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# *Candida albicans* heme oxygenase and its product CO contribute to pathogenesis of candidemia and alter systemic chemokine and cytokine expression

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## Abstract

Mammalian heme oxygenases play important roles in immune regulation by producing immunosuppressive CO. The pathogenic yeast *Candida albicans* encodes a heme oxygenase, Hmx1, that is specifically induced by the host protein hemoglobin, suggesting a role in the pathogenesis of disseminated bloodstream infections. We show that exposing mice to therapeutic levels of CO increases *C. albicans* virulence, whereas a *HMX1* null strain has decreased virulence in murine disseminated candidiasis. Levels of several regulatory cytokines and chemokines are decreased in mice infected with the null strain, and initial lesions in the kidney are more rapidly cleared following PMN infiltration. Reconstitution of one or both alleles restores virulence to the level of wild type. Growth in vitro and initial organ burdens in infected mice are not decreased and host iron overload does not restore virulence for the null strain, suggesting that early growth in the host is not limited by Hmx1-mediated iron scavenging. In contrast, inhaled CO partially reverses the virulence defect of the null strain and restores several host cytokine responses to wild type levels. Collectively, these results show that *C. albicans* Hmx1 expression and CO production limit the host immune response and contribute to the pathogenesis of candidemia.

### Keywords

*Candida albicans*; heme oxygenase; antifungal immunity; kidney; virulence; mouse model; carbon monoxide

## INTRODUCTION

*Candida albicans* is the most common human fungal pathogen and the fourth leading cause of nosocomial infections in the United States [1]. It causes fatal disseminated infections in immunocompromised individuals and superficial infections, including oral thrush and vaginal yeast infections, in immunocompetent individuals. *C. albicans* is a normal component of the human gastrointestinal flora, but imbalances in the normal microbiota or in mucosal and systemic immunity can initiate a switch between its commensal and pathogenic states. Virulence attributes enabling *C. albicans* to adapt and survive in the

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human host include dimorphism, phenotypic switching, expression of host adhesion receptors, and secretion of extracellular proteases [2–3].

Heme oxygenases are enzymes found in bacteria, fungi, and mammals that catalyze the transformation of ferroheme to biliverdin, CO and ferrous iron [4]. Three heme oxygenase isoforms have been identified in higher animals. Production of CO and biliverdin by the inducible mammalian isoform HO-1 plays an important role in controlling inflammatory responses. CO is immunosuppressive, and biliverdin is a potent antioxidant [5–6].

Some bacterial heme oxygenases have been demonstrated to contribute to pathogenesis by scavenging iron from heme, which supports microbial growth and limits host superoxide production [7–8]. Iron deprivation similarly induces expression of the heme oxygenase gene *HMX1* in the pathogenic yeast *C. albicans* [9–10]. Like other heme oxygenases, Hmx1 of *C. albicans* degrades heme to release iron, CO, and biliverdin [10–11]. However, we initially identified Hmx1 as a *C. albicans* gene that is rapidly induced by hemoglobin in an iron-independent manner [10]. This suggested that Hmx1 may play additional roles in hematogenous disseminated infections.

Here we examine the role of *HMX1* in a mouse model of disseminated candidiasis [12] and show that deletion of *HMX1* causes reduced virulence. We show that two products of Hmx1, iron and CO, increase the virulence of *C. albicans* in mice and that exogenous CO partially complements the virulence defect of *C. albicans* lacking *HMX1*. Systemic *C. albicans* infections alter the Th1/Th2 immune balance by altering systemic cytokine and chemokine expression [2], and we further show that deletion of *HMX1* alters the pattern of cytokine and chemokine expression in a manner consistent with CO-mediated suppression of acute host inflammatory responses.

## MATERIALS AND METHODS

### Strains and growth conditions

*C. albicans* strains used for this study are listed in table 1. For challenge of mice, *C. albicans* cells were grown overnight in 50 mL of Yeast Peptone Dextrose (YPD) medium at 30°C with aeration as previously described [13]. Cells were harvested by centrifugation at 5000 rpm for 10 min, washed once with 50 ml of sterile, non-pyrogenic normal saline (Quality Biological Inc. Gaithersburg, MD) and resuspended in 10 ml of saline before quantifying cell numbers using a Petroff-Hausser counting chamber. The cell suspensions were adjusted to the final concentration for parenteral administration using non-pyrogenic sterile saline. For gene expression analysis, *C. albicans* cells were grown for 16 h in 50 ml of yeast nitrogen base (YNB) medium. Fresh cultures were started at 0.2 OD using overnight YNB cultures, and grown up to 0.8 OD (for about 4–5 h) for RNA extraction at 30°C or 37°C. YPD agar plates and YPD agar plates with Nourseothricin (Werner BioAgents, Jena, Germany) were used to grow transformants of *C. albicans*.

### *HMX1* disruption and complementation

We disrupted the *Candida HMX1* gene using the method we published previously [14] according to the strategy reported by Reuss et al. [15] using wild type strain SC5314 [16]. Synthetic oligonucleotides used in this study are listed in Table 2.

### Hemeoxygenase activity assay

Heme oxygenase activity was measured by production of biliverdin. Fresh cultures of WT SC5314, the heme oxygenase null mutant DRL2, and heme oxygenase complemented strain DRL4 were grown in defined GPA (glucose phosphate ammonia) medium with 25 μM Hb

to induce heme oxygenase. Each culture was started from an overnight culture inoculated into 100 ml of medium at $0.6\times10^9$ cells/m l. Biliverdin production was quantified at 5, 10 and 24 h post inoculation with Hb by extracting cell pellets containing $6\times10^9$ cells in triplicate using 1 ml of methanol, vortexing for 30 s, and centrifuging at 5000g at room temperature as reported previously [10].Biliverdin concentrations were calculated from the absorption spectra in methanol at room temperature, which has a maximum at 666 nm with a coefficient of 14,400 $M^{-1}$ $cm^{-1}$ [17].

### Mouse infection with *C. albicans*

Outbred 6–8-week-old (18–20 g) BALB/c female mice obtained from Charles River Laboratories (Wilmington, MA) were randomly allocated to groups of five animals and housed and cared with ad libitum access to filtered water and standard mouse chow. Handing and care of animals under protocol LP-022 was conducted in compliance with the guidelines established by the Animal Care and Use committees of the National Cancer Institute.

Each group of mice was inoculated intravenously in the lateral caudal tail vein using a 27 gauge needle with a volume of 0.1 ml containing $10^6$ *C. albicans* cells [13,18]. Clinical signs of illness in each mouse were evaluated three times daily, and mice that displayed severe signs were euthanized immediately by $CO_2$ inhalation and processed for complete necropsy and collection of tissues for histopathological examination. To longitudinally monitor effects of *C. albicans HMX1* on organ burden and host immune responses, mice were euthanized sequentially from 6 h to 240 h post-inoculation (PI). A total of 40 mice were inoculated with SC5314, 40 were inoculated with DRL2, and 10 control mice received no fungal challenge. Three animals from each group were sacrificed at 6, 12, and 24 h and daily from day two to seven PI for histopathology and cytokine assays. The 10 control animals, i.e., untreated and uninfected, were sacrificed, and the organs and sera were collected. The mean results for these 10 control animals were used as time zero values. Sera separated from the blood collected from individual mice were stored at −80°C until analysis.

### Effect of iron overload on virulence of the *hmx1Δ/hmx1Δ* mutant

To examine the role of iron in the effect of *HMX1* deletion on virulence, mice infected with WT SC5314 and DRL2 mutant were administered 100 μl of iron dextrans (Sigma, St Louis, MO) in normal saline (10 mg iron) i.p. every other day until the end of each experiment. Five mice in a control group received single dose i.v. saline and iron dextran i.p. every other day. The iron overload dose was adopted from previously published iron overload experiments in mice [19–20] In the first experiment, each group of 15 mice received $10^6$ cells of *C. albicans* i.v. In a follow up experiment, each group of 10 mice received $5\times10^5$ cell of *C. albicans* i.v.

### Supplementation of exogenous CO in WT and *hmx1Δ/hmx1Δ* strain infections

To examine whether CO influences the virulence of disseminated Candidiasis and whether CO produced by Hmx1 contributes to virulence, mice infected with wild-type and *HMX1* mutant strains were exposed to established therapeutic levels of exogenous CO. Mice were maintained in an atmosphere of air supplemented with 250 ppm CO continuously in a gas tight chamber as previously published [5,21]. Briefly, mice inoculated with the *C. albicans* wild-type and mutant were exposed to air containing 250 ppm CO (Roberts Oxygen, Rockville, MD) in a 15”×20”×20” gas tight camber (BioSpherix Ltd, Lacona, NY) for up to 7 days. As the oxygen consumption of mice weighing 24 ± 5 g is 2.4 ±0.5 ml/g/hr [22], we supplied 50 ml/min gas flow throughout the experiment and treated 5 mice at a time. In the first experiments two groups of mice were infected with wild-type *C. albicans*, and one group was maintained in the air/CO chamber and the other under room air. Two control

groups of uninfected mice were kept in room air or in the air/CO chamber. In another set of experiments one group was infected with WT and two groups were infected with mutant strain. One group infected with the mutant strain was maintained in the air/CO chamber, and the other group was kept in room air. Uninfected mice were used as a negative control. Moribund mice were immediately euthanized, and necropsies were conducted to confirm death from candidiasis. Cumulative results from 10 mice in each treatment group were analyzed.

To investigate whether exogenous CO complements the inflammatory response, we examined immune responses and organ colonization in infected null mice exposed to 250 ppm CO. Blood was collected from surviving mice by submadibular bleeding until 3 days PI. Sera were collected at 1, 2 and 3 day PI in two group of five mice, each group consisting of five mice infected with DRL2 with or without exposure to CO. At the end of day three, mice were euthanized to study histopathology and assess organ burden and inflammation.

### Necropsy and Histopathology

Immediately after euthanasia, macroscopic changes were recorded, and the brain, heart, lungs, liver, spleen, and right kidneys were immersed in buffered 10% formalin, processed for paraffin embedding, sectioned at 5 μm, and stained with H&E. Grocott's modification of Gomori's methenamine-silver (GMS) stain was used for detection of fungi in situ [23]. Left kidneys of the mice were frozen at −80°C for future DNA extraction to measure fungal burden.

### Microscopy

Histopathology images from sections of formalin-fixed and paraffin-embedded tissues stained with Gomori's methenamine-silver or hematoxylin and eosin were obtained using light microscope (Olympus BX51) fitted with a digital camera (Nikon DXM1200F). Images were processed with Adobe Photoshop.

### Determination of serum cytokines and chemokines

Murine serum was collected f rom sacrificed mice at various time points following infection with *C. albicans* wild- type and mutant strains. A Luminex -bead array (Mouse cytokine/ Chemokine LINCOplex Kit, catalog no. 551287, Linco Research, Inc. St Charles MO) was used for detection of the cytokines IL-4, IL-10, IL12, IL17, and IFN-γ, and Milliplex MAP Kit (catalog no MPXMCYTO-70K, Millipore, Billerica, MA) was used to detect cytokines IL-6, IL-10 IL-17, TNF-α, and chemokines G-CSF, IP-10, MIP1β, MIP1α and RANTES according to the manufacturer's specifications.

### RNA Extraction and Gene expression analysis by RT-PCR

RNA isolation was done using a standard hot phenol procedure [24] and gene expression was done as we reported previously [25]. Synthetic oligonucleotides used in this experiment are listed in table 2.

### DNA Extraction from Infected Kidneys

A qPCR based method was used to quantify organ fungal burden. Total DNA of each frozen kidney was harvested with minor modification of the previously described method [26].

### Determination of Fungal burden

Primers to amplify a highly conserved 150 nucleotide sequences of *C. albicans* ribosomal multicopy genes [27] (Table 2) were used to quantify fungal ribosomal DNA from mouse kidney total DNA. 25 μl of PCR reactions were mixed with 20.5 μl of master mix

containing SYBR green mix and specific primer mix and 4.5 μl total kidney DNA and serially diluted genomic DNA of SC5314 as a standard. Each mixture was transferred to 96-well optical microtiter plates (ABgene, Rockford, IL) and qPCR reaction was performed as described previously. The DNA concentration in each well determined using Opticon I software (BioRad, Hercules, CA) was used to calculate total Candida ribosomal DNA in the respective kidneys.

### Statistics

The probability of survival as a function of time was determined by the Kaplan-Meier method, and significance was determined by the log-rank (Mantel-Cox) test and Jehan-Breslow-Wilcoxon test using GraphPad Prism soft ware. The mixed procedure of the SAS system [28] was used to analyze serum cytokine expression patterns among all treatment groups at various time points. Three randomly selected mice from each group were euthanized at each time point for longitudinal comparisons. Data were analyzed for significant differences by comparing means of each triplicate reading at various time points assuming that the cytokine expression levels within each group of mice are normally distributed. [2]. Cytokine expression in infected mice exposed to CO was analyzed by one way ANOVA with post Bonferroni multiple comparison test. Fungal burden was analyzed using a 2 way ANOVA and t test. Quantitative RT-PCR data were analyzed using the Pfaffl method [25] and were normalized in two steps. First, mean values for the internal CDC36 control were subtracted from the corresponding experimental sample, and the variance was calculated as the square root of the sum of the squares of the standard deviations. Second, each sample group was normalized to the 'medium only' sample followed by calculation of fold change using $2^{-Ct}$ of this normalized Ct value.

## RESULTS

### Creation of the *hmx1Δ/hmx1Δ* mutant

SC5314 *C. albicans* was transformed using a linearized fragment generated from pHMXKO, the deletion cassette containing plasmid (Fig 1a.A). Nourseothricin-resistant colonies were picked after growth at 30°C for approximately 48 h and re-inoculated into YPD liquid medium containing 200 μg/ml nourseothricin. Homologous recombination was confirmed by Southern hybridization. The nouseothricin resistant strain with one inserted *HMX1* knock out cassette was named $DRL1^{NR}$.

$DRL1^{NR}$ was inoculated into liquid medium containing maltose to induce the *MAL2* promoter without selective pressure to allow *caFLP*- mediated excision of the *caSAT1* flipper cassette as described [2,15]. Serially diluted $DRL1^{NR}$ was then spread on YPD plates containing 25 μg/ml of nourseothricin to identify sensitive colonies with excised *caSAT1*. As reported by Reuss et al, the *MAL2* promoter was leaky, and growth in yeast peptone maltose did not yield 100% Flip-mediated excision. However, smaller nouseothricin sensitive CFU could be distinguished from large nouseothricin resistant CFU. These cells were restreaked on YPD containing 200 μg/ml nourseothricin, where lack of growth indicated excision, which was confirmed by Southern hybridization. The resulting strain DRL1 was again transformed with the same ApaI-SacI fragment to make $DRL2^{NR}$ and, following another *caFLP* mediated excision, generated the homozygous *HMX1* knock out mutant DRL2.

### HMX1 complementation

Complementation of the gene was performed by transformation of DRL2 with a *HMX1* complementation cassette comprising the Apa1-Sac1 fragment of pHMXCOMP, yielding

heterozygous knock-in mutants DRL3$^{NR}$ and DRL3 (Fig 1a.B). These were used to create homozygous knock-in strains DRL4$^{NR}$ and DRL4.

### Gene expression analysis

*HMX1* mRNA was not detected in the mutant strain, and when grown at 30°C was restored to 20–70% in several analyses of the single allele complemented strain (DRL3), and 70–150% in the fully reconstituted strain (DRL4) (Fig. 1b.B). Expression was fully restored in both complemented strains when assessed at 37°C (Fig. 1b.A).

The essential gene *HBR1* is located immediately downstream of *HMX1* [10]. Expression of *HBR1* was maintained in DRL2, DRL3 and DRL4 at both 30°C and 37°C (Fig. 1b A & B). The minor differences observed were not reproducible.

### *HMX1* complementation restores heme oxygenase activity

The *HMX1* null mutant DLR2 lacked significant biliverdin production when grown in medium supplemented with Hb (Fig. 2).In contrast, SC3415 WT and the *HMX1* complemented strain DRL4 showed time-dependent biliverdin production in cultures grown up to 24 h in the presence of Hb, confirming restoration of enzymatic activity in the complemented strain.

### *HMX1* deletion does not alter dimorphism or growth rate in vitro

Consistent with previous studies using a different *HMX1* null mutant [10], we did not observe any differences in growth rate for DRL2 in YNB medium or serum-induced germ tube induction at 37°C (data not shown).

### Reduced virulence of DRL2 (*hmx1Δ/hmx1Δ*) in murine disseminated candidiasis

Administration of wild type *C. albicans* strain to mice resulted in significantly lower survival than the *hmx1Δ/hmx1Δ* mutant DRL2 ($p < 0.0001$, Fig. 3a). Mice inoculated with wild type began to die as early as 1 d post-inoculation (PI) and suffered 100% mortality by 7 d PI. No mice inoculated with the mutant died until 10 d PI, and 60% survived at 24 d PI. As expected, control mice inoculated with intravenous saline alone had no mortality through 24 d PI.

In a second experiment, mice inoculated using wild type or the reconstituted DRL3 (*hmx1Δ / hmx1Δ::HMX1*) and DRL4 (*hmx1Δ::HMX1/hmxΔ::HMX1*) strains had similar patterns of mortality and started dying 2 days PI (Fig. 3b). Mice inoculated with *HMX1* null mutant survived longer compared with those receiving wild type ($p< 0.0008$), but *HMX1* complemented strains did not show significant differences in survival from the wild type group (Fig. 3b).

### Iron overload does not complement the *HMX1* deletion phenotype in mice

Because deletion of *HMX1* impairs growth under iron restriction in vitro [10] and iron overload increases the pathogenicity of *C. albicans* and other microbes in mice and humans [29–30], the reduced virulence of DRL2 could be due to impaired iron acquisition and growth in vivo. Preliminary experiments using mice with iron overload induced by administering iron-dextran complex established that a reduced inoculum of $5\times10^5$ cells per mouse is optimal under these conditions. Mice with iron overload receiving the wild type strain began to die at 2 d PI and suffered 100% mortality by 6 d PI (Fig. 4a). Mice with iron overload inoculated with the mutant began to die at 3 d PI, but 30% survived at 7 d PI. As expected, control mice receiving intravenous saline and intraperitoneal iron had no mortality through 7 d PI. Survival of mice inoculated with the WT *C. albicans* strain was significantly

lower than of those receiving the mutant ($p < 0.017$). Therefore, factors other than iron deficiency must contribute to the decreased virulence of the *HMX1* mutant.

### Exogenous CO increases *C. albicans* virulence

Because iron supplementation did not complement the virulence defect of the *HMX1* null mutant, we examined whether another enzymatic product of Hmx1, the immunosuppressive molecule CO [21], was involved. First, we addressed if CO i s a virulence factor for *C. albicans*. CO supplementation at the well documented therapeutic concentration of 250 ppm significantly decreased the survival time of mice infected with wild-type *C. albicans* ($p < 0.0002$) CO (Fig 4b). Exposure of uninfected mice to this level of CO for the same time period had no adverse effects. Therefore, CO is a virulence factor for disseminated *C. albicans* infections.

### Exogenous CO complementation restores virulence to the *hmx1Δ/hmx1Δ* strain

CO supplementation at 250 ppm increased the mortality of mice infected with mutant strain to a level not significantly different from that of WT (Fig. 4c). Both groups began to die as early as 2 day PI and suffered 100% mortality by 7 day PI. Mice in the same experiment not supplemented with CO and infected with the null strain had the expected higher survival through 7 d PI (Fig. 4c, $p < 0.009$). Thus, CO production by Hmx1 at least partially accounts for its effect on virulence.

### *HMX1* deletion does not alter early kidney fungal burden

If Hmx1 activity was primarily limiting for iron acquisition during disseminated candidemia, this should be reflected in decreased early organ burden in mice infected with the null mutant. We measured kidney fungal burden in mice infected with wild type or mutant strains by quantifying *Candida* ribosomal DNA using qPCR. Mice infected with the wild type or the mutant *C. albicans* strain did not show significant difference in kidney fungal burden at early time points (Fig. 5). Only 6 and 7 days PI showed significant differences ($p < 0.03$). This suggests that impaired initial growth does not account for the decreased virulence of the mutant strain and is consistent with the presence of redundant iron uptake systems in *C. albicans* that utilize siderophores and ferric iron chelates as well as heme [31].

### HMX1 alters histopathology of candidiasis in kidney and brain

We also examined whether *HMX1* disruption changes the patterns of fungal colonization in kidney and brain by histological analysis. In addition we monitored inflammatory cell infiltration in infected kidneys to determine whether Hmx1 expression alters the inflammatory response. Fungal growth in these organs was minimal in both groups of mice at 6 h PI, and a few isolated yeast cells starting to develop germ tubes at 12 h PI with no inflammatory reactions (data not shown). Mice euthanized at day 1 PI contained localized areas of mycelia scattered primarily in parenchyma of the kidney and brain tissues. At this time we did not find significant differences in colonization of kidney (Fig. 6a) and brain (Fig. 6c) or in inflammatory reactions (Fig. 6d).

By 2 days PI, the entire kidney parenchyma in mice infected with wild type strain was severely invaded by growing filaments, pseudohyphae, and yeast cells (Fig. 6a). Brain tissues showed scattered predominantly filamentous fungal invasion (Fig. 6c). Mice infected with the mutant strain also showed scattered colonization at 2 days PI, but less condensed compared with mice infected with the wild type (Fig. 6a). Fungal invasion in brain followed a similar pattern (Fig. 6c). Severe inflammatory reactions were observed in kidney parenchyma with polymorphonuclear leucocytes infiltration among necrotic patches

localized with wild type strain colonization, whereas the mutant colonization showed lower inflammatory reactions (Fig. 6d).

Greater differences were seen 3 days PI. SC5314-infected mouse kidneys exhibited fungal cells with large PMN cell infiltration and showed severe phylonephritis (Fig 6a and d). In mice infected with wild-type, we observed fungal colonization and inflammatory reactions in the kidney medulla. Brain fungal invasion were lower in mice infected with the mutant strain. *C. albicans* did not invade the kidney medulla of mice infected with the mutant strain (data not shown).

At 4–7 days PI, some resolution in kidne y parenchymal tissues was observed in both groups, and PMN became focally localized adjacent to the remaining organisms in the cortical region (data not shown). Mice infected with wild type at 5–6 days PI showed efficient cortical tissue clearing, but fungal burden remained in the medulla of the kidney (Fig. 6b). Hyphae were observed in collecting ducts with prominent growth of mycelia in the pelvis. In contrast, we did not observe huge fungal colonies beyond 5 days PI in mice infected with mutant strain (Fig. 6b), and inflammatory reactions were lower in these animals.

### *HMX1* alters serum cytokine and chemokine responses during systemic candidiasis

To further characterize the reduced inflammatory responses in mice infected with the mutant strain, we examined serum levels of several cytokines and chemokines that may participate in immunity against systemic candidiasis in the mice used for histopathology and fungal burden analyses (Fig. 7).

Serum IL-12 levels generally declined following infection but did not differ between wild type and mutant strains (Fig. 7a). IFN-γ and IL-4 levels in all samples were below the detectable limit for the Luminex assay.

TNF-α levels were elevated in all infected mice relative to basal levels in uninfected mice Fig. 7b). Mice infected with wild type had significantly elevated TNF-α levels at 2, and 3 days PI ($p< 0.005$, and 0.04) compared with mice infected with the mutant at the respective time points.

Like TNF-α, IL-6 levels in mice infected with wild type showed early peaks around days 2–3 PI (Fig. 7c). Mice infected with the mutant lacked the early response. IL-6 levels were significantly higher in mice infected with wild type versus mutant strains at 2 and 3 days PI ($p< 0.002$, and 0.0008).

IL-17 levels also showed differences (Fig. 7d). Mice infected with wild type showed significantly lower serum IL-17 at 2,and 3 days PI ($p<0.0001$ and 0.003 compared with control mice and significantly lower IL-17 at 3 days PI compared with respective mutant infected groups ($p< 0.003$).

Serum IL-10 levels differed at the early stage of infection (Fig. 7h). Wild type infected mice at 2 day PI had significantly elevated IL-10 (170 ± 92 pg/ml) compared with the control group (8.6 ± 2.1 pg/ml, $p< 0.004$) or the group infected with mutant strain (3.1 pg/ml, $p<0.009$).

We also examined expression of several chemokines. Serum G-CSF in mice infected with wild type remained significantly elevated compared to control mice in all time points (Fig. 7e). Mice infected with the mutant also had significantly elevated G-CSF at day 1 PI. However, at 2 and 3 days PI these mice showed significantly lower levels of G-CSF

compared with wild type ($p<0.0004$ and 0.004), and levels tended lower than wild type at all time points.

Serum MIP1β showed a response similar to those of IL-6 and TNF-α (Fig. 7f). Wild type infected mice had significantly elevated serum MIP1β levels at 2 and 3 days PI compared to mutant infected groups ($p< 0.002$, and 0.0002.). Mice infected with the mutant lacked the early response for serum MIP1β at 2–3 days PI.

We observed significantly elevated serum RANTES levels at 2 and 3 days PI in wild type infected mice (Fig. 7g) compared with mutant ($p< 0.002$ and 0.007). In contrast, serum IP-10 levels were not significantly affected by *C. albicans* infection and did not differ between wild type and mutant strains (data not shown).

## Exogenous CO supplementation restores WT cytokine responses to a *hmx1Δ/hmx1Δ* infection

To determine whether differential cytokine responses in mice infected with the null strain were due to the absence of CO, mice infected with the mutant strain were exposed to exogenous CO, and expression of selected cytokines and chemokines was compared to infected mice at the same times without CO supplementation (Fig. 8). Exposure to exogenous CO significantly elevated TNF-α levels at day 1 and 2 PI (Fig. 8A, $p< 0.005$ and 0.05 respectively), consistent with the elevated TNF-α at the same time points in mice infected with the WT strain expressing Hmx1. Similarly, the deficit in IL-6 levels one day PI in mice infected with the null mutant was partially reversed by exposing the infected mice to exogenous CO (Fig 8B, $p< 0.003$), as was the deficit in IL-10 at 1 day PI (Fig 8D, $p< 0.01$).

As expected, IL-17 levels differed only at 3 days PI. Exogenous CO significantly lowered serum IL-17 at 3 days PI (Fig. 8C, $p<0.009$), consistent with the lower IL-17 levels observed at this time point in mice infected with the WT strain expressing Hmx1 (Fig. 7d).

Similar to IL-6 and TNF-α, expression of the chemotactic and proinflammatory MIP family chemokine member MIP1-β was decreased in mice infected with the null mutant, and significantly higher levels of serum MIP1-α were seen in mice exposed to CO at 2 and 3 days PI compared with the non exposed group (Fig. 8e, $p< 0.0001$ and 0.001 respectively). Therefore, both cytokine and chemokine responses observed in a WT infection can be recapitulated by exposing mice infected with the null mutant to exogenous CO. This suggests that CO is the relevant enzymatic product of Hmx1 that alters these host responses to infection.

## CO exposure restores organ colonization and inflammation responses to the null mutant

CO supplementation of mice infected with the null mutant delayed the resolution of kidney colonization typically observed at day 3 PI (Fig. 8F upper panels). Similar to a WT infection, CO supplementations resulted in spreading of the null strain into the kidney medulla (data not shown). In addition to prolonging kidney colonization, CO supplementation resulted in an increased inflammatory reaction to the null strain in the kidney cortex that contained abundant polymorphonuclear leucocytes (Fig. 8F lower right). As in previous experiments, infected mice without CO supplementation had only localized inflammatory cell infiltration around candida foci (Fig. 8F lower left panel). CO supplementation also increased brain colonization of the mutant compared with the group infected with mutant alone (data not shown).

## DISCUSSION

The heme oxygenase of *C. albicans* possesses the same enzymatic activity as other microbial and mammalian heme oxygenases but differs in being specifically inducible by the host factor hemoglobin [10]. In addition to their role in iron homeostasis, mammalian heme oxygenases play important roles in redox signaling and immune regulation by producing CO [32]. *HMX1* is not an essential gene for *C. albicans* growth *in vitro* [9–10], but we show here an important role of this gene for pathogenesis in a disseminated candidiasis model. We deleted *HMX1* in the wild-type strain SC5314 and reconstituted the gene by homologous recombination. Complementation restored mRNA expression and Hmx1 enzymatic activity to levels characteristic of the parental WT strain. In the SC5314 background a *HMX1* null mutant exhibited decreased pathogenicity, which was reversed by complementing one (DRL3) or both of the deleted alleles (DRL4) or by exogenous supplementation of CO by inhalation. Hmx1 is not required for the initial growth and or gan colonization in a murine host, but its expression modulates persistence of lesions in kidney and host cytokine responses to *C. albicans*.

Kidney and brain are the predominant colonization sites for disseminated candidiasis in humans and mice [2,33–34]. Brain tissues showed less colonization by the mutant strain from 2 to 5 d PI and were cleared from brain at later time points similar to wild type (data not shown). Wild type infections progressed to medullary invasion of the kidney, but this did not occur with the *HMX1* mutant strain.

We considered several mechanisms by which *HMX1* could contribute to these differences in organ burden and virulence. Disturbance of expression of the adjacent essential gene *HBR1* did not contribute to our results. Rather, the loss of iron scavenging and CO production may each contribute to the virulence defects of this mutant.

Exogenous iron stimulates microbial growth in animals, and bacterial heme oxygenases have been proposed to increase virulence by scavenging iron from heme [7]. Saturating the chelating capacity of endogenous iron binding proteins enhances the severity of microbial infections and impairs host immune responses [35]. Elevated serum iron is correlated with increased incidence of *C. albicans* infections in leukemia patients [36]. If iron acquisition was limiting for growth of the *HMX1* null mutant, organ burdens at early time points should be decreased in mice infected with this mutant. Our failure to see such impairment may be due to the ability of other iron uptake systems in *C. albicans* to mediate iron uptake by the *HMX1* null mutant [31]. Furthermore, establishing iron overload in infected mice increased the virulence of both strains but did not complement the decreased virulence of the null strain. With the caveat that iron overload may have complex effects on the host response to *C. albicans*, these results indicate that iron acquisition via Hmx1 is not sufficient to explain the defects we observed in the mouse candidemia model.

Our observation that kidney colonization in mice infected with the mutant strain decreased after 5 d PI and failed to progress to invasion of the kidney medulla suggested that Hmx1 expression limits clearance of the pathogen, which could be explained by the immunosuppressive activity of CO produced by Hmx1. Supplementation of mice by breathing nontoxic levels (250 ppm) is known to complement several inflammatory response defects of *HO1* null mice [5,21]. CO at this concentration has been administered to humans in clinical trials without toxicity [37–38]. Our results clearly indicate that breathing exogenous CO at this level increases virulence of the *hmx1Δ/hmx1Δ* strain and restores several cytokine responses characteristic of a WT infection. We propose that CO produced by Hmx1, expression of which could be induced by exposure to hemoglobin during

disseminated infection [10], acts as a local immunosuppressive agent to protect the invading pathogen from host immune attack.

Immune regulation is a major physiological function of the inducible mammalian HO-1 but has not been previously reported for microbial heme oxygenases. The enzymatic products CO and biliverdin modulate cytokine levels in various stress models [5–6,39–40]. We consistently observed the expected infiltration of PMN [41] at sites of *C. albicans* colonization in mice infected with the wild type strain, but from 2 d PI onward less PMN infiltrated into kidney lesions of mice infected with the mutant strain lacking *HMX1*. This occurred despite similar fungal burdens at these time points. Mild inflammatory reactions with mononuclear and PMN infiltration persisted beyond 5 d PI, at which time fungi were not detectable in kidneys of mice infected with the mutant (data not shown). Kidneys at this stage appeared to be in the resolution phase, where immune responses eliminate *C. albicans* from kidneys. These findings suggest that deletion of *HMX1* lessens the sub-acute phase of infection despite the infected mouse mounting a decreased early inflammatory reaction.

Candidiasis triggers characteristic patterns of host cytokine and chemokine expression [42]. Resistance to *C. albicans* infection involves Th1/Th17 directed cell mediated immunity [43–44]. Fatal Candida infection is associated with Th2 subset activation and IL-10 responses [41,45–46], and this cytokine is an important inhibitor of Th1 activity in infections [47]. IL-10 levels were significantly higher in mice infected with wild type versus mutant stains at 2 d PI, suggesting that *Candida* Hmx1 expression contributes towards Th2 responses by up-regulating IL-10 expression. Thus, *C. albicans* Hmx1 expression may alter the balance of immune regulation in a manner that is detrimental to the host. We did not observe any changes in IL-12 levels, indicating Th1 differentiation is not initiated by the wild-type or mutant strains [41]. Effects of Hmx1 on Th1 and Th2 subsets could not be evaluated because the respective signature cytokines IFNγ and IL-4 were below the detection limit.

Th17 polarization also mediates protection against disseminated candidiasis, and IL-17 receptor knock-out mice have reduced survival [48], possibly by stimulating immune mediators with fungicidal activity [49]. IL-17 signaling is essential for mucosal host defense against oral candidiasis [50]. In our experiments, mice infected with SC5314 or the *HMX1* mutant supplemented with CO had significantly reduced IL-17A expression compared with uninfected mice and those infected with the *HMX1* mutant. Consistent with our evidence that *C. albicans* Hmx1 expression negatively regulates IL-17A in infected mice, up-regulation of HO-1 expression suppresses IL-17 expression in BALB/c mice [51].

Neutrophils mediate the primary protective innate immune response against *C. albicans* infection [52]; whereas macrophages are involved in regulating cell mediated immunity. The earliest differential responses in our model were significantly decreased levels of RANTES and G-CSF at 2d PI in mice infected with the mutant strain. These chemokines are strong chemoattractants for neutrophils [53], which is consistent with the reduced number of neutrophils in infected kidneys of mice infected with the strain lacking *HMX1*.

TNFα, IL-6, G-CSF and MIP1β are proinflammatory cytokines that induce and recruit neutrophils during candidiasis [54]. Recent studies comparing virulent and impaired strains of *C. albicans* found that such excessive levels of proinflammatory cytokines produced during a virulent infection contribute to the development of sepsis and kidney malfunction [44,55]. Elevated IL-6, G-CSF, TNFα, and MIP1β were associated with infection with more virulent strains, consistent with the increased inflammation by histopathology and higher levels of the same cytokines that we observed 3 d PI in mice infected with wild type versus *HMX1* null strains.

TNFα plays a vital role in host defense in systemic candidiasis [41]. Moderate levels of TNFα induce macrophages to produce microbicidal reactive intermediates [56] and natural killer (NK) cells to produce IFNγ [57], whereas higher levels of TNFα are associated with organ failure and septic shock in experimental Candidiasis in mice [58]. We propose that in the absence of *HMX1* infected mice can maintain moderate levels of TNFα expression that support more effective anti-microbial immunity. Therefore, we suggest that the moderately elevated proinflammatory TNFα and IL-6 levels, elevated MIP1β and RANTES coupled with increased protective IL-17A expression enables a more effective immune response to the *HMX1* mutant.

Although exogenous inhaled CO probably does not have the same tissue distribution as CO produced locally by *C. albicans* Hmx1 in infected mice, exogenous CO supplementation was sufficient to restore kidney colonization and several cytokine responses in mice infected with the null mutant. This supports our conclusion that CO is a crucial enzymatic product of *HMX1* that contributes to Candida virulence (Fig. 8). Among these cytokine changes, we suggest that suppression of IL-17 levels by CO may enhance virulence by limiting the ability of the host to mount a balanced immune response. We have observed similar alterations in host IL-17 responses using other *C. albicans* mutants, and future experiments will further examine the significance of IL-17 as a regulator of host immunity to *C. albicans*.

In summary, Hmx1 is a virulence factor of *C. albicans* in murine candidemia. Hmx1 expression does not affect initial organ colonization but clearly limits clearance from the kidney, which may in turn increase levels of inflammatory cytokines. Consistent with the known ability of CO produced by mammalian HO-1 to limit immune responses [59], we propose that CO produced by *C. albicans* Hmx1 locally limits the ability of the host to clear the initial infection and eventually leads to the immune system being overwhelmed. Early expression of high levels of IL-10 following infection may initiate this immune dysregulation [41], preventing IFNγ expression and subsequent protective cytokine expression. Therefore, we propose that *C. albicans* induction of Hmx1 when in the bloodstream produces CO that shields it from host immune surveillance. Drugs that selectively inhibit Hmx1 could be useful for treating systemic candidiasis by accelerating clearance of the pathogen without initiating an excessive inflammatory response.

Our observation that exposing mice infected with *C. albicans* to therapeutic levels of CO accelerates their death may also be clinically relevant. Inhaled CO is being developed as a treatment to prevent organ transplant rejection [37–38]. Because these patients are immunocompromised, they have increased susceptibility to candidemia, and our studies suggest the CO therapy would be detrimental to survival of such infections.

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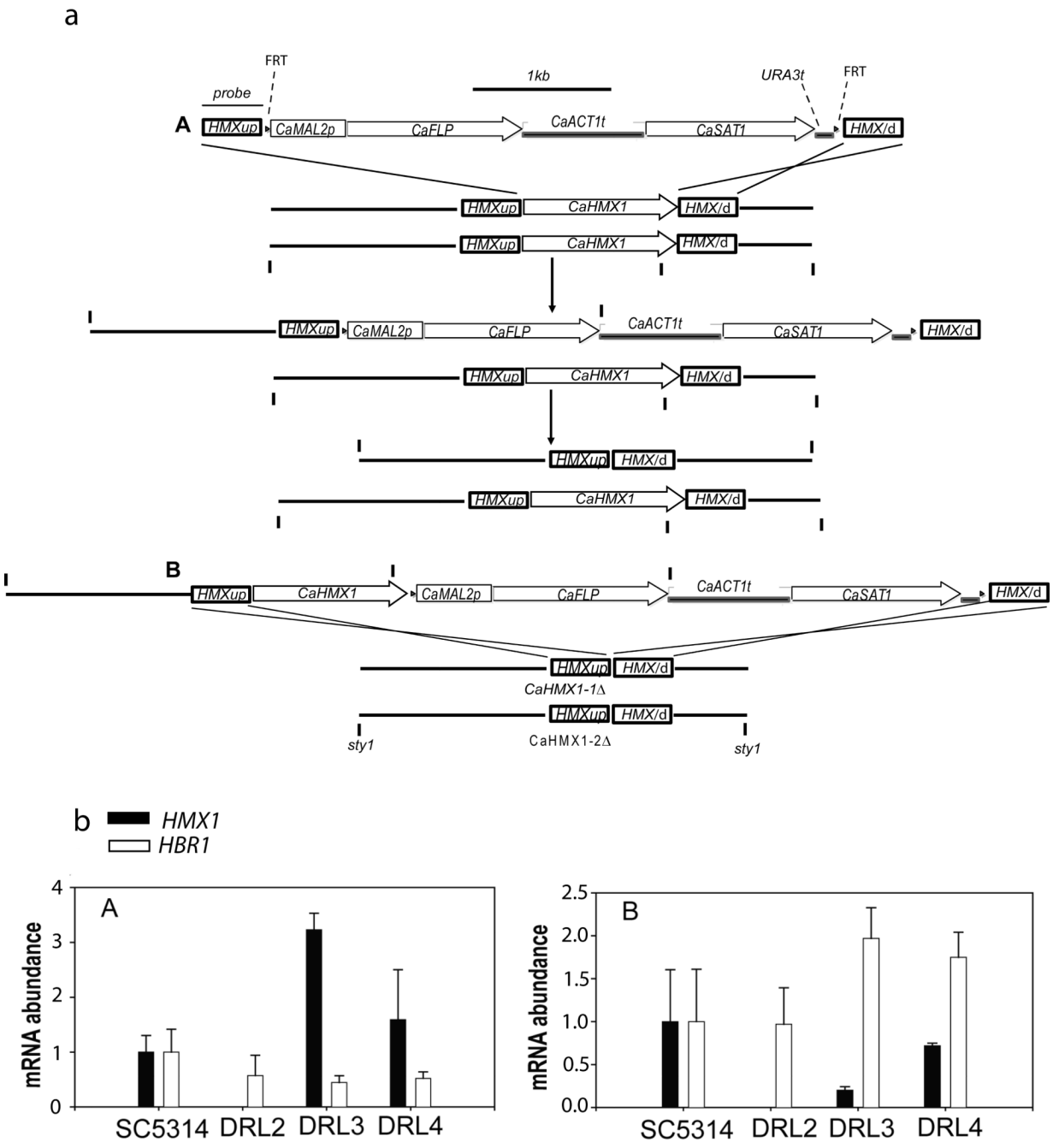


**Fig. 1.**

Disruption and reconstitution of *C. albicans HMX1*. (a). Strategy for deletion and reconstitution of *CaHmx1*. A. The Ca*Hmx*1 knock out was created using the *caSAT1* flipper cassette from plasmid pSFS2A. The *HMX1* knock out cassette contained upstream and downstream fragments flanking either side of the *caSAT1* flipper cassette. The arrowheads are FRT sites for *caFLP*. The *HMX1* flanked by up(*HMX*up) - and downstream (*HMX*/d) regions enabling homologous recombination following transformation. Small vertical lines represent Sty1 cut sites utilized for confirmation of gene deletion and reintegration by Southern hybridization using the upstream probe. C*aFLP* recombinase activation created the *HMX1* deleted allele. B. *HMX1* complementation cassette used for reintegration of an intact

*HMX1*-1 allele into the *HMX1* deleted locus. (b). Expression levels of mRNAs for *HMX1* and the adjacent *HBR1* determined by quantitative real time PCR using RNA harvested at log phase growth in YNB medium. Black columns show HMX1 expression levels, and white columns show *HBR1* expression levels. (A). shows gene expression at 37°C and (B) shows gene expression at 30°C. Results for the deletion strain DRL2 (*hmx1Δ/hmx1Δ*) and reconstituted strains DRL3 and DRL4 are normalized to 1 for the respective mRNA levels in the parental strain SC5314.

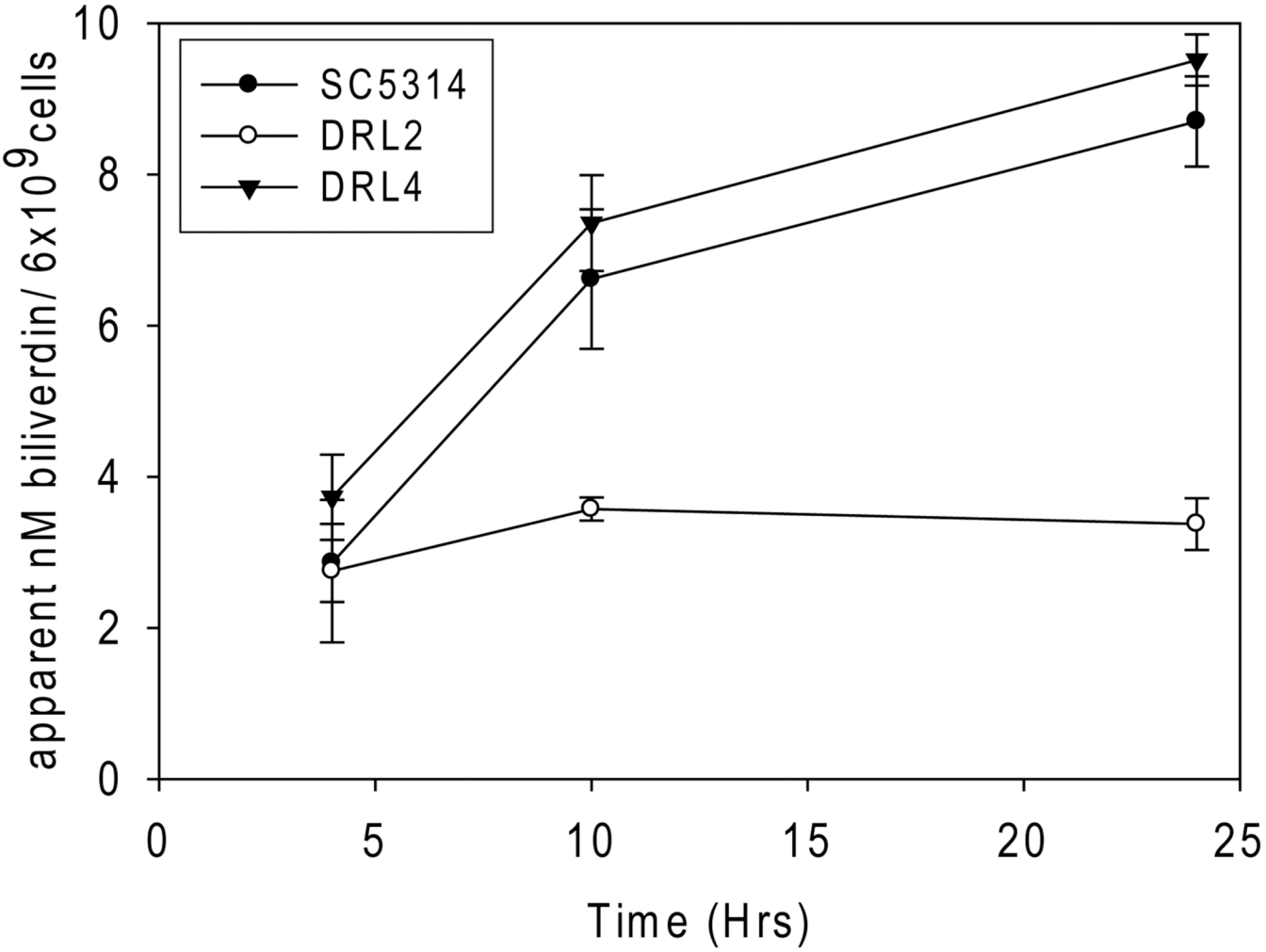


**Fig. 2.**

*HMX1* complementation restores heme oxygenase activity. Closed circles represent biliverdin production by $6\times10^9$ cells of WT SC5314 cells. Opened circles represent mean values for the *hmx1Δ/hmx1Δ* mutant DRL2, and triangles represent the *HMX1* reconstituted DRL4 strain. Data are means ± standard deviation for three samples.

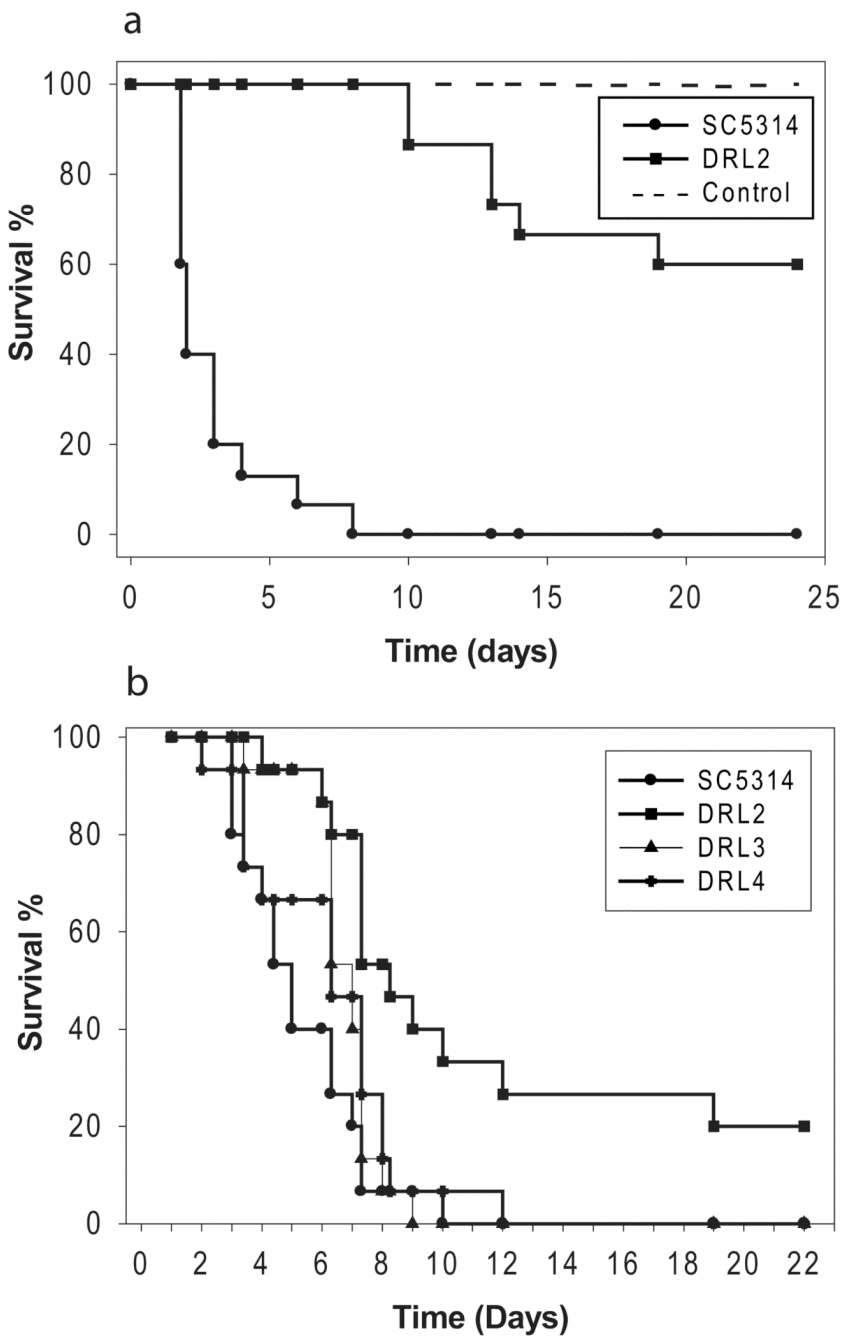


**Fig. 3.**

Altered pathogenicity for *C. albicans* strains with *HMX1* removed and restored. (a) Effect of *HMX1* deletion on mouse mortality following intravenous infection. Survival of mice injected with wild-type *C. albicans* SC5314 (●), DRL2 (*hmx1Δ/hmx1Δ*, ■), and an uninfected control group (- - - -) was assessed daily. Each group contained 15 mice. (b) Restored pathogenicity of *C. albicans* strains with reconstituted *HMX1*. Groups of 15 mice were infected with the wild type strain SC5314 (●), the deletion mutant DRL2 (■), single copy reconstituted DRL3 (▲), and fully reconstituted DRL4; (+).

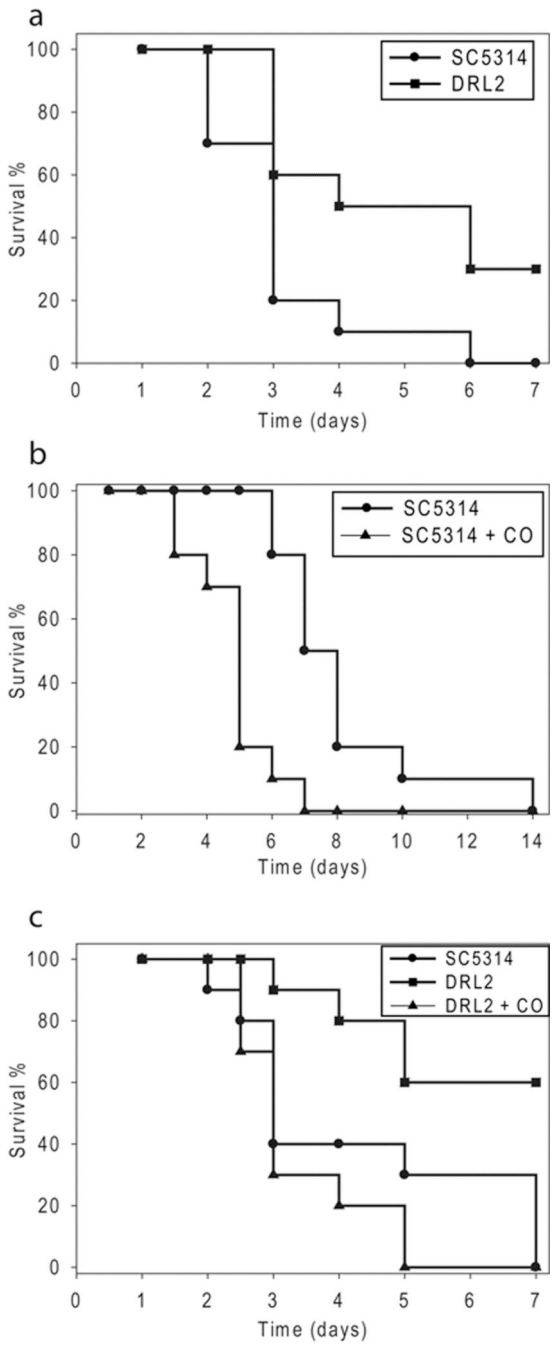


**Fig. 4.**

Exogenous supplementation of iron and CO effect mice mortality in disseminated candidiasis. (a).Effect of iron overload on virulence of the *hmx1Δ/hmx1Δ* strain in mice. Mice were infected with wild-type *C. albicans* SC5314 (•), DRL2 (■) or left uninfected (not shown) and treated every other day with iron administered i.p.. Mice were assessed daily. Each infected group contained 10 mice, and the uninfected control group contained 5 mice. (b). Exogenous CO increases the virulence of wild-type. Mice were infected with wild-type *C. albicans* SC5314 (•) under room air and another group maintained in an atmosphere supplemented with 250 ppm CO (▲). Uninfected control groups maintained in an atmosphere supplemented with 250 ppm CO or in room air had 100% survival. (c). CO

complementation restores the virulence of the *hmx1Δ/hmx1Δ* strain in mice. Mice were infected with wild-type *C. albicans* SC5314 (•), DRL2 (*hmx1Δ/hmx1Δ*, ■), or DRL2 and maintained in an atmosphere supplemented with 250 ppm CO (▲).Uninfected control group had 100% survival. Survival of the infected mice and an untreated uninfected control group (not shown) was assessed daily. Each group contained 10 mice, and the control groups contained 5 mice. Different batches of mice were used for each panel, and the variation in survival curves between experiments is consistent with the known variability of the outbred strain used.

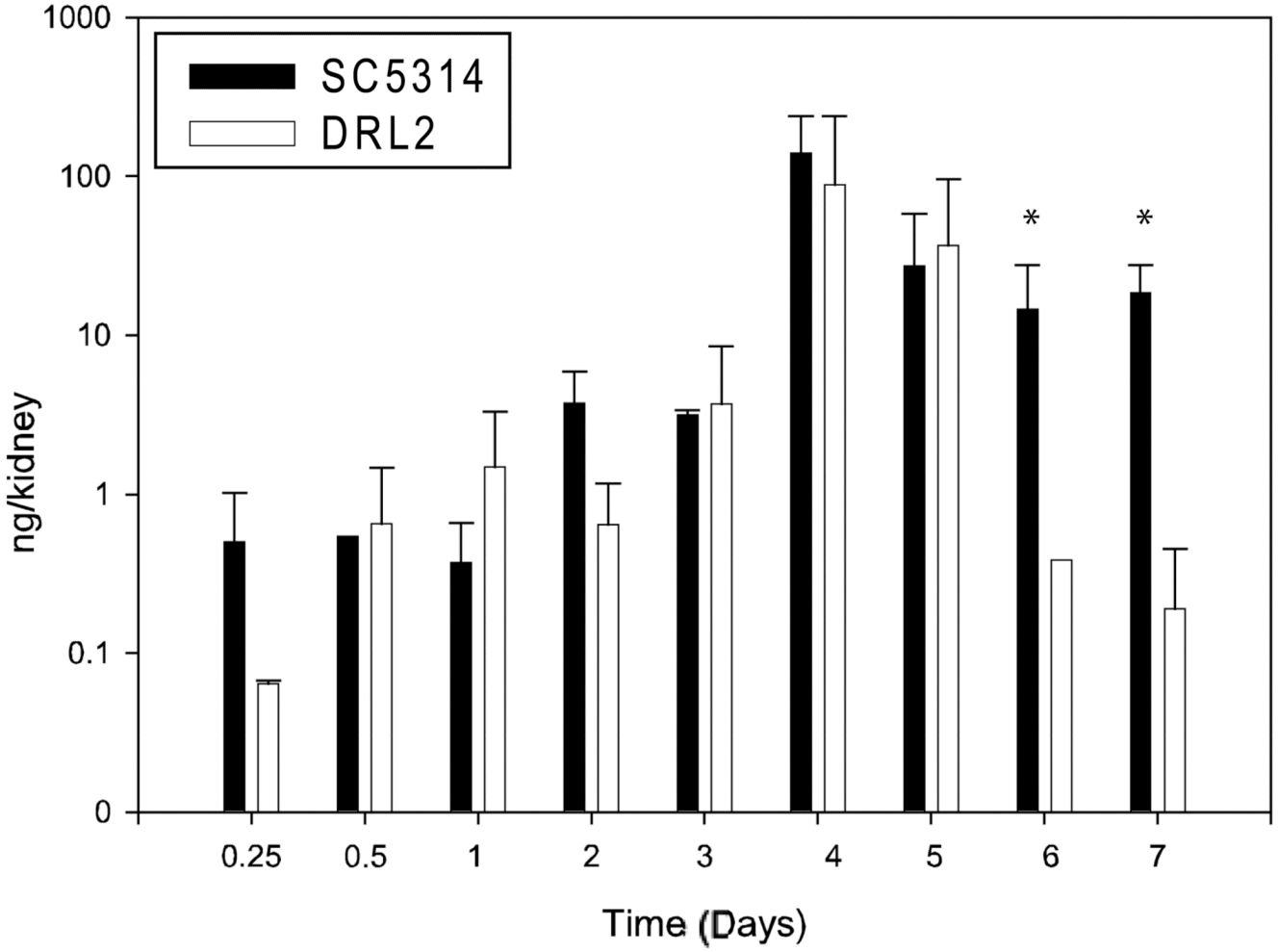


**Fig. 5.**

Kidney fungal burdens of mice infected with parental SC5314 and mutant DRL2 strains. Open bars represent mean quantity of fungal DNA/kidney determined by quantitative PCR for mice infected with DRL2, and closed bars represent mean values for mice administered with SC5314. Data are means ± standard deviation for 3 mice. Six and seven day PI, wild-type infection caused significantly higher fungal burden ($p < 0.03$) in kidneys.

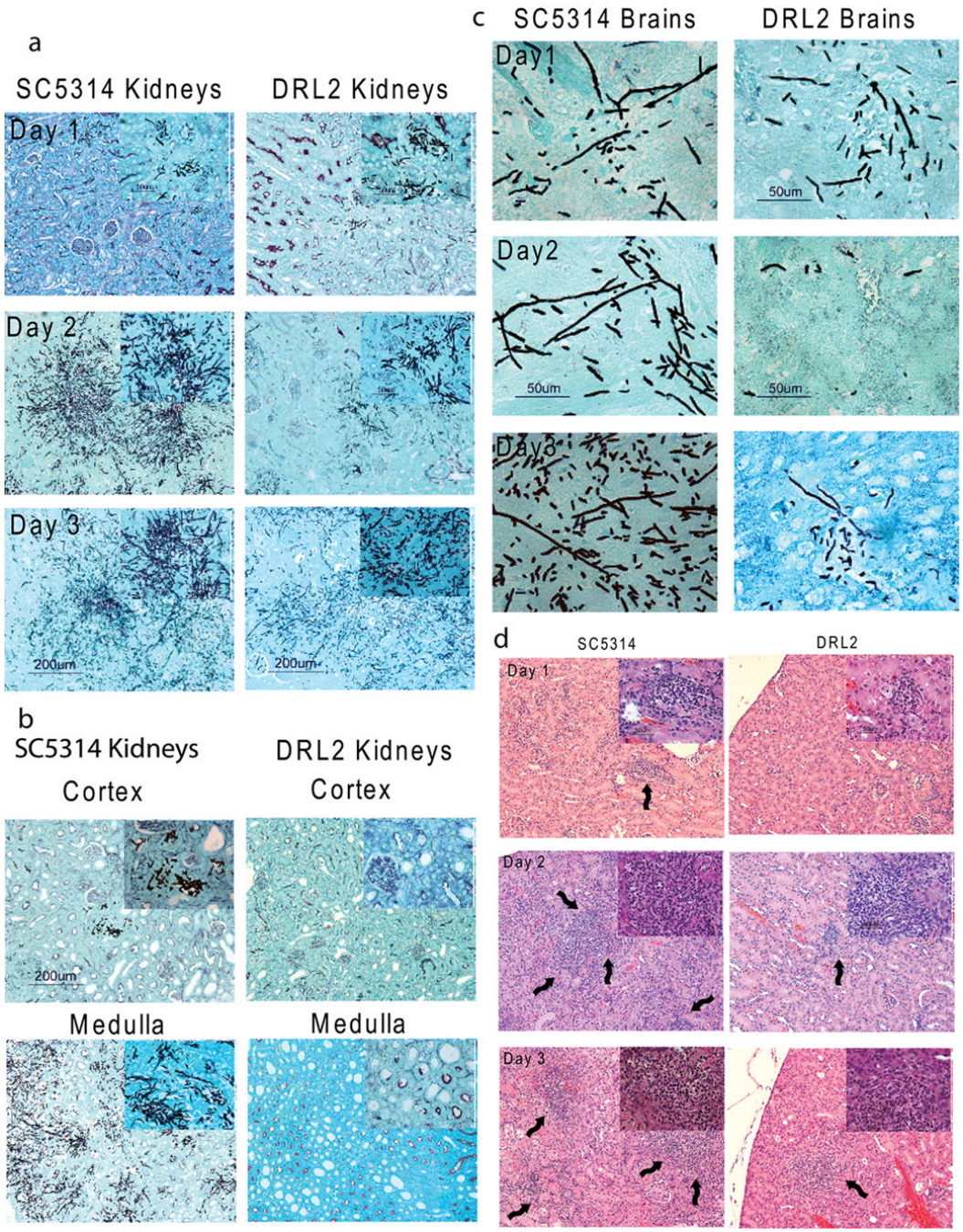


**Fig. 6.**

Comparative histopathology of mice with disseminated candidiasis. (a.) GMS-stained mouse kidney tissue to detect *C. albicans* colonization. Representative sections are shown from kidneys and brains harvested 1 to 3 day PI. Insets show higher magnification images of colonized areas. Scale bars indicate magnification. (b.) GMS-stained mouse kidney tissue 7 day PI. Note the absence of infection in kidney medulla for the mutant strain. (c.) GMS-stained mouse brain tissue harvested 1 to 3 day PI. (d) H&E stained sections of kidneys from SC5314- and DRL2-infected mice to assess inflammatory reactions. Insets show higher magnification fields with inflammatory responses. Arrows indicate severe inflammatory reactions.

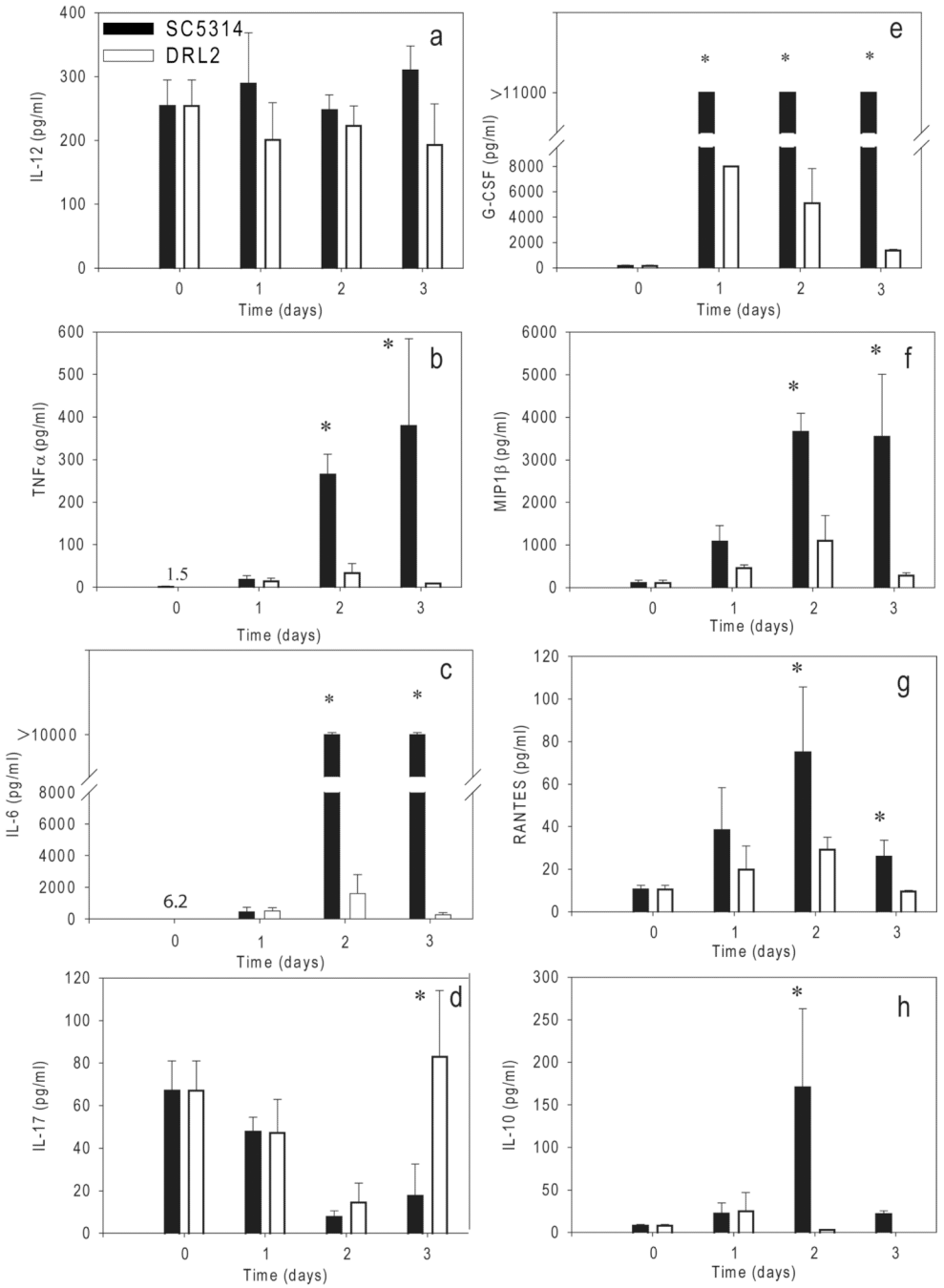


**Fig. 7.**

Concentrations of cytokines and chemokines in sera from *C. albicans*-infected mice at 1 to 3 d PI.. Values at time zero are mean values determined for sera from 10 control mice, and other data are means ± standard deviations for 3 mice at each time point: a, IL-12; b, TNF-α; c, IL-6; d, IL-17; e, G-CSF; f, MIP1β; g, RANTES; and h, IL-10. Closed bars represent mean values for mice infected i.v. using the parental strain SC5314, and opened bars represent mean values for mice infected i.v using the DRL2 strain.

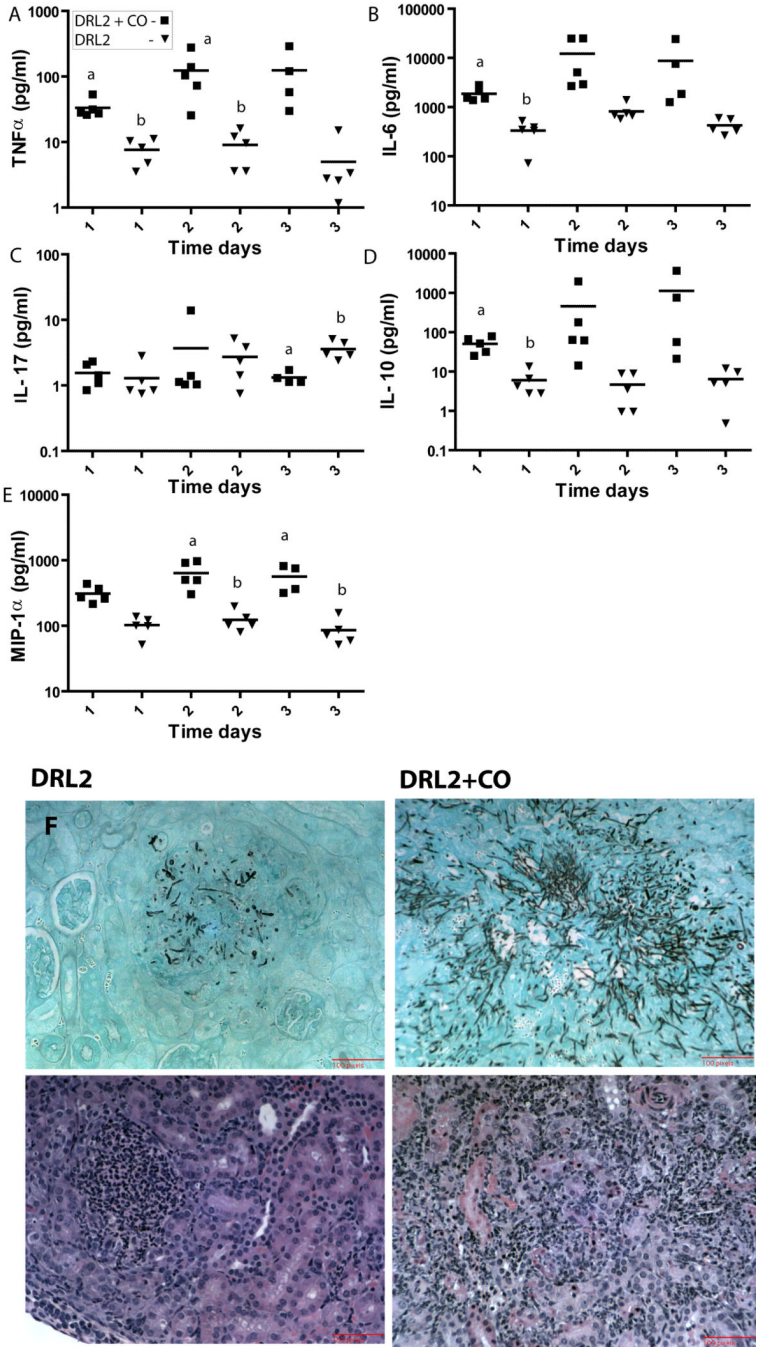


**Fig. 8.**

Exogenous CO reverses altered host cytokine responses in mice infected with the *hmx1Δ/hmx1Δ* strain. Concentrations of cytokines and chemokines were quantified in sera from mice infected with the *C. albicans hmx1Δ/hmx1Δ* mutant and maintained in room air or air supplemented with CO at 250 ppm. Sera were collected at 1,2 and 3 day PI in two groups of five mice, Squares represent five mice infected with DRL2 with CO, and triangles represent five mice infected with DRL2 without exposure to CO: A, TNFα; B, IL-6; C, IL-17; D, IL-10; and E, MIP-1α. 'a' and 'b' indicate where serum cytokine levels differ significantly between groups of infected mice exposed or not exposed to CO as determined by ANOVA.

(F) At the end of day 3, mice were euthanized to study histopathology and assess organ burden (top panels) and inflammation by H&E staining (lower panels).

**TABLE 1**

*C. albicans* strains used and constructed in this study

| ***C. albicans* Strain** | **Genotype** | **Reference** |
|---|---|---|
| SC5314 | Wild Type (*HMX1/HMX1*) | [16] |
| DRL1$^{NR}$* | *hmx1Δ::SAT1-FLP/HMX1* | This study |
| DRL1 | *hmx1Δ/HMX1* | This study |
| DRL2$^{NR}$* | *hmx1Δ/hmx1Δ:: SAT1-FLP* | This study |
| DRL2 | *hmx1Δ/ hmx1Δ* | This study |
| DRL3$^{NR}$* | *hmx1Δ/hmx1Δ::HMX1:: SAT1-FLP* | This study |
| DRL3 | *hmx1Δ/ hmx1Δ::HMX1* | This study |
| DRL4$^{NR}$* | *hmx1Δ::HMX1:: SAT1-FLP/hmx1Δ::HMX1* | This study |
| DRL4 | *hmx1Δ::HMX1/hmx1Δ::HMX1* | This study |

* Nourseothricin resistant

## TABLE 2

Sequences of synthetic oligonucleotides used in this study

| Sequence[a] | Name | Reference |
|---|---|---|
| 5’AGAAAGGGCCCCTTCACAACGAG3’ | HMX1upleft | This study |
| 5’CGATGTAGCTCGAGTCAGTTTGTAT 3’ | HMX1upright | This study |
| 5’GTACTTTTCCCGCGGTTGGCTTTG3’ | HMX1downleft | This study |
| 5’ATAGAATGAGCTCTTGGGTGGAAAC3’ | HMX1downright | This study |
| 5’AACTAAGGGCCCAACTTTTGTGTGTA3’ | HMXcompleft | This study |
| 5’AGGAAAGACTCGAGTACGATGAAGAG3’ | HMXcompright | This study |
| 5’GAGCGTCCAGTATAAATCCACCAC3’ | CDC36 | [25] |
| 5’TCAAGACGGGCTCCACATTACTAT3’ | CDC36 | [25] |
| 5’TGAAATAGCAAAGGAAAGAGACTG3’ | HBR1 | [25] |
| 5’AATATCACAACAATGCCAATCAAC3’ | HBR1 | [25] |
| 5’CTGGTAAAGCCGAGCAAGAC3’ | HMX1 | [25] |
| 5’CAAGTAGGCAAACAAGTTATGG3’ | HMX1 | [25] |
| 5’TGGGTGGTAAATTYCATCTAAAGCTA3’ | Fw Primer | [27] |
| 5’CAAGTKCTTTTCATCTTTCSWTCAC3’ | Rew Primer | [27] |

[a] Underlined segments introduce desired restriction sites in the process of constructing pHMXKO and pHMXCOMP.