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CXCR1-mediated Neutrophil Degranulation and Fungal Killing Promotes *Candida* Clearance and Host Survival

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Abstract

Systemic *Candida albicans* infection causes high morbidity and mortality and is now the leading cause of nosocomial bloodstream infection in the US. Neutropenia is a major risk factor for poor outcome in infected patients; however, the molecular factors that mediate neutrophil trafficking and effector function during infection are poorly defined. Here, using a mouse model of systemic candidiasis, we found that the neutrophil-selective CXC chemokine receptor Cxcr1 and its ligand, Cxcl5, are highly induced in the *Candida*-infected kidney, the target organ in the model. To investigate the role of Cxcr1 in antifungal host defense *in vivo*, we generated *Cxcr1*$^{-/-}$ mice and analyzed their immune response to *Candida*. Mice lacking Cxcr1 exhibited decreased survival with enhanced *Candida* growth in the kidney and renal failure. Surprisingly, increased susceptibility of *Cxcr1*$^{-/-}$ mice to systemic candidiasis was not due to impaired neutrophil trafficking from the blood into the infected kidney but was the result of defective killing of the fungus by neutrophils that exhibited a cell-intrinsic decrease in degranulation. In humans, the mutant CXCR1 allele *CXCR1-T276* results in impaired neutrophil degranulation and fungal killing and was associated with increased risk of disseminated candidiasis in infected patients. Together, our data demonstrate a biological function for mouse Cxcr1 *in vivo* and indicate that CXCR1-dependent neutrophil effector function is a critical innate protective mechanism of fungal clearance and host survival in systemic candidiasis.

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Introduction

Systemic candidiasis, most often caused by the commensal yeast *Candida albicans*, has emerged as the leading cause of nosocomial bloodstream infection in acutely ill and immunocompromised patient populations in the US (1), with an estimated annual cost that exceeds 2 billion dollars (2,3). Fungal vaccines are not available to prevent disease, and despite the availability of antifungal drugs with good *in vitro* and preclinical activity against *Candida*, mortality of affected patients remains 30–40% despite treatment (2,4). Therefore, systemic candidiasis represents an unmet medical condition for which better understanding of the cellular and molecular basis of antifungal immunity is essential to design immune-based strategies for risk stratification, prognostication, prophylaxis and/or treatment of patients.

Neutropenia is a major risk factor for development of candidemia and disseminated candidiasis in patients (2,4). Similarly, neutrophils are indispensable for effective antifungal innate immune responses in the mouse model of systemic candidiasis, as their depletion in the early phase of the infection results in fungal dissemination, uncontrolled fungal proliferation in tissue and increased mortality (4–9).

We previously reported that the chemokine receptor Ccr1 mediates neutrophil recruitment from the blood into the kidney (10), the target organ in the mouse model of the infection (7), during the late phase of systemic candidiasis, when neutrophils promote tissue injury (9,10). However, very little is known about specific molecular factors that mediate early protective trafficking and effector function of neutrophils during systemic candidiasis *in vivo*. Hence, in the present study we focused on CXCR1, the first chemokine receptor cloned in 1991 (11), which has been shown to mediate chemotaxis as well as both oxidative and non-oxidative cytotoxic antibacterial activity in human neutrophils *in vitro* (12,13). Consistent with this, a genetic variant of *CXCR1* named *CXCR1-T276* has been associated with heightened risk of bacterial infection of the kidney in humans (14,15).

Nevertheless, a deeper and broader understanding of the biological role of CXCR1 has been hampered by difficulties identifying and characterizing mouse Cxcr1, which delayed its cloning. Moreover, the human receptor is highly selective for CXCL8/IL-8, a chemokine that is not present in mouse, and only recently mouse Cxcr1 was shown to be a functional receptor for the mouse chemokine Cxcl5/LIX (16). Finally, a Cxcr1 knockout mouse had not been previously available and characterized. Herein, we have developed a Cxcr1-deficient mouse and used it to demonstrate increased susceptibility to systemic candidiasis as a biological phenotype attributable to Cxcr1 deficiency. Importantly, we have also translated this finding to humans by studies with the *CXCR1-T276* mutation.

Results

Cxcr1 and its ligand, *Cxcl5,* are up-regulated after systemic *Candida* infection in mice

Guided by a broad transcriptional screen of the chemoattractant system in the mouse model of systemic candidiasis (10), we aimed to gain insight into the molecular factors that mediate

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protective recruitment and effector function of neutrophils in *Candida*-infected tissues. Thus, we examined the induction of the neutrophil-selective chemokine receptor Cxcr1 and its ligand, Cxcl5, in C57Bl/6 mice infected intravenously with an LD_{50} inoculum of *Candida albicans* at early (day 1), intermediate (day 4) and late (day 7) time-points during the course of the infection (7). We focused on the kidney, the major target organ in the mouse model (4,7), and found that mRNA for *Cxcr1* was significantly and durably induced after infection; a ~70-fold increase was seen at day 1, whereas the receptor was up-regulated ~400-fold and ~750-fold at days 4 and 7 post-infection relative to the uninfected state, respectively (Fig. 1A). In addition, we observed ~130-fold, ~3500-fold and ~5000-fold inductions of mRNA for *Cxcl5* at days 1, 4 and 7 post-infection relative to the uninfected state, respectively (Fig. 1B). Cxcl5 was also significantly induced at the protein level in *Candida*-infected kidneys (Fig. 1C). Taken together, these results identify Cxcr1 as a candidate control factor in systemic candidiasis.

Cxcr1$^{-/-}$ mice are viable and do not manifest developmental or immune defects at steady state

We sought to determine the role of Cxcr1 in host defense during systemic candidiasis *in vivo* by generating Cxcr1-deficient mice. To inactivate Cxcr1, we replaced a 2.7 kb fragment of the *Cxcr1* gene, with a 1.7 kb of neomycin resistance cassette by homologous recombination in 129/Sv embryonic stem cells. The deleted region starts from the middle of the third transmembrane segment predicted from the *Cxcr1* sequence (codons 121–351) plus 2,028 bp of the 3′-untranslated region (Fig. 2A). Chimeric mice were made by injecting targeted 129/Sv cell lines into blastocysts from C57Bl/6 mice. The chimeric mice were mated with C57Bl/6 mice to establish heterozygotes, which were interbred to produce *Cxcr1*$^{+/+}$, *Cxcr1*$^{+/-}$, and *Cxcr1*$^{-/-}$ littermates. The genotypic frequencies for 241 total progeny of 9 *Cxcr1*$^{+/-}$ x *Cxcr1*$^{+/-}$ mating pairs were: 22.4% *Cxcr1*$^{+/+}$, 57.6% *Cxcr1*$^{+/-}$, and 20% *Cxcr1*$^{-/-}$, which is similar to Mendelian expectation for an autosomal gene. *Cxcr1*$^{-/-}$ mice were viable and fertile, and exhibited normal growth, development, anatomy, behavior, and lifespan compared with *Cxcr1*$^{+/+}$ littermates. Importantly, no developmental defects were seen in the kidney at steady state (Fig. S1). Moreover, no abnormalities in the complete blood cell counts or differential white blood cell counts were detected. These mice did not exhibit defects in hemostasis or healing of tail wounds, nor increased susceptibility to spontaneous infection when housed under specific pathogen-free conditions.

Cxcl5 is a selective ligand for mouse Cxcr1

It was previously shown that Cxcl5 is a ligand for mouse Cxcr1 in transfected cells *in vitro* (16). After generating *Cxcr1*$^{-/-}$ mouse, we aimed to verify that Cxcl5 is indeed a ligand for Cxcr1 in mouse neutrophils. Because Cxcl5 is known to bind Cxcr2, we harvested *Cxcr1*$^{-/-}$ and *Cxcr2*$^{-/-}$ mouse neutrophils to examine Cxcl5/Cxcr2 and Cxcl5/Cxcr1 signaling, respectively, using a calcium flux assay. Consistent with Cxcl5 being a known ligand for Cxcr2, *Cxcr1*$^{-/-}$ neutrophils displayed Cxcl5-dependent calcium flux (Fig. S2). Importantly, *Cxcr2*$^{-/-}$ neutrophils exhibited dose-dependent Cxcr1-mediated calcium flux upon addition of Cxcl5 (Fig. S2). These data show that, consonant with prior findings in a transfected cell line, Cxcl5 is a functional ligand for mouse Cxcr1.

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We then evaluated the selectivity of Cxcl5 as a ligand for mouse Cxcr1 by performing calcium flux assays in *Cxcr1*$^{-/-}$ and *Cxcr2*$^{-/-}$ mouse neutrophils using the Cxcr2 ligands Cxcl1/KC and Cxcl2/MIP-2. Consistent with Cxcl1 and Cxcl2 being known ligands for Cxcr2, *Cxcr1*$^{-/-}$ neutrophils displayed Cxcl1- and Cxcl2-dependent calcium flux (Fig. S3). Importantly, *Cxcr2*$^{-/-}$ neutrophils did not exhibit significant Cxcr1-mediated calcium flux upon addition of Cxcl1 or Cxcl2, suggesting that Cxcl5 is a selective ligand among ELR^{+} CXC chemokines for mouse Cxcr1 (Fig. S3).

Cxcr1 is critical for host survival in a mouse model of systemic candidiasis

To determine the impact of Cxcr1 deficiency on susceptibility to systemic candidiasis, we infected *Cxcr1*$^{-/-}$ mice with an LD_{50} inoculum (7) and found that their survival was significantly decreased relative to *Cxcr1*$^{+/+}$ animals (Fig. 2B). Mortality with this inoculum began around day 7 and only 15% of *Cxcr1*$^{-/-}$ mice survived through day 14 post-infection compared to 50% of *Cxcr1*$^{+/+}$ mice. We examined tissue fungal burden in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidneys after infection, which is known to correlate strongly with mortality (4,7). Cxcr1 deficiency resulted in a significant and reproducible ~2-fold increase in renal fungal burden at days 4 and 7 post-infection (Fig. 2C). Accordingly, infected *Cxcr1*$^{-/-}$ mice developed more severe renal dysfunction (Fig. 2D) and more extensive renal histopathology, including increased number of large kidney abscesses post-infection (Fig. 2E and S4). Specifically, the average number of abscesses per kidney measuring >50 μm in greatest dimension in *Cxcr1*$^{+/+}$ mice at day 4 post-infection was 0.73 (SEM, ± 0.27), whereas the number of such abscesses per kidney in *Cxcr1*$^{-/-}$ mice was 1.37 (SEM, ± 0.2)(P=0.0283; n=14–15; 4 independent experiments; Mann-Whitney test). In contrast, *Cxcr1*$^{-/-}$ mice did not exhibit greater fungal proliferation (Fig. S5) or histological abnormalities (Fig. S6) in the spleen, liver or brain post-infection. Thus, for the remainder of our studies, we focused on the effects of Cxcr1 in the kidney.

Neutrophils are the predominant Cxcr1-expressing immune cells in the kidney

To better understand the biological effects of Cxcr1 in the model, we next examined the expression of the receptor by qRT-PCR in various immune cell types that we FACS-sorted from the kidney at steady state and after *Candida* infection (Fig. S7). Neutrophils were by far the predominant Cxcr1-expressing cells in the kidney, whereas significantly lower levels of *Cxcr1* expression were detected in resident and inflammatory macrophages, Ly6C^{hi} inflammatory monocytes and T cells (Fig. 3A). In parallel, we sought to examine cellular expression of the Cxcr1 ligand, Cxcl5. *Cxcl5* was expressed more evenly by all immune cell types tested in the kidney, including neutrophils (Fig. 3B). This finding extends our previous observations that neutrophils serve as major producers of neutrophil-targeted chemokines in the *Candida*-infected kidney (10). We then examined the expression of *Cxcr1* and *Cxcl5* in mouse neutrophils harvested at different time points before and after *Candida* infection and found significant expression of *Cxcr1* and *Cxcl5* in kidney neutrophils both at steady state and during systemic candidiasis (Fig. S8). Based on these results, we focused our analysis of Cxcr1-dependent protective mechanisms in the model on accumulation and function of neutrophils, the predominant leukocyte subset in the *Candida*-infected kidney (7).

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Cxcr1 is dispensable for neutrophil trafficking from the blood into the *Candida*-infected kidney

CXCR1 is known to mediate chemotaxis of human neutrophils (12). Therefore, we reasoned that Cxcr1 promotes mouse survival and control of renal fungal proliferation during infection by mediating neutrophil recruitment from the blood into the kidney early in the course of the infection, when neutrophils are protective in the model (9). We used FACS to quantitatively and temporally assess how Cxcr1 affects neutrophil and other leukocyte subset accumulation in the kidney and blood at days 1, 4 and 7 post-*Candida* infection. We found that Cxcr1 was dispensable for neutrophil accumulation in the kidney, blood and bone marrow at steady state and throughout the course of the infection, as absolute neutrophil numbers (Fig. 4A, S9A and S10A) and percentages of neutrophils within total leukocytes (Fig. 4B, S9B and S10B) were similar in the kidney, blood and bone marrow of $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ mice. In agreement, the induction of neutrophil-targeted chemoattractants was Cxcr1-independent in the *Candida*-infected kidney (Fig. 4C and S11). Besides neutrophils, Cxcr1 deficiency did not impair the renal accumulation of monocytes, macrophages or dendritic cells, which have also been shown to mediate protective immunity in the model (Fig. S12)(17–19). In addition, the induction of other protective pro-inflammatory mediators such as IL-1β, IL-6, Ccl2 and Ccl3 was not impaired in $Cxcr1^{-/-}$ kidneys (Fig. S13)(4,8).

Equivalent accumulation of neutrophils in infected $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ kidneys (Fig. 4A,B) despite greater fungal burden in the knockout kidneys (Fig. 2C) could be interpreted as an abnormal response, reflecting a relative impairment of neutrophil influx to the $Cxcr1^{-/-}$ kidney. To address the potential confounding effects of the differential fungal load in $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ kidneys on neutrophil trafficking and to examine the direct role of Cxcr1 on neutrophil recruitment from the blood into the kidney *in vivo*, we generated mixed bone marrow radiation chimeras. Hence, we adoptively transferred a 1:1 ratio of $CD45.1^{+}Cxcr1^{+/+}$ and $CD45.2^{+}Cxcr1^{-/-}$ bone marrow cells into lethally irradiated $CD45.1^{+}Cxcr1^{+/+}$ recipient mice and allowed reconstitution for 8 weeks before *Candida* infection. We then assessed the relative trafficking of $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ neutrophils toward the same kidney milieu of infected mixed bone marrow chimeras using differential congenic marker detection by FACS. We found that the relative frequency of $CD45.1^{+}Cxcr1^{+/+}$ and $CD45.2^{+}Cxcr1^{-/-}$ neutrophils was unchanged before and after infection and was similar between infected blood and infected kidney, indicating that $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ neutrophils exhibited comparable recruitment from the blood into the infected kidney at days 1 and 4 post-infection (Fig. 4D,E). These data collectively show that Cxcr1 is dispensable for neutrophil recruitment from the blood into the kidney during systemic candidiasis.

Because Cxcr2 is another major neutrophil-targeted chemoattactant receptor that binds CXC chemokines (12), and we found it to be expressed on blood and kidney neutrophils of *Candida*-infected $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ mice (Fig. S14), we reasoned that Cxcr2 may drive renal neutrophil recruitment during infection in the absence of Cxcr1. To test this, we treated $Cxcr1^{-/-}$ mice with vehicle or the selective Cxcr2 antagonist SB225002, which has been successfully used to inhibit Cxcr2-dependent neutrophil trafficking in mouse models of infection and inflammation *in vivo* (20,21). We found that Cxcr2 inhibition did not impair

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neutrophil accumulation in *Candida*-infected kidneys of *Cxcr1*$^{-/-}$ mice (Fig. S15), suggesting that chemoattractant receptors other than Cxcr2 are responsible for neutrophil recruitment into the *Candida*-infected *Cxcr1*$^{-/-}$ kidney.

Cxcr1 deficiency impairs the fungal killing capacity of kidney neutrophils and results in decreased *Candida* hyphal damage in the infected kidney

Since Cxcr1 did not mediate neutrophil recruitment in the kidney during systemic candidiasis, we next investigated the role of Cxcr1 on neutrophil effector function in the infected kidney. Chemokine receptors regulate many leukocyte functions apart from directional cell movement, such as cell survival, proliferation, phagocytosis, and killing (13,19,22), and CXCR1 has been reported to promote oxidative and non-oxidative antibacterial effects in human neutrophils (13). Examination of Periodic acid–Schiff (PAS) kidney stains revealed that neutrophilic abscesses in the kidneys of *Cxcr1*$^{+/+}$ mice contained degraded *Candida* hyphal elements consistent with neutrophil antifungal cytotoxic activity, as previously observed in this model (7,10). Strikingly, *Candida* hyphal elements appeared intact within *Candida*-infected *Cxcr1*$^{-/-}$ renal abscesses (Fig. 5A). We quantified the length of >400 randomly picked individual hyphal elements within abscesses from *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidneys and found significantly longer *Candida* hyphal elements within *Cxcr1*$^{-/-}$ abscesses (Fig. 5B), suggesting that *Cxcr1*$^{-/-}$ neutrophils may be functionally impaired in controlling fungal filamentous growth in the infected kidney.

Indeed, using alamarBlue reduction as a measure of fungal inactivation, as previously described (19), *Cxcr1*$^{-/-}$ neutrophils MACS-sorted from the infected kidney exhibited a significant ~40% defect in their killing capacity of hyphal elements *ex vivo* (Fig. 5C). We examined fungal killing by kidney neutrophils under both opsonized and unopsonized conditions, since opsonization may be suboptimal within neutrophil abscesses (23), and found that the defect in neutrophil killing was independent of fungal opsonization (Fig. 5C). Similarly, *Cxcr1*$^{-/-}$ kidney neutrophils exhibited impaired killing against opsonized and unopsonized *Candida* yeast forms (Fig. S16A). Taken together, these data indicate that Cxcr1 is critical for promoting antifungal killing by neutrophils and inducing *Candida* hyphal damage within neutrophil abscesses at the site of infection.

Cxcr1 mediates neutrophil degranulation

We next investigated the mechanism(s) underlying the decreased neutrophil candidacidal activity in *Cxcr1*$^{-/-}$ kidneys. We found that Cxcr1 deficiency did not result in defective neutrophil killing by adversely affecting neutrophil survival (Fig. 6A), maturation (Fig. 6B), phagocytosis (Fig. 6C) or oxidative burst (Fig. 6D), indicating that Cxcr1-mediated fungal killing in kidney neutrophils occurs via non-oxidative effector mechanisms.

Filamentous fungal element killing by neutrophils occurs predominantly extracellularly due to the large hyphal size that precludes universal internalization by phagocytes (4,8). We found no defect in neutrophil extracellular trap formation of *Cxcr1*$^{-/-}$ neutrophils *ex vivo* (Fig. S17). Hence, we focused our studies on determining whether degranulation was impaired in *Cxcr1*$^{-/-}$ kidney neutrophils, as chemotactic factors have previously been reported to promote neutrophil degranulation (24–27). Indeed, we found that *Cxcr1*$^{-/-}$

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neutrophils MACS-sorted from the infected kidney had a significant ~50% reduction in degranulation capacity as measured by beta-glucuronidase release (Fig. 6E). Consistent with this finding, intracellular levels of another major neutrophil primary granule protein, myeloperoxidase (MPO), were significantly decreased in *Cxcr1*$^{-/-}$ kidney neutrophils (Fig. 6F), and *Cxcr1*$^{-/-}$ neutrophils MACS-sorted from the infected kidney released significantly less MPO upon *Candida* hyphal challenge *ex vivo* (Fig. 6G). Collectively, these data show that Cxcr1 mediates degranulation of neutrophils in the *Candida*-infected kidney.

Cxcr1 deficiency results in a cell-intrinsic defect in degranulation and fungal killing of neutrophils

We next asked whether the impairment in degranulation and fungal killing that we observed in *Cxcr1*$^{-/-}$ kidney neutrophils was due to a cell-intrinsic neutrophil defect or whether it was caused by kidney-specific alterations in the local immunological milieu of *Cxcr1*$^{-/-}$ mice. A recent report showed that Syk-dependent production of IL-23p19 by renal mononuclear phagocytes results in secretion of GM-CSF by NK cells, which provides critical “help” for neutrophil maturation and candidacidal activity locally in the *Candida*-infected kidney (18). Thus, we first examined whether the IL-23p19/GM-CSF axis was impaired in *Cxcr1*$^{-/-}$ kidneys. We found that mononuclear phagocyte (Fig. S12) and NK cell accumulation (Fig. S18A), as well as mRNA induction for *Il-23a* and *Gmcsf* (Fig. S18B,C) were similar in infected *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidneys. Instead, consistent with a cell-intrinsic defect of *Cxcr1*$^{-/-}$ neutrophils, we found that neutrophils isolated from the bone marrows of uninfected *Cxcr1*$^{-/-}$ mice exhibited significantly impaired killing against opsonized and unopsonized *Candida* yeast and hyphal forms relative to *Cxcr1*$^{+/+}$ cells (Fig. 6H and S16B) while oxidative burst was normal (Fig. S19). In addition, *Cxcr1*$^{-/-}$ neutrophils harvested from uninfected bone marrow exhibited significant decreases in (a) degranulation capacity as measured by beta-glucuronidase release (Fig. 6I), (b) and intracellular MPO levels at steady state, suggestive of a Cxcr1-dependent defect in granulogenesis (Fig. 6J). Therefore, our data collectively show the Cxcr1 deficiency results in degranulation and fungal killing defects in neutrophils that are cell-intrinsic and not caused by an impaired kidney-specific local immunological milieu.

CXCR1-T276 is associated with disseminated candidiasis in humans

In humans, the *CXCR1-T276* SNP (rs2234671) caused by a guanine-to-cytosine substitution at nucleotide 827 in exon 2 that changes a serine at position 276 to threonine has been associated with increased susceptibility to bacterial renal infection in two independent cohorts of pediatric patients (14,15), and has been shown to correlate with decreased levels of mRNA and protein expression of CXCR1 in human neutrophils (15,28). To test the hypothesis that the *CXCR1-T276* SNP may increase host susceptibility to systemic candidiasis and/or development of adverse outcome after infection, we genotyped 153 candidemic patients and 151 non-infected control patients of mixed European descent from the US (19)(Table S1).

The genotypes were in Hardy-Weinberg equilibrium in both groups. Although there was no difference in the frequencies of patients carrying the mutant *CXCR1-T276* allele versus the WT genotype among candidemic and uninfected control subjects (Table S2), candidemic

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patients carrying the mutant *CXCR1-T276* allele were more likely to develop disseminated infection (37.5%) than those carrying the WT allele (16.8%)(Table 1; *P*=0.0455; OR, 2.97; 95%CI, 0.98–8.99). This association remained significant in multivariate analysis when controlling for other clinical factors. In the final model, presence of the *CXCR1-T276* allele (*P*=0.0242; OR, 3.95; 95%CI, 1.20–13.03), receipt of total parenteral nutrition (*P*=0.0213; OR, 3.19; 95%CI, 1.19–8.21) and solid organ transplantation (*P*=0.0073; OR, 4.03; 95%CI, 1.46–11.17) were the only factors independently associated with development of disseminated candidiasis. No association of carrying the *CXCR1-T276* SNP was observed with increased mortality among candidemic patients, whereas there was a trend toward development of persistent fungemia in those carrying the mutant allele in multivariate analysis (*P*=0.1110; OR, 2.79; 95%CI, 0.79–9.82)(Table S3).

Neutrophils from healthy individuals with the *CXCR1-T276* SNP exhibit impaired degranulation and fungal killing capacity

Since the mutant *CXCR1-T276* allele was associated with increased susceptibility to disseminated candidiasis in candidemic patients, we next asked whether neutrophils from individuals with the *CXCR1-T276 SNP* exhibit defective effector antifungal function similar to $Cxcr1^{-/-}$ mouse neutrophils. To investigate this question, we screened >50 healthy donors at the NIH Blood Bank and identified individuals with the *CXCR1-WT* and *CXCR1-T276* alleles, from whom we obtained whole blood to harvest neutrophils for functional studies.

In agreement with the comparable neutrophil numbers in $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ mouse blood at steady state, individuals with the *CXCR1-T276* allele did not exhibit decreased absolute neutrophil counts relative to *CXCR1-WT* individuals (Fig. 7A). Remarkably, consistent with our mouse findings, neutrophils from donors with the *CXCR1-T276* allele had impaired fungal killing ability against both opsonized and unopsonized yeast and hyphal elements (Fig. 7B,C), exhibited significantly impaired degranulation capacity as measured by beta-glucuronidase release (Fig. 7D), and had decreased intracellular MPO content (Fig. 7E). Therefore, these data collectively show that neutrophils from donors with the mutant *CXCR1-T276* allele have impaired degranulation and fungal killing capacity.

Discussion

In this study, we present a role for the chemokine receptor Cxcr1 in host defense against systemic fungal infection and show that the mutant *CXCR1-T276* SNP results in impaired neutrophil function and is associated with susceptibility to systemic candidiasis in humans. The receptor appears to act directly by promoting neutrophil degranulation and fungal killing, which mediates *Candida* damage in tissue and fungal clearance. Our conclusions are based on detailed analysis of differences in clinical, microbiological, pathological, immunological and molecular parameters between $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ mice. Our study reveals a biological function for the mouse chemokine receptor Cxcr1 *in vivo* and identifies a neutrophil-targeted chemotactic factor that protects against systemic fungal infection in mice and humans.

We focused on Cxcr1 because (a) neutrophils are the major cellular mediators of the innate immune response against systemic candidiasis and their early recruitment into *Candida*-

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infected mouse tissues promotes *Candida* control and host survival (7–9), and because (b) our broad survey of the chemokine system in the mouse model of systemic candidiasis revealed significant induction of Cxcr1, and its ligand Cxcl5, in infected tissues (10). Notably, the functional importance of Cxcr1 for neutrophils had only been suggested through indirect approaches in humans (12,13) and no study had thus far ascribed any biological function for mouse Cxcr1 *in vivo*; therefore, we generated Cxcr1-deficient mice in order to examine the role of the receptor in antifungal host defense.

We found that Cxcr1 is critical for innate antifungal host defense, as $Cxcr1^{-/-}$ mice have decreased survival and impaired ability to control fungal proliferation in the kidney. We did not find a defect in neutrophil accumulation in $Cxcr1^{-/-}$ mice and our mixed bone marrow chimera experiments revealed no preferential recruitment of $Cxcr1^{+/+}$ over $Cxcr1^{-/-}$ neutrophils in the *Candida*-infected kidney, thus collectively ruling out a role of Cxcr1-dependent neutrophil trafficking in the model. Therefore, yet-unknown chemotactic receptors other than Cxcr1 or Ccr1 (10) mediate early protective trafficking of neutrophils from the blood into the *Candida*-infected kidney. Of interest, $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ mice also had similar neutrophil numbers in the blood at steady state, as opposed to the neutrophil expansion reported in Cxcr2-deficient mice (29); this indicates that Cxcr1 and Cxcr2 play differential roles on mouse granulocytopoiesis and neutrophil homeostasis.

Instead of impaired trafficking, Cxcr1-deficient neutrophils appeared unable to cause damage to *Candida* filamentous elements *in vivo* in the infected kidney. This prompted us to examine the role of Cxcr1 in fungal killing, as human CXCR1 is known to promote oxidative and non-oxidative killing against bacteria (13). Indeed, we found significant cell-intrinsic defects in neutrophil degranulation and anti-*Candida* killing in $Cxcr1^{-/-}$ mice. This observation further expands the previously reported contribution of the neutrophil-targeted chemokine receptors CCR1, CXCR2, LTB4R1, FPR1 and C5aR1 (24–27) in neutrophil degranulation. The decreased basal levels of the major neutrophil granule proteins, myeloperoxidase and beta-glucuronidase, in $Cxcr1^{-/-}$ neutrophils suggest that this degranulation defect lies, at least in part, at the level of granulogenesis. Importantly, our data further underscore the pleiotropic “non-conventional” roles that chemotactic factors play in immunity via modulating cellular functions such as survival, proliferation, differentiation, phagocytosis, killing, degranulation and adhesion, besides their well-established mechanistic role in promoting directional cell movement (13,19,22).

Of interest, oxidative burst of $Cxcr1^{-/-}$ neutrophils isolated from *Candida*-infected kidneys and uninfected bone marrows was intact, suggesting that Cxcr1 likely mediates *Candida* killing via non-oxidative mechanisms. In fact, in contrast to the mechanistic characterization of signaling cascades that underlie oxidative cytotoxic killing in neutrophils during fungal infection (4,8,30), little is known with regard to the molecular mechanisms of neutrophil non-oxidative fungal cytotoxicity.

Our study has implications beyond the importance of defining the role of Cxcr1 in innate anti-*Candida* host defense. Our results present a biological function of mouse Cxcr1 *in vivo* and additional studies will be required to systematically define the spectrum of Cxcr1-dependent effects in non-infectious inflammatory models and in immunity against other

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pathogens including bacteria, viruses, molds and dimorphic fungi. All mouse immunological studies to date have ascribed identified neutrophil-specific functions of mouse CXC chemokines universally to Cxcr2. Our study shows that mouse Cxcr1 is also a functional receptor *in vivo* and the differential contribution of CXC chemokine-Cxcr1 versus CXC chemokine-Cxcr2 signaling in neutrophil functions in various models of infection and inflammation merits investigation.

The mutant *CXCR1-T276* SNP, which has been associated with increased susceptibility to bacterial renal infection in two independent cohorts of pediatric patients (14,15), provided a unique translational opportunity to extend our mouse findings to humans. Hence, we found that the *CXCR1-T276* allele is associated with an increased likelihood for the development of disseminated candidiasis in candidemic patients of mixed European descent. Because the clinical outcome of systemic candidiasis varies substantially among patients with similar clinical and microbiological risk factors, discovery of genetic factors such as *CXCR1-T276* and the previously reported *CX_3CR1-M280* (19) that predispose to disseminated disease could aid in devising individualized prognostication strategies and in identifying candidemic patients in whom intensified diagnostic and therapeutic interventions may be beneficial.

Importantly, we found that neutrophils from individuals carrying the *CXCR1-T276* allele exhibit immunological functional defects similar to those identified in *$Cxcr1^{-/-}$* neutrophils. These defects may account, at least in part, for the increased patient susceptibility to disseminated candidiasis, and it will be important to define the molecular mechanisms by which the *CXCR1-T276* allele impairs CXCR1 signaling and results in defective degranulation and fungal killing. Furthermore, whether similar neutrophil impairments are seen against bacterial pathogens in individuals carrying the *CXCR1-T276* allele merits investigation, as it may provide mechanistic insights on the enhanced susceptibility of these individuals to bacterial renal infection (14,15).

Our study has limitations. Unanswered questions remain with regard to the mechanisms by which Cxcl5 mediate Cxcr1-dependent neutrophil effector function, and the molecular mechanisms involved in Cxcr1-mediated neutrophil granulogenesis and non-oxidative fungal killing. In addition, although the control uninfected patient cohort was matched to the candidemic patient cohort with regard to time and hospital ward of enrollment, the two cohorts were not entirely matched at the level of underlying clinical risk factors. Moreover, a matched patient cohort suffering from non-fungal infection was not available for comparison. Like any genetic association study, it is possible that the association of *CXCR1-T276* with disseminated candidiasis is due to biases at the population stratification level or linkage with another yet-unknown SNP; thus, this finding requires validation in other cohort studies, which should also incorporate patients from other racial backgrounds.

In conclusion, our study identifies CXCR1 as a crucial factor for innate host defense against systemic fungal infection in mouse and man. Mechanistically, CXCR1 protects from fungal infection by promoting neutrophil degranulation and fungal killing. Thus, genetic variation at *CXCR1* is associated with impaired neutrophil effector function and may be useful for risk assessment and prognostication in humans suffering from systemic candidiasis.

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Materials and Methods

Study Design

A previous broad screen of the chemokine system showed that Cxcr1 and its ligand were induced *in vivo* in *Candida*-infected mouse tissue, suggesting that the receptor may play an important role in antifungal immunity. To investigate this, we generated *Cxcr1*$^{-/-}$ mice and found that they were highly susceptible to systemic *Candida* infection as they developed increased mortality and accelerated tissue fungal proliferation. Interestingly, Cxcr1 was dispensable for neutrophil trafficking from the blood into the *Candida*-infected tissue. Instead, Cxcr1 deficiency resulted in a cell-intrinsic defect in fungal killing and degranulation of mouse neutrophils, which were unable to damage fungal elements *in vivo* in the infected tissue. The sample size and replicates of experiments for all mouse studies are included in the legends of the corresponding figures. All mice were maintained under specific pathogen–free housing conditions at an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility at the NIAID and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under a protocol approved by the Animal Care and Use Committee of NIAID.

To translate our mouse findings to humans, we examined the impact of carrying the mutant *CXCR1-T276* allele on susceptibility of human patients to the infection in a cohort of candidemic and control uninfected individuals from the US enrolled at the Duke University Hospital (DUMC) between 2003 and 2009 (see Supplemental Methods for details). There was no blinding or randomization in the cohort enrollment. The study patients were enrolled after informed consent (or waiver as approved by the Institutional Review Board [IRB]) at DUMC. The study was approved by the IRB at DUMC and was performed in accordance with the Declaration of Helsinki. We found that the presence of the *CXCR1-T276* allele was significantly associated with the development of disseminated candidiasis. To examine *CXCR1-T276* neutrophil effector function, we screened >50 healthy donors at the NIH Blood Bank and identified individuals carrying the *CXCR1-WT* or *CXCR1-T276* allele. In agreement with the mouse findings, we found that *CXCR1-T276* neutrophils had impaired degranulation and fungal killing.

Mouse Model of Systemic Candidiasis and *Candida* Strains

Cxcr1$^{-/-}$ mice were generated as described in the Supplemental Methods. *Cxcr2*$^{-/-}$ mice were purchased from The Jackson Laboratories. We used 8–12–week old male *Cxcr1*$^{+/+}$ WT, *Cxcr1*$^{-/-}$ C57Bl/6 and *Cxcr2*$^{-/-}$ C57Bl/6 mice. CD45.1^{+} congenic B6.SJL mice were obtained from Taconic Farms.

Candida albicans strain SC5314 was used for all experiments, except for the neutrophil phagocytosis experiments in which the dTomato-expressing CAF2-1 *C. albicans* strain was used (31). *Candida* was grown as previously described (7), and ~10^5 cells were injected intravenously per mouse. Uninfected and infected mice were sacrificed at days 1, 4 or 7 post-infection and the following analyses were performed (see Supplemental Methods for details): tissue fungal burdens; FACS analyses on bone marrow, blood and kidney cells; histopathology; serological analyses of renal function; quantification of cytokines and

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chemokines by Luminex array; cell sorting; RNA isolation from kidney tissue and bone marrow followed by qRT-PCR analyses; functional analyses of neutrophils isolated from uninfected bone marrow or infected kidney.

Statistics

The mouse experimental data and human neutrophil experimental data were analyzed using unpaired *t*-test, Mann-Whitney test Mann-Whitney test or one-way ANOVA test as appropriate with GraphPad Prism 6.0 and presented as mean values ± SEM. Statistical significance was defined as $P<0.05$.

χ^2 test was used to compare the frequencies of *Candida*-infected and non-infected subjects in order to analyze the impact of the *CXCR1-T276* SNP on human susceptibility to candidemia. Pearson correlation coefficient or Fisher's exact test as appropriate were used to perform univariate analyses, and Odds Ratios (ORs) and 95% confidence intervals (CIs) were reported. Variables with $P<0.2$ were analyzed using multivariable logistic regression by backward elimination. Variables with $P<0.05$ were retained in the final predictive model and OR and 95% CI were reported for the variables that remained significant in the final multivariable model. SAS software version 9.2 was used for the analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Supplementary Materials

Supplementary Methods

Fig. S1. Cxcr1 deficiency does not result in developmental renal defects.

Fig. S2. Cxcl5 promotes dose-dependent Cxcr1-mediated calcium flux in mouse neutrophils.

Fig. S3. Cxcl1 and Cxcl2 do not promote significant Cxcr1-dependent calcium flux in mouse neutrophils.

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Fig. S4. Systemic candidiasis results in increased renal tissue injury in *Cxcr1*$^{-/-}$ mice.

Fig. S5. Cxcr1 deficiency does not impair fungal clearance in the liver, spleen or brain after *Candida* infection.

Fig. S6. Cxcr1 deficiency does not result in histological abnormalities in the liver, brain or spleen after *Candida* infection.

Fig. S7. Gating strategy for FACS-sorting of hematopoietic cells from kidney.

Fig. S8. Neutrophils up-regulate *Cxcr1* but not *Cxcl5* after systemic candidiasis.

Fig. S9. Cxcr1 deficiency does not affect the production of neutrophils in the bone marrow after *Candida* infection.

Fig. S10. Cxcr1 deficiency does not affect the accumulation of neutrophils in the blood after *Candida* infection.

Fig. S11. Cxcr1 deficiency does not impair the induction of *Cxcl5* in the *Candida*-infected kidney.

Fig. S12. Cxcr1 deficiency does not impair the accumulation of mononuclear phagocytes in the kidney after *Candida* infection.

Fig. S13. Cxcr1 does not impair the induction of pro-inflammatory cytokines and chemokines in the kidney after *Candida* infection.

Fig. S14. Cxcr2 is expressed on blood and kidney neutrophils of *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice at steady state and during systemic candidiasis.

Fig. S15. A selective Cxcr2 antagonist does not decrease neutrophil accumulation in the *Candida*-infected kidney of *Cxcr1*$^{-/-}$ mice.

Fig. S16. Cxcr1 deficiency results in a cell-intrinsic defect in killing of *Candida* yeast forms.

Fig. S17. Cxcr1 deficiency does not impair the formation of neutrophil extracellular traps *ex vivo*.

Fig. S18. Cxcr1 deficiency does not impair the accumulation of NK cells or the induction of *Il23a* and *Gmcsf* in the kidney after *Candida* infection.

Fig. S19. Cxcr1 deficiency does not impair the production of reactive oxygen species by bone marrow neutrophils at steady state.

Table S1. Demographic and clinical characteristics of the candidemic and control subjects enrolled in the present study.

Table S2. Association of the mutant *CXCR1-T276* allele with susceptibility to systemic candidiasis in subjects of mixed European descent (n=304).

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Table S3. Association of the mutant *CXCR1-T276* allele with development of persistent fungemia in candidemic patients of mixed European descent (n=153).

Data File 1. Excel file with all data values in tabular format.

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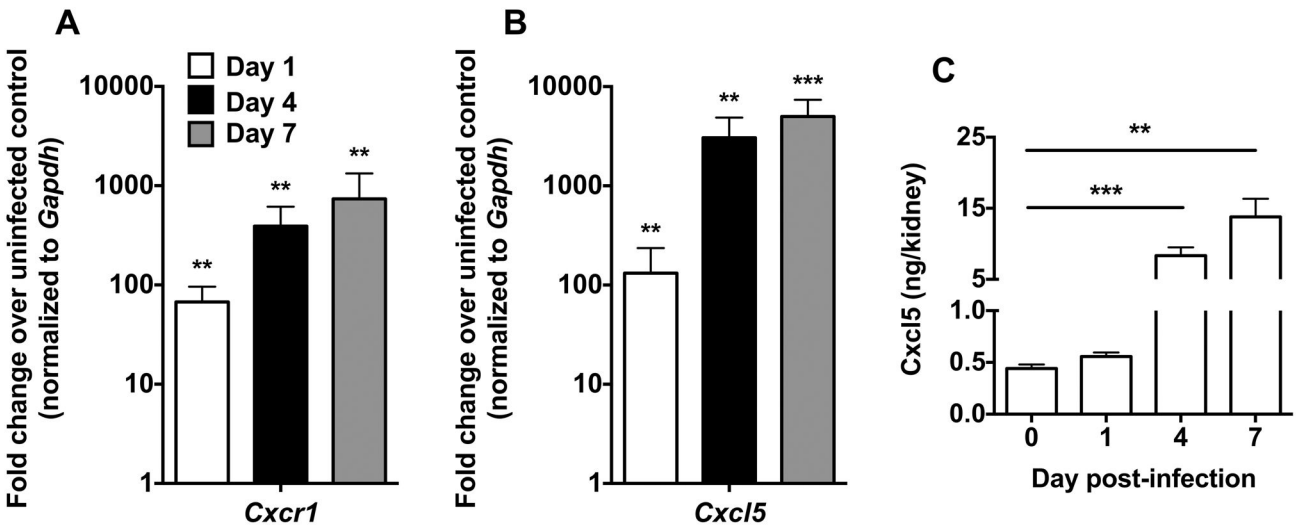

Figure 1. Systemic candidiasis induces the expression of *Cxcr1* and its ligand *Cxcl5*

(A) *Cxcr1* is induced in *Cxcr1*$^{+/+}$ kidneys at days 1, 4 and 7 post-infection. *P*=0.0023, *P*=0.0012 and *P*=0.0022 for *Cxcr1* expression at days 1, 4 and 7 versus day 0, respectively (n=5–8; 2 independent experiments; Mann-Whitney test). **(B)** *Cxcl5* is induced in *Cxcr1*$^{+/+}$ kidneys at days 1, 4 and 7 post-infection. *P*=0.01, *P*=0.0016 and *P*=0.0004 for *Cxcl5* expression at days 1, 4 and 7 versus day 0, respectively (n=5–8; 2 independent experiments; *t*-test). **(C)** Cxcl5 is significantly induced at the protein level in *Cxcr1*$^{+/+}$ kidneys post-infection (n=6–8; 2 independent experiments). ** *P*=0.0012; Mann-Whitney test; *** *P*=0.0003; *t*-test with Welch's correction. Data represent mean ± SEM.

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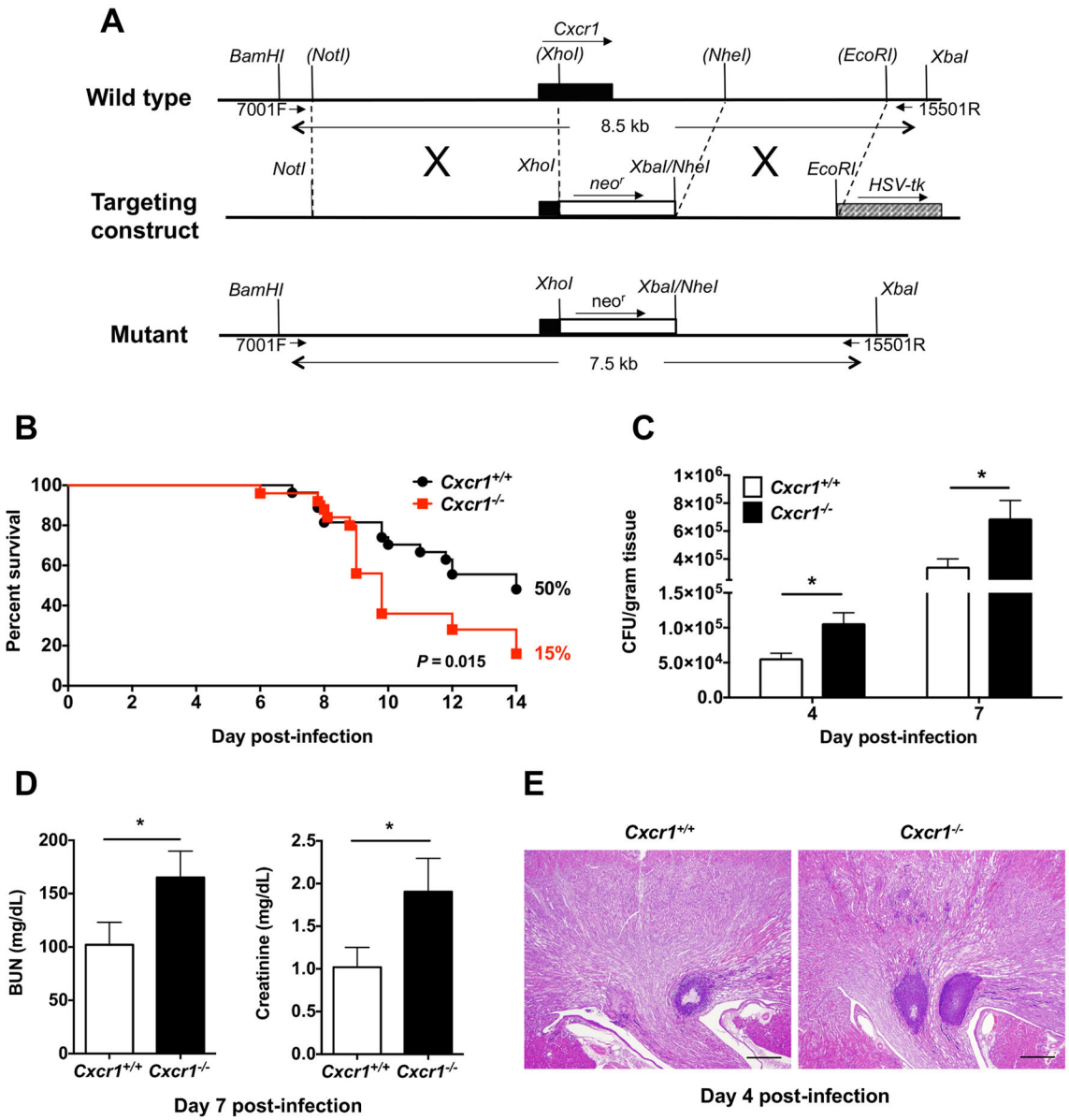

Figure 2. Systemic candidiasis results in increased mortality in *Cxcr1*$^{-/-}$ mice due to enhanced fungal proliferation in the kidney and renal failure

(A) Schematic showing the generation of *Cxcr1*$^{-/-}$ mice by homologous recombination. **(B)** Mortality rates of *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice after intravenous challenge with *Candida* (n=25; summary data of 3 independent experiments). *P*=0.015; Log-rank (Mantel-Cox) test. **(C)** Fungal burden in the kidneys of *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ mice at days 4 (*P*=0.0135; n=14–15; 3 independent experiments; *t*-test with Welch's correction) and 7 (*P*=0.025; n=30–31; 6 independent experiments; Mann-Whitney test) post-infection **(D)** Renal function is significantly compromised in *Cxcr1*$^{-/-}$ mice post-infection. Shown are summary data of serum BUN (*P*=0.0477; Mann-Whitney test) and creatinine (*P*=0.0403; Mann-Whitney test) at day 7 post-infection (n=17–18; 3 independent experiments). **(E)** Histopathology. Representative PAS staining of *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidney sections at day 4 post-

infection. Original magnification, 20x; Bar scale, 500 μm (n=15; 3 independent experiments). All quantitative data represent mean ± SEM.

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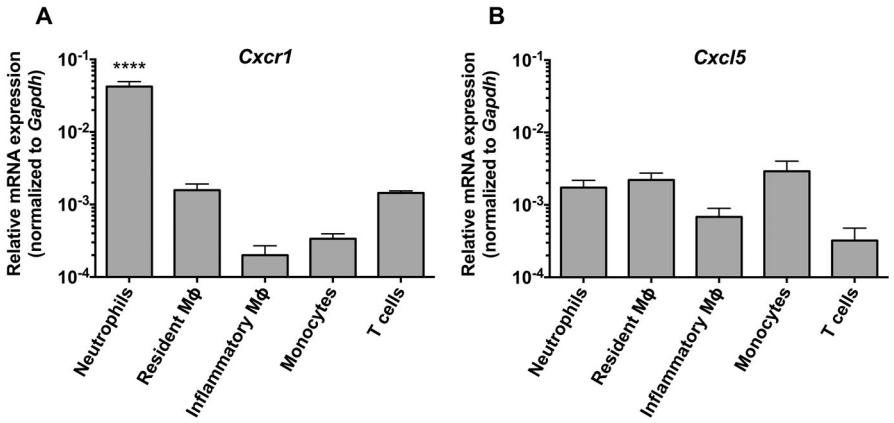

Figure 3. Neutrophils are the major Cxcr1-expressing immune cells in the kidney during systemic candidiasis whereas Cxcl5 is evenly expressed among various immune cell types

(A) Relative expression of *Cxcr1* and **(B)** *Cxcl5* in resident macrophages (day 0) and in neutrophils, monocytes, inflammatory macrophages and T cells (day 4 post-infection) FACS-sorted from *Cxcr1*$^{+/+}$ kidneys (n=3–6; 2 independent experiments). ****P<0.0001 for neutrophil *Cxcr1* expression relative to other immune cells by one-way ANOVA with Bonferroni correction. Data represent mean ± SEM.

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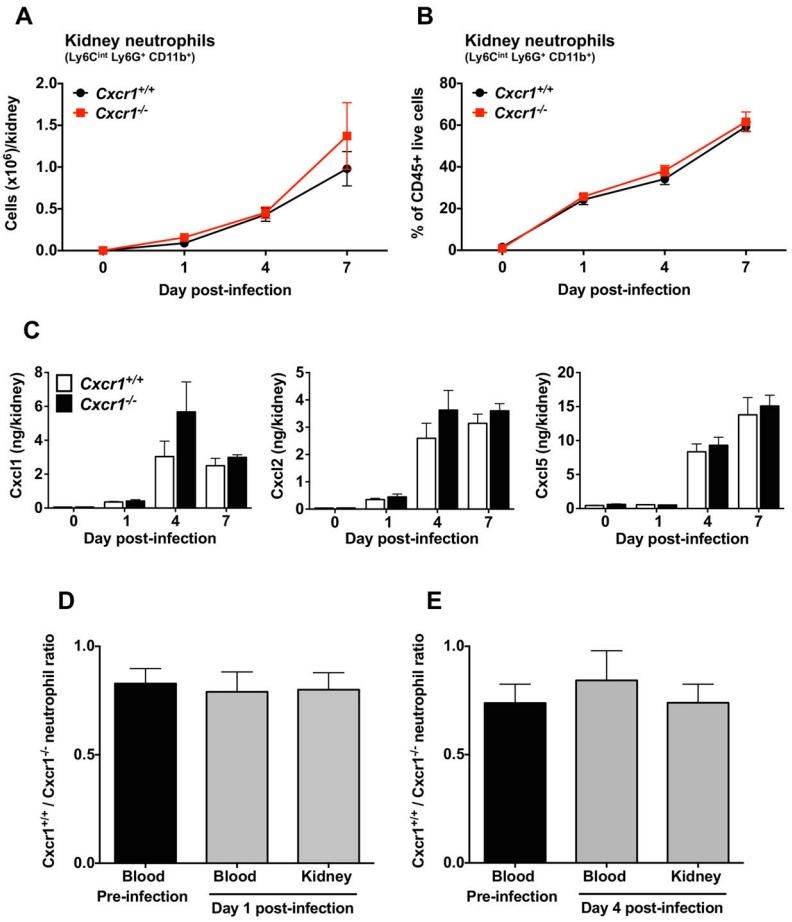

Figure 4. Cxcr1 does not mediate neutrophil trafficking in the kidney during systemic candidiasis

(A, B) Accumulation of neutrophils in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidneys at days 1, 4 and 7 post-infection. **(A)** Number of neutrophils. **(B)** Percent of neutrophils within total CD45^{+} leukocytes (n=6–15; 2–4 independent experiments). **(C)** Induction of neutrophil-targeted CXC chemokines in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidneys after *Candida* infection (n=6–8; 2 independent experiments). **(D,E)** Ratio of CD45.1^{+} *Cxcr1*$^{+/+}$ and CD45.2^{+} *Cxcr1*$^{-/-}$ neutrophils in the blood before infection and in the blood and kidney at days 1 **(D)** and 4 **(E)** after *Candida* infection of mixed bone marrow radiation chimeras (n=6; 2 independent experiments). Data represent mean ± SEM.

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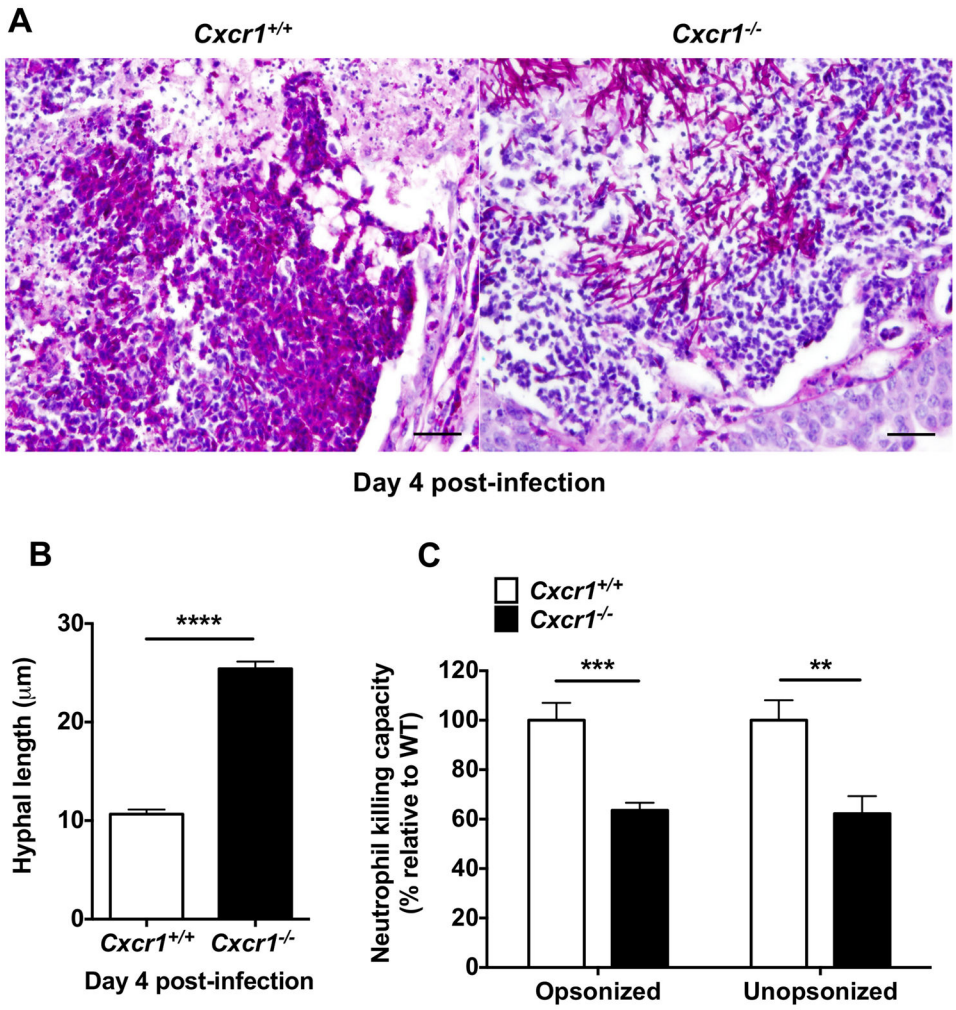

Figure 5. Cxcr1 deficiency impairs the killing capacity of kidney neutrophils against *Candida* and results in decreased hyphal damage in the infected kidney

(A) Representative PAS staining of $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ kidney sections (day 4 post-infection) showing degraded hyphal elements within $Cxcr1^{+/+}$ neutrophil abscesses but intact hyphae within $Cxcr1^{-/-}$ neutrophil abscesses. Original magnification, 400x. Bar scale, 100 μm **(B)** *Candida* hyphal length within $Cxcr1^{+/+}$ and $Cxcr1^{-/-}$ renal neutrophil abscesses at day 4 post-infection (n=452–466 randomly selected hyphal element measurements obtained from 12–13 kidneys per genotype; summary data from 3 independent experiments). **** $P<0.0001$; Mann-Whitney test. **(C)** $Cxcr1^{-/-}$ neutrophils MACS-sorted from the kidney at day 4 post-infection have impaired ability to damage opsonized ($P=0.0009$) and unopsonized ($P=0.0034$) *Candida* hyphae *ex vivo* (n=8; 4 independent experiments; *t*-test). All quantitative data represent mean ± SEM.

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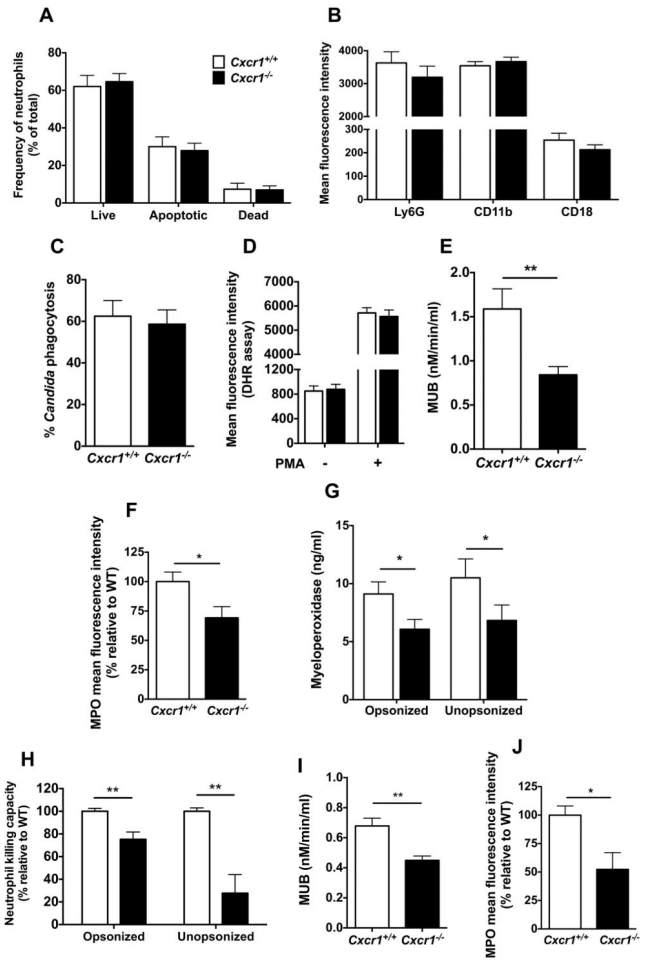

Figure 6. *Cxcr1*$^{-/-}$ neutrophils exhibit cell-intrinsic defects in degranulation and non-oxidative *Candida* killing but no impaired survival, maturation or phagocytosis

(A) Neutrophil survival. Percentage of live, apoptotic and dead neutrophils in the kidney at day 4 post-infection determined by Annexin V and 7-AAD FACS staining (n=8; 2 independent experiments). **(B)** Neutrophil maturation. Mean fluorescence intensity of the maturation markers Ly6G, CD11b and CD18 expressed on the surface of kidney neutrophils at day 4 post-infection (n=4–18; 2–5 independent experiments). **(C)** Neutrophil phagocytosis. *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils isolated from uninfected bone marrows have similar capacity to internalize dTomato-expressing *Candida albicans* (n=4; 2 independent experiments). **(D)** Neutrophil oxidative burst. Mean fluorescence intensity of rhodamine 123 measured by FACS in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils MACS-sorted from the kidney at day 4 post-infection with or without additional *ex vivo* stimulation with PMA (n=4, 2 independent experiments). **(E)** Neutrophil degranulation. β-methylumbelliferone (MUB) amount measured as a marker of release of granule beta-glucuronidase in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils MACS-sorted from the kidney at day 4 post-infection (P=0.004; n=13; 4 independent experiments; Mann-Whitney test). **(F)** Neutrophil MPO content. Mean fluorescence intensity of MPO assessed by intracellular FACS in *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ kidney neutrophils at day 4 post-infection (P=0.0202; n=15; 4 independent experiments; *t*-

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test). **(G)** Neutrophil MPO release. Release of granule MPO from *Cxcr1*$^{+/+}$ and *Cxcr1*$^{-/-}$ neutrophils MACS-sorted from the kidney at day 4 post-infection following *ex vivo* infection with opsonized (P=0.0308; t-test) or unopsonized (P=0.0384; Mann-Whitney test) *Candida* hyphae (n=17; 4 independent experiments). **(H–J)** *Cxcr1*$^{-/-}$ neutrophils isolated from uninfected bone marrows exhibit defects in *Candida* hyphal killing (P=0.0032; n=12; 6 independent experiments; t-test with Welch's correction for opsonized hyphae and P=0.0021; n=9; 5 independent experiments; t-test with Welch's correction for unopsonized hyphae) **(H)**, degranulation capacity measured as beta-glucuronidase release (P=0.0033; n=6; 3 independent experiments; t-test) **(I)**, and intracellular MPO content (P=0.0207; n=8; 4 independent experiments; Mann-Whitney test) **(J)** Data represent mean ± SEM.

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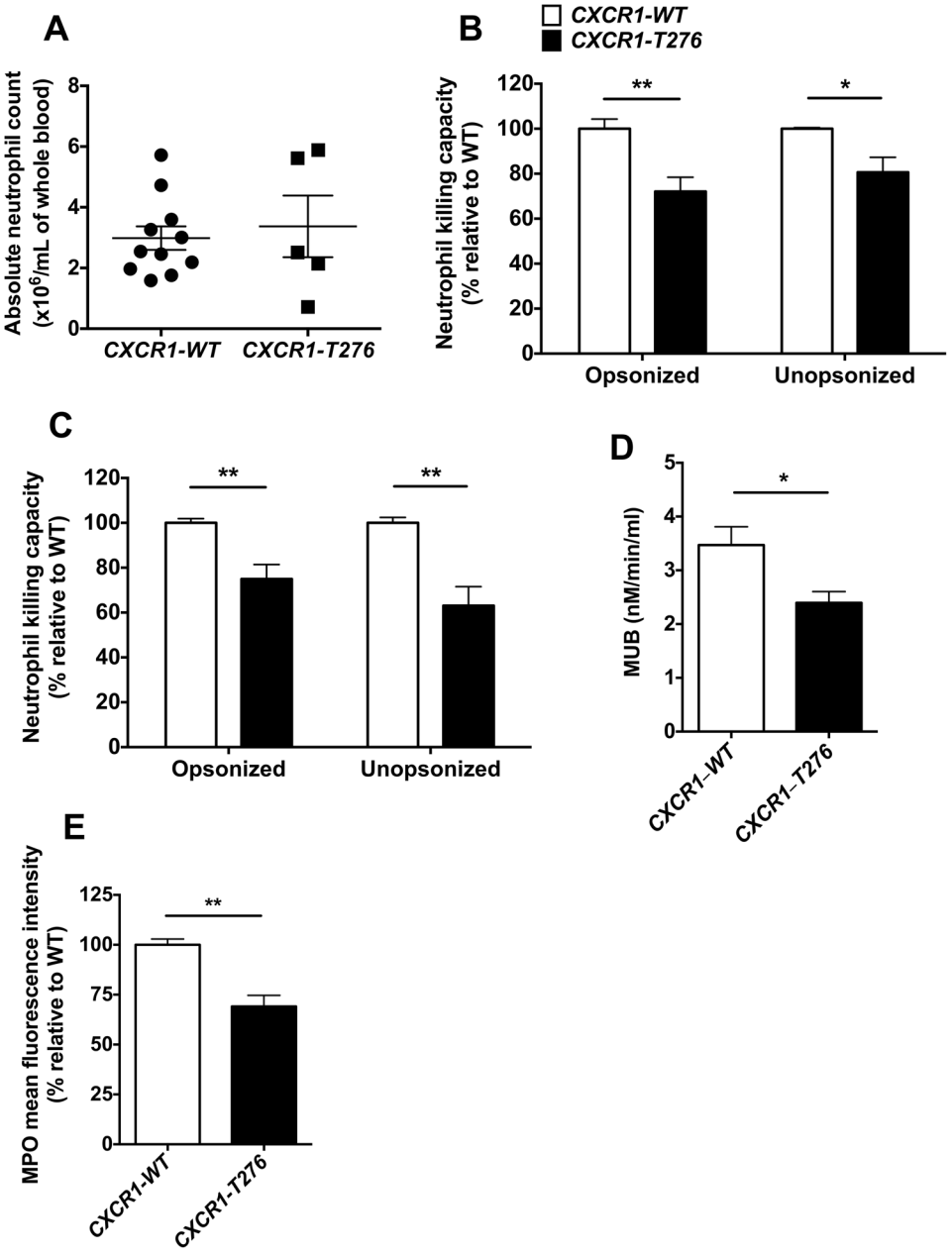

Figure 7. The mutant *CXCR1-T276* allele is associated with impaired fungal killing and degranulation in human neutrophils

(A) Healthy donors carrying the *CXCR1-T276* allele have similar absolute neutrophil counts with *CXCR1-WT* donors (n=5–11). **(B–E)** Neutrophils of healthy donors carrying the *CXCR1-T276* allele exhibit impaired ability to damage opsonized and unopsonized *Candida* yeast **(B;** P=0.0067 for opsonized and P=0.0332 for unopsonized yeast; n=5–6; 5 independent experiments; t-test with Welch's correction) and hyphae **(C;** P=0.0023 for opsonized and P=0.0012 for unopsonized hyphae; n=6–7; 5 independent experiments; Mann-Whitney test) *ex vivo*, defective degranulation as measured by release of beta glucuronidase (P=0.0381; n=4–6; 3 independent experiments; Mann-Whitney test) **(D)**, and

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decreased intracellular MPO content (P=0.0012; n=5–6; 3 independent experiments; t-test) **(E)**.

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Table 1

Association of the mutant *CXCR1-T276* allele with development of disseminated candidiasis after candidemia in patients of mixed European descent (n=153).

Variable	Univariate analysis *P* value	Multivariate analysis OR (95% CI)
CXCR1-T276 allele (CG + GG)	0.0455	3.95 (1.20–13.03)
Male sex	0.5264	
Immunocompromised state	0.2882	
Hematopoietic stem cell transplantation	0.5845 *	
Solid organ transplantation	0.0102	4.03 (1.46–11.17)
Active malignancy	0.3656	
Solid tumor	0.5967	
Leukemia	1.0000 *	
Lymphoma	0.3124 *	
Chemotherapy within past 3 months	0.6836	
Neutropenia (ANC < 500 cells/mm^3)	0.4837	
Surgery within past 30 days	0.102	
Receipt of total parenteral nutrition	0.0891	3.19 (1.19–8.21)
Dialysis dependent	1.0000 *	
Acute renal failure	0.2652	
Liver disease	0.1551	
Intensive care unit admission within past 14 days	0.5278	

* Fisher's exact test; ANC, absolute neutrophil count