Inflammatory Monocytes Mediate Early and Organ-Specific Innate Defense During Systemic Candidiasis

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Candida albicans is a commensal fungus that can cause systemic disease in patients with breaches in mucosal integrity, indwelling catheters, and defects in phagocyte function. Although circulating human and murine monocytes bind *C. albicans* and promote inflammation, it remains unclear whether C-C chemokine receptor 2 (CCR2)- and Ly6C-expressing inflammatory monocytes exert a protective or a deleterious function during systemic infection. During murine systemic candidiasis, interruption of CCR2-dependent inflammatory monocyte trafficking into infected kidneys impaired fungal clearance and decreased murine survival. Depletion of CCR2-expressing cells led to uncontrolled fungal growth in the kidneys and brain and demonstrated an essential antifungal role for inflammatory monocytes and their tissue-resident derivatives in the first 48 hours post-infection. Adoptive transfer of purified inflammatory monocytes in depleted hosts reversed the defect in fungal clearance to a substantial extent, indicating a compartmentally and temporally restricted protective function that can be transferred to enhance systemic innate antifungal immunity.

Keywords. *Candida*; candidiasis; fungus; sepsis; host defense; innate; kidney; renal; brain; yeast; monocyte; chemokine; CCR2; inflammatory; mortality; transfer; graft.

Candida species are a common cause of bloodstream infections and account for 8%–15% of sepsis cases in the United States [1, 2]. The incidence of invasive candidiasis is rising due to advances in medical technology (eg, indwelling catheters, endovascular grafts, and prostheses) and due to increasing numbers of patients with impaired mucosal integrity and defects in innate immune function. The kidney represents the major target organ during candidemia, leading to renal failure as a primary pathophysiologic manifestation of *Candida* sepsis [3].

In humans and mice, 2 circulating monocyte subsets can be distinguished by chemokine receptor and

adhesion molecule expression [4, 5]. Murine CCR2⁺ Ly6C^{hi} (inflammatory) monocytes represent counterparts to human CD14⁺⁺CD16⁻ monocytes, whereas murine CCR2^{lo}Ly6C^{lo} (resident) monocytes represent counterparts to CD14⁺CD16⁺ monocytes [6]. Although human CD14⁺⁺CD16⁺ monocytes display robust proinflammatory cytokine responses and anticandidal activity in vitro [7], their role during primary challenge remains undefined [8]. In candidemic mice, Ly6C^{hi} inflammatory monocytes rapidly infiltrate infected kidneys [9]. However, type I interferon [10] and CCR1 signaling [11] have been linked to pathologic and dysregulated immune responses associated with inflammatory monocytes and neutrophils, respectively. Thus, it remains unclear whether monocytes play a protective or detrimental role during systemic candidiasis.

Herein, we investigated the specific contribution and temporal requirements of inflammatory monocytes during murine disseminated candidiasis. By harnessing 2 transgenic mouse strains that (1) label inflammatory monocytes with green fluorescent protein (GFP; CCR2 reporter mice) and (2) enable systemic depletion of inflammatory monocytes and their derivatives (CCR2

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depleter mice) [12], we determined that inflammatory monocytes are essential in the first 48 hours postinfection to control fungal growth in the kidneys and brain, but not at other sites. Our studies reveal that inflammatory monocytes and their derivatives mediate an essential protective function at the onset of systemic candidiasis and demonstrate that adoptive transfer can provide a benefit in depleted mice.

MATERIALS AND METHODS

Mice

CCR2 reporter (C2R; CD45.1⁺C57BL/6-BAC-Tg[pCCR2-GFP]) [12, 13] and CCR2 depleter (C2D; CD45.2⁺C57BL/6-BAC-Tg[pCCR2-DTR-2A-CFP]) [12], and C57BL/6J (Jackson Laboratories) mice were bred in the Comparative Medicine Shared Resource Facility at Fred Hutchinson Cancer Research Center (FHCRC) under specific pathogen-free conditions. For data shown in Figure 1 and Figure 2, CCR2^(-/-) mice (C2K; Jackson Laboratories) and C2R mice were crossed, yielding CCR2^(-/-) C2R (C2KR) mice that are deficient in CCR2 and express GFP under control of the BAC-encoded CCR2 promoter. For data shown in Figure 3A, the F1 progeny from a cross between C2R and C2D mice were used, resulting in littermates that express the DTR and the GFP transgenes (C2DR mice) and in littermates that express GFP, but not the DTR transgene. Experiments were conducted with sex- and age-matched mice and were approved by the FHCRC Institutional Animal Care and Use Committee.

Fungal Strain and Infection Model

The *Candida albicans* strain SC5314 was grown in yeast extract/peptone/glucose medium at 30°C for 16–18 hours. Mice were infected with 10⁵ blastoconidia via the lateral tail vein. Mice were treated with 10 ng/g body weight diphtheria toxin (DT) intraperitoneally at indicated time points. Mice were monitored for morbidity and euthanized when they showed severe signs of pain, distress, or inability to feed. For colony-forming unit (CFU) determination, the kidneys, brain, spleen, and liver were collected, homogenized in phosphate-buffered saline (PBS) and 0.025% Tween-20, and plated on yeast extract/peptone/dextrose agar plates.

Generation of Single-Cell Organ Suspensions and Flow Cytometry

Cell suspensions were generated by dissociating kidneys through a 100-μm mesh and digesting tissue fragments in PBS, 5% fetal calf serum (FCS), 2 mg/mL collagenase type IV (Worthington), and 20 U/mL DNase I for 30 minutes at 37°C. Samples were lysed of red blood cells (RBCs) and spun over a 40% Percoll cushion (1000g), and the pellet was washed and resuspended in 2 mL PBS and 5% FCS. The number of CD45⁺ leukocytes in each sample was quantified using phycoerythrin-conjugated microspheres (Spherotech). Bone marrow cells were flushed from tibias and

femurs, lysed of RBCs, and resuspended in PBS and 5% FCS, and enumerated using a Beckman Coulter Z2 particle counter.

Cell suspensions were stained with the antibodies anti-Ly6G (1A8), anti-F4/80 (BM8), anti-CD11c (HL3), anti-CD11b (M1-70), anti-Ly6C (AL21), anti-Ly6B.2 (7/4), anti-MHC class II (M5/114.15.2), anti-Ter-119 (TER-119), anti-CD45.1 (A20), and anti-CD45 (30F11). For some experiments, anti-CD3 (145-2C11), anti-CD19 (1D3), anti-CD11c (HL3), anti-NK1.1 (PK136), anti-CD8 (53-6.7), and anti-CD49b (DX5) were used. Flow cytometry was performed on a BD LSR II and cell sorting was performed on a BD FACS Aria II (BD Biosciences).

Histopathology

Perfused kidneys were collected 72 or 144 hours postinfection, fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 2 μm (for hematoxylin and eosin staining) or 4 μm (for periodic acid-Schiff [PAS] staining and methyl green counterstaining) thickness. Images were captured from whole-slide images acquired with the Aperio ScanScope AT using ×20 and ×40 objectives.

In Vitro Candida Killing Assay

Candida albicans (5 × 10⁵ blastoconidia) was incubated with bone marrow (BM) monocytes (1 × 10⁶) in 0.2 mL RP10 (RPMI, 10% FCS, 5 mM HEPES, 1.1 mM L-glutamine, 0.5 U/mL penicillin, 0.5 μg/mL gentamicin, 50 μg/mL streptomycin, and 50 μM 2-mercaptoethanol) in triplicate in a 96-well microplate for 2 hours and 45 minutes at 37°C. Monocytes were lysed with 0.02% Triton-X-100 in water and washed with PBS, and surviving *C. albicans* cells were incubated with 0.2 mL Alamar Blue (diluted 1:40 in PBS) for 16 hours at 37°C prior to absorbance measurement (OD 570 nm, 600 nm) on a Versamax plate reader. A standard curve to determine *C. albicans* killing was constructed by measuring the absorbance of a serial 2-fold dilution of *C. albicans* cells (15.625 × 10³–5 × 10⁵) incubated without cells. *Candida albicans* killing (%) was measured as follows: [1–(number of *C. albicans* incubated with monocytes)/(number of *C. albicans* incubated without monocytes)] × 100.

Statistical Analysis

A Mann–Whitney *U* test (for 2 group comparisons) or a Kruskal–Wallis 1-way analysis of variance followed by Dunn multiple comparison tests (for 3 group comparisons) was used for analyses. Survival data were analyzed by log-rank test. All analyses were performed with GraphPad Prism, version 5.0c.

RESULTS

CCR2 and Renal Inflammatory Monocyte Influx During Candidiasis

To characterize the role of CCR2 on renal cellular influx during systemic candidiasis, renal cell suspensions from naive and *C. albicans*-infected CCR2 reporter (C2R) mice were enumerated

Figure 1. CCR2 and impaired renal monocyte trafficking during systemic candidiasis. *A–F*, CCR2 reporter (C2R) and C2R mice crossed to the CCR2^(-/-) background (C2KR) were infected with 10⁵ *Candida albicans* blastoconidia intravenously and euthanized at the indicated time points for flow cytometric and colony-forming unit analysis of the indicated organs. *A–D*, Representative flow cytometry plots are gated on CD45⁺ renal leukocytes and analyzed for CD11b, Ly6C, CCR2 (GFP), and Ly6G expression. *A* and *B*, Frequency of renal CCR2⁺CD11b⁺Ly6C^{hi}Ly6G⁻ monocytes (Mo) and CCR2⁻CD11b⁺Ly6C^{hi}Ly6G⁺ neutrophils (Ne) are indicated in naive (*A* and *B*) and in day 1 (*C* and *D*). *C. albicans*-infected C2R (*A* and *C*) and C2KR (*B* and *D*) mice. *E* and *F*, Bar graphs show the mean (+SEM) of renal (*E*) and bone marrow (BM) (*F*) inflammatory monocytes at the indicated time points postinfection in C2R and C2KR mice. Data are representative from or pooled from >5 (*A–D*) or 3 (*E* and *F*) experiments and contain 7–12 mice per time point. **P* < .05, ***P* < .01 by Mann–Whitney *U* test.

and examined by flow cytometry. CD11b⁺Ly6C^{hi} inflammatory monocytes (Mo) and CD11b⁺Ly6C^{hi}Ly6G⁺ neutrophils (Ne) each constituted <5% of CD45⁺ renal leukocytes in naive C2R mice (Figure 1*A*). The frequency and absolute number of inflammatory monocytes was reduced in the kidneys of naive CCR2^(-/-) C2R hosts (C2KR mice; Figure 1*B* and 1*E*), as expected from previous studies [14, 15]. Beyond inflammatory monocytes, GFP expression was noted in a subset of CD11b⁺ Ly6C^{variable} renal mononuclear phagocytes (Supplementary Figure 1*A* and 1*B*); these include F4/80⁺ macrophages (approximately 35% GFP⁺; Supplementary Figure 1*C*), CD11c⁺MHC class II⁺ DCs (approximately 80% GFP⁺; Supplementary Figure 1*C*), and NK1.1⁺ cells (approximately 75% GFP⁺; Supplementary Figure 2*A–B*). Unlike inflammatory monocytes, these renal mononuclear cell populations were not significantly reduced in naive CCR2^(-/-) hosts (Supplementary Figures 1*D* and 2*C*).

Systemic candidiasis induced a rapid renal influx of CD11b⁺ Ly6C^{hi} cells and the fluorescent CCR2 reporter was used to distinguish GFP⁻Ly6G⁺ neutrophils and GFP⁺Ly6G⁻ inflammatory monocytes (Figure 1*C*). At 48 hours postinfection, inflammatory monocytes were the dominant GFP⁺ leukocytes

in the kidneys of infected mice (Figure 1*C* and Supplementary Figures 1*E* and 2*D*).

The number of inflammatory monocytes was severely reduced in the kidneys of infected C2KR reporter mice (Figure 1*D*), indicating that CCR2-dependent signals direct inflammatory monocyte trafficking during systemic candidiasis. Although CCR2-independent renal monocyte recruitment was observed starting at day 2 postinfection, this pathway accounted for only 10%–20% of renal inflammatory monocyte influx, given the >80% reduction observed in C2KR mice at all time points examined (Figure 1*E*). In C2KR mice, inflammatory monocytes were retained in the bone marrow of infected mice (Figure 1*F*).

CCR2 and Fungal Clearance During Candidiasis

To determine the impact of CCR2-dependent trafficking steps on the outcome of infection, the renal fungal burden was determined in C2KR mice and compared to C2R mice. Two days postinfection, C2KR mice had a higher renal fungal burden than C2R mice, linking CCR2-dependent inflammatory monocyte trafficking to a defect in renal antifungal activity (Figure 2*A*). At this time point, renal neutrophil influx was similar in CCR2-deficient

Figure 2. Loss of CCR2-dependent inflammatory monocyte trafficking and murine susceptibility to systemic candidiasis. **A**, Plots show renal colony-forming units (CFUs) in C2R (black circles) and C2KR (white circles) mice at day 2 postinfection. The black lines indicate the median values for each group. ** $P < 0.01$ by Mann–Whitney U test. **B**, Bar graphs show the mean (+SEM) number of renal neutrophils at day 2 postinfection in C2R (black bars) and C2KR mice (white bars). Abbreviation: ns, not significant. **C**, Kaplan–Meier survival of C2K mice (CCR2^{−/−}; white circles; $n = 9$) or B6 control mice (black circles; $n = 10$) infected with 10^5 *Candida albicans* blastoconidia. **D** and **E**, Representative micrographs of periodic acid-Schiff (PAS)-stained renal sections from infected C2KR (**D**) or C2R (**E**) mice at day 6 postinfection. The images are shown at $\times 1.5$ – 1.6 (**D** and **E**, scale bar = 1 mm), $\times 10$ (**Di**, scale bar = 200 μ m) or $\times 40$ (**Dii**, **Ei**, and **Eii**, scale bar = 50 μ m) magnification. The arrows in **D** illustrate widespread PAS⁺ *C. albicans* tissue invasion. The boxes in **D** and **E** correspond to the magnified images shown in **Di**, **Dii**, **Ei**, and **Eii**. **D** and **E**, The letter P indicates the renal papilla. Data are representative from 4–6 kidneys per group.

Figure 3. Ablation of CCR2⁺ cells and murine susceptibility to systemic candidiasis. *A*, C2DR (white bars) or C2R (gray bars) littermate mice were treated with 10 ng/g body weight diphtheria toxin (DT) on day −1 and day 0 and infected with 10⁵ *Candida albicans* blastoconidia intravenously. The bar graphs indicate the mean (+SEM) of renal inflammatory monocytes at the indicated time points. ***P* < .01 by Mann–Whitney *U* test. *B*, Kaplan–Meier survival of C2D mice (white circles; *n* = 22) or nontransgenic (non-Tg) littermates (gray circles; *n* = 14) injected with DT or C2D mice injected with phosphate-buffered saline (PBS; black circles; *n* = 9) on days −1 and 0 and infected with 10⁵ *C. albicans* blastoconidia. *C*, C2D and non-Tg littermates were treated with DT (days −1 and 0), infected with 10⁵ *C. albicans* blastoconidia, and euthanized at the indicated time points for colony-forming unit (CFU) analysis. The bar graphs indicate the mean (+SEM) renal CFUs for C2D mice (white bars) and non-Tg littermates (gray bars). Six C2D mice were euthanized prior to day 4 postinfection due to disease severity and were excluded from the analysis. **P* < .05 by Mann–Whitney *U* test. *D* and *E*, Representative micrographs of periodic acid-Schiff (PAS)-stained renal sections from DT-treated (days −1 and 0) C2D mice (*D* and *Di*) or non-Tg littermates (*E* and *Ei*) at day 3 postinfection. The images are shown at ×1.5–1.6 (*D* and *E*, scale bar = 1 mm) or ×40 (*Di* and *Ei*, scale bar = 50 μm) magnification. *D*, Gross areas of PAS⁺ *C. albicans* tissue invasion (arrows). *Di*, Fungal invasion of a glomerulus (G) and tubules (T) within the renal cortex. *D* and *E*, Boxes indicate the magnified regions shown in *Di* and *Ei*. Data are representative from 6–8 kidneys per group.

and -sufficient counterparts (Figure 2*B*). Both CCR2^{−/−} mice (Figure 2*C*) and C2KR mice (data not shown) succumbed to systemic candidiasis more rapidly than C57BL/6 (Figure 2*C*) and C2R mice (data not shown).

Histopathologic examination of PAS- and hematoxylin and eosin (H&E)-stained sections from C2KR mice (6 days postinfection) revealed widespread multifocal and coalescing abscesses

consisting of degenerative and nondegenerative neutrophils and cellular debris. Abscesses surrounded foci of pseudohyphae in the renal cortex and medulla (black arrows in Figure 2*D*, insets in Figure 2*Di* and 2*Dii*; [Supplementary Figure 3A](#) and 3*B*). Severe and diffuse papillary necrosis with abundant pseudohyphal tissue invasion (white arrow in Figure 2*D*; [Supplementary Figure 3A](#) and 3*B*) and extensive degenerative

neutrophil-rich renal parenchymal and tubulointerstitial inflammation were apparent (Supplementary Figure 3A and 3B).

In C2R mice, kidney sections revealed rare microabscesses in the cortex and medulla (Figure 2E and 2Ei), the presence of intratubular neutrophilic infiltrates, and pseudohyphal tissue invasion in the renal papillae (Figure 2Eii and Supplementary Figure 3C and 3D). Sections from C2KR mice consistently displayed more severe and widespread parenchymal inflammation, papillary necrosis, and pseudohyphal tissue invasion than sections from C2R mice, correlating severe pathophysiologic manifestations of invasive candidiasis with loss of CCR2-dependent inflammatory monocyte trafficking.

Depletion of CCR2-Expressing Cells During Systemic Candidiasis and Impact on Infectious Outcome

To address the role of CCR2-independent monocyte trafficking and of CCR2⁺ renal-resident mononuclear phagocytes in host defense, we depleted CCR2⁺ cells systemically using an established DT-dependent cell ablation strategy [12]. To monitor the kinetics of DT-induced cell depletion in infected mice, C2D and C2R mice were crossed to obtain F1 progeny that contain GFP⁺ inflammatory monocytes and either express DTR to enable cell ablation (C2DR mice in Figure 3A) or lack the DTR transgene, rendering CCR2-expressing cells resistant to DT (nontransgenic [non-Tg] littermates in Figure 3A). All mice were treated with 10 ng/g body weight DT on day –1 and 0, infected with *C. albicans*, and euthanized at day 2 and day 4 postinfection. DT treatment reduced the number of renal inflammatory monocytes by >99% in naive and in day 2 infected C2RD mice compared to control littermates (Figure 3A). Although renal inflammatory monocyte numbers recovered by day 4 postinfection in C2DR mice, many infected C2RD mice appeared moribund and were euthanized (Figure 3A).

Kaplan–Meier analysis of DT- or PBS-treated C2D as well as DT-treated non-Tg littermates demonstrated a significant increase in mortality in DT-treated C2D mice compared with both control groups (Figure 3B). The renal fungal burden in DT-treated C2D mice was 1–2 log₁₀ higher than in non-Tg littermates at 2 and 4 days postinfection (Figure 3C), indicating an innate defect in renal antifungal activity linked to the depletion of CCR2⁺ cells.

Histopathologic examination of PAS-stained renal sections isolated from DT-treated C2D mice 72 hours postinfection revealed widespread destruction of renal tubules and glomeruli by invasive fungal filaments and abundant yeast cell forms, with large coalescing foci throughout the cortex and medulla (Figure 3D and 3Di). Widespread and coalescing areas of lytic and coagulative necrosis were observed in H&E-stained sections. The lesions contained degenerative neutrophil-rich inflammatory infiltrates characterized by cellular debris (Supplementary Figure 3E and 3F). Fungal organisms were observed in subcapsular spaces, within blood vessels, and traversing vessel walls (Figure 3Di and Supplementary Figure 3F).

In contrast, PAS-stained sections from DT-treated non-Tg littermates contained scattered fungal foci within the renal pelvis and rare yeast cells and hyphae were noted (Figure 3E and 3Ei). On H&E-stained sections, the medulla contained few focal areas of necrosis and there was mild multifocal tubular degeneration and necrosis with mild multifocal, tubular, and interstitial nondegenerative neutrophilic infiltrates within the cortex and medulla (Supplementary Figure 3G and 3H). Thus, depletion of CCR2-expressing cells is associated with rapid and severe renal inflammation and widespread parenchymal destruction by invasive *C. albicans* filaments.

Timing and Organ Specificity of CCR2-Expressing Cells During Systemic Candidiasis

To define the temporal window of CCR2⁺ leukocyte function, we varied the onset of DT treatment during infection and examined the impact on organ fungal burden (Figure 4A). When DT was administered starting 48 hours after onset of systemic candidiasis, C2D mice did not show a defect in renal or brain fungal clearance compared to non-Tg littermates, unlike C2D mice treated with DT prior to infection (Figure 4B and 4C). These data indicate that CCR2⁺ cells confer protective antifungal activity during the first 48 hours postinfection, using day 4 organ fungal burden as a readout.

Ablation of CCR2-expressing cells in C2D mice led to marginal increases in splenic (Figure 4D) and no increases in hepatic fungal burden compared to control mice, irrespective of DT timing (Figure 4E). Because DT treatment reduced splenic and hepatic inflammatory monocytes by >99% in C2D mice (data not shown), we conclude that inflammatory monocytes and other CCR2⁺ cells do not play a major role in *C. albicans* clearance in these organs. These data indicate that CCR2⁺ cells are essential for antifungal activity in an organ-specific fashion during systemic candidiasis.

Adoptive Transfer of Inflammatory Monocytes and Restoration of Antifungal Activity in Target Organs

To determine whether inflammatory monocytes account for the phenotype observed in mice depleted of CCR2⁺ cells, we examined the candidacidal properties of inflammatory monocytes and conducted adoptive transfer experiments to attempt to restore antifungal activity in DT-treated C2D hosts. Sorted CD45.1⁺ GFP⁺ BM inflammatory monocytes (Figure 5A) were incubated ex vivo with *C. albicans* blastoconidia. Using Alamar Blue reduction as a measure of fungal inactivation, we observed that inflammatory monocytes inactivated approximately 50% of fungal cells using a 2:1 effector to target cell ratio (Figure 5B).

To extend these findings in vivo, we sorted and transferred 10⁶ GFP⁺ BM inflammatory monocytes into DT-treated C2D mice and infected the animals with *C. albicans* (Figure 5C). DT-treated C2D mice that received a monocyte graft had a

Figure 4. Timing and organ specificity of CCR2⁺ leukocyte function during systemic candidiasis. *A*, Summary of experimental scheme. C2D or nontransgenic (non-Tg) littermate mice underwent diphtheria toxin (DT)-induced cell ablation starting at day -1 (C2D, white circle; non-Tg littermate, black circle) or starting at day 2 postinfection (C2D, gray circle) and were infected with 6×10^4 *Candida albicans* (Ca) blastoconidia on day 0. Organs were harvested at day 4 postinfection for colony-forming unit (CFU) analysis. *B–E*, The scatter plots show day 4 CFUs from the kidney (*B*), brain (*C*), spleen (*D*), and liver (*E*) of infected mice. Two C2D mice treated with DT starting at day -1 appeared moribund and were euthanized on day 3 postinfection for CFU analysis (dashed white circles). The lines indicate median values. Data are pooled from 2 experiments. * $P < .05$, Kruskal–Wallis 1-way analysis of variance followed by Dunn test for multiple comparisons.

significantly lower brain and renal fungal burden 4 days postinfection compared to DT-treated C2D mice that did not receive a graft (Figure 5*D* and 5*E*).

In parallel experiments, CD45.1⁺ renal monocytes recovered from infected mice 60 hours postinfection increased surface expression of CD11c, MHC class II, and F4/80 compared to expression levels in the monocyte graft (Figure 5*F*). Thus, in the

context of inflammation, kidney-infiltrating monocytes acquire a flow cytometric phenotype that resembles that of steady-state CCR2⁺ renal mononuclear phagocyte populations, although Ly6C expression remains elevated (not shown). These data indicate that loss of renal antifungal activity in DT-treated C2D mice is largely attributable to depletion of inflammatory monocytes and their derivatives.

Figure 5. Inflammatory monocytes and in vitro *Candida albicans* (Ca) inactivation and in vivo *C. albicans* clearance following adoptive transfer. **A**, Plot shows the purity of sorted GFP⁺CD3⁺CD11c⁺CD19⁺NK1.1⁺Ter119⁺ bone marrow (BM) monocytes used in in vitro *C. albicans* kill assays and adoptive transfer experiments. **B**, Bar graph shows the mean (+SEM) fungal survival of 5×10^4 *C. albicans* blastoconidia incubated with sorted 10^5 BM GFP⁺ monocytes (Mo; black bar) or not (white bar), as measured by Alamar Blue reduction. **C**, Summary of experimental scheme. C2D mice were treated with DT on day -1 and 0 and received 10^6 purified GFP⁺ diphtheria toxin (DT)-resistant BM monocytes (n=6; gray circles) or not (n=8; white circles) 30 minutes before intravenous infection with 10^5 *C. albicans* blastoconidia. For reference, nontransgenic (non-Tg) littermates were treated with DT as above and infected with *C. albicans* (n=3, black circles). **D** and **E**, Scatter plots show brain (**D**) and kidney (**E**) colony-forming units (CFUs) for the indicated groups of mice. Data are pooled from 2 experiments. * $P < .05$, ** $P < .01$ by Mann-Whitney *U* test. **F**, Plots show CD11c, MHC class II, and F4/80 expression on CD45.1⁺GFP⁺ BM monocytes (gray shaded areas; prior to adoptive transfer) and on CD45.1⁺GFP⁺ renal monocytes (black lines) recovered 60 hours post-transfer from *C. albicans*-infected mice as shown in (**C**).

DISCUSSION

Although inflammatory monocyte mobilization to foci of microbial infection is a common feature of the murine immune response to a broad range of pathogens [16, 17], the role of monocytes as innate antifungal effector cells remains poorly understood. In this study, we find that inflammatory monocytes and their derivatives play an early and essential antifungal role and provide a protective benefit in a temporal and compartment-specific fashion during systemic candidiasis. The presence and recruitment of inflammatory monocytes and their derivatives in target organs leads to more effective fungal growth control and to increased host survival compared to monocytopenic control groups. The protective benefit associated with inflammatory monocytes during invasive candidiasis was preserved in an adoptive transfer setting, consistent with an intrinsic innate antifungal effector function.

We examined infectious outcomes in C2K and in C2D mice to distinguish the role of CCR2-dependent mobilization from the role of CCR2⁺ cells in systemic candidiasis. In C2K mice, we observed a minor reduction in renal inflammatory monocyte numbers at baseline (on the order of 10⁴ cells) and a major reduction in renal inflammatory monocyte recruitment during early candidiasis (on the order of 10⁵ cells at 24 hours post-infection); both of these processes likely underlie the enhanced susceptibility of C2K mice compared to CCR2-sufficient controls.

The more prominent phenotype observed in C2D mice compared to C2K mice likely relates to several points. First, inflammatory monocyte renal trafficking is curtailed by 80%–90% in CCR2^(-/-) mice, but it is not fully abolished as it is in C2D mice. Second, resident CCR2⁺ mononuclear phagocytes, including F4/80⁺ macrophages and CD11b⁺ DCs, represent constituents of a mononuclear phagocyte network [18] that likely contributes to candidal defense in a CCR2-independent manner. The finding that tissue-infiltrating inflammatory monocytes upregulate F4/80, CD11c, and MHC class II expression suggests a developmental relationship between circulating and renal-resident mononuclear phagocytes, though further studies are necessary to clarify the ontogeny of renal mononuclear cell populations.

Our data do not preclude a role for CCR2⁺ NK cells in antifungal host defense, as has been demonstrated in a neutropenic model of invasive aspergillosis [19]. However, during early stages of systemic candidiasis, we did not observe numeric expansion of CCR2⁺ NK cells, and adoptive transfer of purified inflammatory monocytes reversed the phenotype of CCR2-depleted mice to a substantial extent.

The paradigm of inflammatory monocyte-dependent protective antimicrobial activity was established in systemic listeriosis, based on the observation that CCR2-dependent trafficking cues are essential for the formation of tumor necrosis factor (TNF)– and iNOS-producing dendritic cells (Tip-DCs) [20], an

inflammatory monocyte-derived population that acts as essential innate effector cell [21]. However, innate antimicrobial properties of inflammatory monocytes and their derivatives can be outweighed by the development of immunopathology, as observed in influenza [22, 23], amebiasis [24], and trypanosomiasis [25]. In these models, loss of CCR2 signaling reduced inflammatory monocyte accumulation at foci of infection, leading to reduced organ damage and enhanced host survival. During trypanosomiasis, IFN- γ and MyD88-dependent signals regulate the maturation of Ly6C^{hi} monocyte-derived Tip-DCs, a process that can be blocked and therapeutically regulated by the action of interleukin 10 [25, 26].

Previous studies in mice with disseminated candidiasis have raised a potential pathologic role of Ly6C^{hi} inflammatory monocytes during the infection process, particularly at late time points. Romani and colleagues demonstrated that murine survival could be ameliorated if leukocytes that express Gr-1 an epitope predominately found on neutrophils but also on Ly6C^{hi} inflammatory monocytes, were targeted for depletion at late time points postinfection [27]. Consistent with the notion that dysregulated phagocyte activity contributes to the pathogenesis of systemic candidiasis, loss of type I interferon improved host survival but did not accelerate renal candidal clearance [10]. Loss of type I interferon signaling induced an early and partial defect in the recruitment and activation of Ly6C^{hi} monocytes into iNOS-producing derivatives and induced a dramatic reduction in renal neutrophil influx at late time points. Administration of the peroxisome proliferator-activated receptor γ ligand pioglitazone, a repressor of CCR2 promoter activity [28], reduced the inflammatory pathology driven by type I interferon signaling [10], linking CCR2 promoter activity to the induction or amplification of pathways that mediate late immunopathology during candidiasis. However, Ly6C^{hi} monocytes were minor constituents of the pathologic effector cell population in the kidneys of mice during the time period when immune-mediated damage led to diminished organ function. The finding that CCR1 signaling led to pathologic renal neutrophil, but not Ly6C^{hi} monocyte, accumulation during the second week of infection reinforces this notion [11]. Whether Ly6C^{hi} monocytes mediate the induction of CCR1 ligands in infected kidneys remains unknown, though our data suggest that disruption of CCR2-dependent monocyte trafficking to infected kidneys did not influence renal neutrophil influx. Thus, dysregulated renal neutrophil influx appears to be the primary mechanism that drives the late immunopathology in mice with systemic candidiasis.

Previous studies in respiratory fungal infection models have illustrated an obligate role for inflammatory monocytes and their derivatives in regulating CD4 T-cell responses. In a pulmonary aspergillosis and in a blastomycosis vaccination model, inflammatory monocytes differentiated into CD11b⁺ monocyte-derived DCs that engulfed and transported fungal cells to organ-draining

lymph nodes [12, 29]. This trafficking step was essential for the priming of fungus-specific CD4T cells. Furthermore, depletion of inflammatory monocytes resulted in a shift from a predominant Th1 to a Th17 differentiation program by regulating T-bet expression in *Aspergillus fumigatus*-specific CD4T cells [30]. During pulmonary cryptococcosis, disruption of CCR2 signaling resulted in the development of a nonprotective Th2-biased response [31] that correlated with impaired monocyte pulmonary influx, reduced numbers of monocyte-derived pulmonary DCs [32], and delayed cryptococcal clearance. Our study extends these findings to identify antifungal properties of monocytes during the earliest stages of disseminated fungal infection. Consistent with this view, our laboratory developed a fluorescent *A. fumigatus* reporter (FLARE) strain that indicates conidial uptake by and viability in host leukocytes with single encounter resolution [33]. In this respiratory fungal infection model, CD11b⁺ monocyte-derived DCs have the capacity to bind, internalize, and inactivate *A. fumigatus* conidia in the lung [33] and represent a major TNF-producing cell population in neutropenic mice [34]. However, it remains to be elucidated whether inflammatory monocytes and their derivatives represent a major and direct fungicidal effector cell population during respiratory fungal infection.

Our studies indicate that inflammatory monocytes can inhibit *C. albicans* growth in vitro and in vivo. The recent development of mouse strains that permit conditional gene knockouts in monocytes [35] will undoubtedly facilitate studies to elucidate the signaling pathways and effector molecules that regulate their antifungal properties in vivo. Given the growing population of immunocompromised patients with functional and numeric deficits in phagocyte activity and the increasing incidence of antifungal drug resistance, pursuing strategies to harness and augment the antifungal activity of monocytes for therapeutic gain is highly relevant for improving patient outcomes.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Author contributions. T. M. H. designed and supervised the study, performed experiments, analyzed data, and drafted the manuscript. L. Y. N., S. K., A. J., S. E. K., and D. K. K. performed experiments, analyzed data, provided scientific input, and assisted with drafting the manuscript.

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Potential conflicts of interest. All authors: No reported conflicts.

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