Control of mucosal candidiasis in the zebrafish swimbladder depends on neutrophils that block filament invasion and drive extracellular trap production

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Running Head: Neutrophils control mucosal candidiasis in zebrafish

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SUPPLEMENTARY DATA and METHODS

Supplementary Figure Legends

Fig. S1. Chemical inhibition of PI3K, but not CXCR2, reduces neutrophil recruitment. Four days post fertilization (4 dpf) *mpx:GFP* zebrafish were incubated in different chemical inhibitors for an hour prior to injection (A and D: pan-PI3K inhibitor: LY294002, 5 µg/mL or 0.05 % DMSO; B, C and E: CXCR2 inhibitor: SB225002, 2 µg/mL or 0.02% DMSO) and injected in the swimbladder with 4 nL of 3 X 10^7 (A,B,D,E) or 10^7 CFU/mL (C) Caf2-dTom or PVP. Fish were immediately screened and fish with 50-100 (A, B, D, E) or 15-30 (C) yeast cells in the swimbladder were selected. Fish were returned to the chemical inhibitor or vehicle, imaged by confocal microscopy at 24 hpi (A, B, D, E) or 4 and 8 hpi (C) and the number of neutrophils counted in the swimbladder region. At least two independent experiments were pooled (A and D: 3 experiments, n= 31 and 35; B and E: 2 experiments, n= 26 and 11; C: 3 experiments, n=46 and 51), medians, box and min-max whiskers are represented. Statistical comparisons were performed with Mann-Whitney test (A: p=0.0002, B: p=n.s. and C: p=n.s. and p=n.s.), and Fisher's exact test (D: p=n.s. and E: p=0.0092).

Fig. S2. CXCR2 inhibition blocks neutrophil recruitment to *Pseudomonas aeruginosa* infection in the otic vesicle. Three days post fertilization (3 dpf) *mpx:EGFP* larvae were incubated for 1 hour in SB225002 (2 µg/mL) or DMSO (0.02%)

and then injected in the otic vesicle with 1 nL of Caf2-dTom yeast cells (2 10^7 CFU/mL in 5% PVP), *Pseudomonas aeruginosa* (10^9 CFU/mL in 5% PVP) or PVP only. Fish were returned to SB225002 or DMSO and imaged by confocal microscopy 2 hours post infection (2 hpi). A: Diagram of the otic vesicle injection model used in this study. Red box indicates area imaged in B. B: Representative images of infected fish in the otic vesicle to illustrate recruitment of EGFP-expressing neutrophils (green) to *P. aeruginosa* (red; top 2 images) or *C. albicans* (red; bottom 2 images) infection with or without CXCR2 inhibition. Images are maximum projections in the green and red channels overlaid only with a single slice in DIC channel (left images) or green and red channels only (right images). The otic vesicle is outlined with blue dots. Scale bar = 100 µm. C: Number of neutrophils recruited to the otic vesicle at 2 hpi. Two independent experiments pooled (n=13/14/26/20/26/21), medians, box and min-max whiskers are represented with Kruskall-Wallis and Dunn's multiple comparison test ($p<0.0001$ and $p=0.0015$).

Fig. S3. CXCR2 inhibition increases the number of macrophages recruited to the swimbladder during mucosal candidiasis. Four days post fertilization (4 dpf) *mpeg1:EGFP* larvae were incubated for 1 hour in SB225002 (2 µg/mL) or DMSO (0.02%) and then injected in the swimbladder with 4 nL of Caf2-dTom yeast cells (10^7

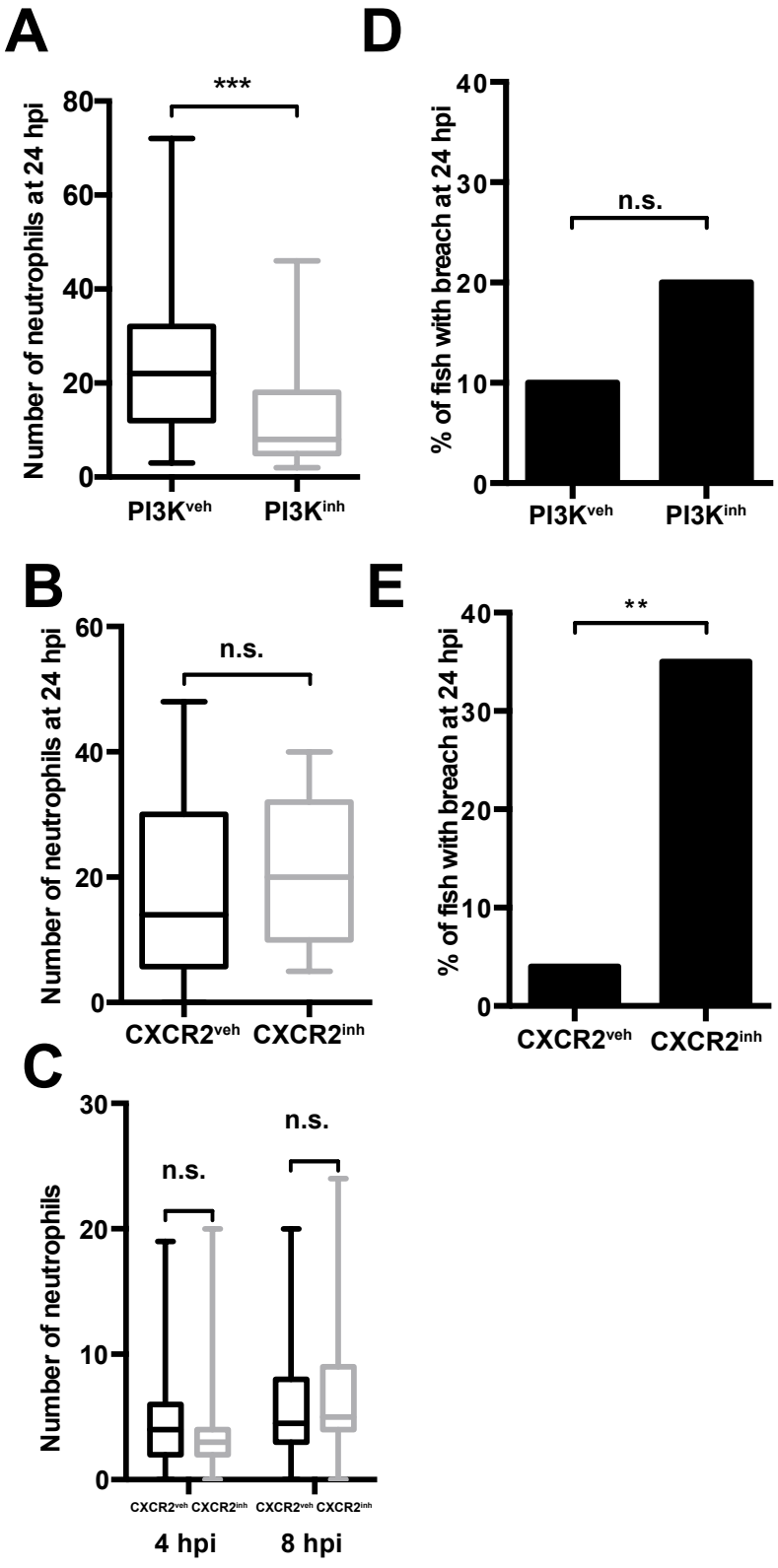
CFU/mL in 5% PVP), or PVP only. Fish were immediately screened and selected for 15-30 yeast cells in the swimbladder. Fish were returned to SB225002 or DMSO and imaged by confocal microscopy at 24 hours post infection (24 hpi). A: Number of macrophages recruited to the swimbladder at 24 hpi. Two independent experiments pooled, medians, box and min-max whiskers are represented with Mann-Whitney test (n=15/21/26/20 and p=0.045). B: Representative images of infected fish to illustrate recruitment of EGFP-expressing macrophages to *C. albicans* infection with or without CXCR2 inhibition. Images are maximum projections in the green and red channels overlaid only with a single slice in DIC channel (left images) or green and red channels only (right images). The swimbladder is outlined with blue dots. Scale bar = 100 μm. C: Percentage of fish with breach of the swimbladder epithelium at 24 hpi; pooled percentage and Fisher's exact test represented. D: *C. albicans* burden measured by percent coverage of the swimbladder surface in juvenile zebrafish after treatment with SB225002 (CXCR2 inh) or DMSO (CXCR2 veh). Medians, box and min-max whiskers are represented with Mann-Whitney test (n=26/27 and $p<0.0001$).

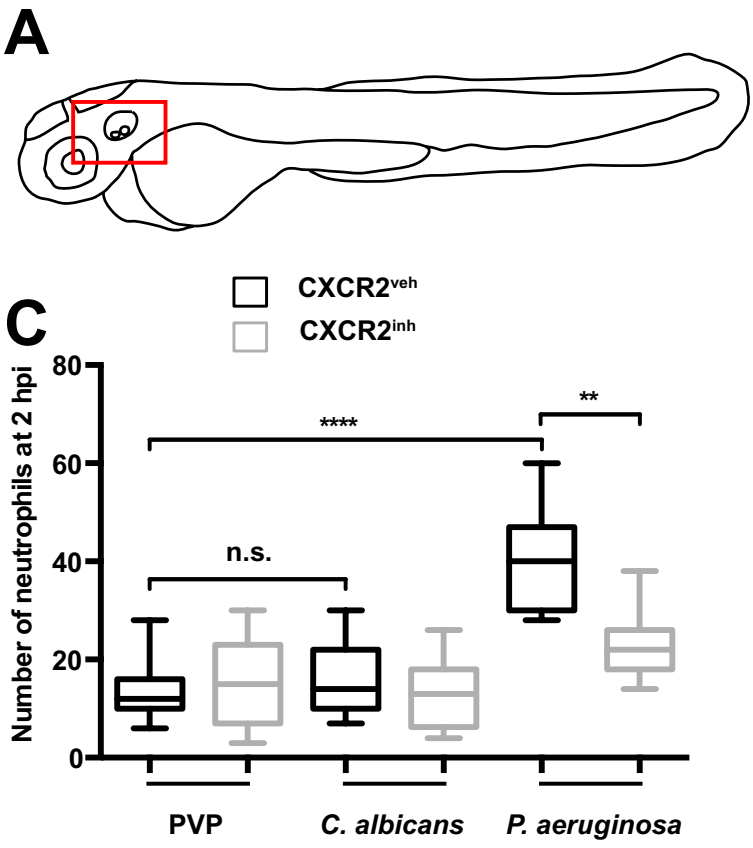
Fig. S4. *C. albicans* burden rank correlates to quantification by surface of the swimbladder coverage. Four days post fertilization (4 dpf) *mpo:Rac2-D57N* larvae were injected in the swimbladder with 4 nL of Caf2-dTom yeast cells (10^7 CFU/mL in

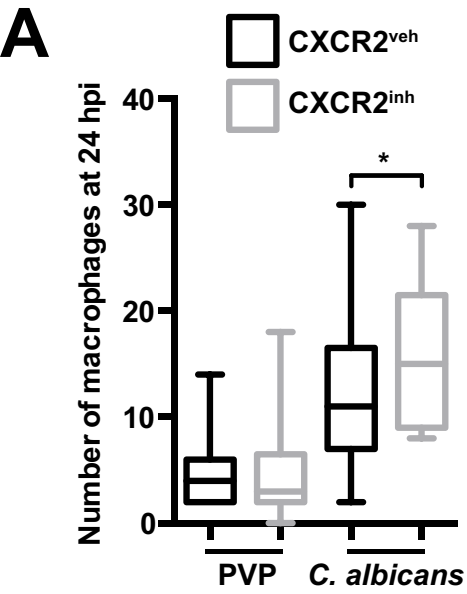
5% PVP). Fish were immediately screened and only fish with 15-30 yeast cells were selected. Fish were imaged by confocal microscopy at 24 hours post infection and both level of infection as well as images were acquired. *C. albicans* burden at 24 hpi was calculated as surface coverage of the swimbladder with ImageJ, and this quantitative data was plotted against the pathology score (n=29).

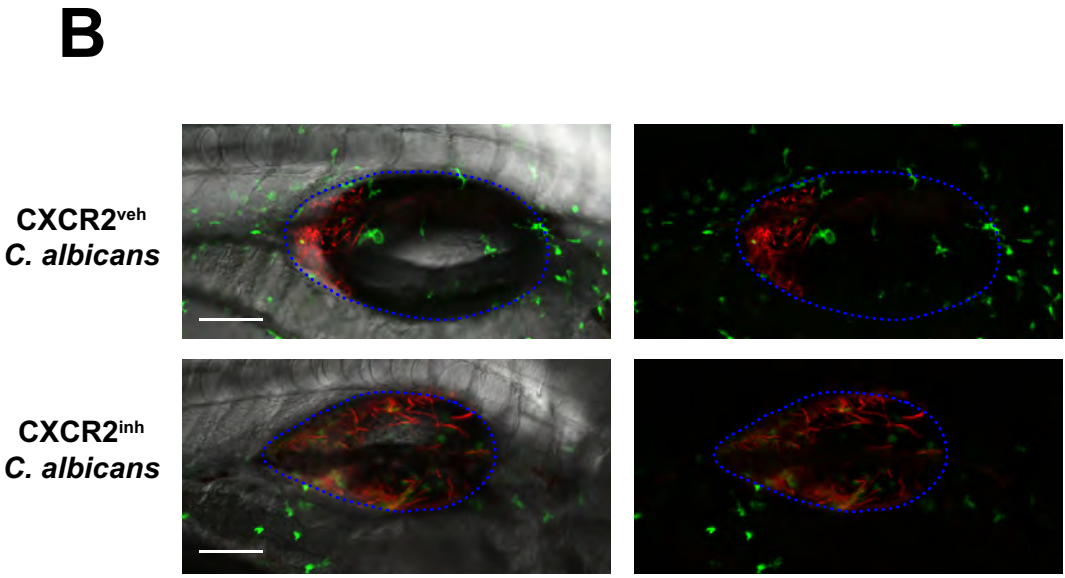
Fig. S5. Adoptive cell transfer of murine neutrophils does not robustly reduce *mpo:Rac2-D57N* susceptibility. Murine PMN were isolated from bone marrow and stained with CFSE for 8 min in HBSS. Cells were then counted and concentration adjusted to 10^7 cells/mL. *mpo:Rac2-D57N* or WT sibling (4 dpf) were injected with 4 nL of Caf2-dTom (10^7 CFU/mL) or Caf2-dTom + PMN. Fish were immediately screened and only fish with 15-30 yeast cells and 5-20 PMN were selected. Fish were incubated at 35°C for 24 hours and imaged by confocal microscopy for burden quantification as well as scored for epithelial breach (High + Breach). A-B. Representative image of murine PMN (green) and *C. albicans* (red) at 4 hpi. Image was processed by overlaying the maximum projection in both red and green channels (A and B) and a single slice in the DIC channel (A). B: Zoom of black box in A with only red and green channels represented. Scale bar represents 100 μm (A) and 20 μm (B). C. Adoptive cell transfer of Murine PMN does not significantly reduce *C. albicans* filament breach in the Rac2-

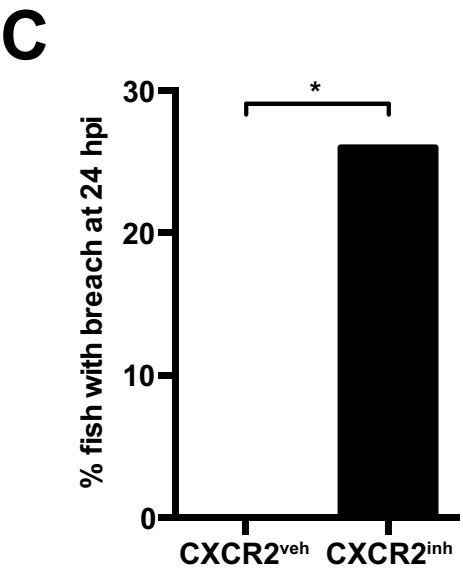
D57N fish. Percent of fish with breach of the epithelial barrier (High + Breach) at 24 hpi. Fisher exact test was used to test for differences. D: Adoptive cell transfer of murine neutrophils does not significantly reduce *C. albicans* burden in the *mpo:Rac2-D57N* fish. Burden at 24 hpi was quantified as *C. albicans* surface coverage of the swimbladder. Data was analyzed with ImageJ. Three independent experiments were pooled, (n=30, 35 and 36), medians, box and min-max whiskers are represented. Mann-Whitney test was used to test for differences (p=n.s.).

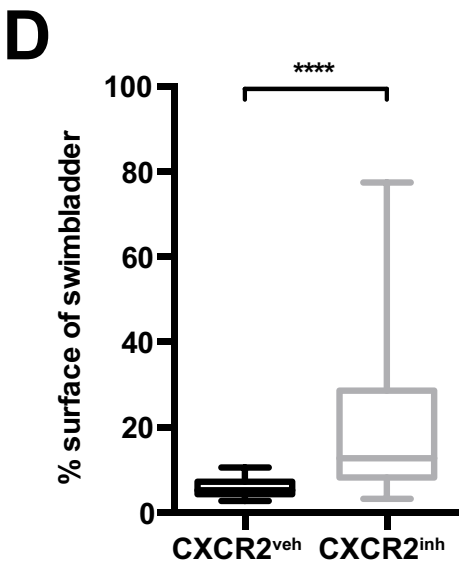

A
***
Number of neutrophils at 24 hpi
80
60
40
20
0
PI3K^veh
PI3K^inh
D
n.s.
% of fish with breach at 24 hpi
40
30
20
10
0
PI3K^veh
PI3K^inh
B
n.s.
Number of neutrophils at 24 hpi
60
40
20
0
CXCR2^veh
CXCR2^inh
E
**
% of fish with breach at 24 hpi
40
30
20
10
0
CXCR2^veh
CXCR2^inh
C
n.s.
n.s.
Number of neutrophils
30
20
10
0
CXCR2^veh CXCR2^inh
CXCR2^veh CXCR2^inh
4 hpi
8 hpi

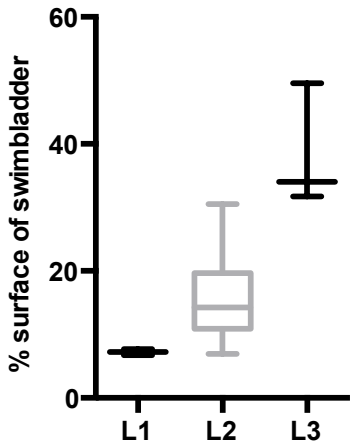

A
B
CXCR2veh
P. aeruginosa
CXCR2inh
P. aeruginosa
CXCR2veh
C. albicans
CXCR2inh
C. albicans
C
CXCR2veh
CXCR2inh
Number of neutrophils at 2 hpi
80
60
40
20
0
****
**
n.s.
PVP
C. albicans
P. aeruginosa


A
CXCR2veh
CXCR2inh
Number of macrophages at 24 hpi
40
30
20
10
0
*
PVP
C. albicans

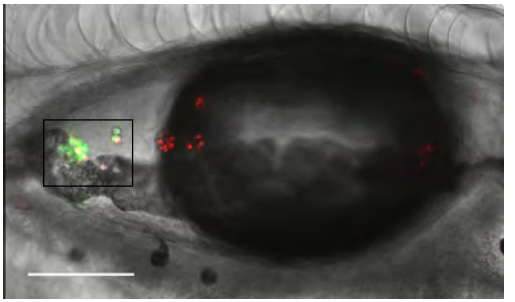

B
CXCR2veh
C. albicans
CXCR2inh
C. albicans


C
% fish with breach at 24 hpi
30
20
10
0
*
CXCR2veh
CXCR2inh

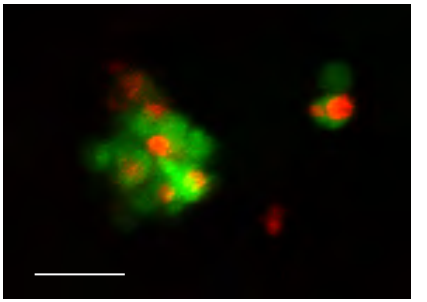

D
% surface of swimbladder
100
80
60
40
20
0
****
CXCR2veh
CXCR2inh


% surface of swimbladder
60
40
20
0
L1
L2
L3

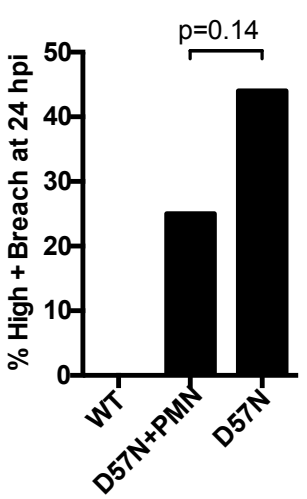
A



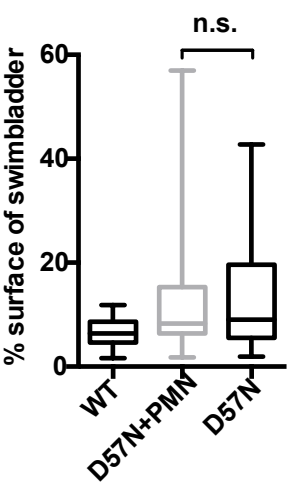
B



C


p=0.14
% High + Breach at 24 hpi
50
40
30
20
10
0
WT
D57N+PMN
D57N

D


n.s.
% surface of swimbladder
60
40
20
0
WT
D57N+PMN
D57N

Supplementary Materials and Methods

Image processing for *C. albicans* burden and extracellular trap quantification

Images acquired for *in vivo C. albicans* quantification were processed using FIJI (NIH). Briefly, maximum projections of the z-stack images was applied, (1) for *C. albicans* burden, the outline of the swimbladder was drawn as an ROI and the number of pixels of fluorescence was determined by using the threshold tool. This number was then compared to the total number of pixels in the swimbladder (ROI) and the ratio was calculated; (2) for Extracellular trap quantification, no ROI was set but the number of pixels in both channels (*C. albicans* in red and ET in green) were measured using the threshold tool, then the ratio of these channels was used; (3) for punctae enumeration, the number of green compact dots colocalizing with filaments were counted by eye on images processed as previously described; this number was then divided by the number of pixels of *C. albicans* to account for different amount of filaments in each images.
To speed the processing up, the batch analysis tool was used with the following steps:

```
run("Z Project...", "projection=[Sum Slices]");
//run("Channels Tool...");
//run("Brightness/Contrast...");
run("8-bit");
setMinAndMax(0, 125);
Stack.setChannel(2);
setMinAndMax(0, 125);
Stack.setChannel(3);
setMinAndMax(0, 125);
```

Then a ROI was drawn outlining the swimbladder using the polygon selection tool and the images saved in a new folder.
Another batch analysis was run on this new folder with the following steps:

```
setAutoThreshold("Default");
//run("Threshold...");
Stack.setChannel(1);
setThreshold(0, 40);
run("Measure");
Stack.setChannel(2);
setThreshold(0, 15);
run("Measure");
Stack.setChannel(3);
setThreshold(0, 15);
run("Measure");
```

Only images with more than 144,352 pixels (10%) in the *C. albicans* channel were used for ET and punctae quantification to avoid non-representative images. If multiple images were acquired for a single fish, results were averaged to get a single value for each fish.