INVENTORY OF SUPPLEMENTAL MATERIALS

1. Supplemental Figures and Legends

Figure S1

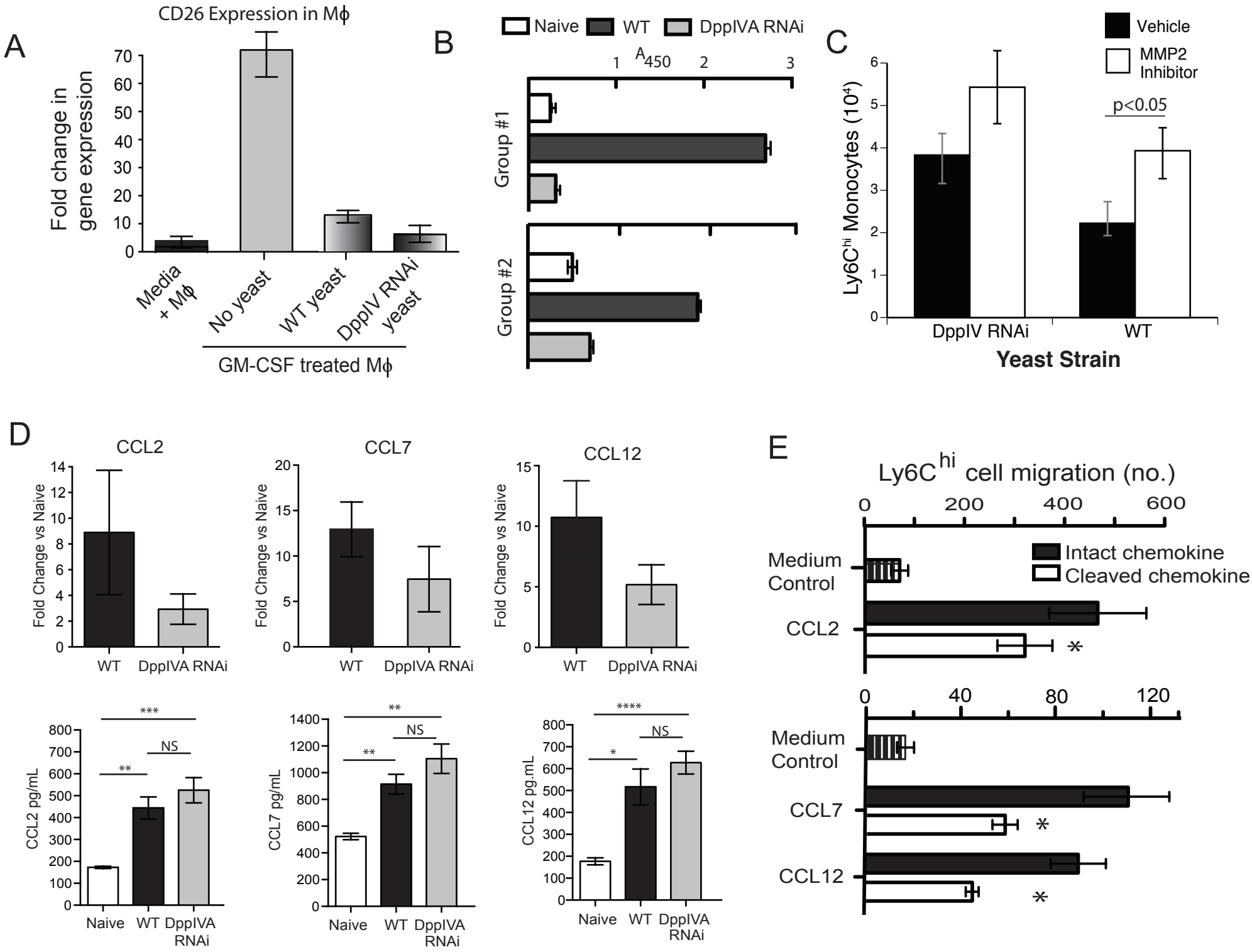

A
CD26 Expression in Mϕ
Fold change in gene expression
Media + Mϕ
No yeast
WT yeast
DppIV RNAi yeast
GM-CSF treated Mϕ
B
Naive
WT
DppIVA RNAi
A450
Group #1
Group #2
C
Vehicle
MMP2 Inhibitor
p<0.05
Ly6Chi Monocytes (10⁴)
DppIV RNAi
WT
Yeast Strain
D
CCL2
CCL7
CCL12
Fold Change vs Naive
WT
DppIVA RNAi
CCL2 pg/mL
CCL7 pg/mL
CCL12 pg.mL
Naive
NS
E
Ly6Chi cell migration (no.)
Intact chemokine
Cleaved chemokine
Medium Control
CCL2
CCL7
CCL12

Figure S2

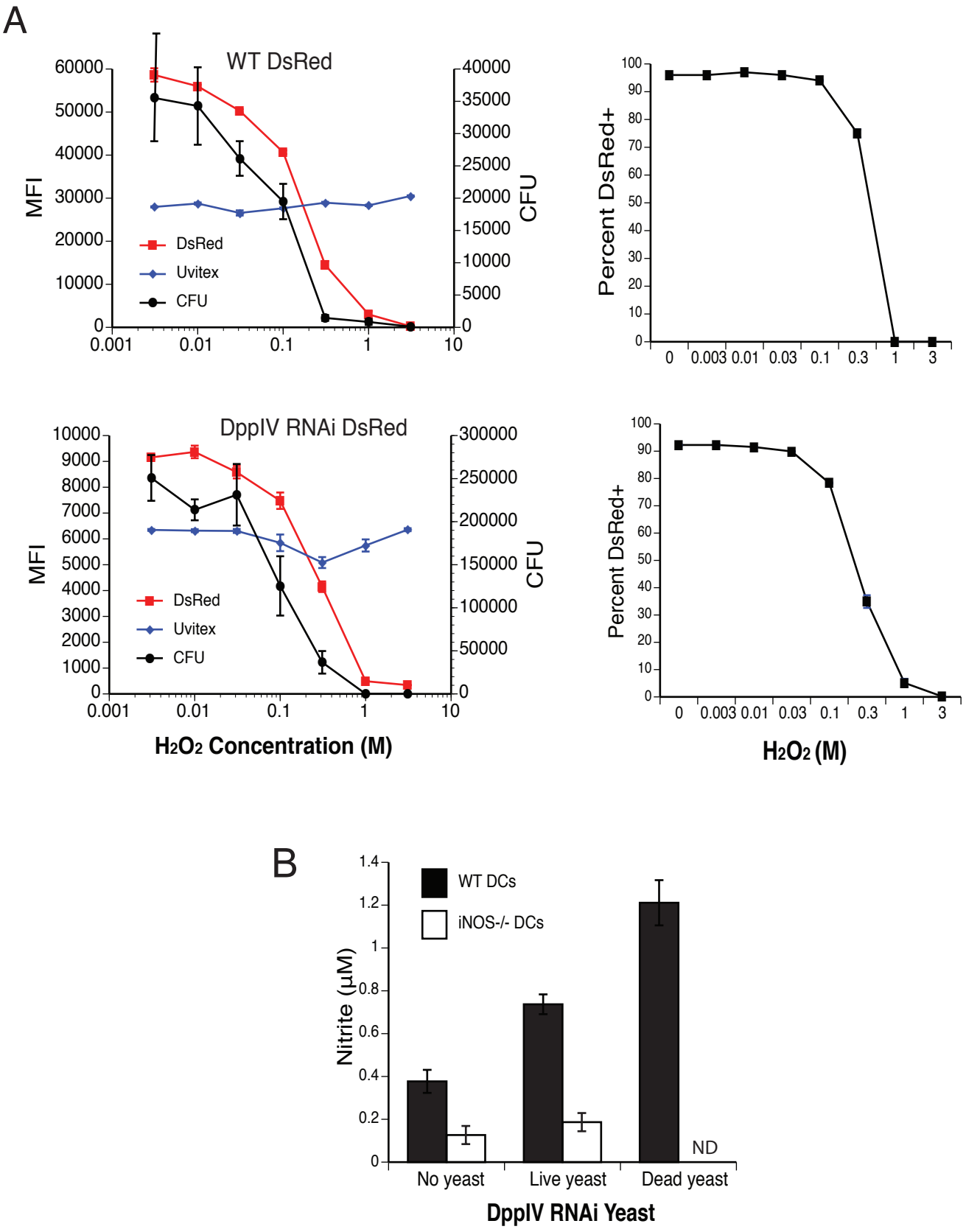

A
WT DsRed
MFI
CFU
DsRed
Uvitex
CFU
Percent DsRed+
DppIV RNAi DsRed
MFI
CFU
DsRed
Uvitex
CFU
Percent DsRed+
H2O2 Concentration (M)
H2O2 (M)
B
WT DCs
iNOS-/- DCs
Nitrite (µM)
No yeast
Live yeast
Dead yeast
ND
DppIV RNAi Yeast

Figure S3

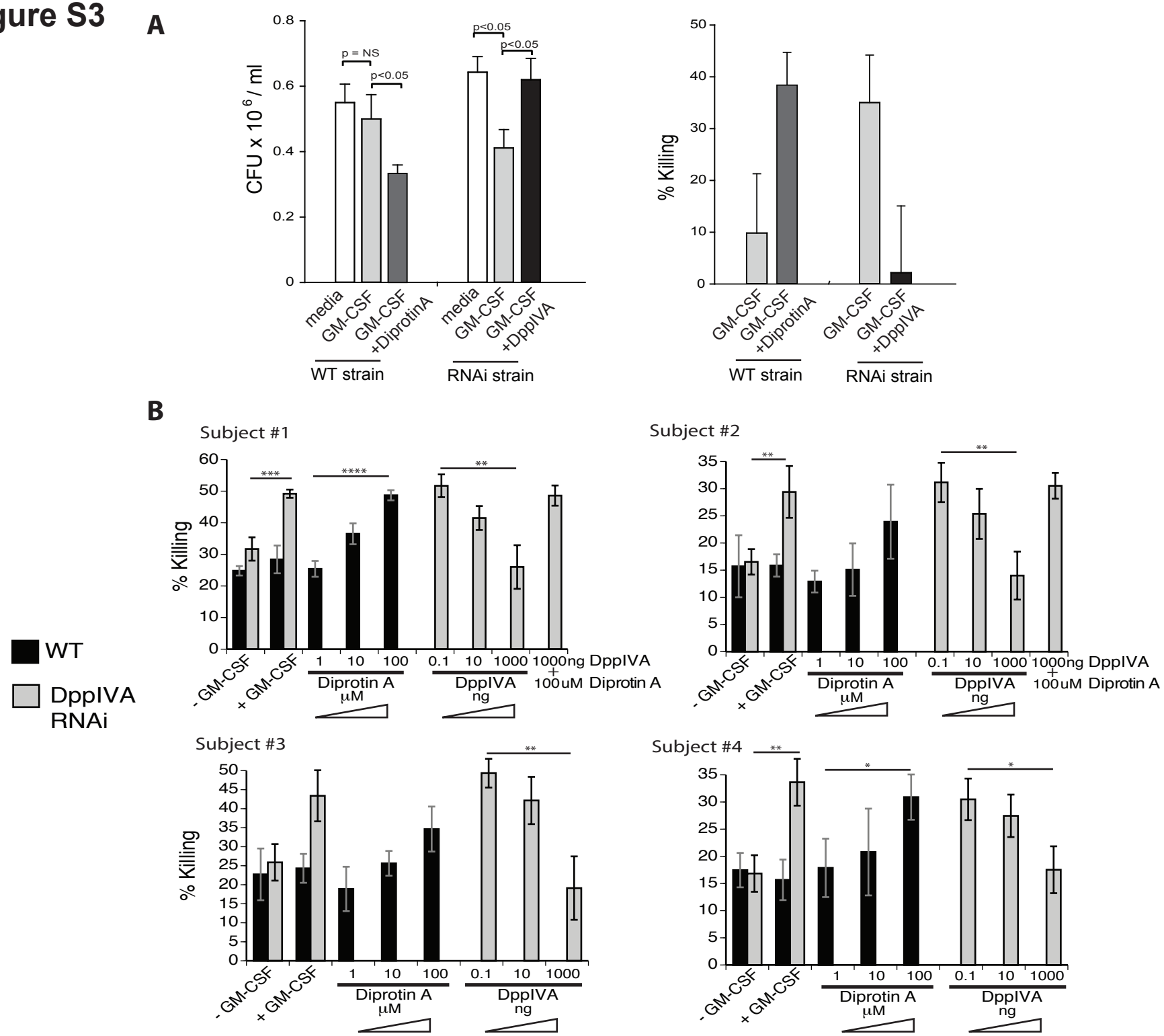

A
CFU x 10^6 / ml
p = NS
p<0.05
p<0.05
p<0.05
media
GM-CSF
GM-CSF +DiprotinA
media
GM-CSF
GM-CSF +DppIVA
WT strain
RNAi strain
% Killing
GM-CSF
GM-CSF +DiprotinA
GM-CSF
GM-CSF +DppIVA
WT strain
RNAi strain
B
WT
DppIVA RNAi
Subject #1
Subject #2
Subject #3
Subject #4
% Killing
- GM-CSF
+ GM-CSF
Diprotin A µM
DppIVA ng
1000ng DppIVA + 100uM Diprotin A

Figure S4

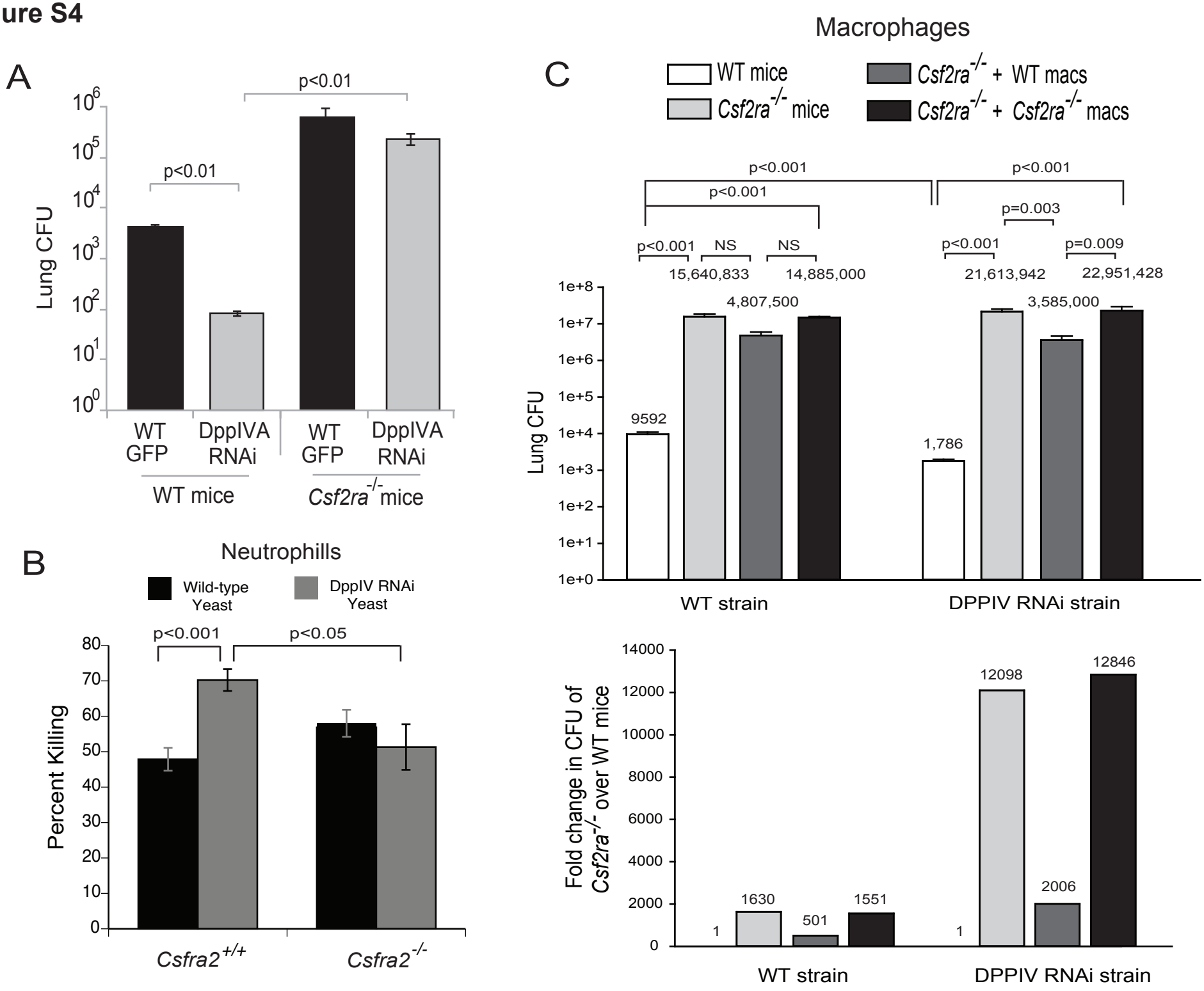

A
p<0.01
p<0.01
Lung CFU
WT GFP
DppIVA RNAi
WT GFP
DppIVA RNAi
WT mice
Csf2ra-/- mice
B
Neutrophills
Wild-type Yeast
DppIV RNAi Yeast
p<0.001
p<0.05
Percent Killing
Csfra2+/+
Csfra2-/-
C
Macrophages
WT mice
Csf2ra-/- mice
Csf2ra-/- + WT macs
Csf2ra-/- + Csf2ra-/- macs
p<0.001
p<0.001
p<0.001
p=0.003
p<0.001
NS
NS
p<0.001
p=0.009
15,640,833
14,885,000
21,613,942
22,951,428
Lung CFU
9592
4,807,500
1,786
3,585,000
WT strain
DPPIV RNAi strain
Fold change in CFU of Csf2ra-/- over WT mice
1
1630
501
1551
1
12098
2006
12846
WT strain
DPPIV RNAi strain

Figure S5

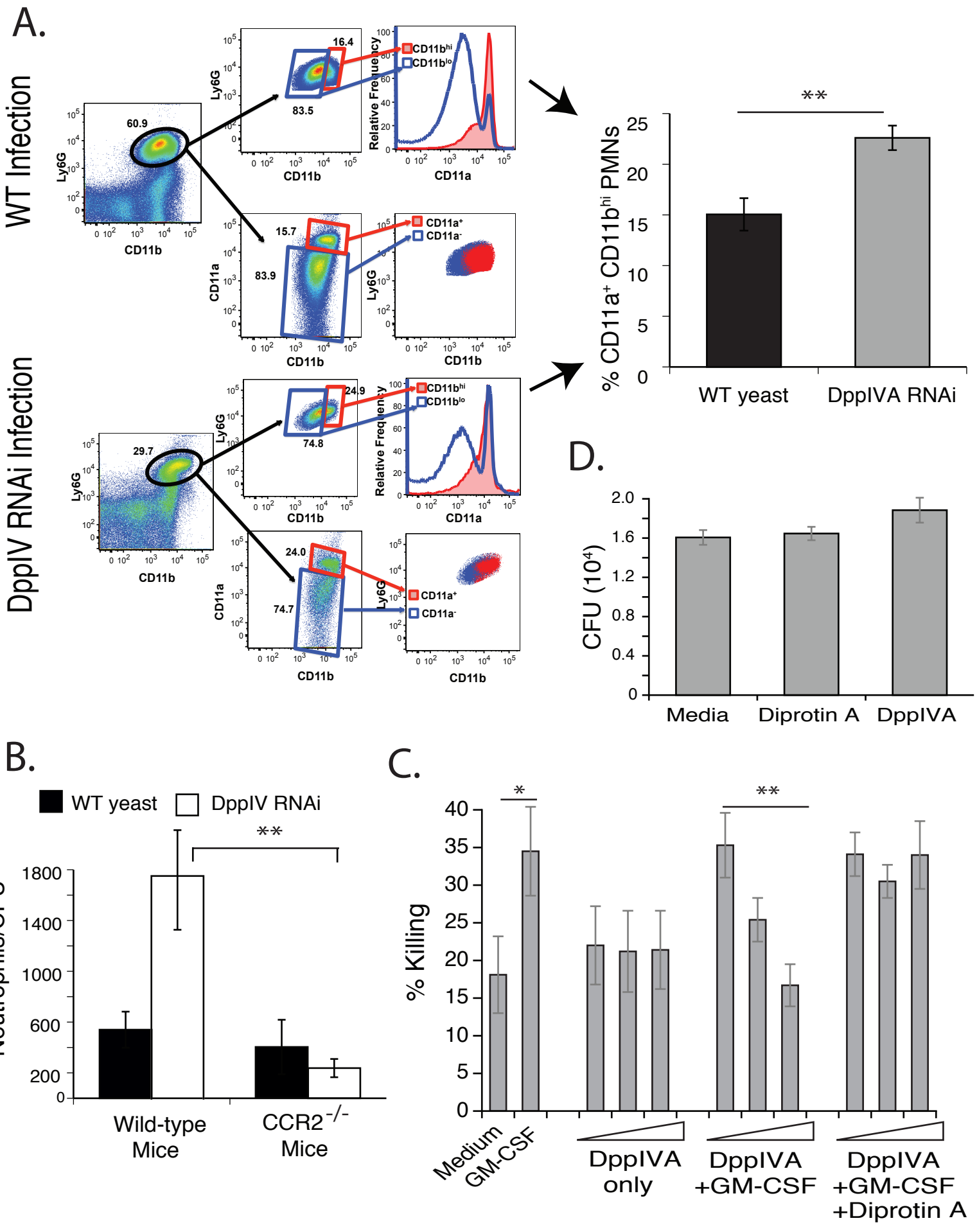

A.
WT Infection
DppIV RNAi Infection
Ly6G
CD11b
CD11a
60.9
16.4
83.5
15.7
83.9
29.7
24.9
74.8
24.0
74.7
CD11b^hi
CD11b^lo
CD11a+
CD11a-
Relative Frequency
% CD11a+ CD11b^hi PMNs
WT yeast
DppIVA RNAi
**
D.
CFU (10^4)
Media
Diprotin A
DppIVA
B.
WT yeast
DppIV RNAi
Neutrophils/CFU
Wild-type Mice
CCR2-/- Mice
C.
% Killing
Medium GM-CSF
DppIVA only
DppIVA +GM-CSF
DppIVA +GM-CSF +Diprotin A

Figure S6

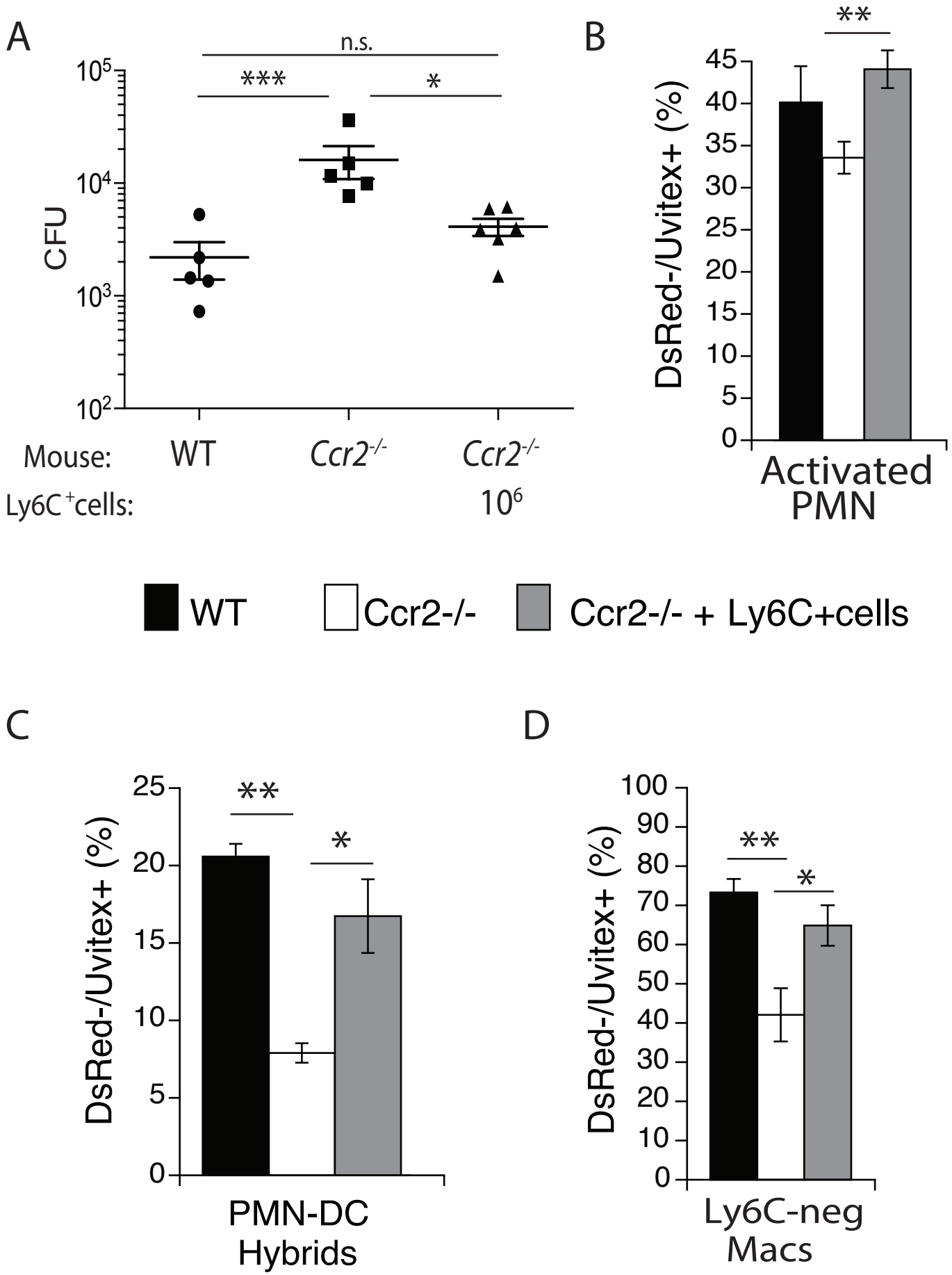
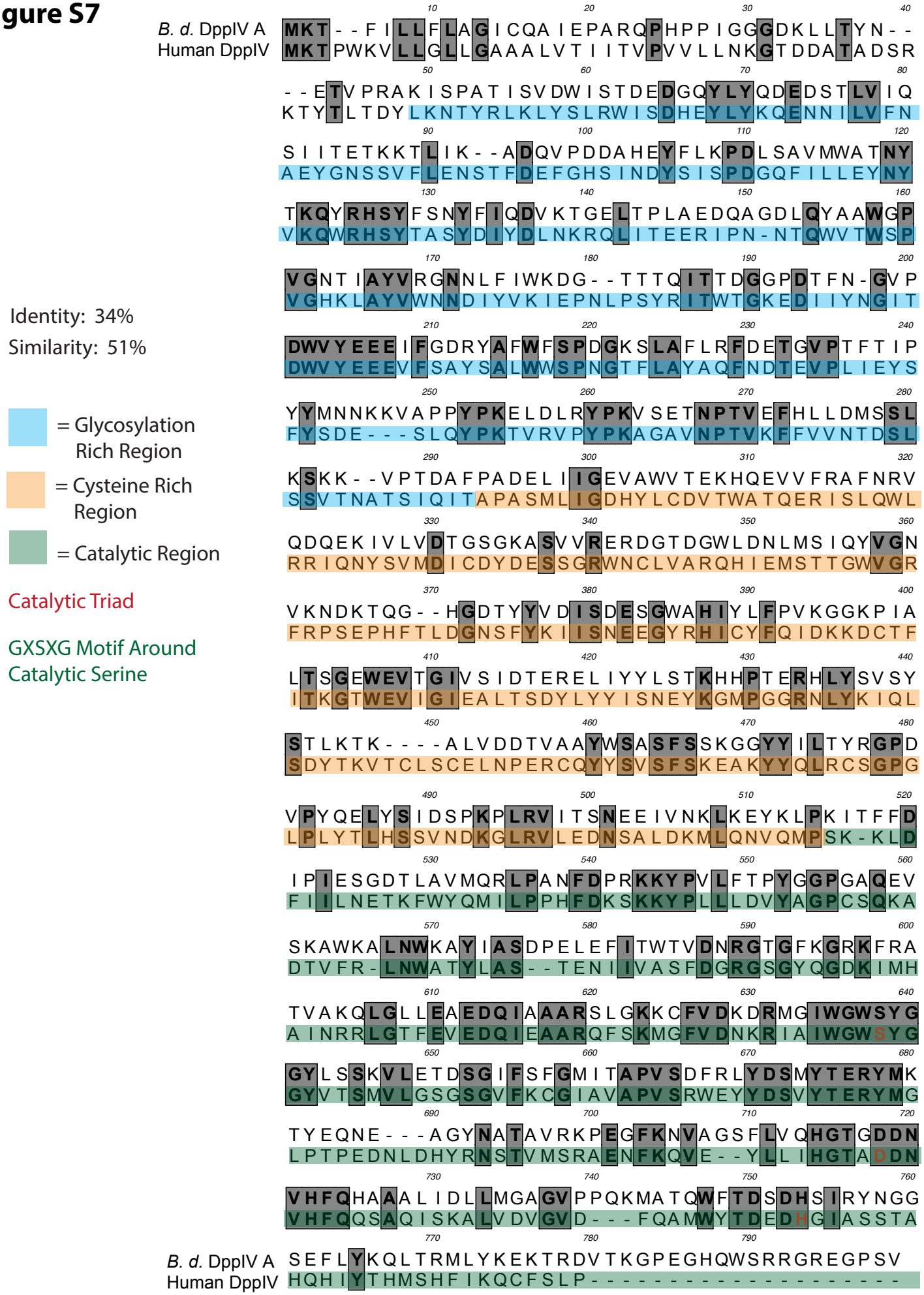

A
n.s.
***
*
10^5
10^4
10^3
10^2
CFU
Mouse:
WT
Ccr2-/-
Ccr2-/-
Ly6C+cells:
10^6
B
**
DsRed-/Uvitex+ (%)
45
40
35
30
25
20
15
10
5
0
Activated PMN
WT
Ccr2-/-
Ccr2-/- + Ly6C+cells
C
**
*
DsRed-/Uvitex+ (%)
25
20
15
10
5
0
PMN-DC Hybrids
D
**
*
DsRed-/Uvitex+ (%)
100
90
80
70
60
50
40
30
20
10
0
Ly6C-neg Macs

Figure S7


B. d. DppIV A
Human DppIV
Identity: 34%
Similarity: 51%
= Glycosylation Rich Region
= Cysteine Rich Region
= Catalytic Region
Catalytic Triad
GXSXG Motif Around Catalytic Serine

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Chemokines that Recruit Ly6c^{hi} cells are Up-regulated during Infection (related to Figures 1 & 2). (A) CD26 expression in bone-marrow derived macrophages co-cultured *in vitro* with yeasts in a ratio of 1:1. Data are the mean ± SEM of two technical replicates. (B) Binding of pre-immune and immune serum (1:64 dilution) to recombinant fungal DppIVA, measured by ELISA. Shown are two groups of mice that were immunized with either wild-type yeast or DppIVA-RNAi yeast as described in Supplemental methods. Data are mean ± SEM of two technical replicates and representative of two experiments. (C) Recruitment of Ly6C^{hi} cells to the lungs 3 days post-infection with wild-type or DppIVA RNAi yeast in mice that received GM6001 (or vehicle control) to inhibit MMP2. Data are mean ± SEM of 5-10 mice per group. (D) CCL2, CCL7, and CCL12 transcript and protein levels in the lung 2 days post-infection. Transcript is fold increase vs. naïve measured by RT-PCR. Protein was detected by ELISA in lung homogenate. Data are mean ± SEM of 5-10 mice/group. Data are representative of two experiments. (E) Transwell migration of Ly6C^{hi} monocytes from murine bone marrow in response to intact or fungal DppIVA-cleaved C-C chemokines. Migration was quantified by flow cytometry after 16-24 hours incubation. Results are representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Fig. S2. DsRed Reporter Strain Features and their use in Interrogating the Host: Pathogen Encounter (related to Figure 4). (A) Expression of DsRed fluorescence correlated with CFU during killing of yeast with H_20_2 to compare wild-type (Fig. 4A) and DppIVA RNAi strains Wild-type DsRed strain and DppIVA RNAi DsRed strain show similar features. The mean fluorescence intensity (MFI) and CFU is shown on left, and the percent of yeast positive for DsRed and considered living is shown on right. (B) TipDCs were generated from wild-type and *iNOS*$^{-/-}$ mice and the cells were co-cultured with DsRed reporter yeast. Nitrate levels in culture supernatant were measured after 24 hours of co-culture using the Griess reaction. ND, not done.

Fig. S3. Activity of Human Macrophages and Neutrophils against Yeast *in vitro* (related to Figures 5 & 6). (A) Blood monocyte-derived Mϕs from two healthy subjects were tested for killing of wild-type and DppIV RNAi yeast upon co-culture for 24 hours *in vitro* with GM-CSF, recombinant DppIVA (10mg/ml), DiprotinA (100mM) alone or in combination. Effector to target ratio was 1:1. (B) Peripheral blood neutrophils from four healthy subjects were tested for growth inhibition of wild-type and DppIV RNAi yeast upon co-culture for 6 hours *in vitro* with GM-CSF, recombinant DppIVA, DiprotinA alone and in combination. Effector to target ratio was 10:1. Percent killing was assessed by CFU plating of yeast. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. S4. The Role of GM-CSF in Macrophage Killing of Yeast *in vivo* (related to Figure 3). (A) *Csf2ra*$^{-/-}$ (GM-CSF receptor-null) mice were infected with isogenic DppIVA-sufficient or -deficient yeast. Lung CFU were counted 7 days post infection. Data are the mean ± SEM of 5-10 mice per group, and representative of 3 experiments. (B) Percentage of wild-type and DppIVA-deficient yeast killed after incubation with neutrophils from wild-type mice or *Csf2ra*$^{-/-}$ mice. Cell were obtained from five mice per group. Data are the mean ± SEM and representative of 2 experiments (C) Adoptive transfer of wild-type or *Csf2ra*$^{-/-}$ peritoneal Mϕs into wild type or *Csf2ra*$^{-/-}$ mice infected with either wild-type or DppIVA RNAi yeast. 0.5 x 10^6 cells were transferred into each recipient. Mice were sacrificed seven days post-infection. The numbers over each histogram bar represent CFU (upper graph) or fold-change in CFU relative to wild-type mice (lower), and are representative of three experiments.

Fig S5. Activation and Action of Murine Neutrophils against Yeast (related to Figure 6). (A) Analysis of neutrophil activation using CD11a in tandem with CD11b. Neutrophils (black gate: Ly6G$^+$ CD11b$^+$) from mice were assayed 4d after infection for activation by gating CD11b^{hi} neutrophils (top panels) or CD11a$^+$ neutrophils (bottom panels). CD11b^{hi} neutrophils express more CD11a, and CD11a$^+$ neutrophils express higher CD11b (right panels). Determination of neutrophil activation using CD11a in tandem with CD11b show similar results as gating with CD11b alone; more neutrophils are CD11a$^+$ CD11b^{hi} in mice infected with DppIVA RNAi vs. wild-type (far right panel, **p<0.01). (B) Ratio of neutrophils: yeast cells in lung tissue 7 days after infection. (C) The percentage of DppIVA RNAi yeast killed after culture for 6 hours with neutrophils and combinations of GM-CSF, recombinant DppIVA, and DiprotinA alone or together. Effector to target ratio was 10:1 *p<0.05, **p<0.01. (D) CFU of DppIV RNAi yeast after culture for 6 hours with only DppIVA or DiprotinA, in the absence of neutrophils.

Fig. S6. Impact of Ly6C^{hi} Monocytes on Leukocyte Killing of Yeast (related to Figures 1 and 6). (A) Lung CFU were measured 7d after infection with DppIV RNAi yeast in wild-type or *Ccr2*$^{-/-}$ mice that received adoptive transfer of Ly6C$^+$ cells. (B-D). Killing of yeast was determined by quantifying the number of DsRed$^-$ yeast events among the

total yeast-associated events (Uvitex$^+$) for each leukocyte population. Activated neutrophils (B) were defined as Ly6G$^+$ CD11a$^+$ CD11b^{hi} CD11c$^-$. Neutrophil-DC hybrids (C) were defined as Ly6G$^+$ CD11b$^+$ CD11c$^-$. Ly6C-negative (neg) Mϕs (D) were defined as Ly6C$^-$ CD11c$^+$ Ly6G$^-$ and distinguished from DCs by autofluorescent properties. *p<0.05, **p<0.01, ***p<0.001, n.s. p>0.05.

Fig. S7. Whole Protein Alignment of the Primary Amino Acid Sequences of DppIV (related to Figure 7). *B. dermatitidis* DppIV A (Gene ID: BDFG_07406, Uniprot ID: T5BJB9) and human DppIV (Uniprot ID: P27487) sequences are compared, which are 34% identical and 51% similar as determined by NCBI blastp (protein-protein BLAST). Identical amino acids are bolded and boxed in gray.

2. SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Mice were housed and cared for according to guidelines from the University of Wisconsin Animal Care and Use Committee and the Department of Laboratory Animal Medicine, University of Cincinnati, accredited by the American Association for Accreditation of Laboratory Animal Care (Frederick, MD), who approved this work. Their guidelines are in compliance with Health and Human Services Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act guidelines of National Institutes of Health.

Fungi

Blastomyces yeast were taken in log-phase growth from 7H10 slants, suspended in PBS, aspirated through a 26-G needle, and passed through a 40 μm filter to reduce aggregation. In some experiments, yeast were heat-killed at 65°C for one hour in PBS. *Histoplasma* yeast were taken in log growth from HMM plates and suspended in PBS. Yeast CFU were counted from brain-heart infusion plates or *Histoplasma*-macrophage medium (HMM) (Sigma-Aldrich, St. Louis, MO) plates after five to seven days of growth at 37°C. Yeast growth assays were for 5 days in liquid HMM containing 100 units penicillin, and 100 μg streptomycin (Hyclone) shaking in flasks at 37°C. Growth was measured by OD600.

DppIV GLO Assay

DppIV Glo-Protease assays were performed following the manufacturer's protocol (Promega, Madison,WI). *B. dermatitidis* yeast were grown in HMM liquid media with penicillin and streptomycin. Inoculated liquid cultures were grown for 24hr at 37ºC shaking at 240 rpm before being passaged to a starting O.D. of A_{600} 0.1. Every 24hr, O.D A_{600} of each culture was taken and 1 mL aliquots were collected, centrifuged at 1.2 x g for 10 min., and supernatant filtered through a 0.22 μm filter and stored at -20ºC.

Generation of GFP-sentinel silenced strains

DppIV knockdown (RNAi) and control strains were engineered utilizing the *B. dermatitidis* GFP-sentinel system (Krajaejun et al., 2007). Two non-overlapping regions of DppIV (BDFG_ 07406) were targeted for silencing. A 5' region of DppIV was amplified utilizing the following primers: 5'-
GGGGACAAGTTTGTACAAAAAAGCAGGCT CCGCGTTTGTCACTCCACA-3' and
5'- GGGGACCACTTTGTACAAGAAAGCTGGGT AGGAGTATAAATGTCTTCA-3'
and a 3' region of DppIV was amplified utilizing the following primers:
5'-GGGGACCACTTTGTACAAGAAAGCTGGGT CGGCGACACTTACTATGTTG-3' and
5'- GGGGACAAGTTTGTACAAAAAAGCAGGCT TCCTTGTCGACGAAGCATTT-3' from *B. dermatitidis* genomic DNA using either Elongase® (Invitrogen, Grand Island, NY) or Herculase®(Agilent Technologies, Madison, WI). After gel purification, the amplified DNA from the 5' and 3' target regions of DppIV were separately cloned into the silencing plasmid pFANTAi via the *att*sites present on the amplicons (underlined region of primers) using the Gateway cloning system® (Invitrogen). These and other vectors were separately transformed into *Agrobacterium tumefaciens*. Then, utilizing *A. tumefaciens*-mediated transformation (Sullivan et al., 2002), the 26199-GFP strain was individually transformed with a non-silencing plasmid (pBTS4) to generate the non-silencing control strain; the pFANTAi plasmid to generate the GFP-silencing control strain; the pFANTAi-5' DppIVRNAi plasmid two independent times to generate the two independent 5' DppIVRNAi silenced strains; and the pFANTAi-3' DppIVRNAi plasmid to generate the 3' DppIVRNAi silenced strain. Transformants were selected on 3M plates containing 50μg/mL hygromycin B at 37°C. DppIV silenced transformants were initially identified by the loss of

GFP fluorescence and confirmed by reduction in extracellular DppIV activity using the DppIV-Glo Protease Assay (Promega, Madison, WI).

DsRed strains

Agrobacterium binary vector, pCAMDsRed#2 (Eckert et al., 2005), was obtained from the Fungal Genetics Stock Center plasmid collection (http://www.fgsc.net). The T-DNA region of this plasmid harbors the DsRed-Express gene (Clontech; Mountain View, CA) under the control of the *Aspergillus nidulans Gpd* promoter, and the *TrpC* terminator, together with the *E. coli hph* gene under the control of the *A. nidulans TrpC* promoter (Hyg^r). *Agrobacterium*-mediated transformation (AMT) of *B. dermatitidis* strain, 26199, with pCAMDsRed#2 and selection for hygromycin resistance were done as described (Sullivan et al., 2002). For the DppIVA RNAi strain, which is already hygromycin resistant, AMT was accomplished by co-cultivation with two different *A. tumifaciens* strains - one harboring pCAMDsRed#2 and one harboring pCTS59. pCTS59 was generated by Gibson Assembly (New England Biolabs, Ipswich, MA; Cat. No. E2611) using the binary vector pFANTAi4 (Krajaejun et al., 2007) digested with *BamHI* and *PacI,* and PCR fragments for the *A. nidulans Gpd* promoter and the *Neomycin phosphotransferase* (*NPTII*) gene from transposon *Tn5,* which were amplified from the plasmid pBTS92 (Sullivan et al., 2002) using primer pairs tdsP858 (TTTCAGTAACGTTAAGTGGATCCTCAGAAGAACTCGTCAAG) plus tdsP859 (ACATCACCATGATTGAACAAGATGGATTG), and tdsP860 (GTTCAATCATGGTGATGTCTGCTCAAGCG) plus tdsP861 (GATAGTTTAAACCCTTAATTAAGTCGACCCATCCGGTGCT), respectively. Transformants were selected for growth on plates with 200 µg/ml G418. For both the wild-type and DppIVA RNAi strains, transformants were screened for DsRed expression using the BioRad Versadoc 5000 (Hercules, CA; Green LED, 605 nm filter). For the DppVIA RNAi strain about one in 50 of the $G418^r$ colonies were also DsRed-expressing (indicating co-transformation with both binary vector T-DNAs). For both the wild-type and the DppIVA RNAi strains, red fluorescent colonies were streaked on selection medium and isolated colonies were re-screened with the Versadoc, and analyzed by fluorescent microscopy and flow cytometry to confirm uniformly fluorescent cell populations.

Treatment of mice with DppIV or MMP2 inhibitors

Mice were treated with MMP2 inhibitor GM6001 or vehicle control as described in (Wüthrich et al., 2012), and infected i.t. with 2 x 10^4 wild-type or DppIVA RNAi yeast. At 3 days post-infection, lungs were harvested and leukocytes were stained for monocyte and neutrophil markers. In some experiments, mice that were infected with wild-type yeast were treated daily with Sitagliptin (Cayman Chemical), diprotin A (Sigma). Sitagliptin-treated mice received 20 µL (10mg/mL) via intubation and 500 µL (10 mg/mL) via the intraperitoneal (i.p.) route. Diprotin A treated mice received 20 µL (4 mg/mL) via intubation and 500 µL (3.45mg/mL) i.p. Mice were analyzed for lung burden 7 days post-infection.

$Ly6C^{hi}$ Cells Purification, Culture, and Adoptive Transfer

$Ly6C^{hi}$ cells were purified from bone marrow using a 2-step purification on the AutoMACS system (Miltenyi, San Diego, CA). The first step negatively selects the unwanted cells while the second step positively selects for $Ly6C^{hi}$ cells. Bone marrow was collected from the femurs and tibias of wild-type or $iNOS^{-/-}$ mice and RBCs were lysed as above. To deplete neutrophils, the bone marrow suspension was mixed with biotinylated anti-Ly6G (clone 1A8). Lymphocytes were depleted using anti-Th1.2 (clone 30-H12) and anti-B220 (clone RA3-6B2) biotinylated antibodies because some lymphocytes are $Ly6C^+$. Cells that bind biotinylated antibodies are removed from the cell suspension when the biotin binds to streptavidin beads (Miltenyi). The magnetic streptavidin beads are held in a column with the labeled cells while unlabeled cells flow through to a collection tube. In the second step, we positively selected for $Ly6C^+$ cells by adding biotinylated anti-Ly6C (clones AL-21 or HK1.4) to the cells remaining after the depletion in step 1. $Ly6C^+$ cells were positively selected in the second step using the method described above except that the cells attached to the beads were collected after washing. Enrichment of $Ly6C^+$ cells was confirmed by flow cytometry. $Ly6C^{hi}$ monocytes ($CD11b^+$, $CD11c^-$, $Ly6C^{hi}$, $Ly6g^-$) were >90% pure and viability was >98%. The purified $Ly6C^{hi}$ monocytes were used for *in vivo* and *in vitro* experiments.

$Ly6C^{hi}$ cell-derived macrophages and inflammatory DCs were differentiated from Auto-MACS sorted bone marrow $Ly6C^+$ cells in cRPMI with 20 ng/mL GM-CSF at 37° C. Adherent $CD11b^+$, $CD11c^-$, $Ly6g^-$ macrophages were obtained after 5-7 days of culture, and non-adherent $CD11b^+$, $CD11c^+$, $Ly6g^-$ inflammatory DCs were obtained after 10-14 days of culture. Ly6C leaves the cell surface as cells differentiate over time so was not used in characterization. Cell types were confirmed by flow cytometry. TipDCs generated from $Ly6C^{hi}$ derived inflammatory DCs produced nitric oxide and TNF-α in response to exposure to yeast (Fig. S2b and data not shown) as measured by Griess reaction (Nagano, 1999) and/or respective transcript, consistent with a TipDC phenotype (Serbina 2003)(Shi

and Pamer, 2011). To investigate yeast killing *in vitro* by TipDCs or Ly6C-derived macrophages, these cells were cultured with wild-type or DppIVA RNAi yeast for 24 hours at 37° C (in the absence of GM-CSF) at an effector to target ratio of 3:1. To measure killing by undifferentiated Ly6C^{hi} monocytes, these monocytes (CD11b^{+}, Ly6C^{hi}, Ly6G^{-}) were sorted to >99% purity from bone marrow using FACS Aria II Sorter (BD Bioscience) and cultured with yeast under the same conditions. Monocytes were purified using FACS because AutoMACS sorting of bone marrow contained some immature neutrophils; these neutrophils did not survive differentiation of inflammatory DCs and Mϕs, as confirmed by flow cytometry.

For adoptive transfer into *Ccr2*$^{-/-}$ mice, 10^5 to 10^6 cells of Ly6C^{+} populations derived *in vitro* were administered to mice i.t. by intubation in 20 μL of PBS. If Ly6C^{hi} cells for transfer were elicited *in vivo,* "donor" mice received i.t. inoculation of 4 x 10^5 heat-killed yeast; on day four post-inoculation, lungs, regional lymph nodes and spleens were harvested and characterized as described (Wüthrich et al., 2012) for transfer of 10^5 to 10^6 cells per recipient.

Macrophages

Bone marrow derived macrophages (BMDM) were differentiated using 10 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ). After 7 days, BMDM were harvested by trypsinization of adherent cells. The phenotype of the cells is F4/80^{+} CD11b^{hi} CD208^{lo} PDL2^{int} CD301^{lo}. For *in vitro* killing assays, BMDM were plated at 10^5 cells per well in 100μl cRPMI in a 96-well plate. Macrophages were activated with 10 ng/ml GM-CSF for 24h. The next day, media was changed, fresh GM-CSF was added and macrophages were added to yeast at a ratio of 1:1. In some assays, macrophages were treated with diprotin A (Enzo Lifesciences, Farmingdale, NY) or rDPPIV at the time of infection.

***Csfra2*$^{-/-}$ mouse experiments, including adoptive transfers of peritoneal macrophages**

Peritoneal macrophages were obtained by peritoneal lavage from mice using Hank's balanced salt solution (HBSS) (Corning, Cellgro). Peritoneal exudate was plated overnight in RPMI 1640 medium (HyClone, Logan, Utah) with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA), 100 units penicillin, and 100 μg streptomycin and cultured at 37°C and 5% CO_2. Floating cells were removed by washing with HBSS, and adherent peritoneal macrophages were dissociated using enzyme-free cell dissociation buffer (Gibco Life Technologies, Grand Island, NY). For adoptive transfer studies, peritoneal macrophages were obtained as above, washed and re-suspended in HBSS with 0.1% FBS, and numbers transferred as described in the Methods.

Neutrophils

Neutrophils were purified from the bone marrow of 6-8 week old wild-type mice. Red blood cells (RBCs) were lysed in 0.2% NaCl for 5 seconds, then neutralized with 1.2% NaCl. The suspension was filtered through a 40 mm mesh. Cells were separated using a Percoll density gradient with cells layered over 5 mL of 62% then over 2 mL of 80%. Cells were centrifuged for 30 min at 1000g at room temperature without braking. Neutrophils were collected at the 62/80 interface and washed twice in cRPMI. Purity was checked by flow cytometry and was greater than 95% in all experiments. Experimental wells contained $2.5x10^5$ neutrophils, $2.5x10^4$ yeast, 10 ng/mL GM-CSF, *Blastomyces* recombinant DppIV, and/or diprotinA in cRPMI. Cells were cultured for 6 hours at 37°C and 5% CO_2. Neutrophils were lysed in distilled H_20 and yeast washed in PBS before plating on BHI. Each condition had 6 to 8 replicates. The percent killing was defined as (1-(# of yeast in a condition/# of yeast without neutrophils)) x 100.

Flow cytometry

Cells were washed with FACS buffer, fixed with 2% paraformaldehyde solution for 20 min, and washed and suspended in FACS buffer for analysis. Centrifugation was done at 1500 rpm for 5 min and 4°C in an Eppendorf 5825 centrifuge. Events were gated on FCS and SSC to exclude debris and LIVE/DEAD Fixable Yellow® (Molecular Probes, Grand Island, NY) negative cells to exclude dead cells. Alveolar macrophages were defined as CD11c^{+}, CD11b^{-}, Ly6g^{-}, Mac3^{+} and autofluorescent; neutrophils as CD11b^{+} and Ly6g^{+} (clone 1A8), CD11c^{-}; dendritic cells (DCs) as CD11c^{+}, CD11b^{+} and Ly6G^{-}; neutrophil-DC hybrids as Ly6G^{+}, CD11b^{+} and CD11c^{+}; Ly6C^{hi} monocytes as CD11b^{+}, Ly6C^{hi} and Ly6g^{-}; TipDCs as CD11b^{+}, Ly6C^{hi}, CD11c^{+}, Ly6g^{-}, and expression of NOS2 and TNFα^{+}; and inflammatory monocyte-derived phagocytes as, Ly6C^{+}, CD11b^{+}, CD11c^{+} and Ly6G: macrophages were distinguished from DCs in this population by autofluorescence (Geng et al., 2013; Misharin et al., 2013; Serbina and Pamer, 2006; Serbina et al., 2003; Zaynagetdinov et al., 2013). Activated neutrophils were measured as CD11b^{hi}, Ly6g^{+} (Kuijpers et al., 1991; van Eeden et al., 1999). Reactive oxygen species (ROS) were measured with dihydrorhodamine 123 (DHR) (Sigma) 0.5 mg/mL in 500uL RPMI and 10% FBS with 2 x 10^6 cells in a 24-well plate at 37° C for 4 hours, unstimulated. TNF-α was measured with intracellular staining for 4 hours at 37° C in 500μL cRPMI

with 2 x 10^6 cells in a 24-well plate. Antibodies were conjugated to biotin or the following fluorophores: FITC, PE, PerCP, PE-Cy7, APC, A700, or APC-Cy7, eFluor450 or BUV395 from BD Biosciences (San Jose, CA), eBiosciences (San Diego, CA) BioLegend (San Diego, CA) or Miltenyi. Yeast were identified by GFP or with 10 ng/mL Uvitex 2B (PolySciences, Inc., Warrington, PA). For *in vivo* analysis of yeast association with leukocytes, cells were permeablized before staining with uvitex 2B. Yeast viability was measured by the fluorescence of DsRed. Data was collected on a LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Lungs were processed by pressing them through a 40 μm filter with the plunger of a 5-mL syringe. Homogenates were digested using 1 mg/mL Collagenase D (Roche, Nutley, NJ) and 10 ng/mL DNAse I (Sigma) for 20 min. at 37°C. RBCs were lysed with ACK buffer and the remaining cells were washed with 2 mM EDTA and 0.5% BSA in PBS (FACS buffer). Markers, gating and instruments are described above.

Cell Migration assays

To measure cell migration, 24-well plates and 5 μm porous transwells (Corning, Tewksbury, MA) were coated with a 10 μg/mL solution of fibrinogen (Sigma-Aldrich) and blocked with 2% BSA. When assaying migration of murine 300-19 cells transfected with human CCR2 (a gift of Bernhard Moser (Thedor-Kocher Institute, Bern, Switzerland), 2 x 10^5 cells in cRPMI were loaded into each transwell, while the bottom chamber received 500 μl of intact or cleaved chemokine resuspended in cRPMI. Migration of cells was allowed to proceed for 16-24 hours at 37°C in 5% CO_2. Cell counts from the bottom well were determined by flow cytometry based on events in the appropriate FSC/SSC gate. To assay migration of primary, murine Ly6c^{hi} cells, bone marrow was collected from C57BL/6 mice, RBCs were lysed, and 10^6 bone marrow cells in cRPMI were loaded into a transwell. The bottom chamber was loaded with chemokine and cells were permitted to migrate as above. Cells that migrated into the bottom chamber were stained for viability, CD11b, Ly6g and Ly6c. Ly6c^{hi} cell counts in the chamber were assayed by flow cytometry based on CD11b, Ly6c^{+}, Ly6g- events.

Microscopy

Differential fluorescence microscopy was performed using an Olympus BX60 microscope. Images were captured with an Exi Aqua Camera (QImaging, Surrey, BC) and QCapture Pro 6.0 image software. Images were processed using Adobe Photoshop.

Immunization of mice:

To assess whether DppIVA is expressed *in vivo*, we administered yeast to mice subcutaneously since infection with wild-type yeast is better controlled at this site. Mice received 10^6 live wild-type yeast or DppIVA RNAi yeast subcutaneously four times, two weeks apart. The final administration of yeast was given with incomplete freund's adjuvant. Mice were bled ten days after the last boost and analyzed for anti-DppIV antibody detection by ELISA.

ELISA

After infection, mice were sacrificed and bronchoalveolar lavage fluid was collected by injecting and immediately collecting 1 mL of PBS. Lung homogenate supernatant was collected in total of 2 mL PBS after the lungs were ground through a 70 μm filter via plunger, spun at 1500 rpm for 5 min, and supernatant removed. ELISA assays for CCL2, CCL12 (both from R&D, Minneapolis, MN) and CCL7 (Peprotech) was performed according to the manufacturer's protocol using 1:5 dilution of sample.

To detect antibody responses to fungal DppIVA expressed *in vivo* by yeast, sera from immune mice were assayed by ELISA. Microtiter wells were coated with 10μg/mL recombinant *B. dermatitidis* DppIVA protein, washed with buffer (PBS +0.05% Tween 20) and blocked with blocking buffer (PBS + 2% BSA) for 1 hour. After washing, wells were coated for 1 hour with 100μL of naïve, pre-immune serum, or serum from mice immunized with wild-type *B. dermatitidis* or DppIV A RNAi yeast as above. Sera were tested at 1:2 serial dilutions. Treated wells were washed with buffer and coated with anti-mouse horse-radish peroxidase antibody for 1 hour (1:1000 dilution). After washing, wells were treated with 3,3',5,5'-tetramethylbenzidine substrate and incubated until color change was observed. A stop solution of 1M HCl was then added to wells and A_{450} was measured.

Recombinant DppIVA

Expression of *B. dermatitidis* rDppIVA was performed using EasySelect*Pichia* Expression Kit (Invitrogen, Grand Island, NY). The DppIVA ORF was amplified from genomic DNA with primers

JLL189:GGCTGAAGCTGAATTCACCCATCATCATCATCATCATATTGAACCCGCGCGCCAGCC
JLL188:TTTTGTTCTAGAAAGCTGGCTTAAACGCTCGGCCCCTCAC and cloned into the pPICZ**α**A vector using a *PmlI* restriction site via Gibson assembly reaction following the standard protocol (New England Biolabs). A 6X-His tag (underlined) was included as well. *P. pastoris* was transformed with this vector and transformants were selected on LB Zeocin plates.

RT-PCR
After infection, mice were sacrificed and lung tissue was collected in RNAlater (Qiagen, Valencia, CA). Lung tissue RNA was purified using a Qiagen midiRNA extraction kit following the manufacturer's protocol. cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol. Real-time PCR was performed with SYBR Green (Bio-Rad) and iCycler (Bio-Rad) with the following protocol: 95°C 3min., 40 cycles of 95°C 1min. and 60°C 1 min., 95°C 1min., 60°C 1 min., and for the melt curve 71 cycles of 60°C 10sec. The amount of transcript was normalized to 18srRNA. The following primers (IDT) were used:
CCL2: Jll238: TTAAAAACCTGGATCGGAACCAA, Jll239**:** GCATTAGCTTCAGATTTACGGGT
CCL7: Jll244:CCAATGCATCCACATGCTGC, Jll245:GCTTCCCAGGGACACCGAC,
CCL12: Forward: CTTCCGGACGTGAATCTTCT, Reverse: AGTCCTCAGGTATTGGCTGG,
18srRNA Forward: CGCCGCTAGAGGTGAAATTCT, Reverse: CGAACCTCCGACTTTCGTTCT
Mammalian CD26: 10^6 BMDM were plated in a 12 well plate and stimulated with 10 ng/ml GM-CSF for 24 hours in RPMI media. The next day, media was changed, cells were restimulated with GM-CSF and infected with 10^6 *B. dermatitidis* yeast for 24 hours. RNA was extracted after elimination of genomic DNA using RNeasy Mini Kit (Qiagen) and cDNA was prepared using Reverse Transcription System Kit (Promega, WI) to assess mammalian DppIV expression in macrophages. A mammalian CD26-specific (proprietary) primer probe set for Taqman assay was obtained from Applied Biosystems (Mm00494549_m1) spanning exon boundaries 20-21.

Mass Spectrometry
Recombinant CCL-2, -7, and -12, and GM-CSF (Sigma) that was non-glycosylated and lacked methionine at the N-terminus, were incubated with recombinant *Blastomyces* DppIVA. One microgram of target was incubated with 10 ng of DppIVA in 20mM HEPES, pH 7.6, 40mM NaCl at 37°C for 0 to 6 hrs; 5mM diprotin A or 2% fetal bovine serum (FBS) was included in some reactions. Wild type yeast were cultured *in vitro* in HMM with 2% FBS containing 20 μg GM-CSF. After 72 hours, yeast were removed. All reactions were terminated by freezing on dry ice, and were kept frozen until immediately prior to mass spectrometric analysis at the UW biotechnology center.

MALDI TOF analysis. Protein samples were cleaned/concentrated using solid phase extraction C18 cartridges (ZipTip-C18, EMD Millipore, Billerica, MA) according to manufacturer's protocol, but eluted with 1μl solution of 60%Acetonitrile/39%H_2O/1%TFA. Half of the protein sample (0.5μl volume) was directly deposited onto the OptiTOF™ 384 well plate (Applied Biosystems, Foster City, CA) and re-crystalized with 0.4μl of matrix [5mg/ml of α-Cyano-4-Hydroxycinnamic acid in acetonitrile/H_2O/TFA (70/30/0.1)]. Mass spectrum was acquired on a 4800 Matrix-Assisted Laser Desorption/Ionization-Time of Flight-Time of Flight (MALDI TOF-TOF) mass spectrometer (Applied Biosystems) scanning 5,000-40,000 Da mass range using 1000 shots acquired from 20 randomized regions of the sample spot at 3,800 intensity and 0.88V Detector Voltage Multiplier of OptiBeam™ on-axis Nd:YAG laser with 200Hz firing rate and 3 to 7ns pulse width in Positive Linear Mid Mass mode. External calibration with CytochromeC protein standard was performed to validate mass accuracy.

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