^{-/-}$ mice. Another possibility is that CCR5 may be involved in chemotaxis of non-NK cells that contribute to fungal clearance. Further studies will be needed to clarify this aspect of CCR5 function during systemic *C. albicans* infection.

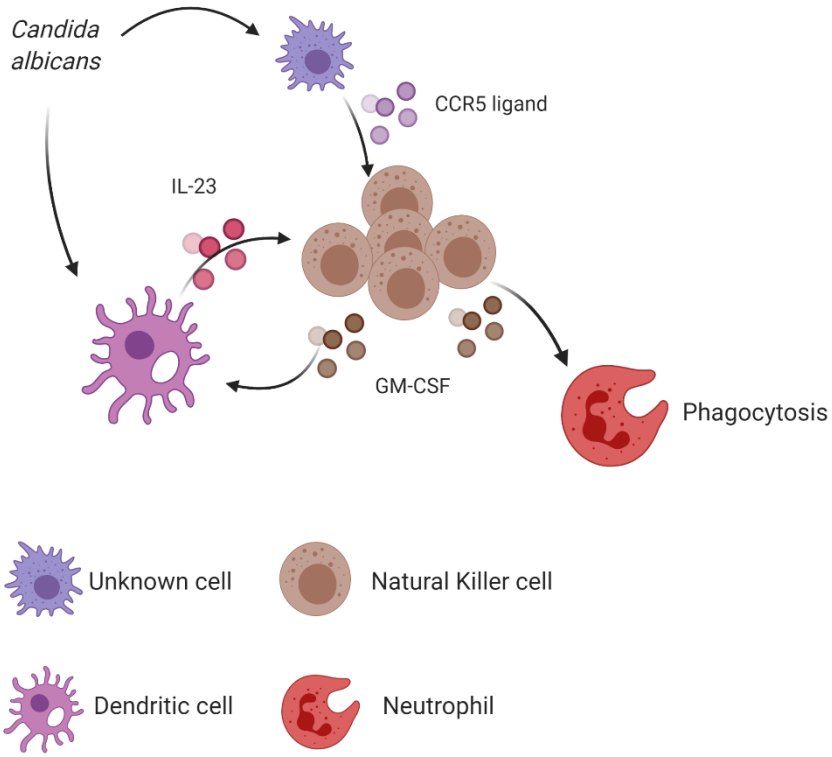

Figure 1-8. A schematic diagram showing CCR5 function in host defense against systemic *C. albicans* infection.

CCR5 ligands are released by unidentified cells in response to *C. albicans* infection and induce recruitment of NK cells into the kidney. On the other hand, *C. albicans* triggers secretion of DC IL-23, which in turn stimulates NK cell to produce GM-CSF. Release of GM-CSF is amplified by a positive feedback regulation of IL-23 production by DCs. GM-CSF increases the phagocytic activity of neutrophils.

Chapter 2: IL-33 COORDINATES INNATE DEFENSE TO SYSTEMIC CANDIDA ALBICANS INFECTION BY REGULATING IL-23 AND IL-10 IN AN OPPOSITE WAY.

2.1 ABSTRACT

Invasive candidiasis has high mortality rates in immunocompromised patients, causing serious health problem. In mouse models, innate immunity protects the host by rapidly mobilizing a variety of resistance and tolerance mechanisms to systemic *Candida albicans* infection. We have previously demonstrated that exogenous IL-33 regulates multiple steps of innate immunity involving resistance and tolerance processes. In this study, we systematically analyzed the in vivo functions of endogenous IL-33 using *Il33*$^{-/-}$ mice and in vitro immune cell culture. Tubular epithelial cells mainly secreted IL-33 in response to systemic *C. albicans* infection. *Il33*$^{-/-}$ mice showed increased mortality and morbidity, which were due to impaired fungal clearance. IL-33 initiated an innate defense mechanism by co-stimulating dendritic cells to produce IL-23 after systemic *C. albicans* infection, which in turn promoted the phagocytosis of neutrophils through secretion of GM-CSF by NK cells. The susceptibility of *Il33*$^{-/-}$ mice was also associated with increased levels of IL-10 and neutralization of IL-10 resulted in enhanced fungal clearance in *Il33*$^{-/-}$ mice. However, depletion of IL-10 overrided the effect of IL-33 on fungal clearance. In *Il10*$^{-/-}$ mouse kidneys, MHCII^{+}F4/80^{+} macrophages were massively differentiated after *C. albicans* infection and these cells were superior to MHCII^{-}F4/80^{+} macrophages that were preferentially differentiated in WT mouse kidneys, in killing

of extracellular hyphal *C. albicans*. Taken together, our results identify IL-33 as critical early regulator controlling a serial downstream signaling events of innate defense to *C. albicans* infection.

2.2 INTRODUCTION

The innate immune system directs the host protection mechanisms against systemic *C. albicans* infection by two main ways: elimination of invading fungi and inhibition of fatal infection-related immunopathology. $Ly6G^+$ neutrophils play a pivotal role in killing fungi in the bloodstream and organs and thereby prevent fungal dissemination and growth [34–36]. Mononuclear phagocytes also are indispensable for host defense against systemic *C. albicans* infection [9, 14, 27]. For example, $Ly6C^+$ monocytes and $CX3CR1^+$ macrophages can not only directly phagocytose fungi [9, 14, 27] but also help neutrophils eliminate fungi indirectly by mediating tissue inflammation [10, 13]. Dysregulation of these inflammatory cells is associated with uncontrolled, lethal tissue inflammation independently of resistance mechanisms [13]. Recent studies have well established immune cell-cytokine networks linked to neutrophils' fungal control activities [11–13, 37]. A tissue inflammatory milieu lacking anti-inflammatory mediators is also favorable for effective elimination of fungi. IL-10 is a prototype of cytokines that control both tissue inflammation and anti-pathogen immunity [38]. Although the protective role of IL-10 in candidiasis has been known for long time [39, 40], which factor regulates IL-10 during candidiasis has not been revealed in detail.

IL-33 is a multifaceted cytokine released rapidly by injury and infection, which subsequently participate in initiation and amplification of immune responses [41]. Using an IL-33 infusion model, IL-33 has been shown to play a critical role in many steps of the neutrophil-mediated resistance mechanisms to *C. albicans*, ranging from recruitment through fungal killing [19]. IL-33 also induces polarization of renal macrophages toward an M2 type, which promotes resolution of inflammation caused by *C. albicans* infection [42]. However, a physiological function of endogenous IL-33 remains to be clarified. In this study, we demonstrated that renal $CD11b^+$ DCs of *Il33*$^{-/-}$ mice released lower levels of IL-23, a cytokine that stimulates GM-CSF production by NK cells. As a consequence, reduced levels of GM-CSF in *Il33*$^{-/-}$ mice resulted in lowering a neutrophils' ability to inhibit proliferation of *C. albicans*. By contrast, *Il33*$^{-/-}$ mice secreted higher levels of IL-10 which played a role in impairing killing extracellular fungi by neutrophils and macrophages. Our results reveal a hitherto unappreciated host protection mechanism of IL-33 during disseminated candidiasis, which may be of relevance to the understanding of human candidiasis.

2.3 MATERIALS AND METHODS

2.3.1 Mice

C57BL/6 mice were purchased from Orient Bio-Charles River. *Il33*$^{-/-}$ and *Il10*$^{-/-}$ mice with a C57BL/6 background were purchased from the Jackson Laboratories and maintained in a specific pathogen-free facility and used between 7-10 wk of age. All experiments were conducted according to the regulations of the Animal Committee of the University of Ulsan.

2.3.2 Production of recombinant IL-33 protein

Recombinant IL-33 protein was produced as previously described [19, 42].

2.3.3 Fungal strains and growth conditions

C. albicans (ATCC26555) was grown in peptone dextrose extract at 30°C overnight, and aliquots were frozen at -80°C. To obtain hyphal forms, yeast forms were suspended in RPMI medium at a final concentration of 5 x 10^6 cells/ml and were further cultured on a rotary shaker at 37ºC for 120 min. To kill *C. albicans* hyphae, organisms were harvested by centrifugation, and pellets were washed twice in sterile PBS and resuspended at a density of 1 x 10^8 cells/ml before heat killing at 90°C for 30 min.

2.3.4 Experimental systemic candidiasis

C. albicans yeast were inoculated i.v. into the lateral caudal tail veins with 3×10^5 CFUs (lethal dose) or 1×10^5 CFUs (sublethal dose).

2.3.5 Counting CFUs

Mice were euthanized, and kidneys were removed aseptically to determine fungal burden. Harvested kidneys were homogenized in 2 ml PBS, and serial dilutions of homogenates were plated on Sabouraud agar and incubated at 37°C for 24 h. Colonies were counted, and results were expressed as $\log_{10}$(CFUs/organ).

2.3.6 Preparation of kidney cells

Kidneys were perfused, minced, and placed in DMEM (Life Technologies) containing 1 mg/mL collagenase IA and 100 ng/ml DNAse I (Sigma-Aldrich) at 37°C for 30 min. Digested kidney tissues were passed through a 40-µm cell strainer (BD Falcon), and the cell suspension obtained was centrifuged at 300 x g for 10 min. Cells were washed in PBS containing 2% BSA, suspended in 36% Percoll (Amersham Pharmacia Biotech), and gently overlaid onto 72% Percoll. After centrifugation at 900 x g for 30 min at room temperature, cells were retrieved from the Percoll interface and washed twice in DMEM and once with staining buffer (PBS containing 2% BSA and 0.1% sodium azide).

2.3.7 Flow cytometry

The following FITC-, PE-, PE-Cy5 (PE-cytochrome 5), PerCP- or APC (allophycocyanin)-conjugated mAbs to mouse proteins were purchased from BD Biosciences or eBioscience and used for cell staining: anti-CD45, anti-CD11b, anti-Ly6G, anti-Ly6C, anti-Gr-1, anti-F4/80, anti-MHCII, anti-CD11c, anti-IL-23p19, and anti-Rat IgG2a κ chain. Prepared cells were blocked with 2.4G2 mAb in a staining buffer at 4^0C for 20 min. Before washing twice with a staining buffer, cells were incubated with the relevant mAb at 4^0C for 30 min. For intracellular cytokine staining, after staining of surface markers, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/wash buffer (BD Biosciences), followed by staining with mAbs to mouse anti-GM-CSF and anti-IL-23p19 (eBioscience). Flow cytometric analysis was performed using a FACS Canto II (BD Biosciences) cytometer and data were analyzed using FACS Diva software (BD Biosciences) and FlowJo software (Tree Star).

2.3.8 Phagocytosis assay

BM cells were collected from femurs and tibias, suspended in RPMI medium (Welgene) supplemented with 10% FBS, penicillin/streptomycin (100 U/mL), 2 mM L-glutamine (GIBCO), and 50 μM 2-mercaptoethanol. To purify neutrophils from BM cells, anti-Ly6G MACS beads were used according to the manufacturer's

instructions (Miltenyi Biotech). Purified neutrophils were resuspended in RPMI medium and adjusted to a concentration of 2 x 10^6 cells/ml. The cells were preincubated with GM-CSF (10 ng/ml) and/or 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 hyphal form *C. albicans* (MOI = 10), the mixtures were incubated with slow rotation at 37°C for the indicated times. 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 in a quenching solution containing 0.04% trypan blue and 1% formaldehyde, and cells containing fungi were analyzed using flow cytometry. Phagocytosis was expressed as the percentage of neutrophils phagocytosing FITC-labeled *C. albicans*.

2.3.9 Cell sorting

Kidney cells were harvested at 0-, 1- or 3-d PI. $CD11b^+$ myeloid cells were enriched by MACS purification with anti-CD11b-PE/anti-PE microbeads (Miltenyi Biotech). Positively selected cells were stained with anti-CD45, anti-Ly6C, anti-Ly6G, anti-MHCII, anti-F4/80, and anti-CD11c mAbs. Cells were sorted using the BD FACSAria fusion cell sorter (BD Biosciences). Sorted cells were used for RNA extraction and killing assays. To purify TECs, anti-CD45 and anti-EpCAM MACS beads were used according to the manufacturer's instructions (Miltenyi Biotech).

The purified $CD45^{-}EpCAM^{+}$ TECs reached over 92% purity.

2.3.10 Nuclear and cytoplasmic extraction

The nuclear extraction was prepared using an NE-PER Nuclear Cytoplasmic Extraction Reagent Kit (ThermoFisher Scientific) according to the manufacturer's instruction. Briefly, TECS cells were washed twice with cold PBS and centrifuged at 500 x *g* for 5 min. The cell pellet was suspended in 200 µl of cytoplasmic extraction reagent I (CER I) containing proteinase and phosphatase inhibitor cocktatil ThermoFisher Scientific) by maximum-speed vortexing. The suspension was incubated on ice for 10 min and added with 11 µl of a second cytoplasmic extraction reagent II (CER II). The suspension was vortexed, incubated on ice for 1 min and centrifuged at 16,000 x *g* for 5 min. The supernatant fraction (cytoplasmic extract) was transferred immediately to a pre-chilled tube. The insoluble pellet fraction was resuspended in 100 µl of nuclear extraction reagent containing proteinase and phosphatase inhibitor cocktail by the highest-speed vortexing for 15 s. The sample was placed on ice and vortexed for 15 s every 10 min for a total of 40 min. After centrifuging at 16,000 x *g* for 10 min, the supernatant (nuclear extract) fraction was immediately transferred to a clean pre-chilled tube. Protein concentrations were determined by the BCA Protein Assay Kit (ThermoFisher Scientific).

2.3.11 Western blotting

Proteins (10μg) were seperated by electrophoresis using 12% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). Anti-IL-33 (Abcam), anti-α-Tubulin (Cell Signaling), anti-PARP1 (Cell Signaling) rabbit polyclonal Abs were used at 1:1,000 dilution and incubated overnight at 4^{o}C. Anti α-Tubulin and anti-PARP1 were used as the loading control for cytoplasmic and nuclear extract, respectively. HRP-conjugated anti-rabbit IgG secondary Ab (Abcam) were incubated at room temperature for 1 h. Immunoreactivity was detected using the ECL detection system (Bio-Rad).

2.3.12 Measurement of ROS

Sorted $CD45^{+}CD11b^{+}Ly6G^{+}$ neutrophils were seeded in 96-well plate (3 x 10^{5} cells/well) and added with opsonized *C. albicans* hyphae (MOI = 10) After 1-h incubation, 20 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma) were added and the cells were further incubated for 0, 10, 30 and 60 min. After washing with FACS buffer, fluorescence was measured using FACS.

2.3.13 Killing assay

Sorted $CD45^{+}CD11b^{+}Ly6G^{+}$ neutrophils, $CD45^{+}CD11b^{+}Ly6G^{-}Ly6C^{hi}$ monocytes, $CD45^{+}CD11b^{+}Ly6C^{-}Ly6G^{-}$ macrophage-enriched cells were incubated with *C. albicans* hyphae in 20:1 ratio for 3 h. CFUs was determined as described

previously [43]. The percentage of killing (%) was calculated at [1-(CFUs after incubation/CFUs at the start of incubation)] x100. In some experiments, 10μM diphenyleneiodonium chloride (DPI) (Sigma), an ROS inhibitor, was added to wells containing neutrophils 30 min before exposure to *C. albicans.*

2.3.14 Histology

Kidneys were fixed in 10% (v/v) formalin, embedded in paraffin, sectioned (5 μm), stained with H&E and analyzed.

2.3.15 Immunohistochemistry

Kidneys were harvested, rapidly embedded in optimal cutting temperature (OCT) compound (Sakura) and frozen in liquid nitrogen. Frozen sections (8μm) were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.25% Triton X-100 for 10 min, and blocked with 2% BSA in PBS for 1 h. Sections were then stained for 1:100 diluted FITC-conjugated anti-CD326 (EpCam) (eBiosciences) and anti-IL-33 Abs (Santa Cruz Biotechnology) for 2 h at room temperature and then treated with PE-conjugated donkey anti-rabbit IgG secondary Ab (1:100; Santa Cruz Biotechology) for 1 h at room temperature in the dark. All specimens were mounted with Prolong Antifade reagent (Molecular Probes). Slides were examined under a laser-scanning confocal microscope (Olympus).

2.3.16 In vivo administration of cytokines and Abs

IL-33 (1 μg/mouse) was i.p. injected into mice 1 d before and 1 d after infection. IL-23 (1 μg/mouse; Biolegend) was injected daily starting immediately after infection and GM-CSF (5 μg/mouse) was injected at 24 h and 36 h after infection. Neutralizing anti-IL-23 (100 μg/mouse; clone G23-8), anti-GM-CSF (100 μg/mouse; clone MP1-22E9), and anti-IL-10 (100 μg/mouse; clone JES5-2A5) Abs were purchased from BioXcell and injected at different times (anti-IL-23, days 0 and 2; anti-GM-CSF, day 2; anti-IL-10, immediately before infection). Anti-NK1.1 mAb was purified from PK136 hybridoma and i.p. injected into mice 1 d before and 2 d after infection to deplete NK cells (200 μg/mouse).

2.3.17 Real-time RT-PCR

Total RNA was extracted from kidneys or cultured cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Whole tissues were homogenized with a TissueLyzer tissue homogenizer (QUAGEN), and cDNA was prepared with SuperScript reverse transcription (Invitrogen). Real-Time PCR was performed using SYBR Green PCR Master Mix (Qiagen) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The primers used in the experiments were as follows. *Il33*: 5'-CCTCCCTGAGTA CATAACATGACC-3' (forward) and 5'-GTAGTAGCACCTGGTCTTGCTCTT-3' (reverse). *Csf2*: 5'-CGTTCCCCTGGT

CAGTGTC-3’ (forward) and 5’-CCGCTGGCCTGGATCTTC-3’ (reverse); *Il23a*: 5’-CCAGCAGCTCTCTCGGAATC-3’ (forward) and 5’-TCATA GTCCCGCTGGTGC-3’(reverse); *Il12b*: 5’-CCTGGTTTGCCATCGTTTTG-3’ (forward) and 5’-TCAGAGTCTCGCCTCCTTTGTG-3’(reverse); *18S rRNA*: 5’-AGACAAATCGCTCCACCAAC-3’ (forward) and 5’- CTAAACACGGGAAAC CTCAC -3’ (reverse). *Il10*: 5’-AGGGTTACTTGGGTTGCCAA -3’ (forward) and 5’- CACAGGGGAGAAATCGATGA -3’ (reverse); All PCRs were performed in triplicate and normalized to internal control *18S rRNA* mRNA. Relative expression was presented using the $2^{-\triangle\triangle CT}$ method.

2.3.18 Culture and stimulation of BMDCs

BM cells were collected from *Il33*$^{-/-}$ mouse femurs and tibias and suspended in RPMI medium (Welgene) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml), 2 mM L-glutamine (GIBCO), 50 μM 2-mercaptoethanol and FLT3 ligand (FLT3L) (150 ng/ml). Cells were cultured in 6-well plate at a density of 2 × 10^6 cells/ml for 7 d. GM-CSF (10 ng/ml) was added at day 7 and cells were cultured for additional 2 d. Mature BMDCs were stimulated with combination of IL-33 (150 ng/ml) and HK *C. albicans* hyphae (MOI = 10) for 24 h. For adoptive transfer, mature *Il33*$^{-/-}$ BMDCs were primed with combination of IL-33 and HK *C. albicans*

for 24 h. Primed or unprimed *Il33*$^{-/-}$ BMDCs (1 x 10^6 cells/mouse) were i.v. injected into WT and *Il33*$^{-/-}$ mice 2 h before infection.

2.3.19 Primary TEC culture

TECs were cultured as previously described [21]. In brief, TECs were obtained by digeting kidney with collagenase IA for 30 min. Cells were grown in complete DMEM:Ham's F-12 (50:50) culture medium (Invitrogen), supplemented with 5% (vol/vol) FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, hormone mix (5 μl/ml insulin, 5 μl/ml transferrin, 1.25 ng/ml prostaglandin E1 (PGE1), and 1.73 ng/ml sodium selenite), and 25 ng/m of epidermal growth factor (EGF). Cells were trypsinized before each passage. Three-passage TECs (1 x 10^6 cells/well) used for experiments. Cells were incubated in 6-well flat-bottomed plates for 6 h prior to being challenged by HK *C. albicans* yeasts (MOI = 10) or hyphae (MOI = 10). After culture for 24 h, harvested TECs and culture medium were used for RT-PCR or ELISA assay, respectively.

2.3.20 Measurement of cytokines and chemokines

Cytokines and chemokines present in total kidney homogenate and cell culture supernatant were measured using a Cytometric Bead Array kit (BD Biosciences) or by ELISA (eBioscience or R&D system), respectively, according to the

manufacturers' protocols.

2.3.21 Adoptive transfer of neutrophils

Neutrophils were isolated from BM cells using anti-Ly6G microbeads. Purified neutrophils were resuspended in RPMI medium (Welgene) and adjusted to a concentration of 2 x 10^6 cells/ml. The cells were preincubated with combination of GM-CSF (10 ng/ml) and IL-33 (150 ng/ml) at 37˚C for 2–3 h. Cells were harvested, washed, resuspended in PBS and injected to mice (1 x 10^7 cells per mouse) 2 h before infection.

2.3.22 Statistical analysis

All data were analyzed using GraphPad Prism Software version 5. Unpaired Student t-test or one- or two-way ANOVA with posthoc analysis were used to compare differences between the groups. The 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 below 0.05 was considered statistically significant.

2.4 RESULTS

2.4.1 TECs release IL-33 after systemic C. albicans infection

The kidney is a main site for proliferation of *C. albicans*. We first investigated the kinetics of IL-33 production in the kidney after systemic *C. albicans* infection. Levels of *Il33* transcripts increased from 1-d PI, reached a peak at 3-d PI, and declined thereafter (Fig. 2-1A). Production of IL-33 protein followed a similar pattern as expression of *Il33* mRNA (Fig. 2-1B). Immunohistochemical and FACS analysis showed that IL-33 was detected mainly in $EpCAM^+$ TECs at 3-d PI. (Fig. 2-1, C and D). Endothelial cells, mast cells, and inflammatory monocytes barely expressed IL-33 at 3-d PI but levels of IL-33 were mildly increased in renal macrophages at 3-d PI (Fig. 2-1E). We confirmed that isolated TECs increased transcription and release of IL-33 in response to yeast or hyphal forms of *C. albicans* (Fig. 2-1F). To further confirm that TECs release IL-33 at 3-d PI, we investigated the cellular localization of IL-33 in TECs. At 3-d PI, full-length IL-33 was detected in the nucleus of purified TECs, whereas either full-length or cleaved, mature IL-33 was localized in the cytoplasm (Fig. 2-1G). These results suggest that IL-33 is processed in the cytoplasm and released after cleavage.

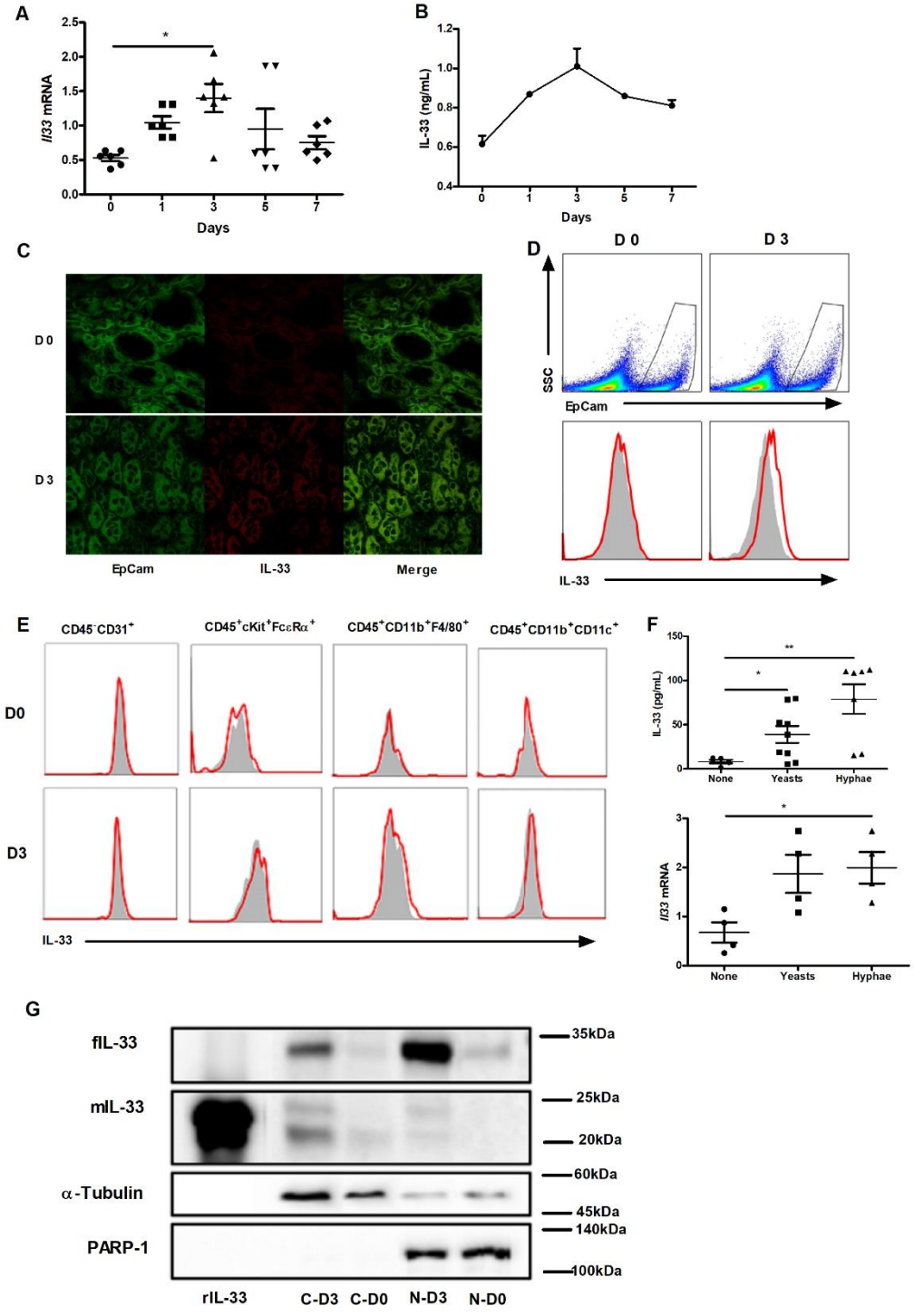

Figure 2-1 Upregulation of IL-33 in TECs after systemic *C. albicans* infection.

(A-D) C57BL/6 (WT) mice were i.v. injected with a sublethal dose of *C. albicans* yeasts and the kidneys were harvested at the indicated time points. (A) Levels of *Il33* mRNA in kidney tissues were determined by RT-PCR (n = 6 mice/group). (B) The concentrations of IL-33 were measured in kidney lysates using ELISA (n = 3 mice/group). (C) Immunohistochemical analysis of IL-33 in kidneys. (D, E) Identification of kidney cells producing IL-33 by intracellular staining. (F) Cells were cultured for 24 h in the presence of HK *C. albicans* yeasts or hyphae. Levels of IL-33 released from TECs were measured by ELISA (*Upper panels*) (n = 5-7 replicas/group) and levels of *IL-33* mRNA in TECs were determined by RT-PCR (*Lower panel*s) (n = 4 replicas/group). (G) Identification of the intracellular localization of full-length IL-33 (fIL-33) and mature IL-33 (mIL-33) in TECs. Cytoplasmic extract (C) and nuclear extract (N) were obtained from isolated TECs before (D0) and 3 d after infection (D3) and Western blot analysis was performed. Recombinant IL-33 (rIL-33) was run as reference. One-way ANOVA test was used for statistical analysis. Results are representative of 2-3 experiments and are presented as the mean ± SEM. $*P < 0.05$; $**P < 0.01$.

2.4.2 *Il33*$^{-/-}$ mice are susceptible to systemic *C. albicans* infection

To define the role of endogenous IL-33 during systemic candidiasis, *Il33*$^{-/-}$ mice were challenged with *C. albicans* and monitored for survival. *Il33*$^{-/-}$ mice succumbed to a sublethal dose of *C. albicans* infection rapidly, showing a higher mortality rate than WT mice (Fig. 2-2A). Consistently, *Il33*$^{-/-}$ mice experienced more severe body weight loss at 5-d PI (Fig. 2-2B), and their kidneys appeared more swollen and had more severe edema with a greater weight gain than WT mouse kidneys at 3-d PI and 5-d PI (Fig. 2-2, C and D). Gross observations also indicated that *Il33*$^{-/-}$ kidneys had many more distinguishable nodules than WT kidneys (Fig. 2-2C). All of these gross observations indicated that fungi proliferated more rapidly and induced more extensive abscess formation in *Il33*$^{-/-}$ vs WT kidneys. Indeed, *Il33*$^{-/-}$ mice had markedly increased fungal burden in the kidney at 3- and 5-d PI compared with WT mice (Fig. 2-2E). Histopathological analysis showed that *Il33*$^{-/-}$ kidneys had numerous multifocal areas of abscess formation than WT kidneys (Fig. 2-2F). PAS staining clearly revealed more prominent hyphae within abscesses of *Il33*$^{-/-}$ versus WT kidneys (Fig. 2-2F).

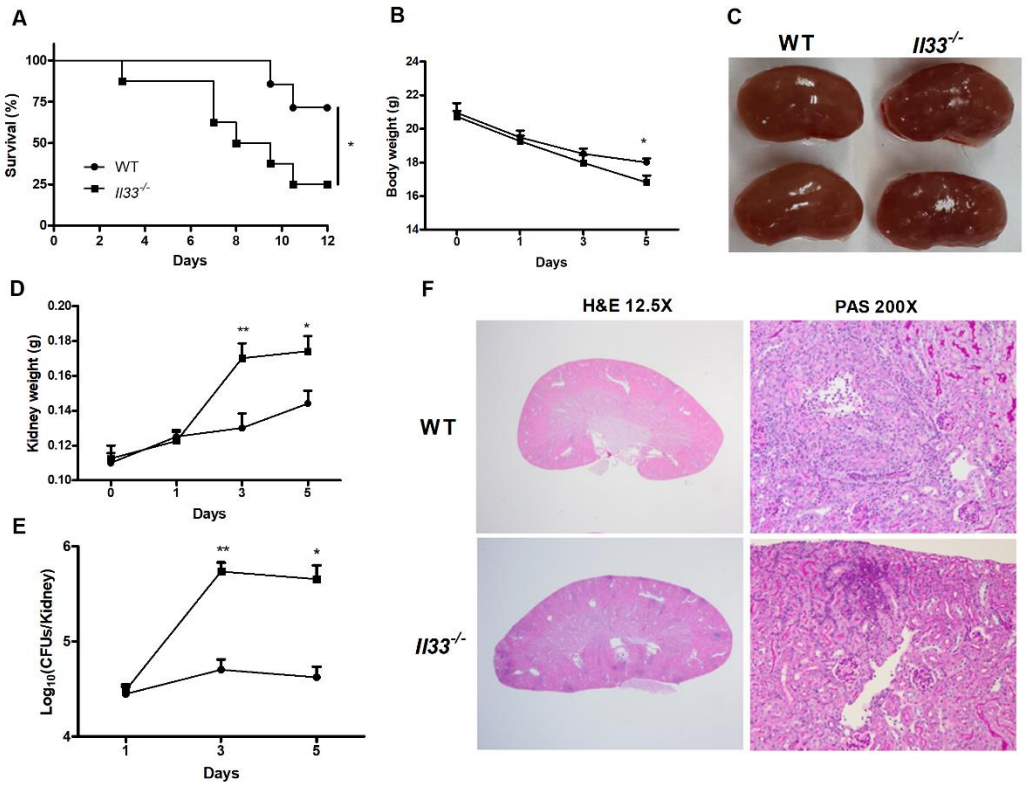

Figure 2-2 *Il33*$^{-/-}$ mice are susceptible to systemic candidiasis.

WT and *Il33*$^{-/-}$ mice were injected with a sublethal dose *C. albicans*. (A) Survival curves (n = 7-8/group). The log-rank test was used. (B) Changes in body weight (n = 6/group). (C) Gross morphology of kidneys at 3-d PI. (D) Changes in kidney weight (n = 3-5 kidneys/group). (E) Fungal burden in kidneys (n = 5-6/group). Two-way ANNOVA test was used (B, D, E). (F) H&E and PAS staining for kidney sections. Magnification: 12.5X (*left panels*); 200X (*right panels*). Results are representative of 2-4 experiments and are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

2.4.3 IL-33 is crucial for IL-23 production by CD11b$^+$ DCs

DCs play a critical role in clearance of *C. albicans* through IL-23 which mediates sequential downstream signaling events involving NK cell production of GM-CSF and consequent enhancement of neutrophil's phagocytic and fungicidal activities [11, 12, 37]. We examined the involvement of IL-33 in this process. *Il33* expression in the kidney was transiently increased at 12-h PI (Figure 2-3A). By contrast, expression of *Il23a*, *Il12b*, and *Csf2* showed a continuous increasing pattern from 12 h after infection (Fig. 2-3, B-D). These gene expression patterns suggest that IL-33 may trigger expression of the downstream genes including *Il23a*, *Il12b*, and *Csf2* during the early phase of *C. albicans* infection. As IL-23 is produced by CD11b$^+$ DCs, we investigated whether there are changes in cell numbers of renal CD11b$^+$ DCs after infection. We did not observe a difference in the absolute number of CD103$^-$CD11b$^+$ DCs in the 3-d PI kidney between *Il33*$^{-/-}$ and WT mice (Fig. 2-4A). Analysis for intracellular staining of IL-23p19 showed that there were lower levels of intracellular IL-23p19 in CD11b$^+$ DCs isolated from 3-d PI *Il33*$^{-/-}$ mouse kidneys (Fig. 2-4B). In a similar context, injection of IL-33 resulted in no changes in total numbers of CD11b$^+$ DCs (Fig. 2-4C) but it increased intracellular levels of IL-23p19 (Fig. 2-4D). Consistent with this, total concentrations of IL-23 in the kidney were significantly lower in *Il33*$^{-/-}$ mice at 3-d PI (Fig. 2-4E), while being

significantly greater in IL-33-injected WT and *Il33*$^{-/-}$ mice (Fig. 2-4E). IL-33-mediated increase of IL-23 was associated with more effective inhibition of fungal proliferation in either WT or *Il33*$^{-/-}$ mice (Fig. 2-4F). Next, we explored whether CD11b^{+} DCs can produce IL-23 directly in response to IL-33 and/or *C. albicans*. CD11b^{+} DCs were isolated from BMDCs and stimulated with a combination of IL-33 and HK *C. albicans* hyphae for 24 h. We then measured levels of *Il12b* and *Il23a* transcripts in cultured BMDCs and protein levels of IL-23 in culture supernatant. Although IL-33 alone barely induced expression of either *Il12b* nor *Il23a*, HK *C. albicans* hyphae markedly increased expression of either *Il12b* or *Il23a* and IL-33 potentiated their action on the expression of these genes (Fig. 2-4G). Measurement of IL-23 in culture supernatant confirmed the potentiating effect of IL-33 on production of IL-23 by CD11b^{+} DCs (Fig. 2-4H). To further support the involvement of IL-23 in the IL-33-mediated defense against *C. albicans* infection, we infused IL-23 into *Il33*$^{-/-}$ mice before systemic *C. albicans* infection to see whether IL-23 can lower fungal burden. Indeed, injection of IL-23 significantly reduced fungal burden in *Il33*$^{-/-}$ mice (Fig. 2-4I). By contrast, neutralization of IL-23 resulted in increase in fungal burden in WT mice (Fig. 2-4J). In aggregate, our results indicate that IL-33 acts on CD11b^{+} DCs to increase their ability to produce IL-23 in response to *C. albicans* and consequently more effectively inhibit fungal proliferation.

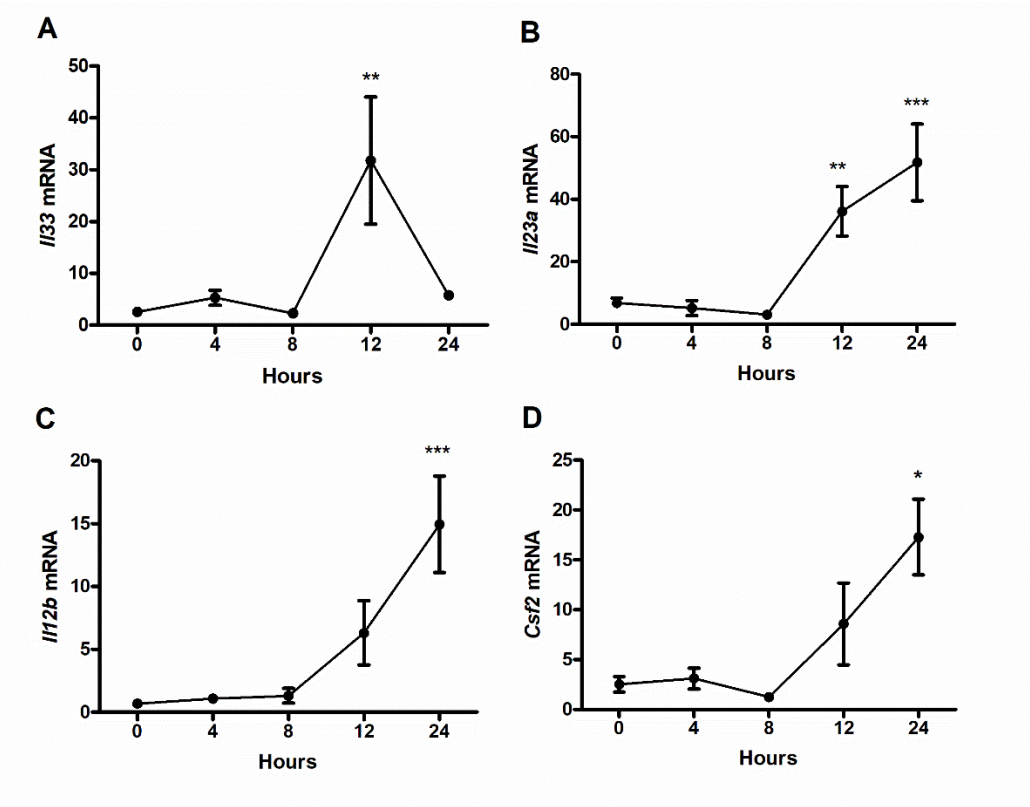

Figure 2-3 Kinetics of cytokine expression.

WT mice were injected with alethal dose of *C. albicans* and kidneys were harvested at the indicated time points. Transcriptional levels of *Il33* (A), *Il23a* (B), *Il12b* (C), and *Csf2* (D) (n= 6/ group). One-way ANOVA test was used. Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

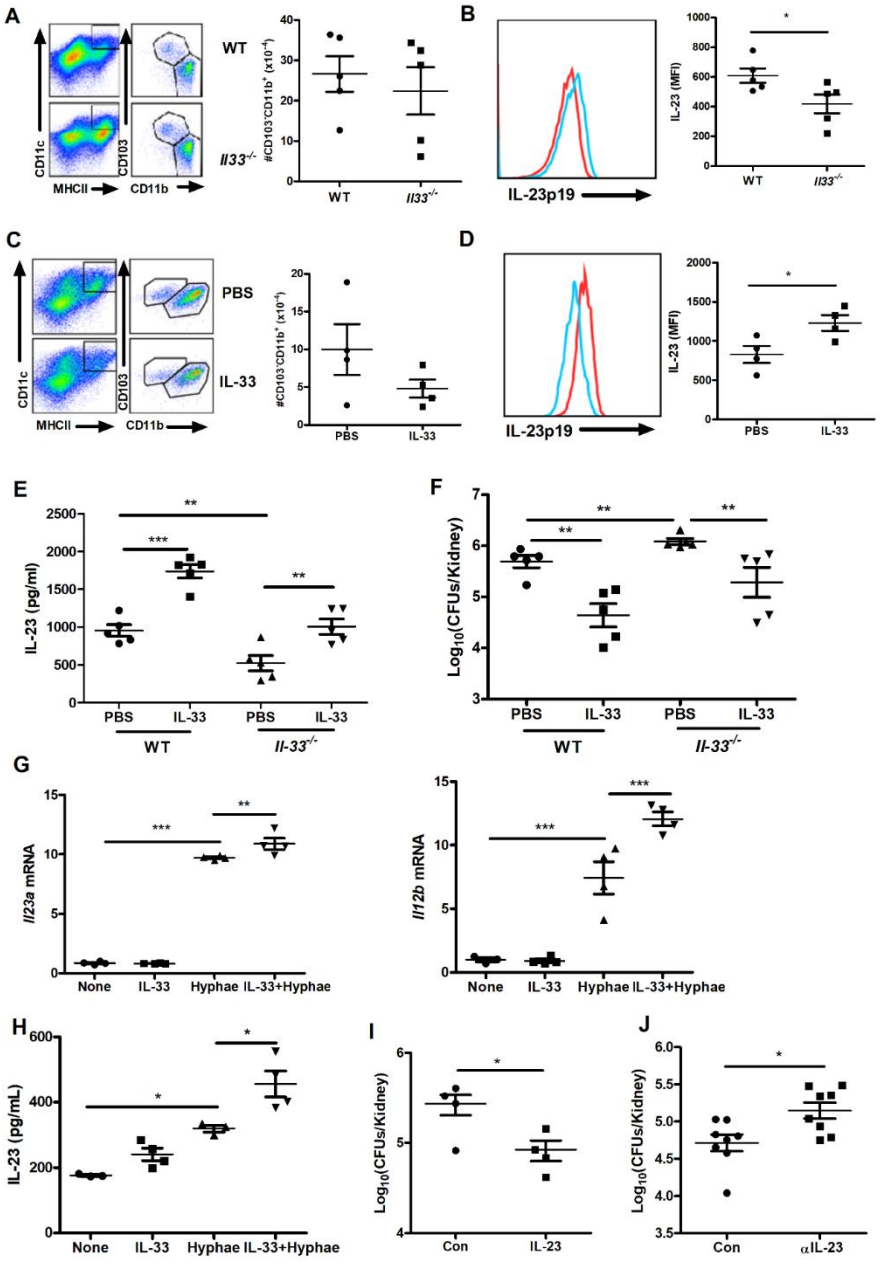

Figure 2-4 IL-33 is crucial for IL-23 production by CD11b$^+$ DCs.

(A-F) WT and *Il33*$^{-/-}$ were injected with a lethal dose of *C. albicans* and kidneys were harvested at 3-d PI. IL-33 was injected into WT mice 1 d before and 1 d after infection (C, D, F). (A, C) Kidney cells were stained with anti-CD45, anti-MHCII,

anti-CD11c, anti-CD103, and anti-CD11b mAbs. $CD11c^{+}MHCII^{+}$ DCs were gated from $CD45^{+}$ cells. Representative dot plots for $CD11b^{+}CD103^{-}$ and $CD11b^{-}CD103^{+}$ DC subsets and the absolute number of $CD11b^{+}CD103^{-}$ cells were presented (n = 4-5/group). (B, D) Representative histograms for intracellular IL-23p19 staining in gated $CD11b^{+}CD103^{-}$ DCs were presented. The mean fluorescence intensity (MFI) for IL-23p19 staining was also presented (n = 3-4/group). Blue line, WT; red line, *Il33*$^{-/-}$ in *B*. Blue line, PBS; red line, IL-33 in *D*. (E, F) Concentrations of IL-23 (E) and fungal burden (F) were presented (n = 5/group). Two-way ANOVA test was used. (G, H) BMDCs were cultured in the presence of combination of IL-33 and HK *C. albicans* hyphae for 24 h. Levels of *Il23a* and *Il12b* mRNA in BMDCs (G) and concentrations of IL-23 in culture supernatant (H) were presented (n = 4/group). One-way ANOVA test was used. (I) *Il33*$^{-/-}$ mice were injected daily with IL-23 (1 μg/mouse) starting immediately before infection. CFUs were counted from kidney lysate at 3-d PI (n = 4 kidneys/group). (J) WT mice were injected with anti-IL-23 neutralizing mAb at days 0 and 2 (100 μg/mouse). CFUs were counted at 3-d PI. (n= 8/ group). Unpaired Student t-test was used. Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

2.4.4 GM-CSF is indispensable for IL-33-mediated fungal clearance

We next investigated whether IL-33 affects GM-CSF production after systemic *C. albicans* infection. *Il33*$^{-/-}$ mouse kidneys contained significantly lower levels of GM-CSF at 3-d PI (Fig. 2-5A), while its renal levels in WT mice were significantly increased by injection of IL-33 (Fig. 2-5B). Consistent with this, infusion of GM-CSF into *Il33*$^{-/-}$ mice significantly decreased CFUs of *C. albicans* in the kidney (Fig. 2-5C) but neutralization of GM-CSF had an opposite effect on fungal burden in WT mice (Fig. 2-5D). In addition, adoptive transfer of GM-CSF-primed neutrophils into *Il33*$^{-/-}$ mice augmented fungal clearance (Fig. 2-5E). Priming of neutrophils by IL-33 also increased their effector function for fungal clearance, as previously shown[19, 42] , but IL-33-primed neutrophils did so less potently than those primed by GM-CSF (Fig. 2-5E). There was no synergistic effect of IL-33 and GM-CSF on the neutrophil activity for fungal clearance (Fig. 2-5E). This result seems to indicate that IL-33-mediated increase of GM-CSF is largely responsible for fungal clearance by neutrophils. In support of this hypothesis, our in vitro analysis showed that priming of neutrophils with GM-CSF promoted their phagocytic activity for either opsonized or nonopsonized *C. albicans* (Fig. 2-5F) but priming of neutrophils with IL-33 did so for only opsonized fungi (Fig. 2-5F). There was no synergistic effect of IL-33 and GM-CSF on the phagocytic activity of neutrophils for either opsonized or

nonopsonized *C. albicans* (Fig. 2-5F). In sum, our results suggest that although IL-33 is able to directly activate neutrophils, IL-33-mediated fungal clearance occurs largely through GM-CSF. As expected, injection of IL-23 into *Il33*$^{-/-}$ mice increased the concentration of GM-CSF at 3-d PI kidneys (Fig. 2-5G), whereas neutralization of IL-23 led to reduced levels of GM-CSF (Fig. 2-5H). To further investigate whether lower levels of GM-CSF in *Il33*$^{-/-}$ mouse kidneys are linked to CD11b^{+} DC production of lower levels of IL-23 during systemic *C. albicans* infection, *Il33*$^{-/-}$ mice were adoptively transferred with IL-33 plus HK *C. albicans*-primed DCs. Primed WT DCs, but not unprimed DCs, increased renal production of GM-CSF equally in either WT or *Il33*$^{-/-}$ mice (Fig. 2-5I). We next investigated whether production of GM-CSF by NK cells is a signaling event downstream of IL-33 signaling. Injection of anti-NK1.1 mAb (PK136) effectively depleted NK1.1^{+} PBMCs (Fig. 2-6A) and subsequently abrogated the effect of IL-33 on GM-CSF production in WT mice (Fig. 2-6B). Taken together with other studies [11, 12, 37], these results indicate that NK cells produce GM-CSF after systemic *C. albicans* infection and injection of IL-33 further elevated levels of GM-CSF. In addition, IL-33-mediated increment of fungal clearance was significantly abolished when NK cells were depleted (Fig. 2-6C). In aggregate, our data suggest that the IL-33 → IL-23 → GM-CSF axis is critical in fungal clearance. It is worthwhile noticing that

there were a greater number of neutrophils infiltrating into the kidney of *Il33*$^{-/-}$ mice at 3-d PI (Fig. 2-6D), which was associated with more severe renal inflammation (Fig. 2-6F), Taken together our results indicate that levels of IL-23 or GM-CSF were not reduced sufficiently to severely impair survival of neutrophils in *Il33*$^{-/-}$ mice [44].

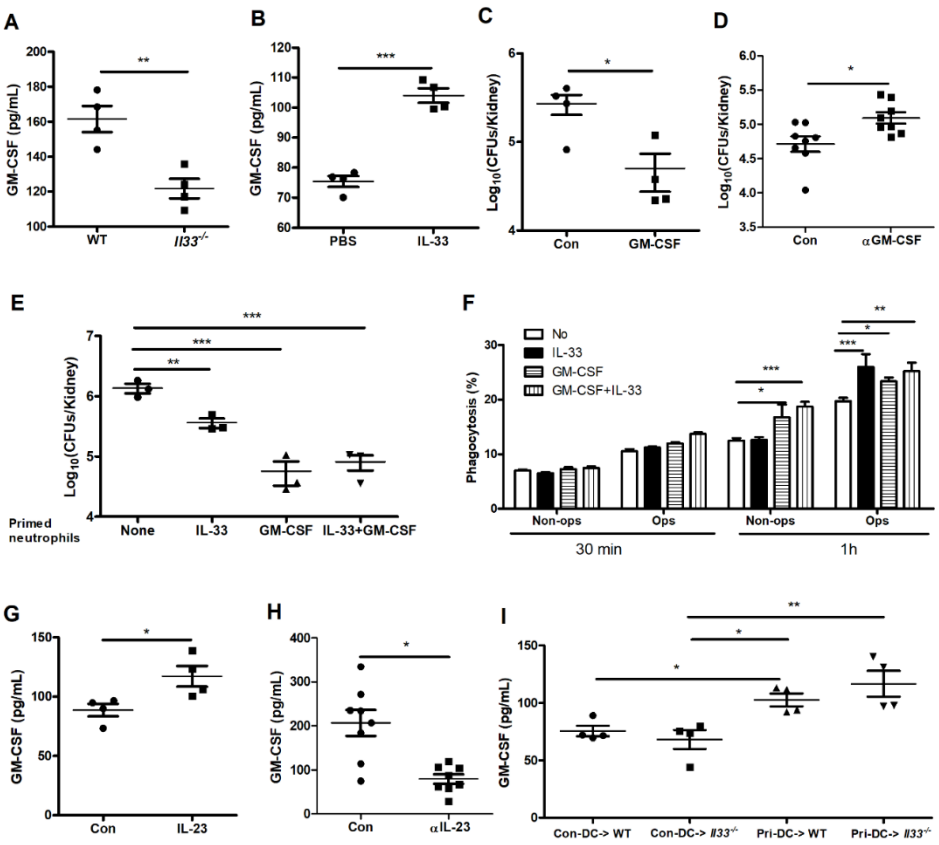

Figure 2-5. GM-CSF is indispensable for IL-33-mediated fungal clearance.

(A) Levels of GM-CSF were measured in 3-d PI kidney lysate of WT and *Il33*$^{-/-}$ mice that received a lethal dose of *C. albicans* (n = 4/group). (B) IL-33 was injected into WT mice 1 d before and 1 d after infection with a lethal dose of *C. albicans*. Levels of renal GM-CSF at 3-d PI (n = 4/group). (C, D) *Il33*$^{-/-}$ and WT mice were injected with GM-CSF (5 μg/mouse) 24 h and 36 h after infection or with anti-GM-CSF neutralizing mAb 2 d after infection (250 μg/mouse), respectively. CFUs were counted in 3-d PI kidney lysate (n = 4-8/group). Unpaired Student t-test was used. (E) Isolated WT neutrophils were primed with combination of IL-33 and GM-CSF

for 2–3 h and adoptively transferred to WT mice just before infection. CFUs were counted in 3-d PI kidney lysate (n = 3 kidneys/group). One-way ANOVA test was used. (F) Isolated neutrophils were pre-incubated with combination of IL-33 and GM-CSF for 2 h. Phagocytosis assays were performed by adding either opsonized (ops) or non-opsonized (non-ops) HK *C. albicans* for 30 min or 1 h (n = 4/groups). (G, H) *Il33*$^{-/-}$ and WT mice were injected with PBS or IL-23 (1 µg/mouse) 1 d before and 1 d after infection and neutralizing anti-IL-23 mAb (100µg/ mouse) 2 d after infection, respectively. Levels of GM-CSF were measured in 3-d PI kidney lysate (n = 4-8/groups). Unpaired Student t-test was used. (I) *Il33*$^{-/-}$ BMDCs were primed with conbination of IL-33 and HK *C. albicans* for 2 h and adoptively transferred to WT or *Il33*$^{-/-}$ mice 2 h before infection (1 x 10^6 cells/mouse). Levels of GM-CSF were determined in 3-d PI kidney lysate (n = 4/groups). Results are representative of 2-4 experiments and are presented as the mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001

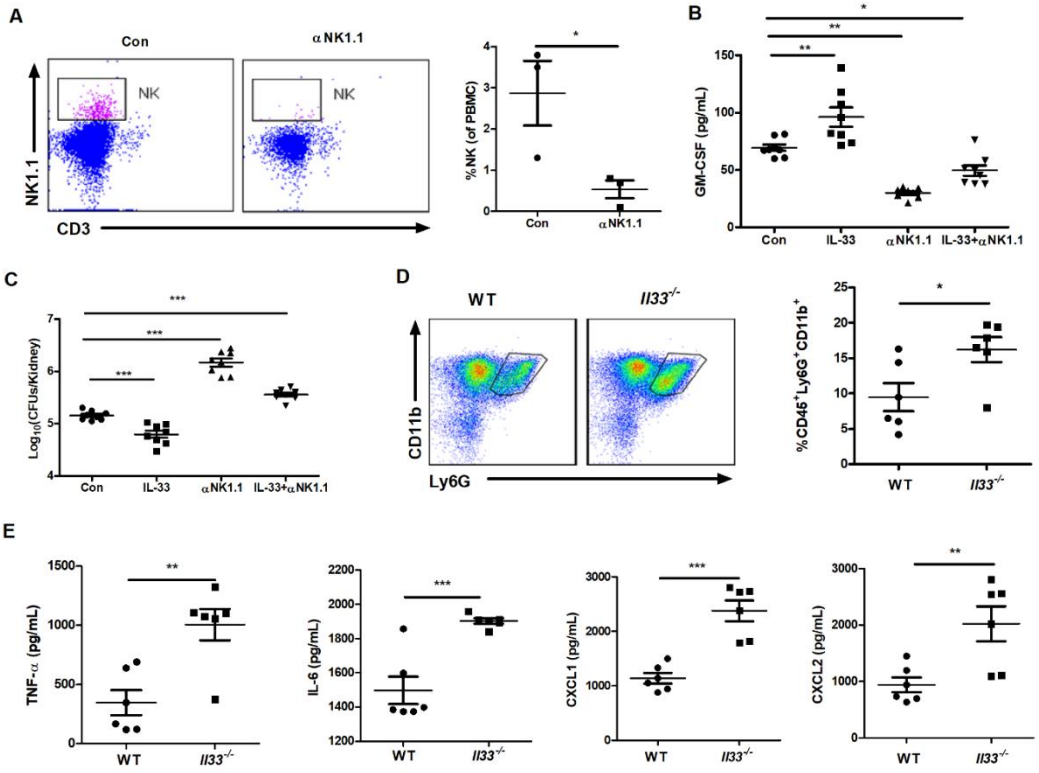

Figure 2-6 NK cell GM-CSF is required for IL-33-mediated fungal clearance.

(A-C) WT mice received depleting anti-NK1.1 mAb and IL-33 at 1 d before and 2 d after infection. PBMCs and kidneys were harvested at 3-d PI. (A) Representative dot plots for $CD3^{-}NK1.1^{+}$ NK cells and their percentages in PBMCs were presented. Unpaired Student t-test was used (n = 3/group). (B, C) Levels of GM-CSF (C) and fungal burden were determined in kidney lysate. One-way ANOVA test was used (n = 8/group). (D, E) WT and *Il33*$^{-/-}$ mice were infected with a lethal dose of *C. albicans* and kidneys were harvested at 3-d PI. (D) Representative dot plots for $CD45^{+}Ly6G^{+}CD11b^{+}$ neutrophil and their percentages were presented. (E) Levels of IL-6. TNF-α, CXCL1, and CXCL2 was measured from kidney lysates.

Unpaired Student t-test was used (n = 6/group). Results are representative of 2-4 experiments and are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

2.4.5 IL-10 can control fungal clearance in an IL-33-independent way

We detected higher levels of IL-10 in *Il33*$^{-/-}$ mouse kidneys 1 d after infection (Fig. 2-7A). In WT mice, Ly6C^{-}Ly6G^{-}CD11b^{+} MHCII^{-}F4/80^{+} macrophages expressed higher levels of *Il10* mRNA, while its expression levels were very low in Ly6C^{+}Ly6G^{-}CD11b^{+} monocytes (Fig. 2-7B). There were medium levels of *Il10* mRNA in WT Ly6G^{+}CD11b^{+} neutrophils and MHCII^{+}F4/80^{+} macrophages. Deficiency of *Il33* resulted in increased transcription of *Il10* in Ly6G^{+}CD11b^{+} neutrophils and MHCII^{+}F4/80^{+} macrophages (Fig. 2-7B) but expression of *Il10* was mildly reduced in *Il10*$^{-/-}$ MHCII^{-}F4/80^{+}macrophages (Fig. 2-7B). As expected, neutralization of IL-10 lowered fungal burden in either WT or *Il33*$^{-/-}$ mice (Fig. 2-7C). Unlike WT mice, *Il10*$^{-/-}$ mice failed to decrease fungal burden in response to injected IL-33 (Fig. 2-7D). These results seem to suggest that IL-33 no longer affect defense mechanisms in the renal microenvironment created by the absence of IL-10. To test this hypothesis, we first investigated whether a change in the renal microenvironment occurs in *Il10*$^{-/-}$ mice after infection. The kidneys of *Il10*$^{-/-}$ mice displayed a couple of characteristic features of myeloid cell compositions (Fig. 2-8,

A and B): 1) they contained significantly reduced percentages of $Ly6G^+$ neutrophils and $Ly6C^+$ monocytes; 2) a $F4/80^+$ macrophage phenotype was mainly $MHCII^+$ in *Il10*$^{-/-}$ kidneys, while being $MHCII^-$ in WT kidneys. Except for $MHCII^+F4/80^+$ macrophages, injection of IL-33 did not induce a change in percentages of myeloid cells either in WT or *Il10*$^{-/-}$ mice, including monocytes, neutrophils, $MHCII^-$ macrophages, and $CD11c^+F4/80^-MHCII^+$ DCs (Fig. 2-8, A and B). Finally, we assayed the killing activities of $Ly6C^+$ monocytes, $Ly6G^+$ neutrophils, and $Ly6C^-$ $Ly6G^-$ macrophage-enriched myeloid cells of infected WT or *Il10*$^{-/-}$ mice that received PBS or IL-33. *Il10*$^{-/-}$ neutrophils and macrophages were superior to WT counterparts in killing extracellular hypha-phase *C. albicans*, regardless of IL-33 injection (Fig. 2-8, C and D). Monocytes were equal in their killing activity in all the four groups (Fig. 2-8E). Treatment of *Il10*$^{-/-}$ neutrophils with opsonized *C. albicans* induced higher levels of ROS (Fig. 2-8F). *Il10*$^{-/-}$ neutrophils also killed extracellular *C. albicans* hyphae more effectively (Fig. 2-8G). However, ROS inhibitor diminished the killing activity of neutrophils of WT and *Il10*$^{-/-}$ mice to a similar base line (Fig. 2-8G), indicating that more active production of ROS is linked to effective fungal killing of *Il10*$^{-/-}$ neutrophils. Taken together, these results supported the hypothesis that IL-33 did not further augment the antifungal activity of *Il10*$^{-/-}$ neutrophils and macrophages.

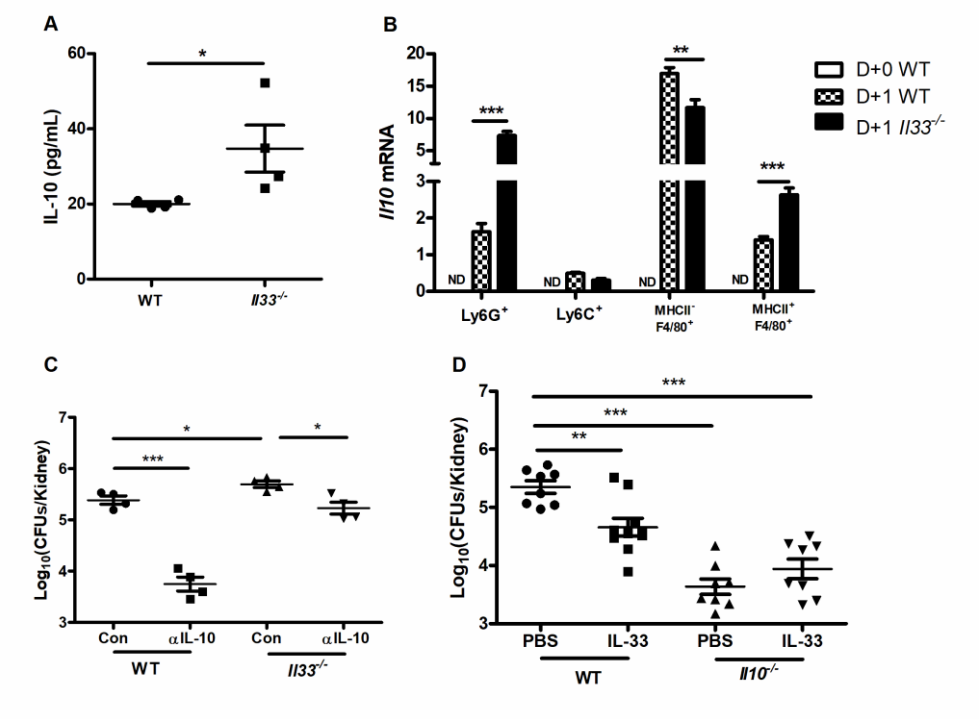

Figure 2-7 Depletion of IL-10 increases fungal clearance in an IL-33-independent manner.

WT and *Il33*$^{-/-}$ mice were infected with a lethal dose of *C. albicans*. (A) Level of IL-10 were measured from 1-d PI kidney lysate. Unpaired Student t-test was used (n = 4/group). (B) At 1-d PI, renal myeloid cells were sorted using FACSAria after enrichment with anti-CD11b-PE/anti-PE microbeads. Sorted cells are: CD45^{+}CD11b^{+}Ly6G^{+} neutrophils, CD45^{+}CD11b^{+}Ly6C^{+} monocytes, CD45^{+}CD11b^{+}Ly6G^{-}Ly6C^{-}F4/80^{+}MHCII^{+} macrophages, and CD45^{+}CD11b^{+}Ly6G^{-}Ly6C^{-}F4/80^{+}MHCII^{-} macrophages. RT-PCR was performed to measure levels of *Il10* mRNA (n = 6 mice/group). (C) WT and *Il33*$^{-/-}$ mice received anti-IL-10 neutralizing mAb (100 µg/mouse) immediately before infection with a lethal dose

of *C. albicans*. CFUs were counted in 3-d PI kidney lysate (n = 4/group). (D) WT and *Il10*$^{-/-}$ mice were injected with PBS or IL-33 1 d before and 1 d after infection. CFUs were counted in 3-d PI kidney lysate (n = 8/group). Two-way ANOVA test was used. Results are representative of 2-4 experiments and are presented as the mean ± SEM. ***P* < 0.01; ****P* < 0.001.

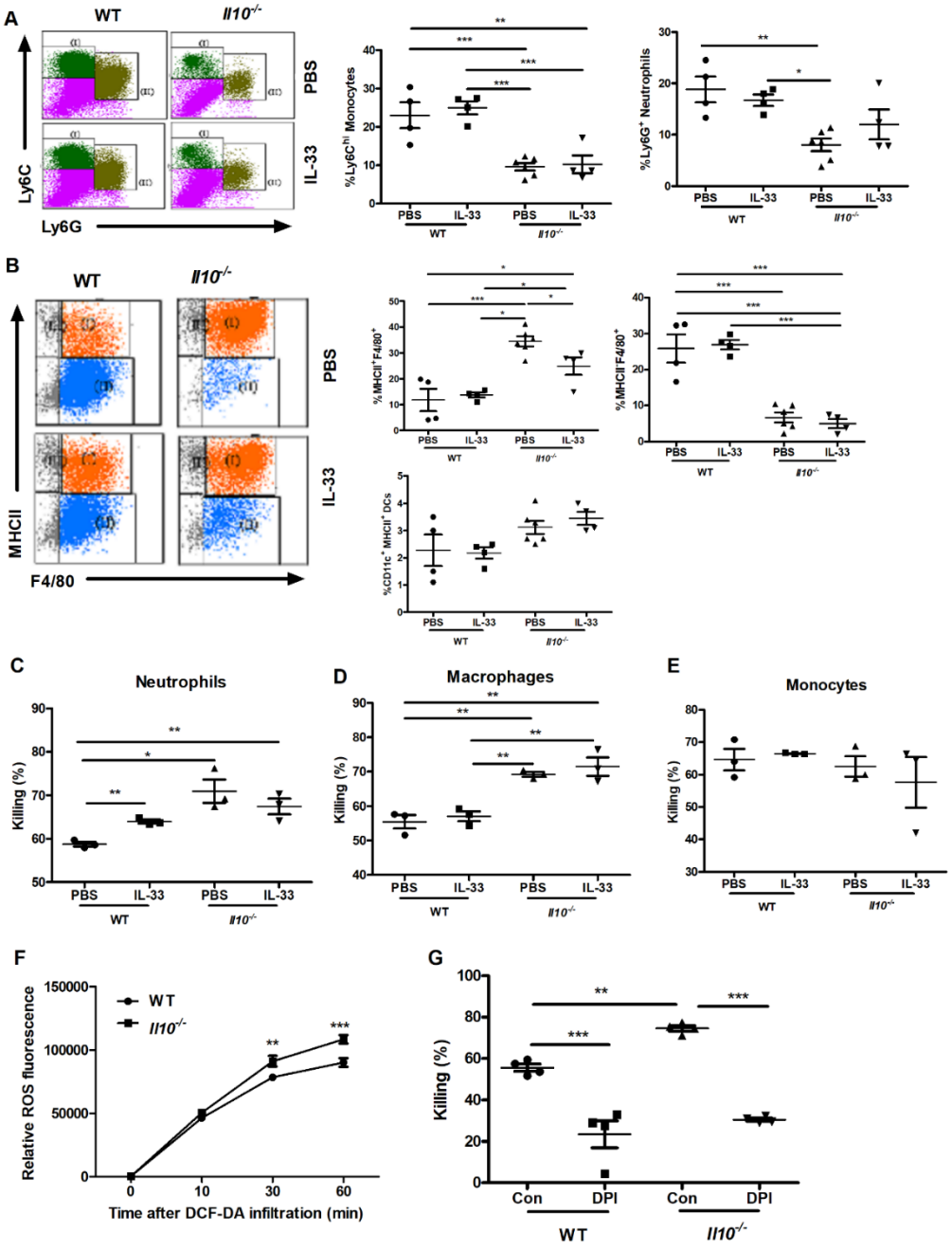

Figure 2-8 *Il10*$^{-/-}$ myeloid cells have a greater killing activity to *C. albicans*.

(A-E) WT and *Il10*$^{-/-}$ mice were injected with PBS or IL-33 from 1 d before and 1 d after infection with a lethal dose of *C. albicans*. Kidneys were harvested at 3-d

PI. Kidney cells were stained with anti-CD45, anti- MHCII, anti-CD11c, anti-F4/80, anti-CD11b, anti-Ly6C, anti- Ly6G mAbs. $Ly6C^+$ monocytes and $Ly6G^+$ neutrophils were gated from $CD45^+CD11b^+$ leukocytes (A). $MHCII^-F4/80^+$ macrophages, $MHCII^+F4/80^+$ macrophages, and $F4/80^-MHCII^+$ DCs were gated from $Ly6C^-Ly6G^-CD11c^+$ cells (B). Representative FACS plots and percentages of myeloid subsets in $CD45^+$ leukocytes were presented (n = 4-6/group). (C-E) $Ly6G^+$ neutrophils, $Ly6C^+$ monocytes, and $Ly6G^-Ly6C^-$ macrophage-enriched cells were sorted from 3-d PI kidneys. Killing assays were performed by adding *C. albicans* hyphae to sorted cells (n = 3/group). Two-way ANOVA test was used. (F) Isolated neutrophils were incubated with opsonized HK *C. albicans* hyphae for 1h. Levles of ROS were measured by FACS at the indicated times after treatment with DCF-DA. (G) Isolated neutrophils were treated with DPI 30 min before exposure to *C. albicans.* Killing percentages was calculated by counting CFUs in medium. Two-way ANOVA test was used (n = 4/group). Results are representative of 2-4 experiments and are presented as the mean ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

2.5 DISCUSSION

In this study, we characterized the mechanism of IL-33-mediated protection to systemic *C. albicans* infection. IL-33 is produced from early on after infection and takes part in two critical points of resistance processes. First, IL-33 action on $CD11b^+$ DCs is a critical point where the IL-33 → IL-23 → GM-CSF resistance axis is initiated. Second, IL-33 seems to suppress expression of *Il10*, an anti-fungal cytokine gene, in $Ly6G^+$ neutrophils and $MHCII^+F4/80^+$ macrophages. IL-10 is required for IL-33-mediated fungal clearance, as this is because the IL-33 → IL-23 → GM-CSF resistance axis functions only in an IL-10-replete renal microenvironment. $Il10^{-/-}$ neutrophils and $MHCII^+F4/80^+$ macrophages infiltrating into the infected kidney display a superior fungicidal activity which is not affected by IL-33 (Fig. 2-9). Considering cell numbers, $MHCII^+F4/80^+$ macrophages are likely to be the major effector cell in restricting fungal growth in $Il10^{-/-}$ mice. This interpretation may provide an adequate explanation for why IL-33 cannot co-op the IL-33 → IL-23 → GM-CSF axis in $Il10^{-/-}$ mice.

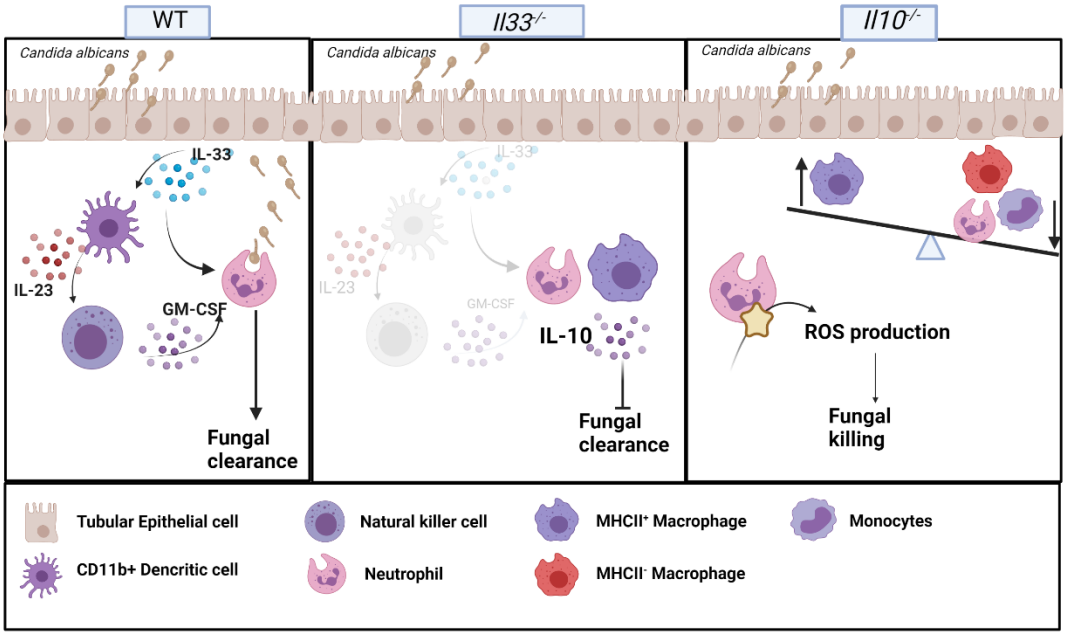

Figure 2-9. A schematic diagram presenting endogenous function of IL-33 in host defense against systemic *C. albicans* infection.

The releasing pattern of IL-33 from TECs after infection is a typical characteristic of alarmin [45]. Early target cells of IL-33 are myeloid cells including DCs (in this study), monocytes, macrophages and neutrophils [19, 42]. Our results indicate that IL-33 co-stimulates *C. albicans*-recognizing cell surface receptors to induce expression and of *Il23a* and *Il12b* in DCs (Fig. 2-4G). Although IL-33 has been shown to augment the phagocytic activity of neutrophils and macrophages directly [19, 42], our results from this study indicates that the IL-33 → IL-23 → GM-CSF axis lies in a central position for defense mechanisms against systemic *C. albicans* infection. If this axis is impaired, as seen in *Il33*$^{-/-}$ mice, excess fungal growth results in uncontrolled inflammation and fatal immunopathology (Fig. 2-6, D and E).

IL-33 promotes DC maturation. IL-33-activated DCs induce differentiation of Th2 cells [46], although the mechanism behind this is still unknown. They also drive naïve CD4^{+} T cells or Treg cells to differentiate into Th17 cells through release of IL-6 and IL-1β [47, 48]. However, little has been known regarding the involvement of IL-33-activated DCs in activating cells of the innate arm of immunity. In this study, IL-33 is shown to be required for production of IL-23 by DCs during *C. albicans* infection. Although IL-23 is a key player for Th17 expansion and maintenance, CD4^{+} T cells are dispensable for protection from systemic *C. albicans*

infection [49, 50]. Instead, the protective role of IL-23 is linked to the augmentation of neutrophils' phagocytosis through production of GM-CSF by NK cells. Another study has demonstrated that IL-23 protects the host from *C. albicans* infection by increasing neutrophils' survival [44]. As IL-23 action on myeloid cells occurs in an autocrine but not cell-intrinsic manner [44] and GM-CSF modulate a broad range of neutrophil activities, it is likely that the IL-33 → IL-23 → GM-CSF axis increase both survival and phagocytosis of neutrophils. However, a greater number of neutrophils infiltrate into the kidney of *Il33*$^{-/-}$ mice after *C. albicans* infection and more severe renal inflammation occurs in these mice (Fig. 2-6, D and E). This seems to indicate that impaired fungal clearance is linked directly to severe renal inflammation that overcomes impaired survival of neutrophils caused by decreased levels of IL-23 and GM-CSF in *Il33*$^{-/-}$ mice.

Ly6C^{+} inflammatory monocytes infiltrating into the kidney are generally believed to be polarized to M1 macrophages during the acute phase of renal inflammation [51]. *Il10*$^{-/-}$ macrophages were mostly MHCII^{+} in the infected kidney, whereas WT macrophages were MHCII^{-}. MHCII^{+}F4/80^{+} macrophages were superior in killing extracellular *C. albicans*. As MHCII^{+}F4/80^{+} macrophages are a major cell population that produces IL-10, they may have an anti-inflammatory feature, as seen in the intestine [52, 53]. Taken together, renal macrophages seem to

become potent phagocytes in the absence of IL-10 during *C. albicans* infection, presumably due to loss of negative feedback regulation for renal inflammation.

IL-33 seems to be unique in immune responses to systemic *C. albicans* infection, as it simultaneously induces sustained host mechanisms of defense and tolerance. In general, pro-inflammatory mediators released during acute inflammation results in severe tissue inflammation and repel pathogens. In addition, their negative feedback regulation for inflammation frequently sacrifices infection clearance function [54, 55]. A well-known example is type I IFNs. Similar to IL-33, IFN-β stimulates production of GM-CSF by NK cells through monocyte secretion of IL-15 [13, 56]. Thus, signaling via IL-33 and IFN-β is converged on NK cells to exert their protection mechanism to *C. albicans* infection. In summary, our results provide a novel insight into protective innate immune networks wherein IL-33 plays a central role.

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PUBLICATIONS

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