Supporting information

Cholesterol and sphingomyelin are critical for Fcγ receptor-mediated phagocytosis of *Cryptococcus neoformans* by macrophages

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Running title: Lipid rafts are critical for phagocytosis of *C. neoformans*

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Figure S1. Cholesterol and sphingomyelin are important for antibody-mediated phagocytosis. A) Macrophages (J774.1) treated with either 10 or 30 mM methyl-beta-cyclodextrin (MβCD) were co-incubated with antibody-opsonized *C. neoformans* H99 at a 1:1 ratio and allowed to interact for 2 hours. Cells were then fixed and stained with Giemsa and phagocytic index was calculated by microscopic observation (n=3). **B)** Macrophages (J774.1) treated with bacterial sphingomyelinase (bSMase) as indicated were co-incubated with antibody-opsonized *C. neoformans* H99 at a 1:1 ratio and allowed to interact for 2 hours. Cells were then fixed and stained with Giemsa and phagocytic index was calculated by microscopic observation (n=3). Error bars represent the standard error of the mean (SEM), and statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. **P* < 0.05 compared to the untreated control. All *P*-values were adjusted for multiplicity.

Figure S2. Structures of key lipids in this study. A) d18:1/16:0 sphingomyelin (C16 SM) **B)** d18:1/16:0 ceramide (C16 Cer) **C)** cholesterol **D)** 7-dehydrocholesterol **E)** coprostanol **F)** Azobenzene-sphingomyelin (AzoSM) is synthesized through a design principle called "azologization" (denoted with arrow 'a'). The structures of the cis and trans isomers of AzoSM upon light exposure are shown.

Figure S3. Repletion with raft-altering sterols affects GPMV levels. A) Macrophages (MH-S) were pre-treated with either 10 mM MβCD or 30 mM MβCD to deplete cholesterol. Monolayers were then washed and subject to giant plasma membrane vesicle formation using 25 mM PFA and 2 mM DTT. Nanodomains were assessed via FRET with DPH as the FRET donor and ODRB as the FRET acceptor. The ratio of DPH fluorescence intensity in the presence vs. absence of ODRB was calculated (F/F_0). Raw F/F_0 values for Figure 4A are reported (n=3). Error bars represent SEM. Similar unnormalized F/F_0 values at high temperature indicate that the amount of FRET acceptor bound to GPMV was similar in the samples. **B)** Macrophages were pre-treated with 10 mM MβCD to deplete cholesterol and then washed and incubated with 2.5 mM MβCD loaded with 0.2 mM of indicated sterol. Monolayers were then washed and subject to giant plasma membrane vesicle formation using 25 mM PFA and 2 mM DTT. Nanodomains were assessed via FRET with DPH as the FRET donor and ODRB as the FRET acceptor. The ratio of DPH fluorescence intensity in the presence vs. absence of ODRB was calculated (F/F_0). Raw F/F_0 values for Figure 4B are reported (n=3). Error bars represent SEM. Similar unnormalized F/F_0 values at high temperature indicate that the amount of FRET acceptor bound to GPMV was similar in the samples.

Figure S4. Treatment with bacterial sphingomyelinase does not affect nanodomain stability. Macrophages (MH-S) were pre-treated with either 250 mU/ml or 500 mU/ml bacterial sphingomyelinase (bSMase). Monolayers were then washed and subject to giant plasma membrane vesicle formation using 25 mM PFA and 2 mM DTT. **A)** Nanodomain stability was assessed via FRET with DPH as the FRET donor and ODRB as the FRET acceptor. The ratio of DPH fluorescence intensity in the presence vs. absence of ODRB was calculated (F/F_0). F/F_0 values were normalized to the final F/F_0 value at 64°C (n=3). Error bars represent SEM. **B)** Relative domain levels were estimated using the polynomial fits from A as described under *Experimental procedures*. Error bars represent SEM. **C)** Raw F/F_0 values are reported for Figure S4A (n=3). Error bars represent SEM. Similar unnormalized F/F_0 values at high temperature indicate that the amount of FRET acceptor bound to GPMV was similar in the samples.

Figure S5. Lipid depleting treatments do not decrease amount of FcγR. Median fluorescence intensity for 50,000 live macrophage cells for untreated control, 10 mM MβCD, 30 mM MβCD, 250 mU/ml bSMase, and 500 mU/ml bSMase were labeled with CD45-PE Cy7, CD16/32-APC, and CD11b-BV510 and quantified using FlowJo (n=3). Error bars represent SEM.

Figure S6. BSA-IgG immune complexes induce Fc receptor γ chain phosphorylation. Macrophages (MH-S) were stimulated with BSA-IgG immune complex (IC) for 5-30 min. Fc receptor γ chain (FcRγ) was immunoprecipitated (IP) and evaluated for phosphorylation of the tyrosine residues. IP samples or lysates (lys)

were probed for FcRγ or phospho-tyrosine (p-Tyr). IP control defined as IP buffer with IP antibody without cell lysate. Representative blot from 2 experiments.

Figure S7. Exclusion of sphingomyelin from rafts affects Fcγ receptor-mediated signaling. A) Macrophages (MH-S) treated with the AzoSM exchange protocol as described in *Experimental Procedures* were either unexposed, exposed to UV light, or exposed to UV light followed by blue light. Control samples were mock treated. Cells were then stimulated with BSA-IgG immune complex (IC) stimulation for 5 min. Fc receptor γ chain (FcRγ) were immunoprecipitated and evaluated for phosphorylation of the tyrosine residue. Lysates and IP samples were probed for phospho-tyrosine (p-Tyr) and/or FcRγ. Detection was performed using ECL, a chemiluminescent substrate. Representative immunoblots are shown. **B)** Immunoblots were quantified using ImageJ software; p-Tyr was normalized by FcRγ (n=4 experiments). Error bars represent the standard error of the mean and statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001. All *P*-values were adjusted for multiplicity.

Figure S8. MαCD-based exchange protocol successfully introduces AzoSM into cells. Endogenous C16 sphingomyelin (SM) was exchanged for azobenzene-sphingomyelin (AzoSM) in macrophages (MH-S) using MαCD as described in *Experimental Procedures*. AzoSM in cellular extracts were then measured using high-performance lipid chromatography (HPLC) at λ=365. **A)** Representative HPLC readout for untreated control sample. **B)** Representative HPLC readout for sample following AzoSM exchange. AzoSM elutes at ~10.6 minutes.

Figure S1

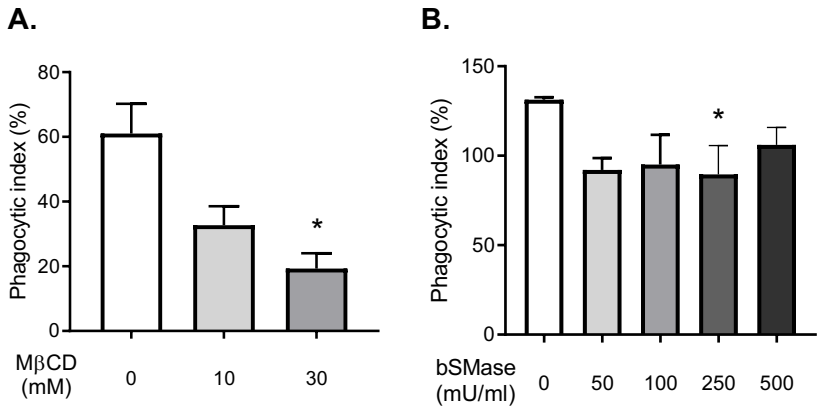

A.
Phagocytic index (%)
80
60
40
20
0
*
MβCD (mM)
0
10
30
B.
Phagocytic index (%)
150
100
50
0
*
bSMase (mU/ml)
0
50
100
250
500

Figure S2

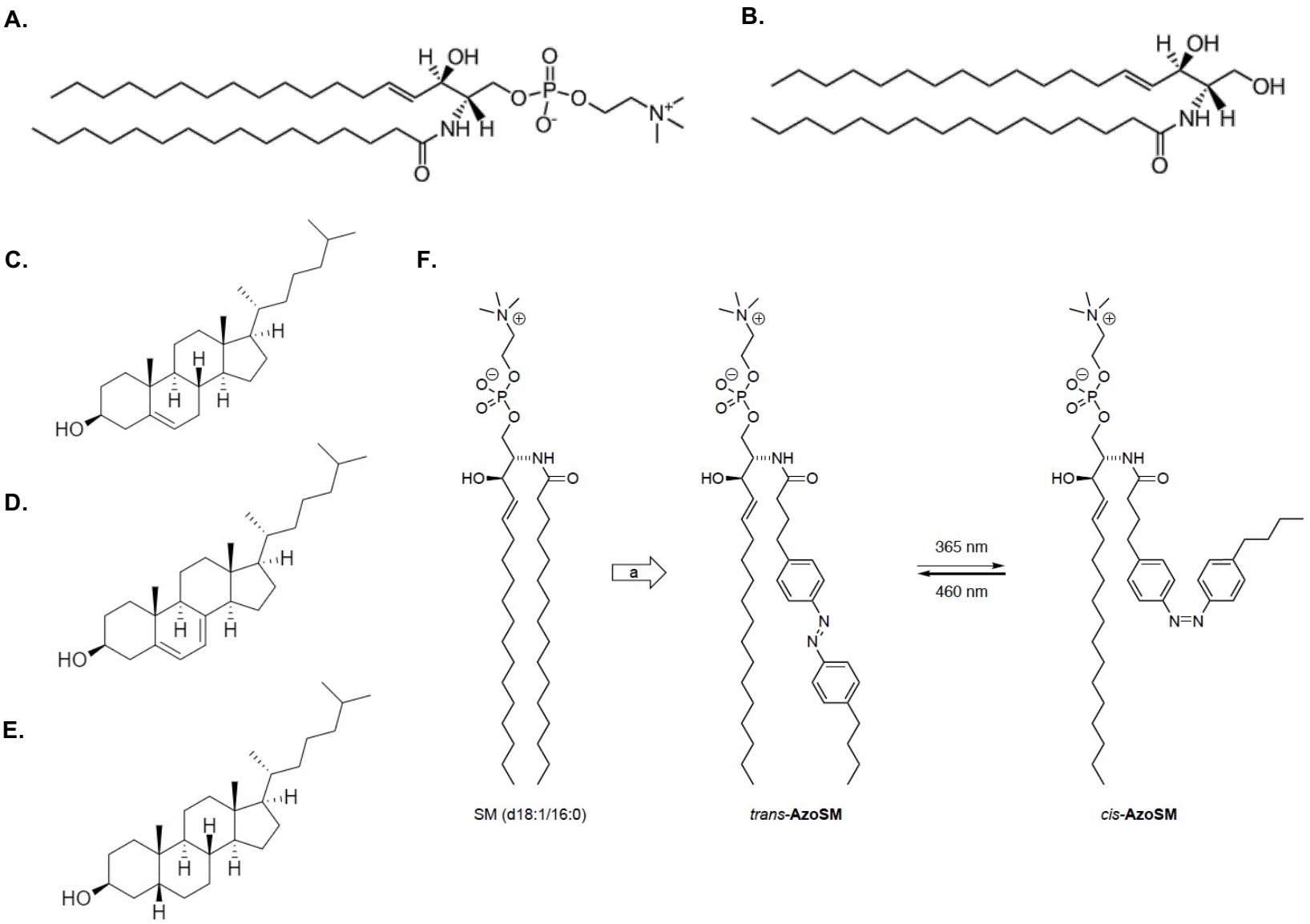


Figure S3

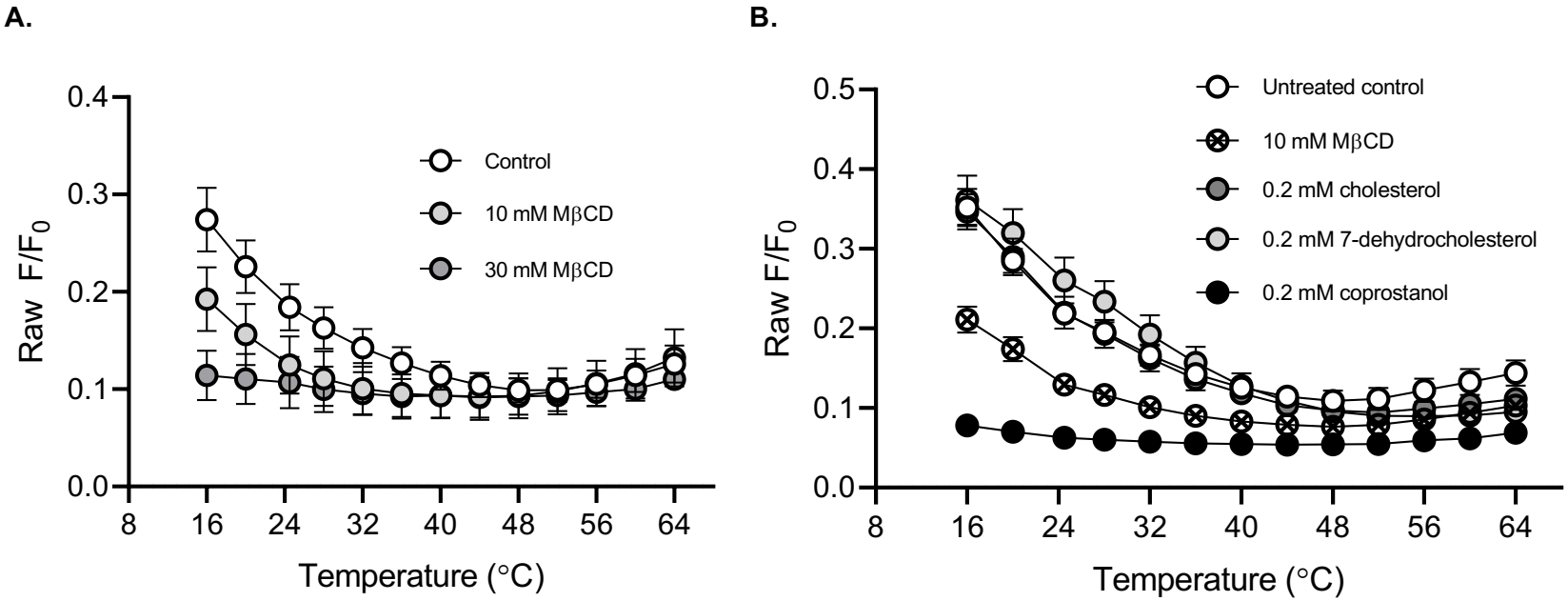

A.
Raw F/F0
0.4
0.3
0.2
0.1
0.0
8
16
24
32
40
48
56
64
Temperature (°C)
Control
10 mM MβCD
30 mM MβCD
B.
Raw F/F0
0.5
0.4
0.3
0.2
0.1
0.0
8
16
24
32
40
48
56
64
Temperature (°C)
Untreated control
10 mM MβCD
0.2 mM cholesterol
0.2 mM 7-dehydrocholesterol
0.2 mM coprostanol

Figure S4

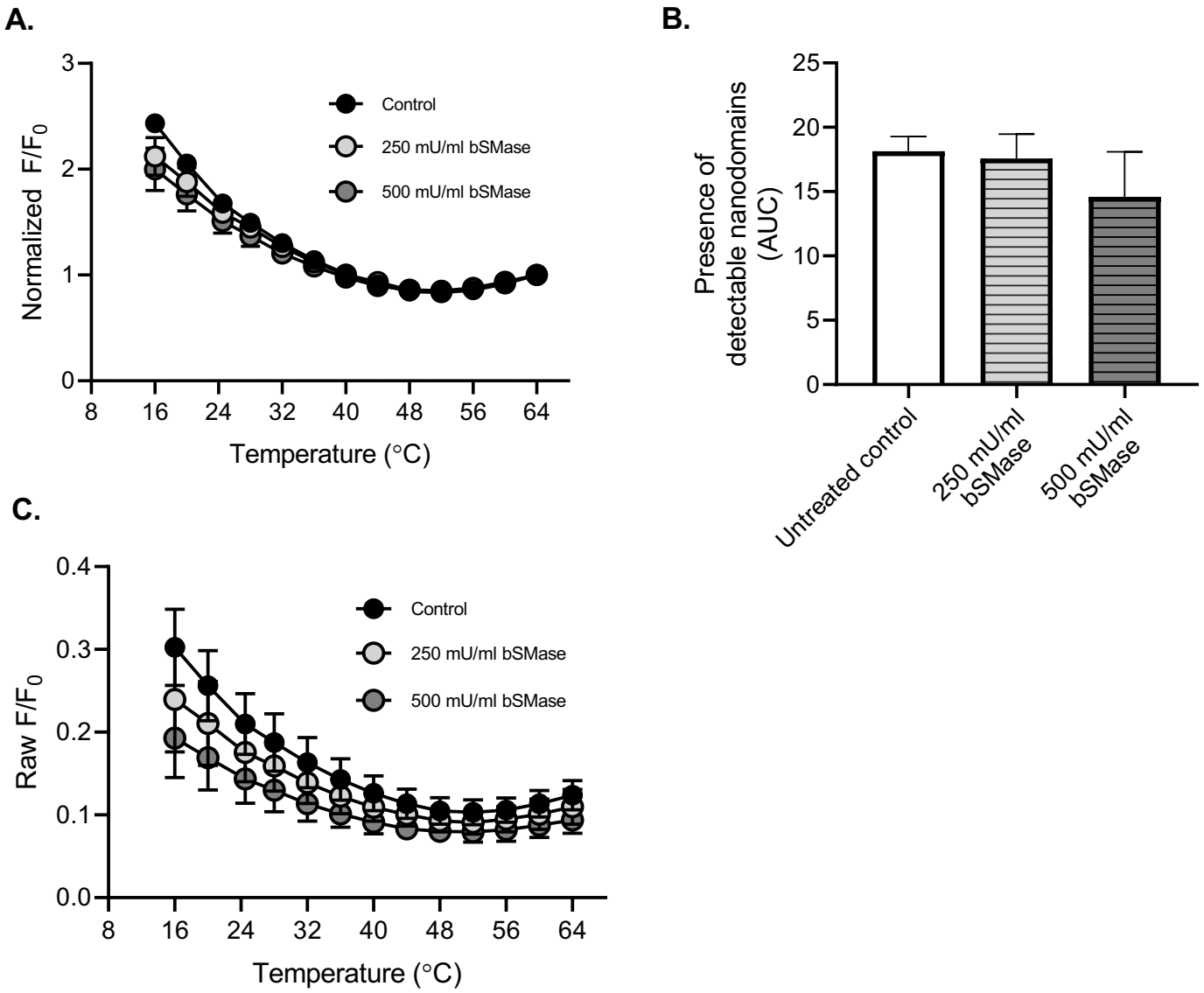

A.
Normalized F/F0
Control
250 mU/ml bSMase
500 mU/ml bSMase
Temperature (°C)
B.
Presence of detectable nanodomains (AUC)
Untreated control
250 mU/ml bSMase
500 mU/ml bSMase
C.
Raw F/F0
Control
250 mU/ml bSMase
500 mU/ml bSMase
Temperature (°C)

Figure S5

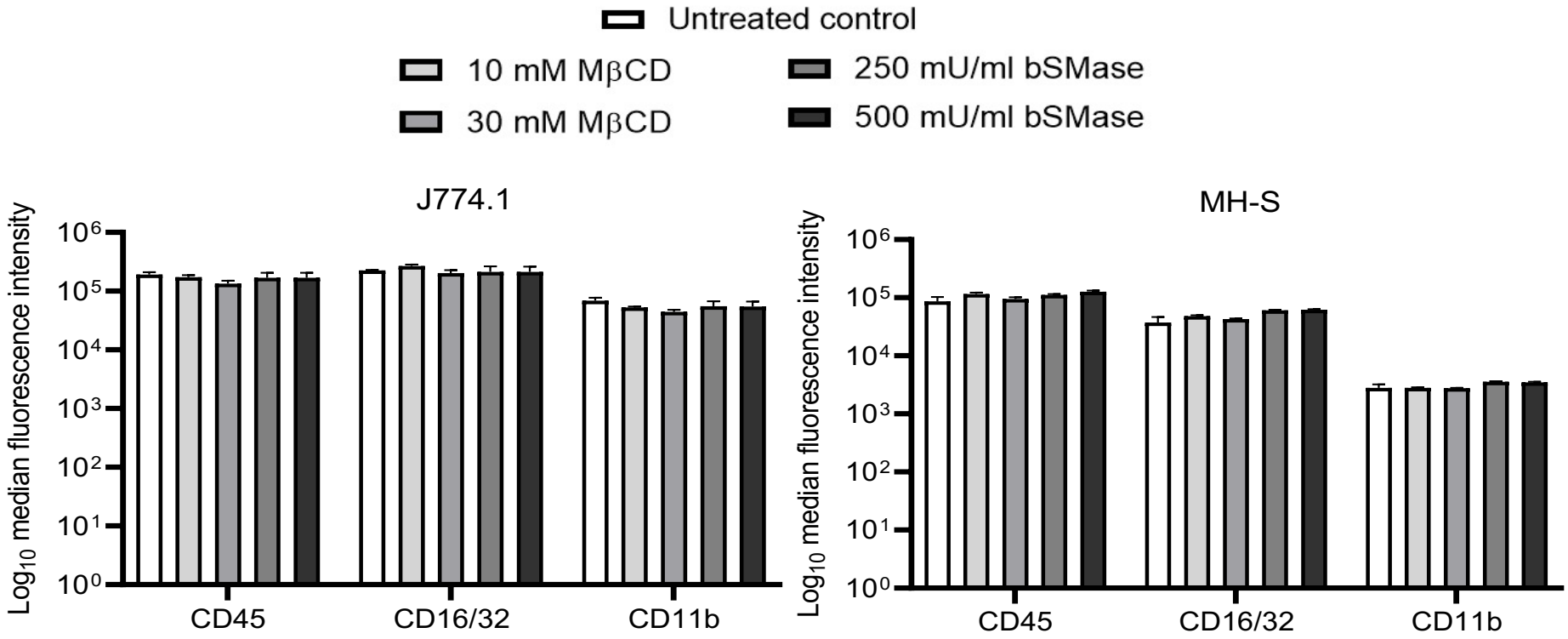

Untreated control
10 mM MβCD
30 mM MβCD
250 mU/ml bSMase
500 mU/ml bSMase
J774.1
MH-S
Log10 median fluorescence intensity
10^6
10^5
10^4
10^3
10^2
10^1
10^0
CD45
CD16/32
CD11b

Figure S6

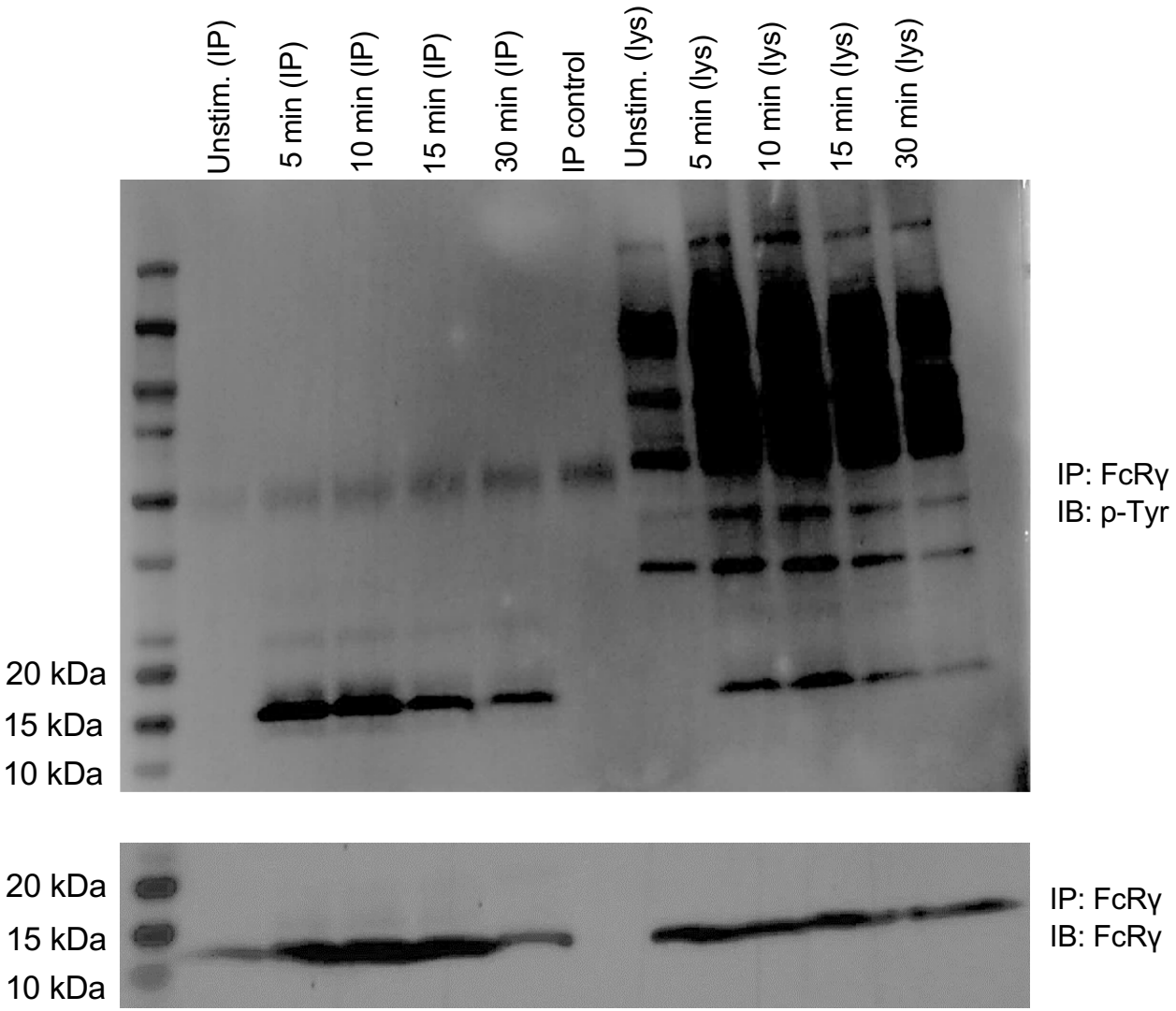

Unstim. (IP)
5 min (IP)
10 min (IP)
15 min (IP)
30 min (IP)
IP control
Unstim. (lys)
5 min (lys)
10 min (lys)
15 min (lys)
30 min (lys)
20 kDa
15 kDa
10 kDa
IP: FcRγ
IB: p-Tyr
20 kDa
15 kDa
10 kDa
IP: FcRγ
IB: FcRγ

Figure S7

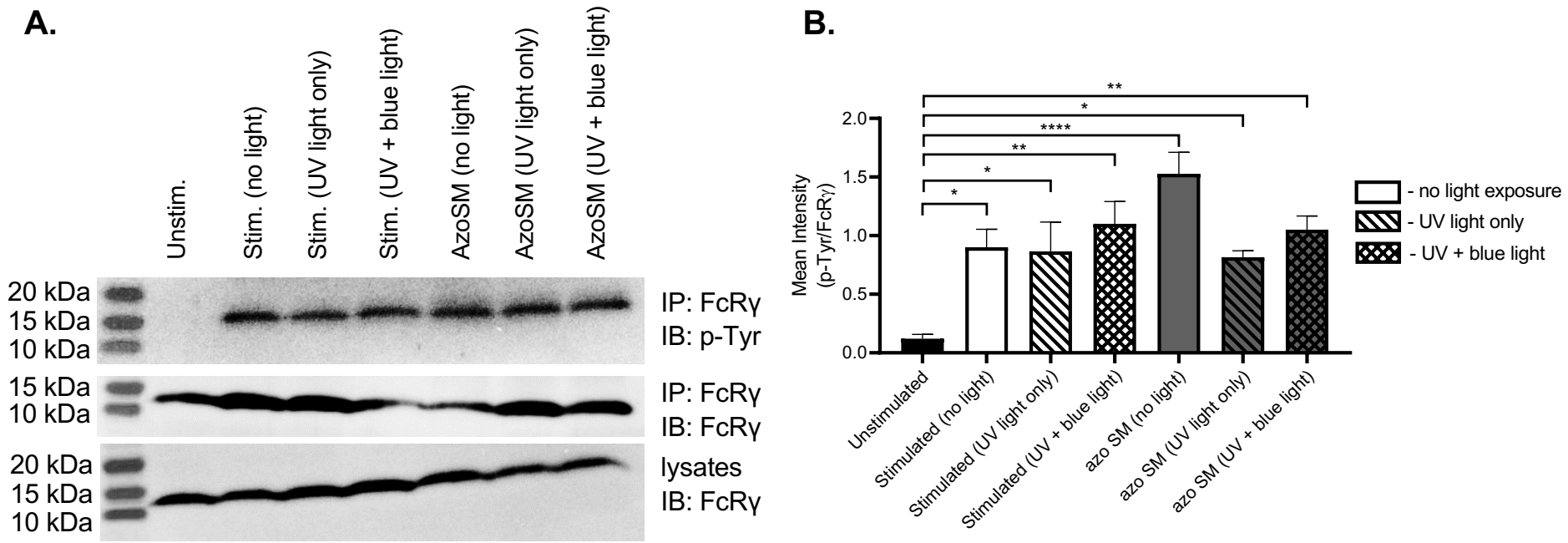
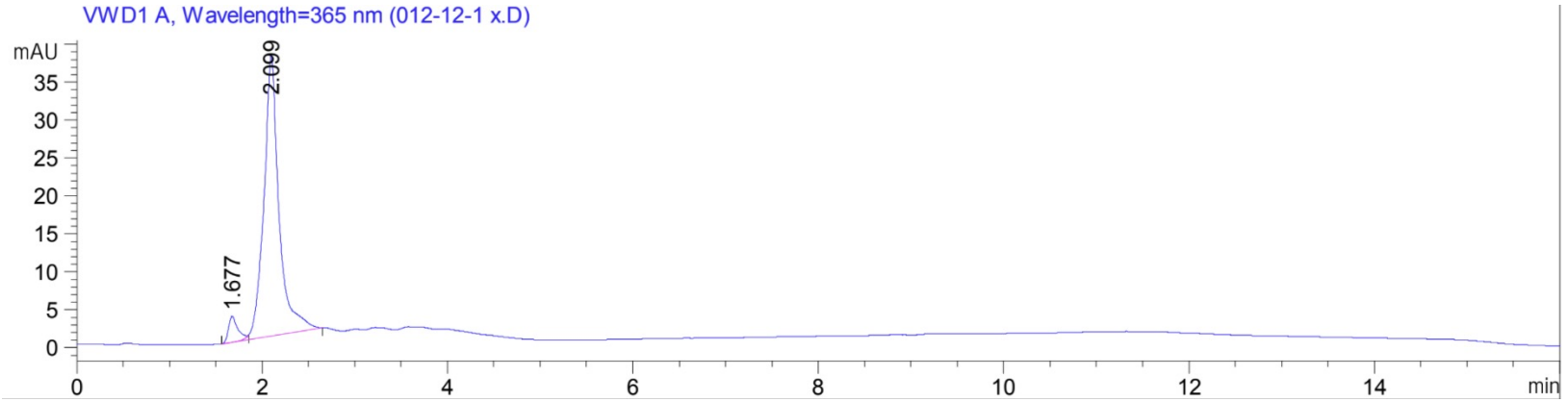
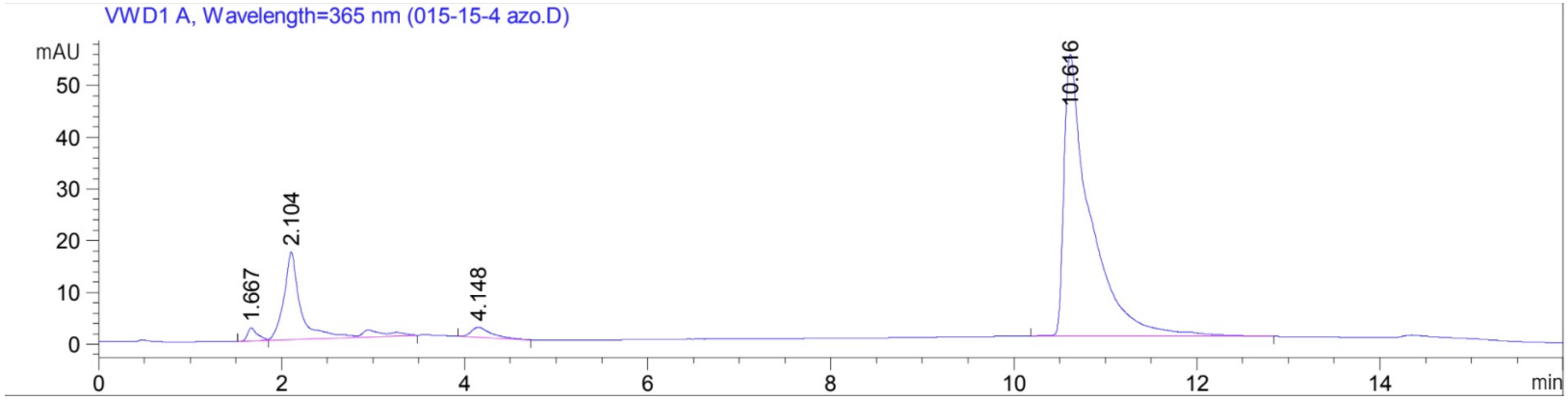

A.
Unstim.
Stim. (no light)
Stim. (UV light only)
Stim. (UV + blue light)
AzoSM (no light)
AzoSM (UV light only)
AzoSM (UV + blue light)
20 kDa
15 kDa
10 kDa
IP: FcRγ
IB: p-Tyr
15 kDa
10 kDa
IP: FcRγ
IB: FcRγ
20 kDa
15 kDa
10 kDa
lysates
IB: FcRγ
B.
Mean Intensity (p-Tyr/FcRγ)
2.0
1.5
1.0
0.5
0.0
**
*
****
**
*
*
Unstimulated
Stimulated (no light)
Stimulated (UV light only)
Stimulated (UV + blue light)
azo SM (no light)
azo SM (UV light only)
azo SM (UV + blue light)
- no light exposure
- UV light only
- UV + blue light

Figure S8

A.


VWD1 A, Wavelength=365 nm (012-12-1 x.D)
mAU
35
30
25
20
15
10
5
0
2.099
1.677
0
2
4
6
8
10
12
14
min

B.


VWD1 A, Wavelength=365 nm (015-15-4 azo.D)
mAU
50
40
30
20
10
0
10.616
2.104
1.667
4.148
0
2
4
6
8
10
12
14
min