

Original Article



CCR5-mediated Recruitment of NK Cells to the Kidney Is a Critical Step for Host Defense to Systemic *Candida albicans* Infection

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

BMDC, bone marrow-derived dendritic cell; BUN, blood urea nitrogen; CCR, C-C

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⁺ dendritic cells (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 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.

Keywords: *Candida albicans*; CCR5; NK cells; IL-23; Dendritic cells; GM-CSF

INTRODUCTION

Candida 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

chemokine receptor; CFU, colony-forming unit; DC, dendritic cell; HK, heat-killed; ILC, innate lymphoid cell; MOI, multiplicity of infection; MPO, myeloperoxidase; PAS, periodic acid-Schiff; RPMI, Rosewell Park Memorial Institute; TEC, tubular epithelial cell; WT, wild-type.

Author Contributions

Conceptualization: Nguyen NZN, Tran VG, Kwon B; Data curation: Tran VG, Kang SW, Kim J, Kwon B; Formal analysis: Nguyen NZN, Tran VG, Kwon B; Funding acquisition: Cho HR, Kwon B; Investigation: Nguyen NZN, Tran VG, Lee S, Kim M, Kang SW, Kim J, Kim HJ; Project administration: Kwon B; Supervision: Cho HR, Kwon B; Validation: Lee JS, Kwon B; Writing - original draft: Nguyen NZN, Tran VG, Kwon B; Writing - review & editing: Nguyen NZN, Kwon B.

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 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 underlie 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 clearly 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, dendritic cells (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 define 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 recruitment of immune cells to the infection site. Although trafficking of Ly6C⁺ monocytes to the kidney is known to depend upon C-C chemokine receptor type 2 (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 (TECs) 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.

MATERIALS AND METHODS

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 Ulsan University Hospital (UUH-0120-03)).

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, Gyeongsan, Korea) at a final concentration of 5×10⁶ cells/ml and were further cultured on a rotary shaker at 37°C 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 1×10⁸ cells/ml before heat killing at 90°C for 30 min.

Experimental systemic candidiasis

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

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°C for 24 h. Colonies were counted, and results were expressed as \log_{10} (CFUs/organ).

Phagocytosis assay

Bone marrow cells were collected from femurs and tibiae, suspended in RPMI-1640 medium (Wegene) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml), 2 mM L-glutamine (GIBCO, Grand Island, NY, USA), and 50 μ M 2-mercaptoethanol. To purify neutrophils from bone marrow cells, anti-Ly6G magnetic activated cell sorting beads were used according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified neutrophils were resuspended in RPMI-1640 medium and adjusted to a concentration of 2×10^6 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 (HK) *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*.

Diff-Quit staining of neutrophils

Purified neutrophils were resuspended in PBS. Specimens were prepared using the Cytospin™ 4 Cyto centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The centrifuged specimens were then stained with a Diff-Quit™ staining set (Siemens Healthcare Diagnostics, Deerfield, IL, USA) and examined under microscope.

Histology

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

Depletion of neutrophils and NK cells

Neutrophil depletion was achieved by injecting 200 μ g anti-Gr-1 (RB6-8C5) mAb intraperitoneally into mice 2 days before *C. albicans* infection. For NK cell depletion, anti-NK1.1 (PK136; eBioscience, San Diego, CA, USA) mAb was injected 1 day before and 2 days after infection.

Real-time RT-PCR

Total RNA was extracted from kidneys or cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Whole tissues were homogenized with a TissueLyzer tissue homogenizer (QUAGEN, Caldwell, NJ, USA), and cDNA was prepared with SuperScript reverse transcription (Invitrogen). Real-Time PCR was performed using SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems,

Foster City, CA, USA). The primers used in the experiments were as follows. *Ccr5*: 5'-AGATCTCTGCAGCTGCCCTCA-3' (forward) and 5'-GGAGCACCTGCTGCTGGTGTAG-3' (reverse); *Il23a*: 5'-CCAGCAGCTCTCTCG GAATC-3' (forward) and 5'-TCATA GTCCCGCTGGTGC-3' (reverse); *Il12b*: 5'-CCTGGTTTGCCATCGTTTTG-3' (forward) and 5'-TCAGAGTCTCGCCTCCTTTGTG-3' (reverse); *Actb*: 5'-CATTACTGCTCTGGCTCCTACC-3' (forward) and 5'-GACTCATCGTACTCCTGCTTGC-3' (reverse). All PCRs were performed in triplicate and normalized to internal control *Actb* mRNA. Relative expression was presented using the $2^{-\Delta\Delta CT}$ method.

Measurement of cytokines, chemokines, and myeloperoxidase

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

Preparation of kidney cells

Kidneys were perfused, minced, and placed in DMEM (Life Technologies, Carlsbad, CA, USA) containing 1 mg/ml collagenase IA and 100 ng/ml RNase I (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Digested kidney tissues were passed through a 40- μ m cell strainer (BD Falcon, San Diego, CA, USA), and the cell suspension obtained was centrifuged at 300 \times g for 10 min. Cells were washed in PBS containing 2% BSA, suspended in 36% Percoll (Amersham Pharmacia Biotech, Little Chalfont, UK), and gently overlaid onto 72% Percoll. After centrifugation at 900 \times 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).

Flow cytometry

The following FITC-, PE-, PE-Cy5, PerCP- or allophycocyanin-conjugated 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, TCR β chain, and Rat IgG2a κ chain. Prepared cells were blocked with 2.4G2 mAb in a staining buffer at 4°C for 20 min. Before washing twice with a staining buffer, cells were incubated with the relevant Ab at 4°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 mAb 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, Ashland, OR, USA).

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, Hayward, CA, USA).

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 μ M 2-mercaptoethanol and Flt3L (150 ng/ml), were distributed into 6-well plates at a density of 1×10^6 cells/well for 7 days. At day 7, GM-CSF (10 ng/ml) was added, and cells were harvested at day 9. Cells were stimulated with GM-CSF (10 ng/ml) and HK *Candida albicans* hyphae (MOI=10).

Statistical analysis

All data were analyzed using GraphPad Prism Software version 4 (GraphPad Software, Inc., San Diego, CA, USA). Unpaired Student *t*-test or 1- or 2-way ANOVA with *post hoc* 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.

RESULTS

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. 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. 1B). The percentages of CCR5⁺ macrophages were significantly greater at either day 1 or day 3 after infection (Fig. 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. 1C).

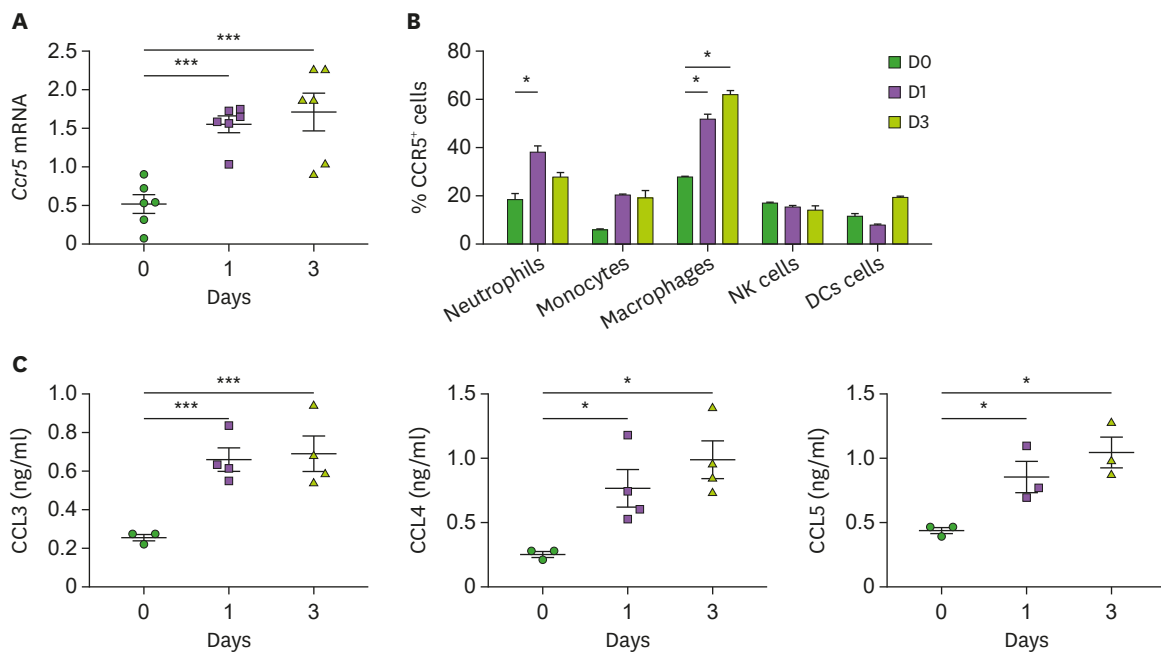


Figure 1. Upregulation of CCR5 and its ligands in the kidney during systemic candidiasis. C57BL/6 mice were intravenously injected with 3×10^5 CFUs of *C. albicans* and the kidneys were harvested at the indicated time points. (A) Levels of *Ccr5* mRNAs in the kidneys were determined by RT-PCR (n=6 kidneys/group). (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*<0.001.

***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 wild-type (WT) mice (Fig. 2A). *Ccr5*^{-/-} mice also experienced more severe body weight loss (Fig. 2B), and their kidneys appeared less pink, more swollen (Fig. 2C), and were significantly heavier than WT kidneys at day 3 post-infection (Fig. 2D). Gross observations also indicated that *Ccr5*^{-/-} kidneys had many more distinguishable nodules than WT kidneys (Fig. 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. 2E). Histopathological analysis showed that *Ccr5*^{-/-} kidneys had numerous multifocal areas of abscess formation as compared to WT kidneys (Fig. 2F). In particular, PAS staining revealed more prominent hyphae within abscesses of *Ccr5*^{-/-} kidneys compared with those of WT mice (Fig. 2F).

The data presented in Fig. 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 (Fig. 3A-D). Consistent with these results, there was greater infiltration of CD45⁺ leukocytes, predominantly CD11b⁺Ly6G⁺ neutrophils, into infected *Ccr5*^{-/-} kidneys (Fig. 3E and F). In addition, higher levels of serum creatinine and BUN indicated that *Ccr5*^{-/-} mice had more severe impairment of kidney

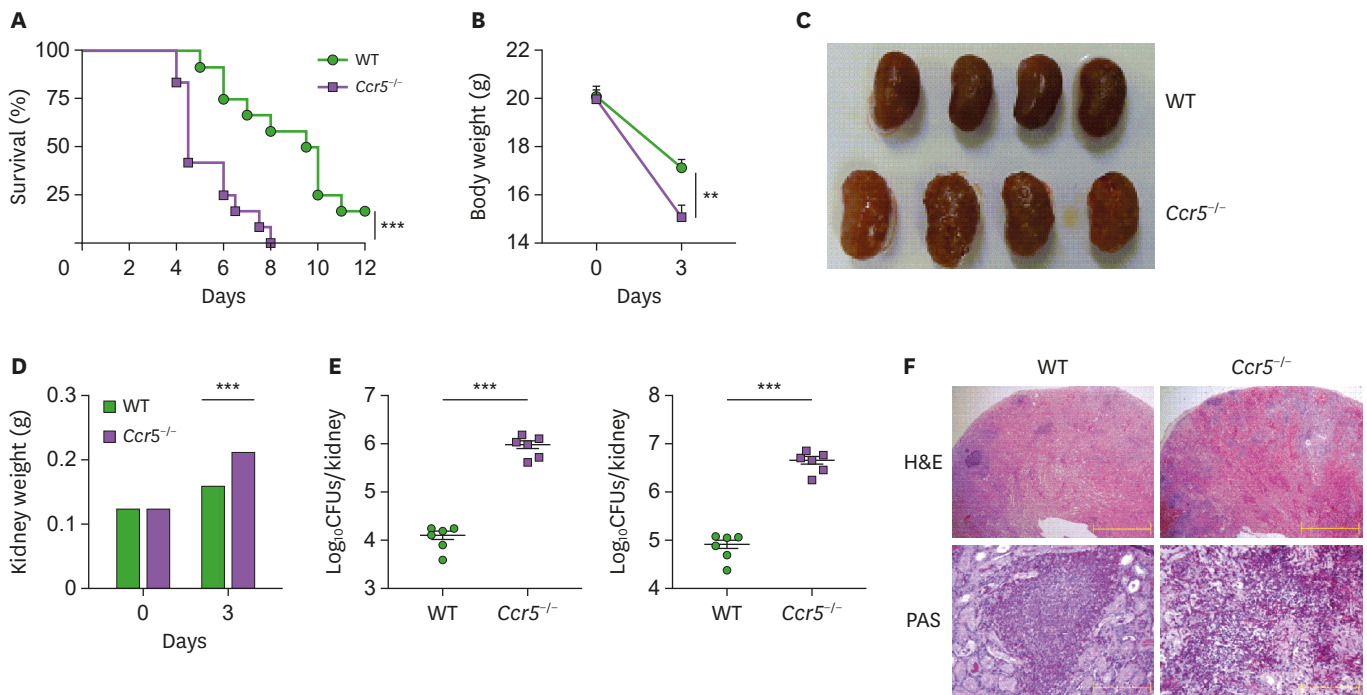


Figure 2. CCR5^{-/-} mice are susceptible to systemic candidiasis. WT and CCR5^{-/-} mice were intravenously injected with 3×10⁵ CFUs of *C. albicans*. (A) Survival curves (n=12 mice/group). The log-rank test was used. (B) Changes in body weight (n=4 or 5 mice/group). Unpaired Student *t*-test was used. (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*<0.01; ****p*<0.001.

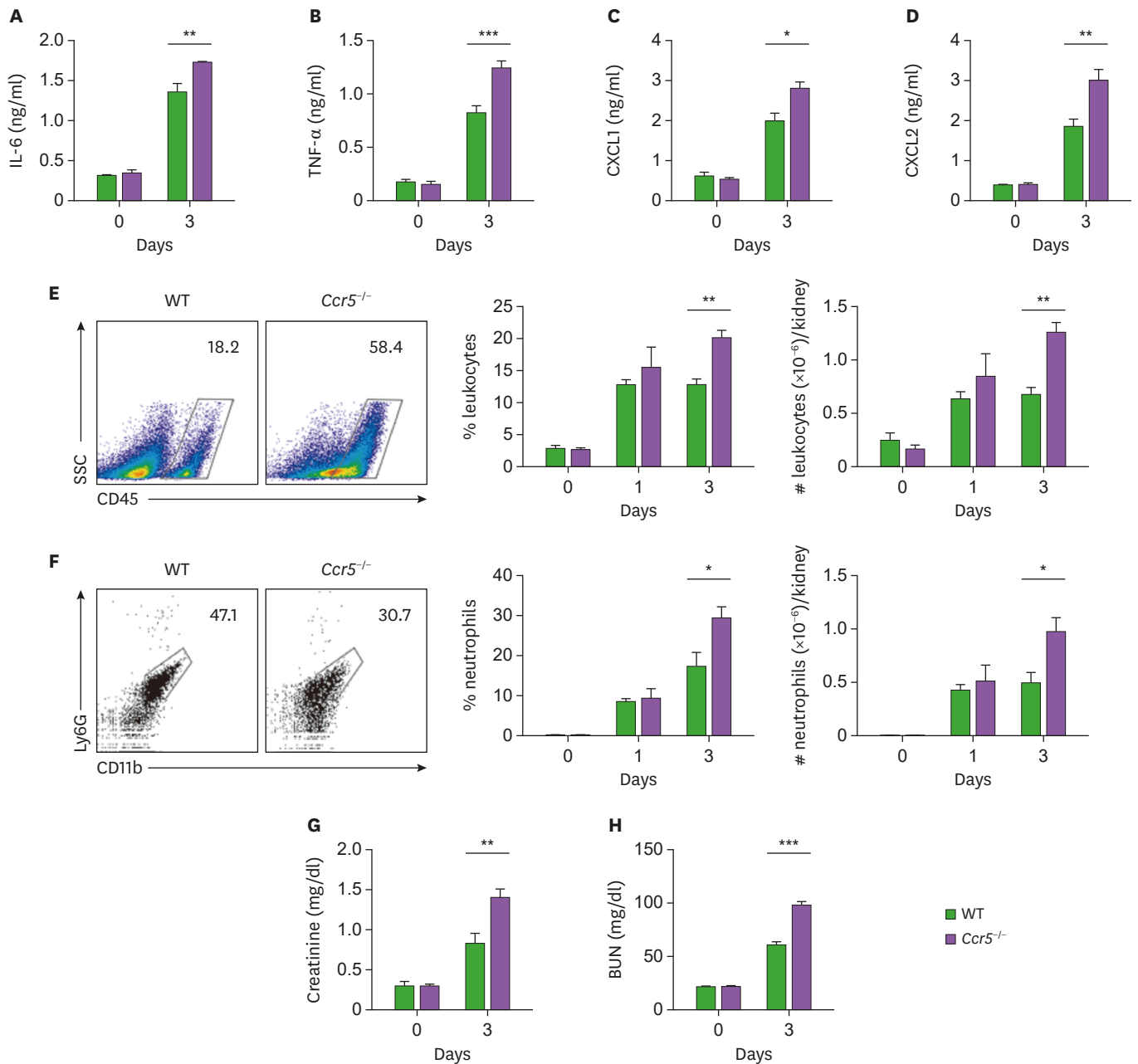


Figure 3. *Ccr5*^{-/-} mice have increased inflammatory responses to systemic *C. albicans* infection. WT and *Ccr5*^{-/-} mice were intravenously injected with 3×10⁵ CFUs of *C. albicans*. (A-D) Levels of IL-6 (A), TNF-α (B), CXCL1 (C), and CXCL2 (D) in kidney lysates at day 3 post-infection (n=4 or 6 kidneys/group). (E, F) Kidney cells were isolated, and stained with anti-CD45, anti-CD11b, and anti-Ly6G Abs. (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, 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.

function at day 3 post-infection (**Fig. 3G and H**). Taken together, our results suggest that uncontrolled fungal proliferation in *Ccr5*^{-/-} mice might cause severe renal inflammation linked to fatal immunopathology.

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 azurophilic granules necessary for generation of ROS, a key component of the neutrophils' killing arsenal (13,18) (Fig. 4A). Taking their numbers into account, the concentration of MPO per cell was approximately 2-fold lower compared to WT kidney neutrophils (Fig. 4B). Depletion of neutrophils using anti-Gr-1 Ab completely abrogated differences in survival and fungal clearance between *Ccr5*^{-/-} and WT mice (Fig. 4C 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. albicans* infection. Either WT or *Ccr5*^{-/-} neutrophils isolated from the bone marrow had a similar premature neutrophil's nuclear morphology (Fig. 4E). Treatment with GM-CSF induced nuclear segmentation of either type of neutrophils to a similar extent (Fig. 4E). Consistent with this result, there was no difference in their phagocytic activity and MPO production after treatment with GM-CSF (Fig. 4F and G). However, it seems that *Ccr5*^{-/-} bone marrow neutrophils have a lower phagocytic capacity in steady state (Fig. 4F).

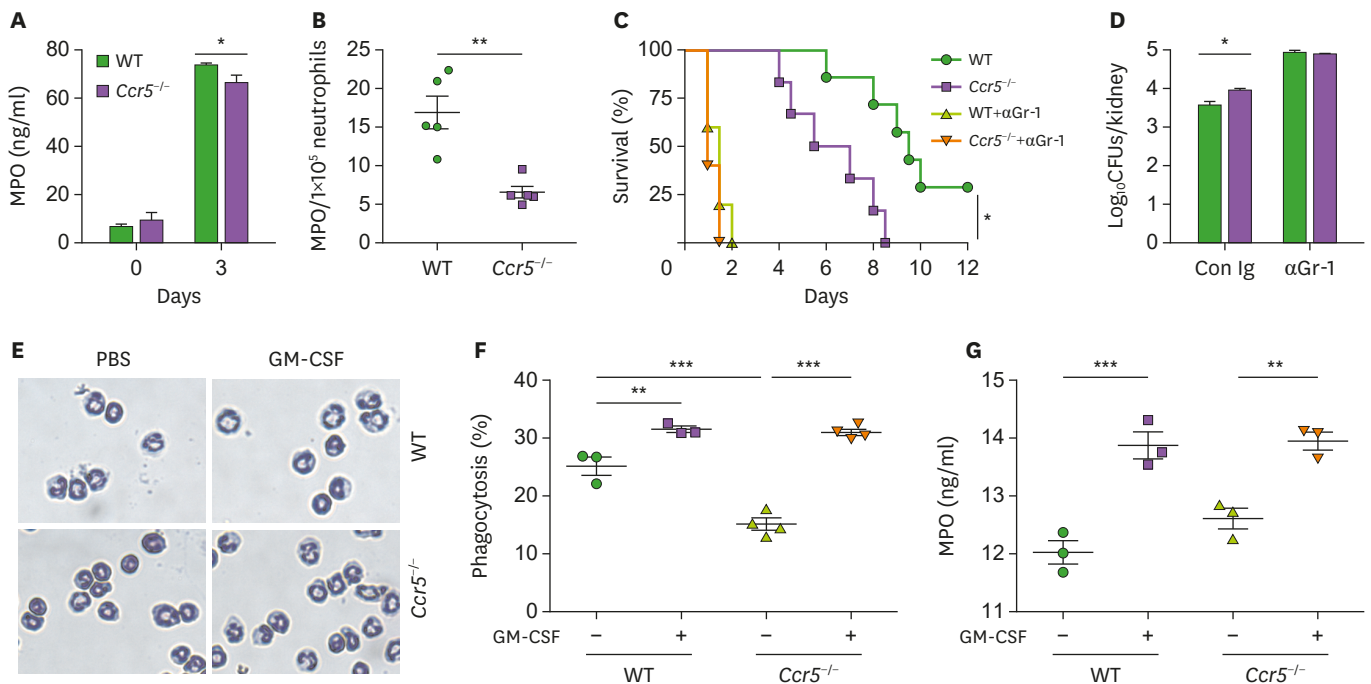


Figure 4. Impaired recruitment of NK cells to the kidney results in characteristic phenotypic changes of *Ccr5*^{-/-} mice after *C. albicans* infection. (A, B) Levels of MPO in kidney lysates obtained at day 3 post-infection. Data are presented as a concentration per kidney or normalized to 1×10⁵ renal neutrophils (n=4 or 5 kidneys/group). (C, D) WT mice were administered anti-Gr-1 mAb (200 µg/mouse) or control IgG Ab 2 days 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.

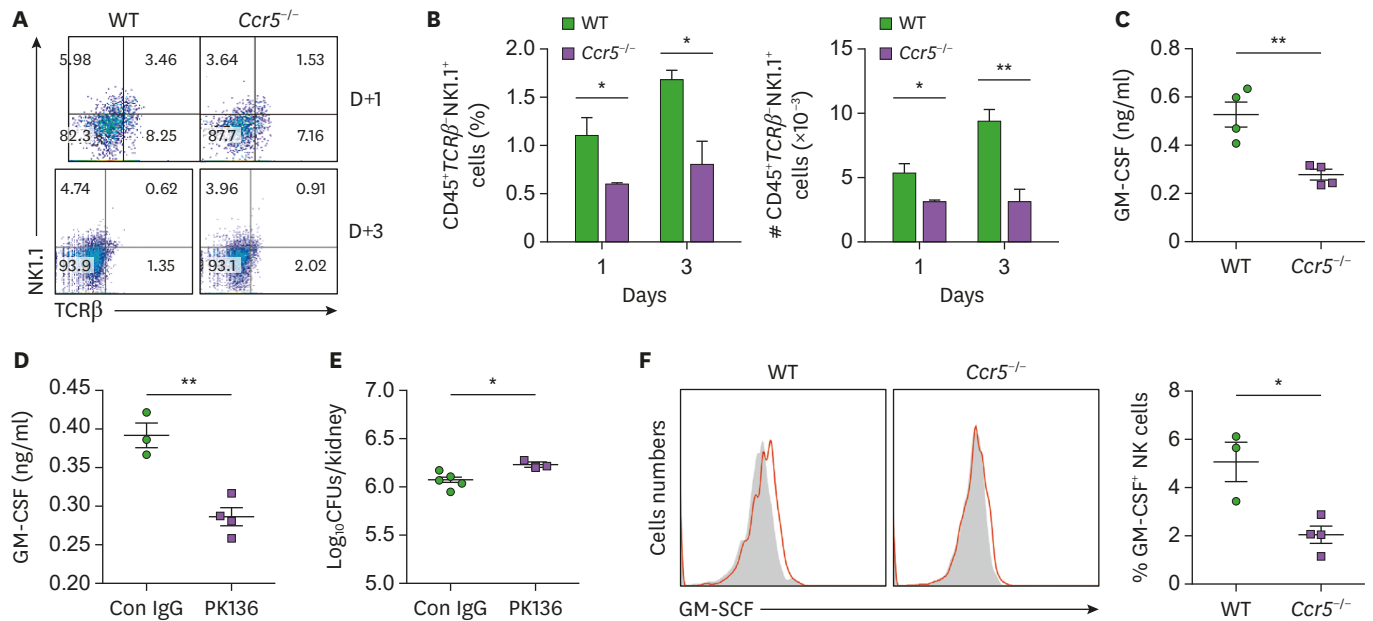


Figure 5. Impaired recruitment of NK cells and their GM-CSF production in *Ccr5*^{-/-} mice. (A, B) Renal cells were stained with anti-CD45, anti-TCRβ, and anti-NK1.1Abs. (A) CD45⁺ leukocytes were gated and analyzed for expression of NK1.1 and TCRβ. Representative FACS dot plots and percentages of CD45⁺TCRβ⁺NK1.1⁺ NK cells in leukocytes were presented. (B) Absolute numbers of CD45⁺TCRβ⁺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, E) Mice were depleted of NK cells using anti-NK1.1 Ab (PK136). Kidneys 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-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.

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. 5A 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. 5C). We further showed that depletion of NK cells in WT mice reduced levels of renal GM-CSF (Fig. 5D) and fungal clearance to some extent (Fig. 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. 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. 6A) but in our hands, exogenous GM-CSF didn't reduce 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. 6B 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.

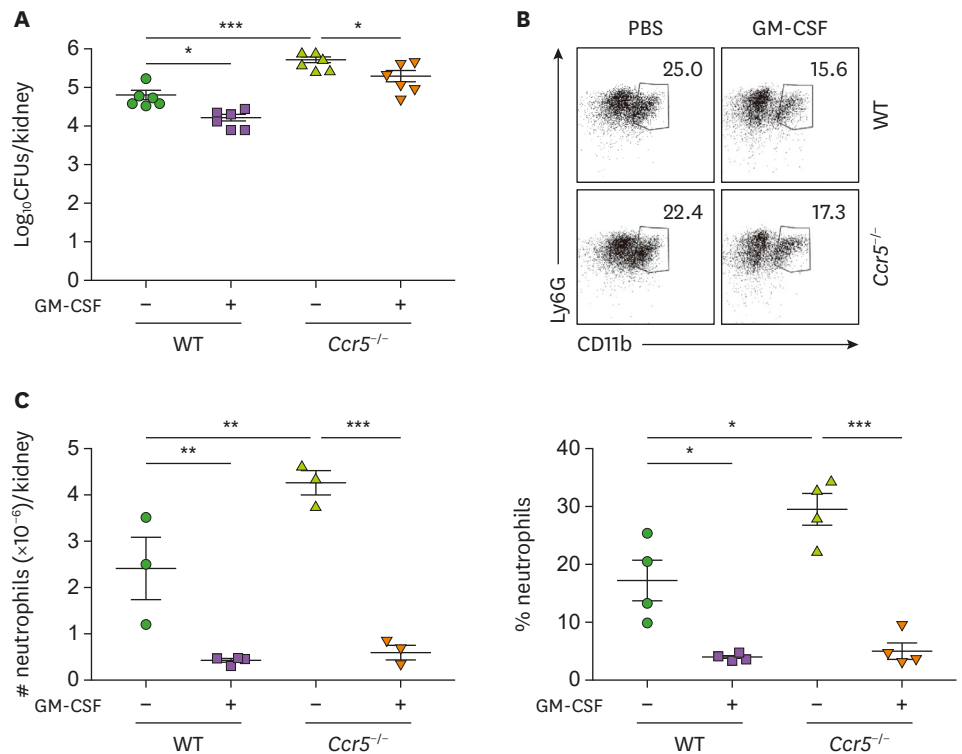


Figure 6. GM-CSF augments fungal clearance but reduces renal inflammation. Mice were intraperitoneally injected with GM-CSF (5 μ g/mouse) 24 h and 36 h after infection. (A) Fungal CFUs were counted in kidneys at day 3 post-infection (n=6 kidneys/group). (B) Representative FACS dot plots for CD45⁺CD11b⁺Ly6G⁺ 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 \pm SEM. *p<0.05; **p<0.01; ***p<0.001.

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-day post-infection kidneys (Fig. 7A-C). We noticed that expression of *Il12b* was reduced in *Ccr5*^{-/-} mouse kidneys to larger extent than that of *Il23a* (Fig. 7A 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. 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 HK *C. albicans* hyphae. Combined treatment had a synergistic effect on IL-23 production compared to GM-CSF or HK *C. albicans* alone (Fig. 7E). There was no difference in renal DC numbers between naïve WT and *Ccr5*^{-/-} mice (Fig. 7F). Thus, GM-CSF can increase its own production indirectly through DC stimulation of IL-23 production during systemic *C. albicans* infection.

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

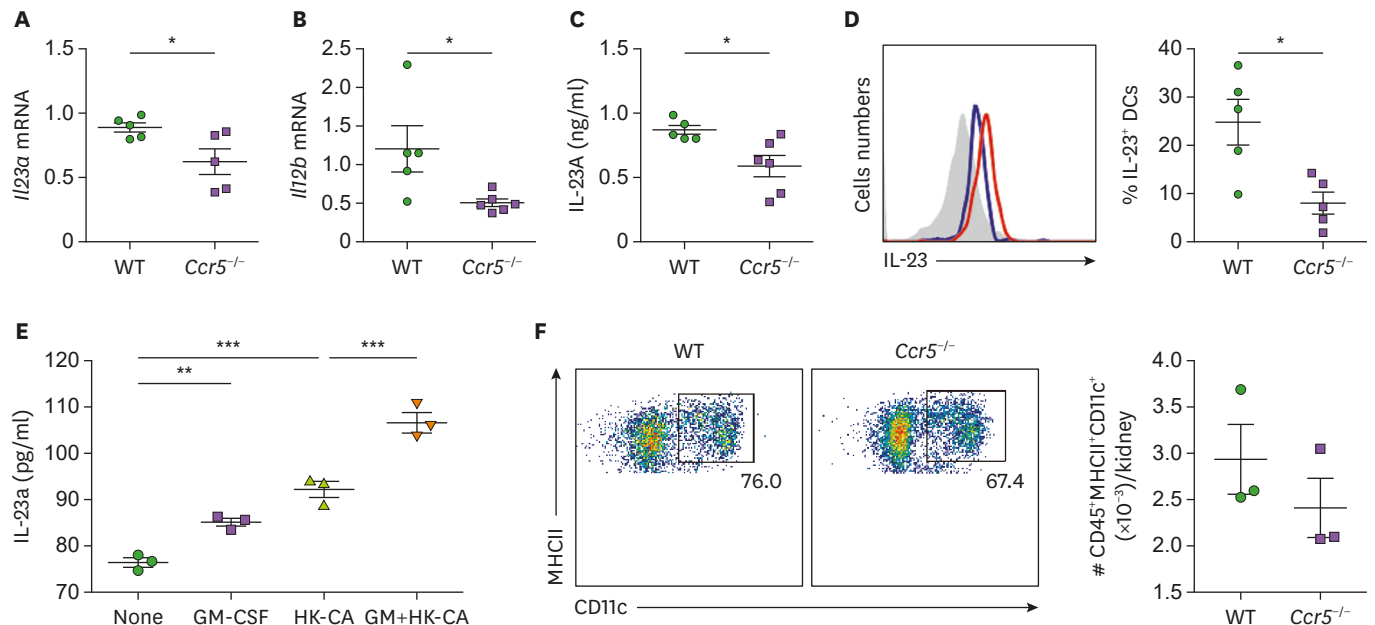


Figure 7. GM-CSF enhances IL-23 production by DCs. (A-D) Kidneys were harvested at day 3 post-infection and their expression of IL-23 was investigated. (A, B) Transcriptional levels of *Il23a* and *Il12b* (n=4 or 5 kidneys/group). (C) Concentrations of renal IL-23 protein (n=5 or 6 kidneys/group). (D) FACS histograms for IL-23A intracellular staining in renal CD45⁺MHCII⁺CD11c⁺ DCs and percentages of IL-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-killed (*HK*) *C. albicans* for 24 h. Concentrations of IL-23 in culture supernatants 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.

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* infection. Although the percent of CCR5-expressing renal NK cells was lower compared to neutrophils and macrophages (Fig. 1B), CCR5 was highly specific for recruiting NK cells to the kidney (Fig. 5A 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. 7).

NK cells are a particularly important regulator for acute renal inflammation, regardless of its causes. During ischemia-reperfusion kidney injury, 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 (21). 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 2 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 *Cx3cr1* 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 NKT 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. 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. 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. 8). Nonetheless, injection of GM-CSF in *Ccr5*^{-/-} mice did not induce fungal clearance as effectively as in WT mice (Fig. 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.

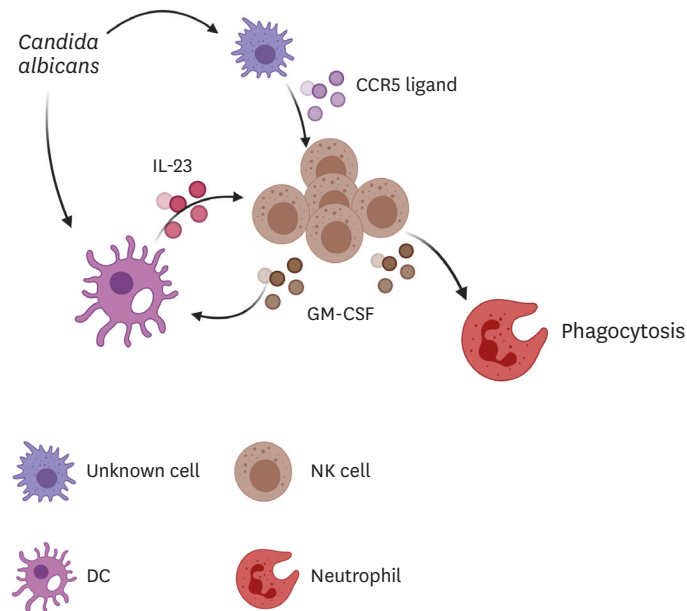


Figure 8. A schematic diagram showing CCR5 function in host defense against systemic *C. albicans* infection. CCR5 ligands are released by unidentified cells in response to *C. albicans* infection and induce recruitment of NK cells into the kidney. On the other hand, *C. albicans* triggers secretion 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.

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