Cell Host & Microbe, Volume *30*

Supplemental information

A dissemination-prone morphotype enhances extrapulmonary organ entry by *Cryptococcus neoformans*

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Supplemental Figure 1: Flow cytometry tracks *C. neoformans* populations shifting toward smaller cell size in the lungs. Related to Figure 2. Representative flow cytometry plots from mice inoculated with 10^4 KN99-mCherry cells / mouse.

Supplemental Figure 2: DNA content of *ex vivo C. neoformans* populations. Related to Figure 2. Percentage of cells with 1C, 2C, and >2C DNA content in the indicated population. Values correspond to the representative flow cytometry plot in Figure 2E. Bar graphs display the mean.

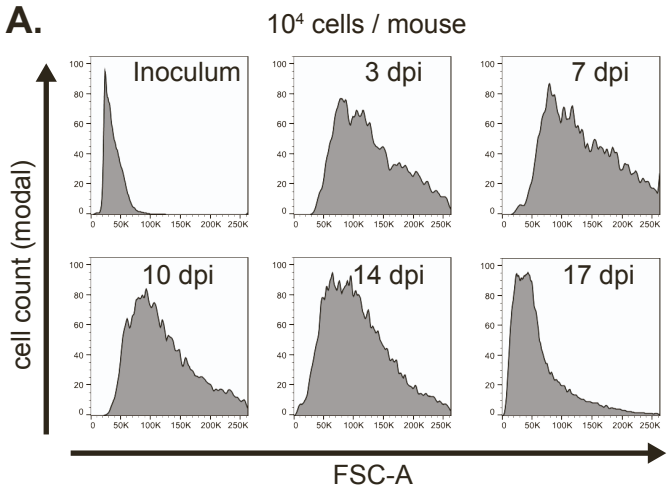
Supplemental Figure 3: Representative flow cytometry plots for opsonization and macrophage depletion efficiency. Related to Figure 3. **(A)** Representative flow cytometry plot showing single cell gating performed prior to analyzing opsonization of *ex vivo* cell populations (**Fig. 3A**). **(B)** Flow cytometry plots for small, intermediate **(C)**, and large **(D)** *ex vivo* cells stained with lectins or antibodies to detect cell surface binding. **(E)** Representative flow cytometry plots displaying the frequency of $CD45^+$, $F4/80^+$ macrophages in the liver and spleen after treatment with either PBS (control) liposomes or clodronate liposomes. **(F)** Quantification of macrophage depletion measured as the ratio of macrophages in clodronate-treated mice to PBS-treated mice. **(G)** Colony forming units (CFU) of data in **Figure 3H**. Mann-Whitney U-test, compared to PBS control; N=6 mice per group. ns: not significant *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Bar graphs display the mean. Inset (box) is CFU in the brain.

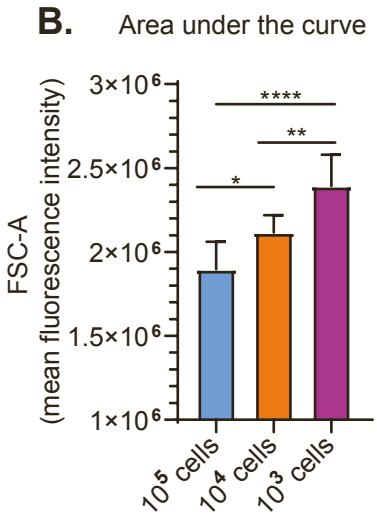
Supplemental Figure 4: Depletion of platelets or neutrophils does not affect the dissemination of small *ex vivo* cells. Related to Figure 3. **(A)** Fungal burden in mice, measured by colony forming units (CFU), following treatment with PBS (control) or anti-GPIbα antibody to deplete platelets prior to intravenous inoculation with 10^5 small *ex vivo* cells / mouse. **(B)** Platelet count (K: units) in the blood before and after treatment with PBS (control) or anti-GPIbα antibody. **(C)** Quantification of platelet depletion in Figure S4B. **(D)** Fungal burden in mice treated with PBS (control) or anti-Ly6G antibody to deplete neutrophils prior to intravenous inoculation with 10^5 small *ex vivo* cells / mouse. **(E)** Representative flow cytometry plots displaying the frequency of $CD45^+$, $Ly6C^+$, $Ly6G^+$ neutrophils in the blood after treatment with either PBS (control) or anti-Ly6G antibody. **(F)** Quantification of neutrophil depletion measured as the ratio of neutrophils in anti-Ly6G antibody-treated mice to PBS-treated mice. All comparisons in Figure S4 were analyzed using unpaired t-tests (ns: not significant *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). All bar graphs display the mean.

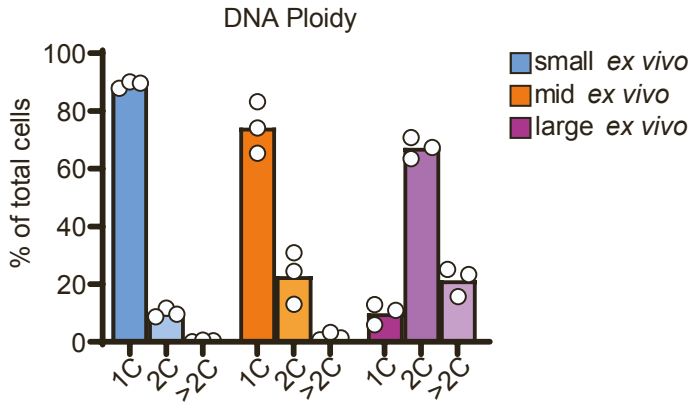
Supplemental Figure 5: Screen for factors that induce cell size reduction. Related to Figure 6. **(A)** Fungal cell size measured by forward scatter-area (FSC-A). Cells were cultured in capsule inducing medium (CAP medium) buffered to pH 7.4 or 6.3 before subculture (1:1) in fresh medium with 10% of the final volume being the indicated supplement solubilized in H_2O (CM: conditioned medium; GXM: glucuronoxylomannan; YNB: minimal YNB medium; PBS: phosphate buffered saline). Comparisons in Figure S5A were analyzed using one-way ANOVA and uncorrected Fisher's LSD (ns: not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). All bar graphs display the mean and error bars indicate standard deviation. **(B)** Cell body diameter and **(C)** capsule thickness of cells cultured in capsule inducing medium (CAP medium) buffered to pH 7.4 or 6.3 before subculture (1:1) in fresh medium with the addition of a putative inducing factor. For H_2O, CM, or YNB, the inducing factor was 10% of the final volume. (CM: conditioned medium; YNB: minimal YNB medium). **(D)** Cell body diameter and **(E)** capsule thickness measurements at 3, 10, and 17 days post-intranasal inoculation (dpi) of mice (Mann-Whitney U test; N=6 mice per time point; 50, 100, and 150 wild-type cells measured per mouse at days 3, 10, and 17 respectively. 50, 50, and 100 *pho4*Δ mutant cells measured per mouse at days 3, 10, and 17 respectively). Solid lines in the violin plots indicate the median and dotted lines mark quartiles. All comparisons in Figure S5B-E were analyzed using Mann-Whitney U-tests; N=200 fungal cells (ns: not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). **(F)** Representative India ink images of fungal cells quantified in Fig. 6G. Scale bars represent 20 μm. **(G)** Nanomoles of phosphate cells grown in YNB, CAP-medium (pH 6.8 or pH 7.4)

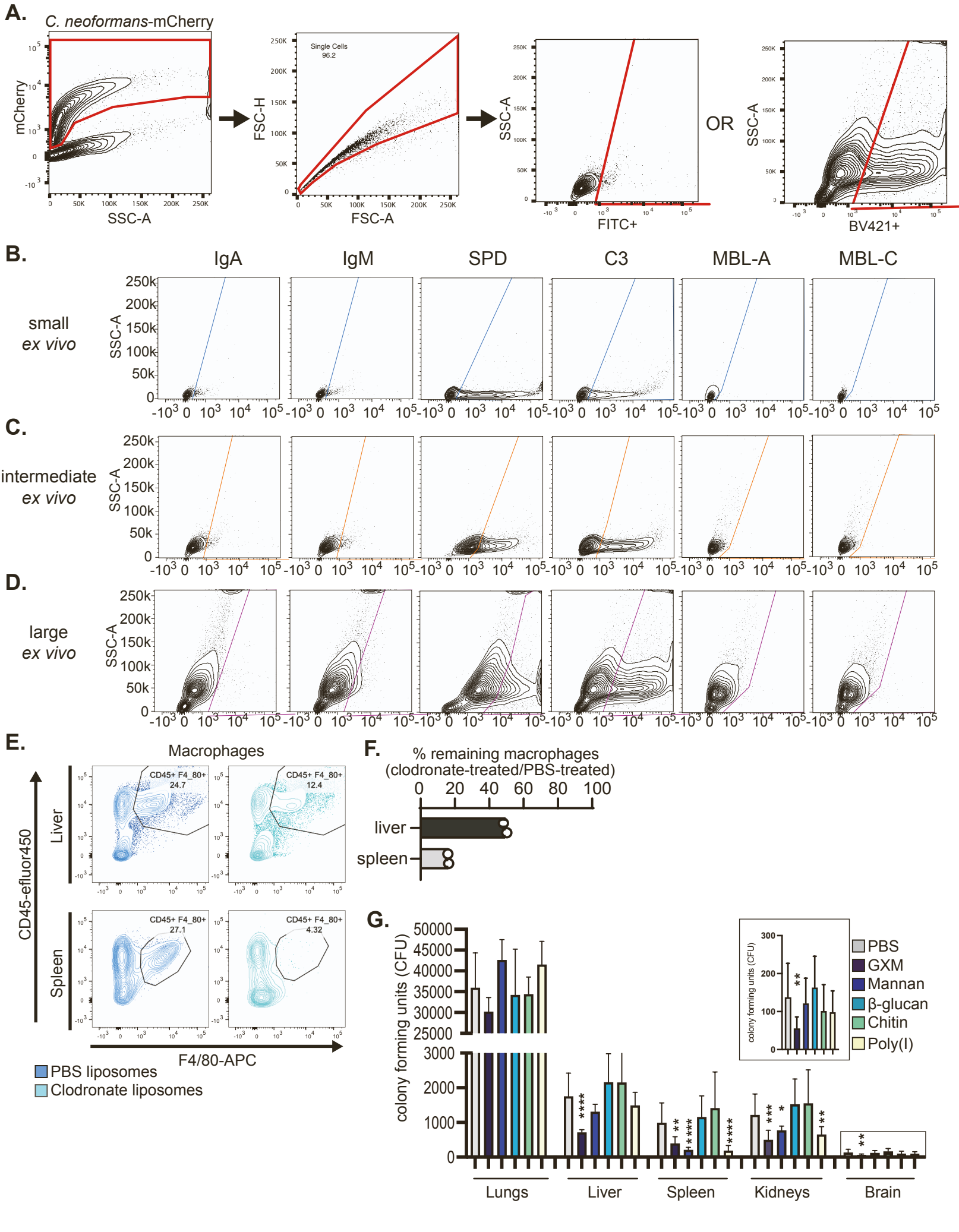
Supplemental Figure 6: Multiple sources of phosphate are sufficient to induce cell body diameter and capsule thickness reduction. Related to Figure 7. Total diameter measurements of CAP-grown cells (pH 6.8 in **A**, pH 7.4 in **B**) after exposure to ATP, CTP, GTP, UTP at 200 μM or a pool of NTPs sy 200 μM total concentration (50 μM each of adenine, cysteine, guanine, and uridine triphosphates). **(C)** Capsule thickness (calculated using the formula (total diameter – cell body diameter)/2)) of CAP-grown cells (pH 6.8 on left, pH 7.4 on right) induced to form small cells in guano medium. **(D)** Cell body diameter of CAP-grown cells (pH 6.8 on left, pH 7.4 on right) induced to form small cells in guano medium. **(E)** Total diameter measurements of wild-type or *pho4*Δ cells grown in capsule-inducing medium (pH 7.4) or induced to form small cells (+ guano). P-values calculated using Mann-Whitney test; N = 3 biological replicates per experiment and >100 cells measured per replicate. The solid line represents the population median and the dotted lines the 25^{th} and 75^{th} percentiles. The solid

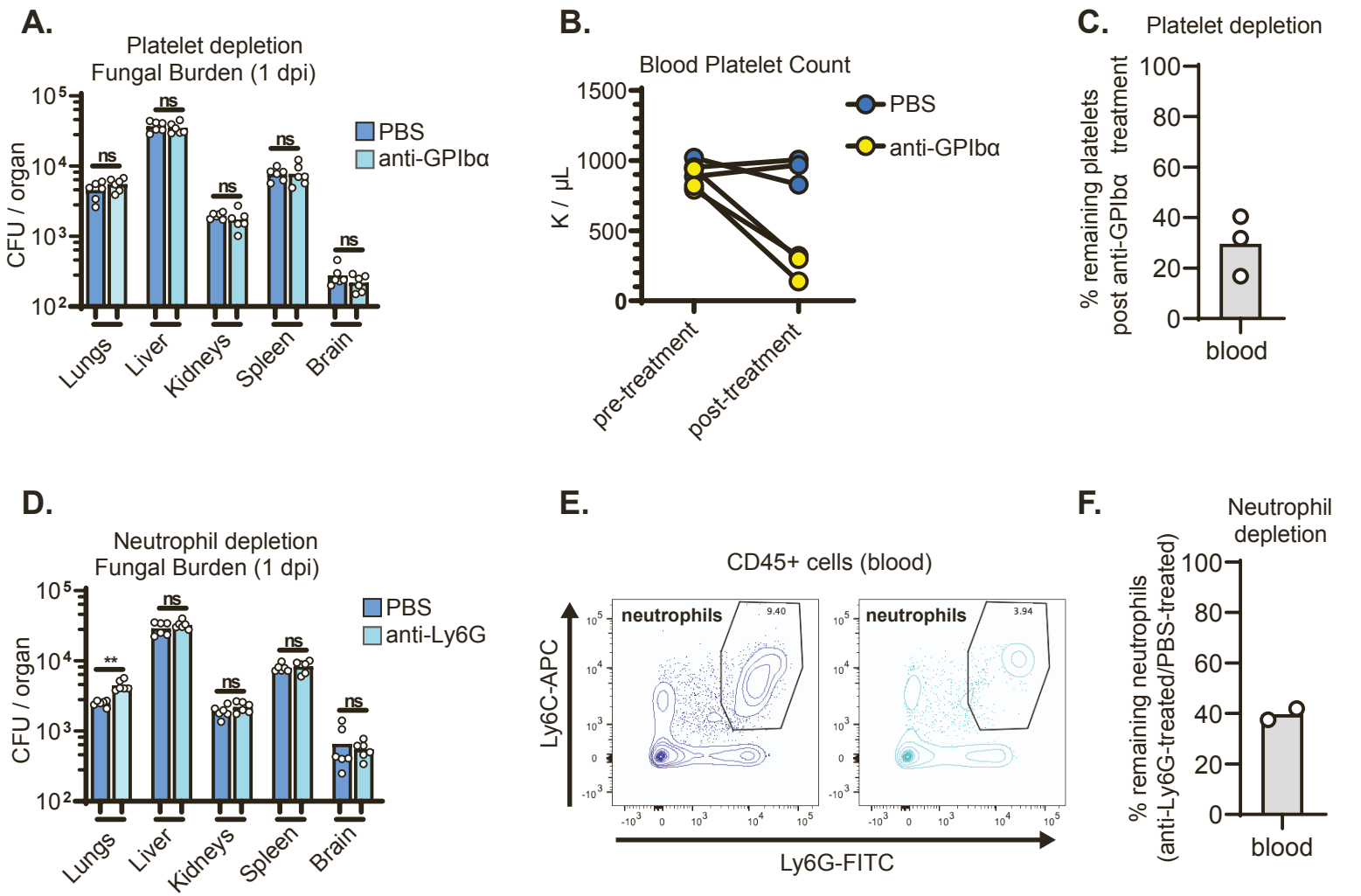
line represents the population median and the dotted lines the 25th and 75th percentiles. All comparisons marked as significant represent populations for which the 95% confidence interval does not overlap. ****p<0.0001.

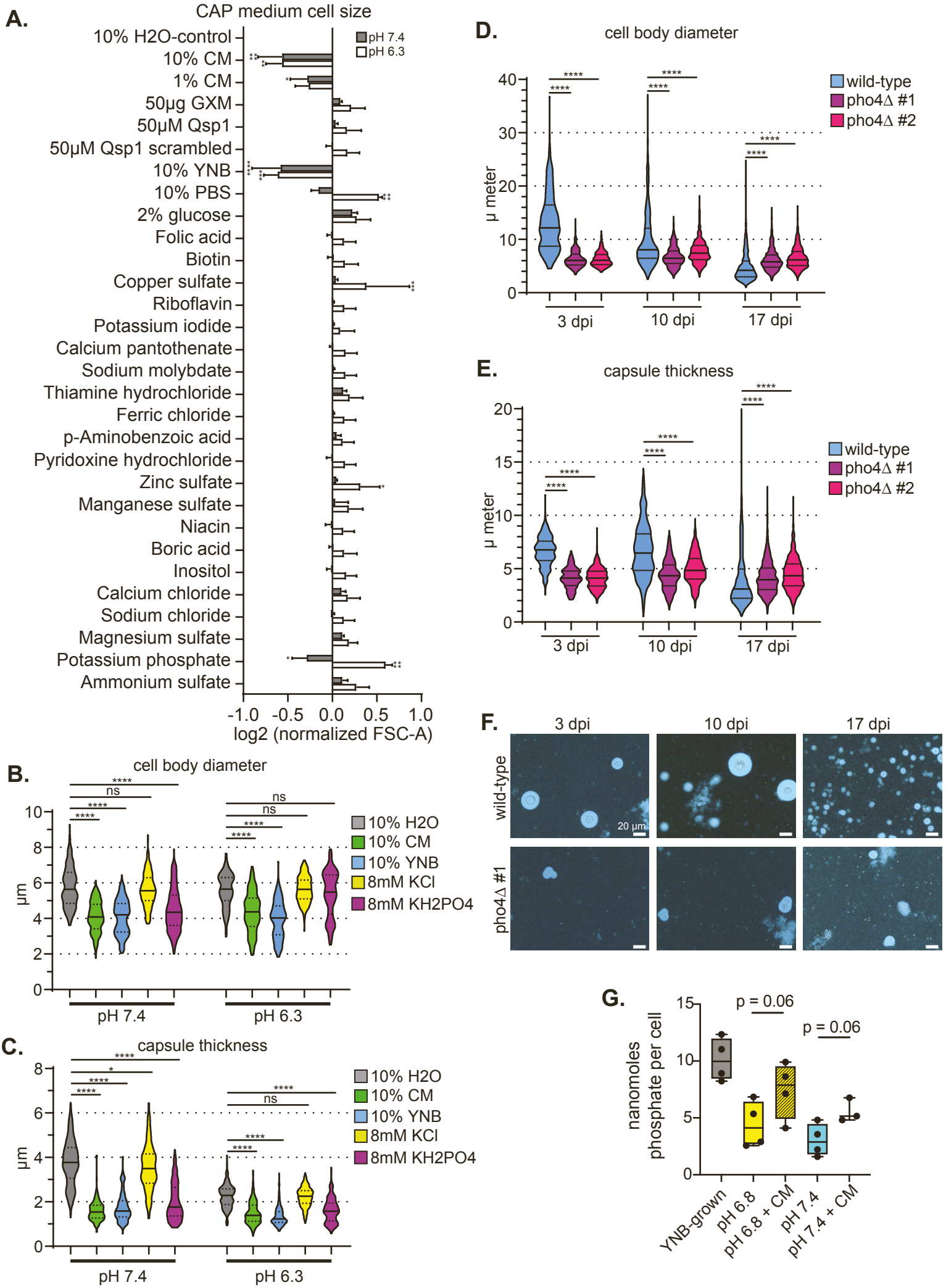

A.
10⁴ cells / mouse
Inoculum
3 dpi
7 dpi
10 dpi
14 dpi
17 dpi
cell count (modal)
FSC-A

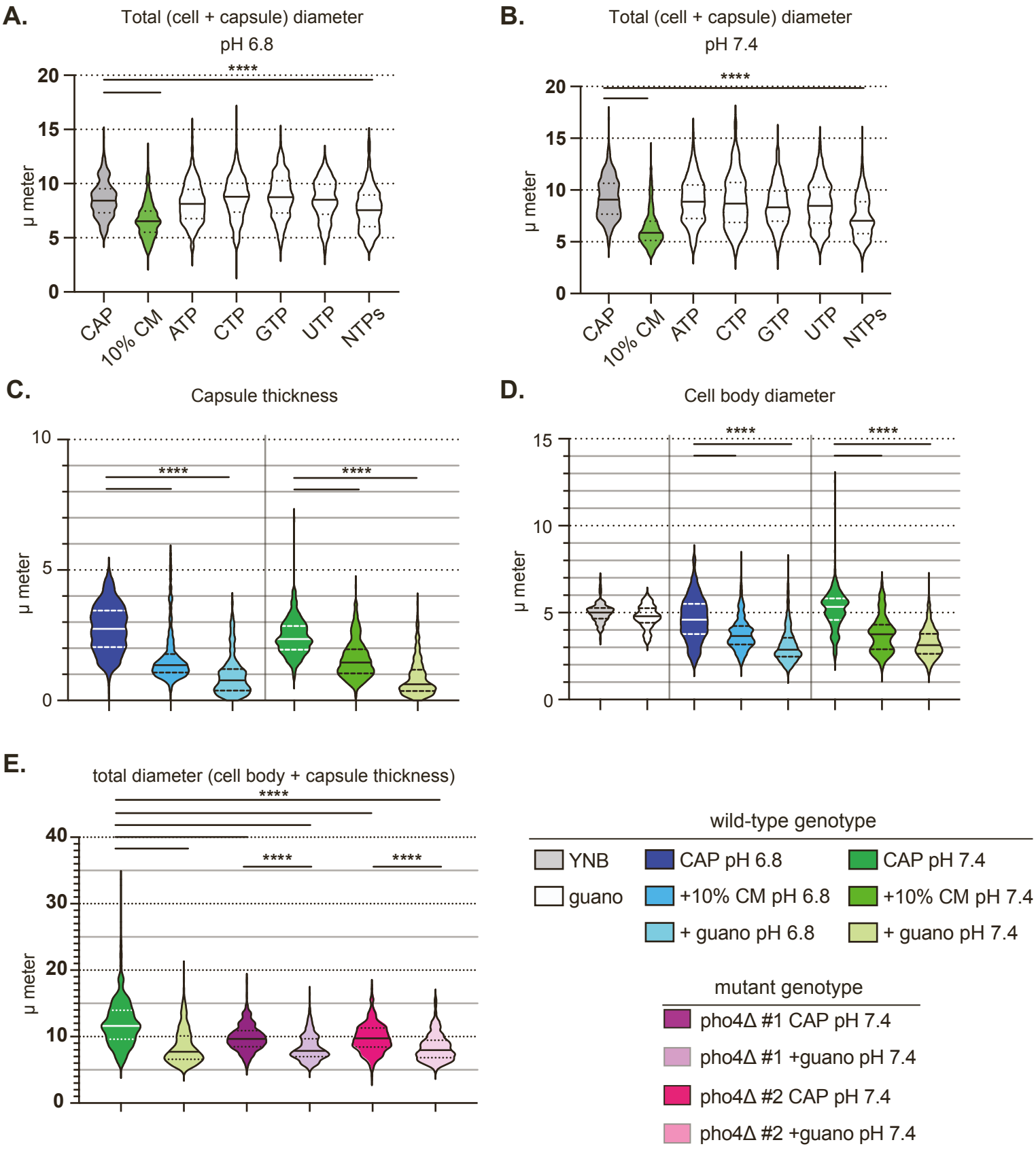

B.
Area under the curve
FSC-A
(mean fluorescence intensity)
3×10⁶
2.5×10⁶
2×10⁶
1.5×10⁶
1×10⁶
****
**
*
10⁵ cells
10⁴ cells
10³ cells


DNA Ploidy
% of total cells
100
80
60
40
20
0
1C
2C
>2C
1C
2C
>2C
1C
2C
>2C
small ex vivo
mid ex vivo
large ex vivo


A.
C. neoformans-mCherry
mCherry
SSC-A
Single Cells
96.2
FSC-H
FSC-A
SSC-A
FITC+
OR
SSC-A
BV421+
B.
IgA
IgM
SPD
C3
MBL-A
MBL-C
small
ex vivo
SSC-A
C.
intermediate
ex vivo
D.
large
ex vivo
E.
Macrophages
CD45-efluor450
Liver
Spleen
CD45+ F4_80+
24.7
CD45+ F4_80+
12.4
CD45+ F4_80+
27.1
CD45+ F4_80+
4.32
F4/80-APC
PBS liposomes
Clodronate liposomes
F.
% remaining macrophages
(clodronate-treated/PBS-treated)
0 20 40 60 80 100
liver
spleen
G.
colony forming units (CFU)
PBS
GXM
Mannan
β-glucan
Chitin
Poly(I)
Lungs
Liver
Spleen
Kidneys
Brain


A.
Platelet depletion
Fungal Burden (1 dpi)
CFU / organ
PBS
anti-GPIbα
ns
Lungs
Liver
Kidneys
Spleen
Brain
B.
Blood Platelet Count
K / µL
PBS
anti-GPIbα
pre-treatment
post-treatment
C.
Platelet depletion
% remaining platelets post anti-GPIbα treatment
blood
D.
Neutrophil depletion
Fungal Burden (1 dpi)
CFU / organ
PBS
anti-Ly6G
**
ns
Lungs
Liver
Kidneys
Spleen
Brain
E.
CD45+ cells (blood)
Ly6C-APC
Ly6G-FITC
neutrophils
9.40
neutrophils
3.94
F.
Neutrophil depletion
% remaining neutrophils (anti-Ly6G-treated/PBS-treated)
blood


A.
CAP medium cell size
pH 7.4
pH 6.3
10% H2O-control
10% CM
1% CM
50µg GXM
50µM Qsp1
50µM Qsp1 scrambled
10% YNB
10% PBS
2% glucose
Folic acid
Biotin
Copper sulfate
Riboflavin
Potassium iodide
Calcium pantothenate
Sodium molybdate
Thiamine hydrochloride
Ferric chloride
p-Aminobenzoic acid
Pyridoxine hydrochloride
Zinc sulfate
Manganese sulfate
Niacin
Boric acid
Inositol
Calcium chloride
Sodium chloride
Magnesium sulfate
Potassium phosphate
Ammonium sulfate
-1.0 -0.5 0.0 0.5 1.0
log2 (normalized FSC-A)
B.
cell body diameter
µm
pH 7.4
pH 6.3
10% H2O
10% CM
10% YNB
8mM KCl
8mM KH2PO4
C.
capsule thickness
µm
pH 7.4
pH 6.3
10% H2O
10% CM
10% YNB
8mM KCl
8mM KH2PO4
D.
cell body diameter
µ meter
3 dpi
10 dpi
17 dpi
wild-type
pho4Δ #1
pho4Δ #2
E.
capsule thickness
µ meter
3 dpi
10 dpi
17 dpi
wild-type
pho4Δ #1
pho4Δ #2
F.
3 dpi
10 dpi
17 dpi
wild-type
pho4Δ #1
20 µm
G.
nanomoles phosphate per cell
p = 0.06
p = 0.06
YNB-grown
pH 6.8
pH 6.8 + CM
pH 7.4
pH 7.4 + CM


A.
Total (cell + capsule) diameter
pH 6.8
μ meter
20
15
10
5
0
****
CAP
10% CM
ATP
CTP
GTP
UTP
NTPs
B.
Total (cell + capsule) diameter
pH 7.4
μ meter
20
15
10
5
0
****
CAP
10% CM
ATP
CTP
GTP
UTP
NTPs
C.
Capsule thickness
μ meter
10
5
0
****
****
D.
Cell body diameter
μ meter
15
10
5
0
****
****
E.
total diameter (cell body + capsule thickness)
μ meter
40
30
20
10
0
****
****
****
wild-type genotype
YNB
guano
CAP pH 6.8
+10% CM pH 6.8
+ guano pH 6.8
CAP pH 7.4
+10% CM pH 7.4
+ guano pH 7.4
mutant genotype
pho4Δ #1 CAP pH 7.4
pho4Δ #1 +guano pH 7.4
pho4Δ #2 CAP pH 7.4
pho4Δ #2 +guano pH 7.4