SUPPLEMENTAL FIGURE LEGENDS

Macrophage hypophagia as a mechanism of innate immune exhaustion in mAb-induced cell clearance

Jonathan J. Pinney, Fátima Rivera-Escalera, Charles C. Chu, Hannah Whitehead, Karl VanDerMeid, Ashley M. Nelson, Michelle C. Barbeau, Clive S. Zent, and Michael R. Elliott

Supplemental Figure 1. ADCP and hypophagia in BMDM and hMDM.

(A) Representative images from live-cell imaging ADCP experiments showing macrophages labeled with cell-permeant fluorescent tracer dyes TAMRA SE (BMDM) and Cell Tracker Deep Red (hMDM). BMDM were co-cultured with thymocytes and αCD90.2. hMDM were co-cultured with CLL cells and ALM mAb. Internalized targets are visible in the macrophages as dye voids, which peak around 1 hr and are largely absent by 6 hr; scale bar, 25μm.

(B) Comparison of void- versus pH-sensitive dye-based methods to quantify ADCP. *Left y-axis:* Void detection was carried out by live-cell imaging as described in Figure 1A-E for BMDM co-cultured with thymocytes and αCD90.2. *Right y-axis:* Dye-based detection of phagocytosis was carried out using live-cell imaging of BMDM co-cultured with pHrodo Red STP-labeled thymocytes (a pH-sensitive dye that increases fluorescence upon internalization and acidification in the phagolysosomal pathway).

(C) Confirmation of void-based detection to analyze target cell clearance resulting from ADCP. Live-cell imaging and void analysis of ADCP was carried out using BMDM co-cultured with αCD90.2-opsonized thymocytes as in Figure 1A-E (*left y-axis*). Additionally, at the indicated time points, the free/unengulfed thymocytes were collected and counted by flow cytometry. Data on the *right y-axis* show the percentage of the initial input cells that remained in the co-culture wells at the indicated times.

(D) Live-cell imaging and phagocytosis quantification of BMDM that were initially challenged as indicated ("initial") for 2 hrs and subsequently re-challenged with αCD90.2-opsonized thymocytes.

(E) Representative images demonstrating capacity of hypophagic macrophages to bind mAb-opsonized targets. BMDM and hMDM were induced to undergo hypophagia as described in Figure 1E-H, for 2 hrs. After washing to remove free targets and mAb, macrophages were re-challenged with fresh targets and mAb for 2 hrs. Dye labeled images (above) indicate the lack of phagocytosis. Merging this image with phase contrast channel (below) shows the presence of multiple target cells in close apposition to these dye-labeled macrophages; scale bar, 25μm.

Supplemental Figure 2. Free IgG does not affect ADCP or hypophagia.

(A) Murine BMDM were pre-treated ±20 μg/mL non-specific IgG for 1hr in the presence of thymocytes. Following pre-treatment ADCP assay was performed by spiking in 10 μg/mL anti-CD90.2 and quantified by live-cell imaging as described in Figure 1A-E. n=3, mean ±SEM are shown.

(B) Quantification of ADCP experiments in (A) for n=3, mean ±SEM, unpaired two-tailed Student's t-test. No significant differences between groups were observed.

(C) Human MDM were pre-treated ±20 μg/mL non-specific IgG for 1hr in the presence of CLL cells. Following pre-treatment ADCP assay was performed by spiking in 10 μg/mL ALM and quantified by live-cell imaging as described in Figure 1A-E. n=3, mean ±SEM are shown.

(D) Quantification of ADCP experiments in (C) for n=3, mean ±SEM, unpaired two-tailed Student's t-test. No significant differences between groups were observed.

Supplemental Figure 3. ADCP in hCD2-iCre-tdTomato mice.

(A) Representative flow cytometry data from liver, spleen, and blood of untreated hCD2-iCre-tdTomato mice showing expression of tdTomato is largely restricted to lymphoid populations ($CD19^+$, $CD3^+$) rather than myeloid populations ($Ly6C^+$, $F4/80^+$). Pregating: single, live, $CD45^+$ cells; $tdTomato^+$ and $tdTomato^-$ KC are shown in red and blue, respectively.

(B) Quantification of $CD19^+$ B cell percentages (of total $CD45^+$ cells) in blood, spleen, and liver of hCD2-iCre-tdTomato mice at the indicated times following i.v. treatment with 25 μg αCD20. Data shown are n=3, ±SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, unpaired one-way ANOVA.

(C) Thymocytes from C57BL/6J mice were induced to undergo apoptosis by treatment with 10 μM dexamethasone for 4 hrs. Left, representative flow cytometry analysis of Annexin V staining of treated thymocytes. Right, percentage of apoptotic (Annexin V^+) thymocytes 4 hrs ± dexamethasone treatment. n=3.

Supplemental Figure 4. Mechanisms of ADCP hypophagia.

(A) BMDM were co-cultured with thymocytes ± αCD90.2 for 30 and 120 min and supernatants collected and applied to unfed BMDM prior to addition of fresh thymocytes and αCD90.2. ADCP was measured by live-cell imaging as described in Figure 1A-E.

(B) Flow cytometry analysis of FcγRIIa/b and FcγRIIIa surface levels on hMDM co-cultured with CLL cells in the absence (grey) or presence (color) of ALM mAb for 2 hrs. Unstained control shown as dashed line. Bar graphs to the right show quantification of relative MFI for each receptor for n=3, mean ±SEM, ****p<0.0001, unpaired two-tailed t-test.

Supplemental Figure 5. Relative surface expression of FcγRI and FcγRIIb 6hrs post-hypophagia induction.

(A) Representative histogram showing BMDM that were co-cultured with thymocytes ±anti-CD90.2 for 2 hrs to induce hypophagia, then targets and free antibody were washed away and BMDM were cultured in fresh medium for 6 hrs. Surface levels of FcγRI were then measured by flow cytometry. Unstained control shown as dashed line.

(B) MFI quantification for (A) are shown for n=5, mean ±SEM, *p<0.05, unpaired two-tailed t-test.

(C) Representative histogram showing BMDM treated as described in (A). Surface levels of FcγRIIb were then measured by flow cytometry. Unstained control shown as dashed line.

(D) MFI quantification for (C) are shown for n=5, mean ±SEM, *p<0.05, unpaired two-tailed t-test.

Supplemental Figure 6. Representative histograms for FcγRI and FcγRIIb intracellular flow cytometry staining.

(A) Representative histogram of intracellular staining and flow cytometry for surface levels of FcγRI as shown in Figure 4A. Unstained control shown as dashed line.

(B) Representative histogram of intracellular staining and flow cytometry for total (intracellular + surface) levels of FcγRI as shown in Figure 4A. Unstained control shown as dashed line.

(C) Representative histogram of intracellular staining and flow cytometry for surface levels of FcγRIIb as shown in Figure 4B. Unstained control shown as dashed line.

(D) Representative histogram of intracellular staining and flow cytometry for total (intracellular + surface) levels of FcγRIIb as shown in Figure 4B. Unstained control shown as dashed line.

Supplemental Figure 7. Blocking FcγR degradation does not affect hypophagia.

(A) BMDM were pretreated ± 100 nM Bafilomycin A1 for 3 hrs prior to addition of thymocytes and αCD90.2. ADCP was measured by live-cell imaging and the phagocytic index calculated as described in Figure 1A-E.

(B) Flow cytometry analysis of FcγRI surface levels on unfed and hypophagic (αCD90.2-opsonized thymocytes, 2 hrs) BMDM that were treated with 25 μM MG132 3 hrs prior to addition of fresh thymocytes and αCD90.2. n=3, ***p<0.001, unpaired one-way ANOVA.

(C) Flow cytometry analysis of FcγRIIb surface levels on unfed and hypophagic (αCD90.2-opsonized thymocytes, 2 hrs) BMDM that were treated with 25 μM MG132 3 hrs prior to addition of fresh thymocytes and αCD90.2. n=3, ****p<0.0001, unpaired one-way ANOVA.

Supplemental Figure 8. Comparison of unfed and hypophagic macrophage ADCP after 24 hr recovery in presence of recombination IFNγ or C5a.

(A) Murine BMDM were incubated with thymocytes ±anti-CD90.2 for 2hr in the presence of thymocytes. Free thymocytes and antibody were then washed away and BMDM were cultured 24 hrs ± recombinant IFNγ (50 ng/mL). After 24 hrs BMDM were re-challenged with fresh thymocytes and 10μg/mL αCD90.2 for another 2 hours and ADCP was measured by live cell imaging as in Figure 1E. Data shown are mean ±SEM, n=3.

(B) Murine BMDM were incubated with thymocytes ±anti-CD90.2 for 2hr in the presence of thymocytes. Free thymocytes and antibody were then washed away and BMDM were cultured 24 hrs ± recombinant C5a (50 ng/mL). After 24 hrs BMDM were re-challenged as in (A) and ADCP was measured by live cell imaging as in Figure 1E. Data shown are mean ±SEM, n=3.

(C) Quantification of ADCP experiments in A-B for n=3, mean ±SEM, **p<0.01, unpaired two-tailed Student's t-test.

SUPPLEMENTAL VIDEO LEGENDS

Macrophage hypophagia as a mechanism of innate immune exhaustion in mAb-induced cell clearance

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Supplemental Video 1.

Representative video showing ADCP by murine BMDM, labeled with Cell Tracker Deep Red, upon primary challenge (1st challenge) with αCD90.2 opsonized thymocytes. Cell Tracker Deep Red and Phase Contrast channels are shown together to illustrate interaction between macrophages and target cells. Scale bar, 25μM.

Supplemental Video 2.

Representative video showing ADCP by murine BMDM, labeled with Cell Tracker Deep Red, upon primary challenge (1st challenge) with αCD90.2 opsonized thymocytes. Cell Tracker Deep Red channel is shown alone to illustrate formation and processing of "dye voids." Scale bar, 25μM.

Supplemental Video 3.

Representative video showing ADCP by hypophagic murine BMDM, labeled with Cell Tracker Deep Red, upon secondary challenge (2nd challenge) with αCD90.2 opsonized thymocytes. Cell Tracker Deep Red and Phase Contrast channels are shown together to illustrate interaction between macrophages and target cells. Scale bar, 25μM.

Supplemental Video 4.

Representative video showing ADCP by hypophagic murine BMDM, labeled with Cell Tracker Deep Red, upon secondary challenge (2nd challenge) with αCD90.2 opsonized thymocytes. Cell Tracker Deep Red channel is shown alone to illustrate formation and processing of "dye voids," which is greatly reduced in hypophagic macrophages. Scale bar, 25μM.

Supplemental Video 5.

Representative video showing ADCP by human MDM, labeled with Cell Tracker Deep Red, upon primary challenge (1st challenge) with ALM opsonized CLL cells. Cell Tracker Deep Red and Phase Contrast channels are shown together to illustrate interaction between macrophages and target cells. Scale bar, 25μM.

Supplemental Video 6.

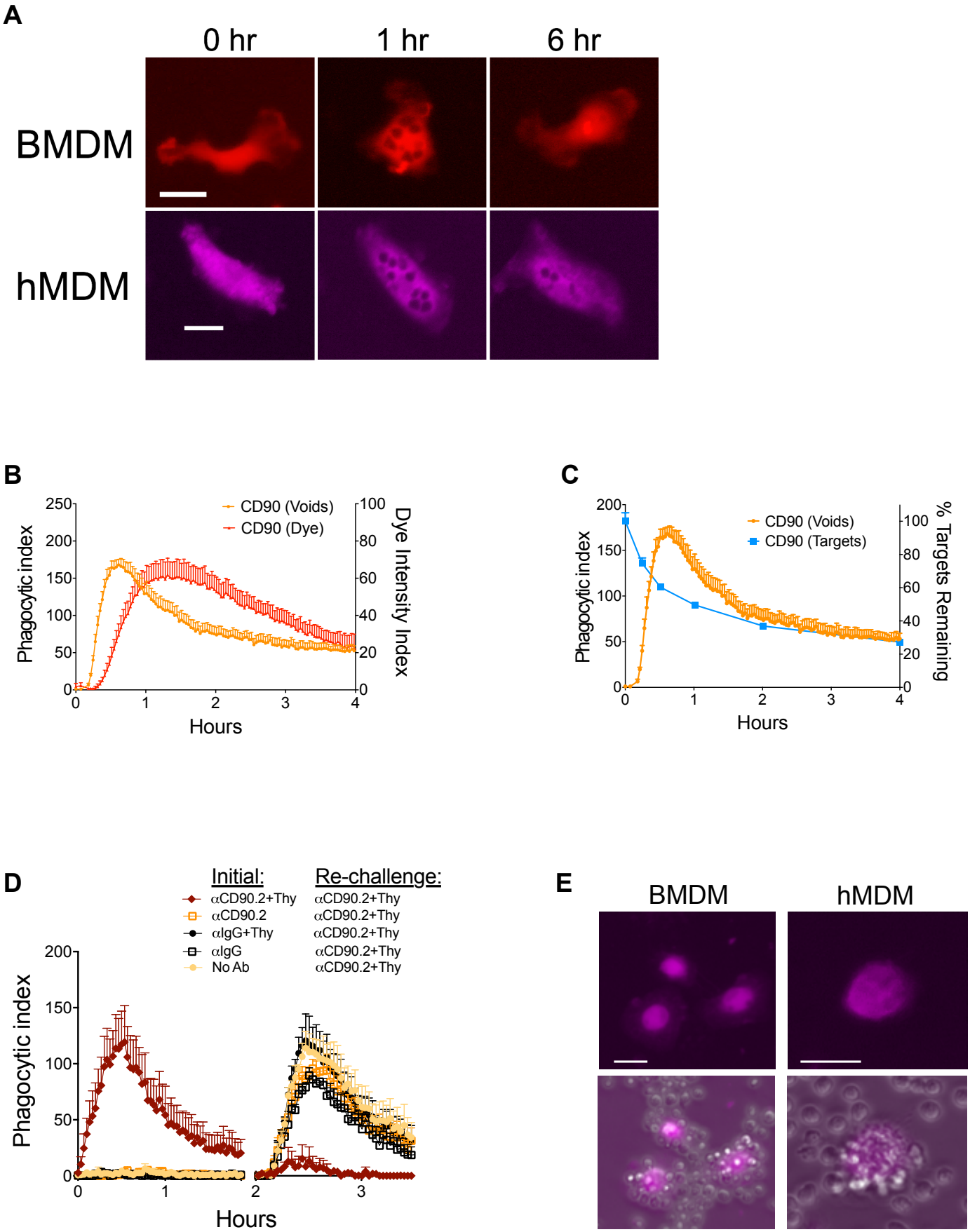
Representative video showing ADCP by human MDM, labeled with Cell Tracker Deep Red, upon primary challenge (1st challenge) with ALM opsonized CLL cells. Cell Tracker Deep Red channel is shown alone to illustrate formation and processing of "dye voids." Scale bar, 25μM.

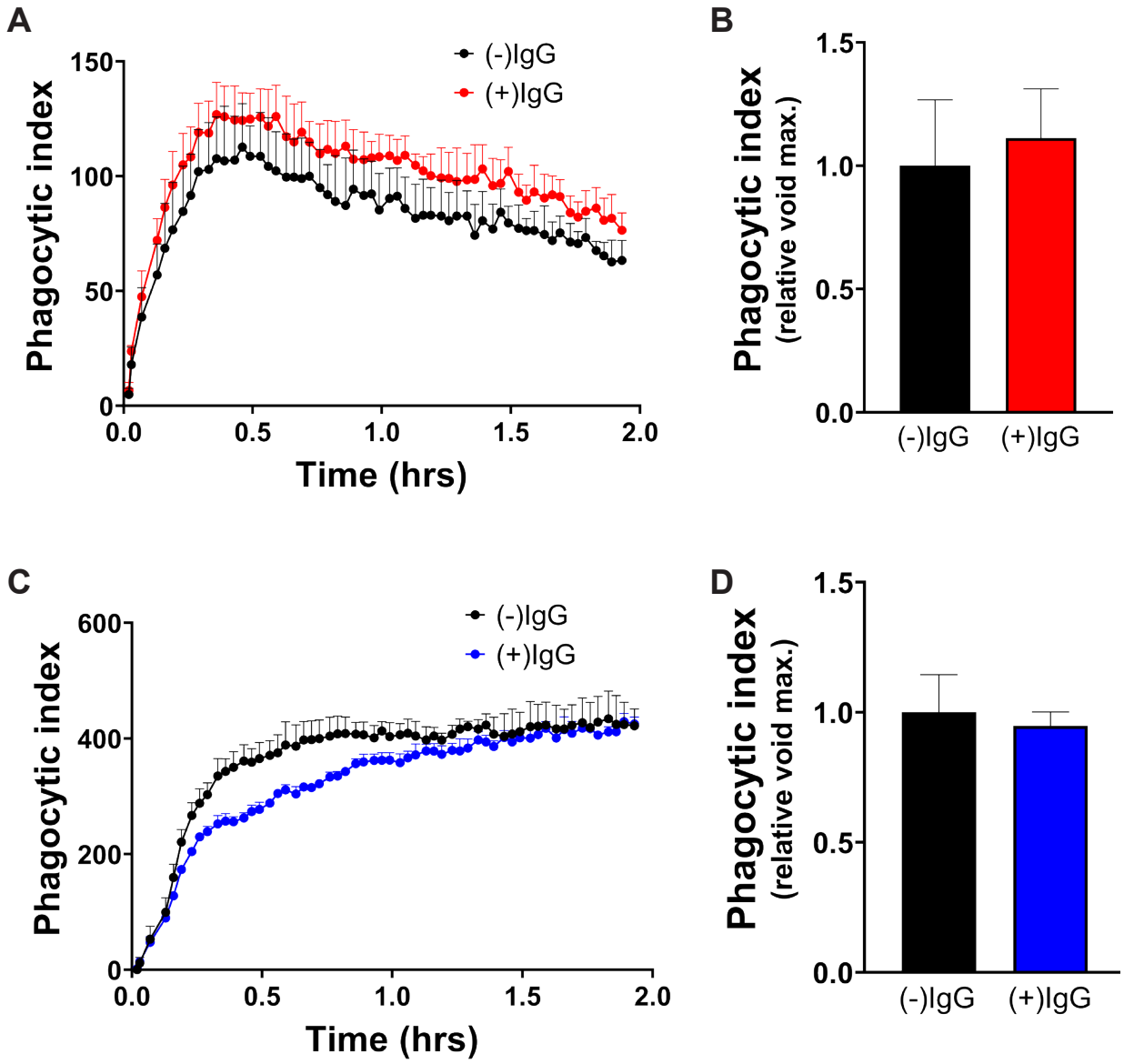
Supplemental Video 7.

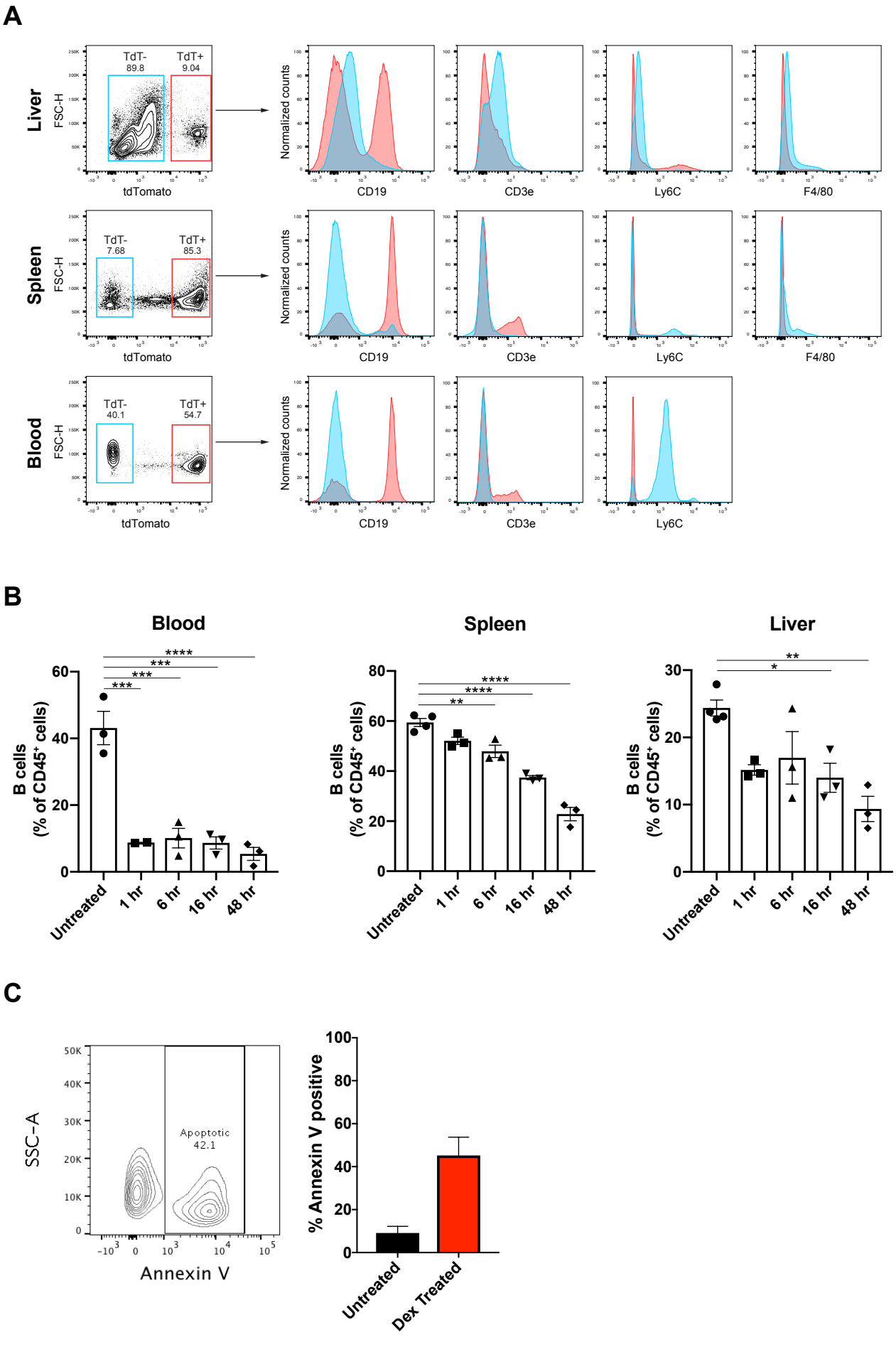
Representative video showing ADCP by hypophagic human MDM, labeled with Cell Tracker Deep Red, upon secondary challenge (2nd challenge) with ALM opsonized CLL cells. Cell Tracker Deep Red and Phase Contrast channels are shown together to illustrate interaction between macrophages and target cells. Scale bar, 25μM.

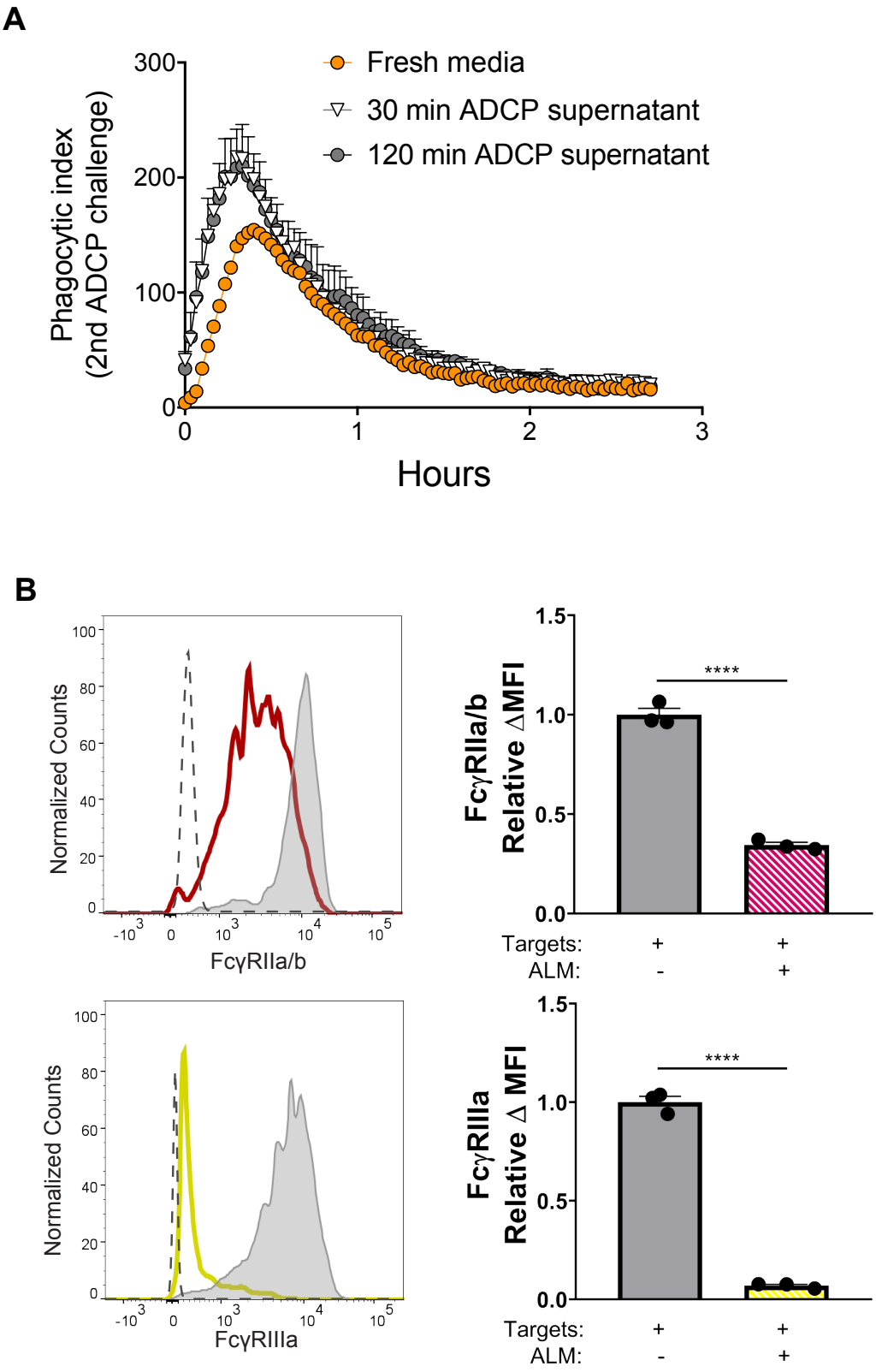
Supplemental Video 8.

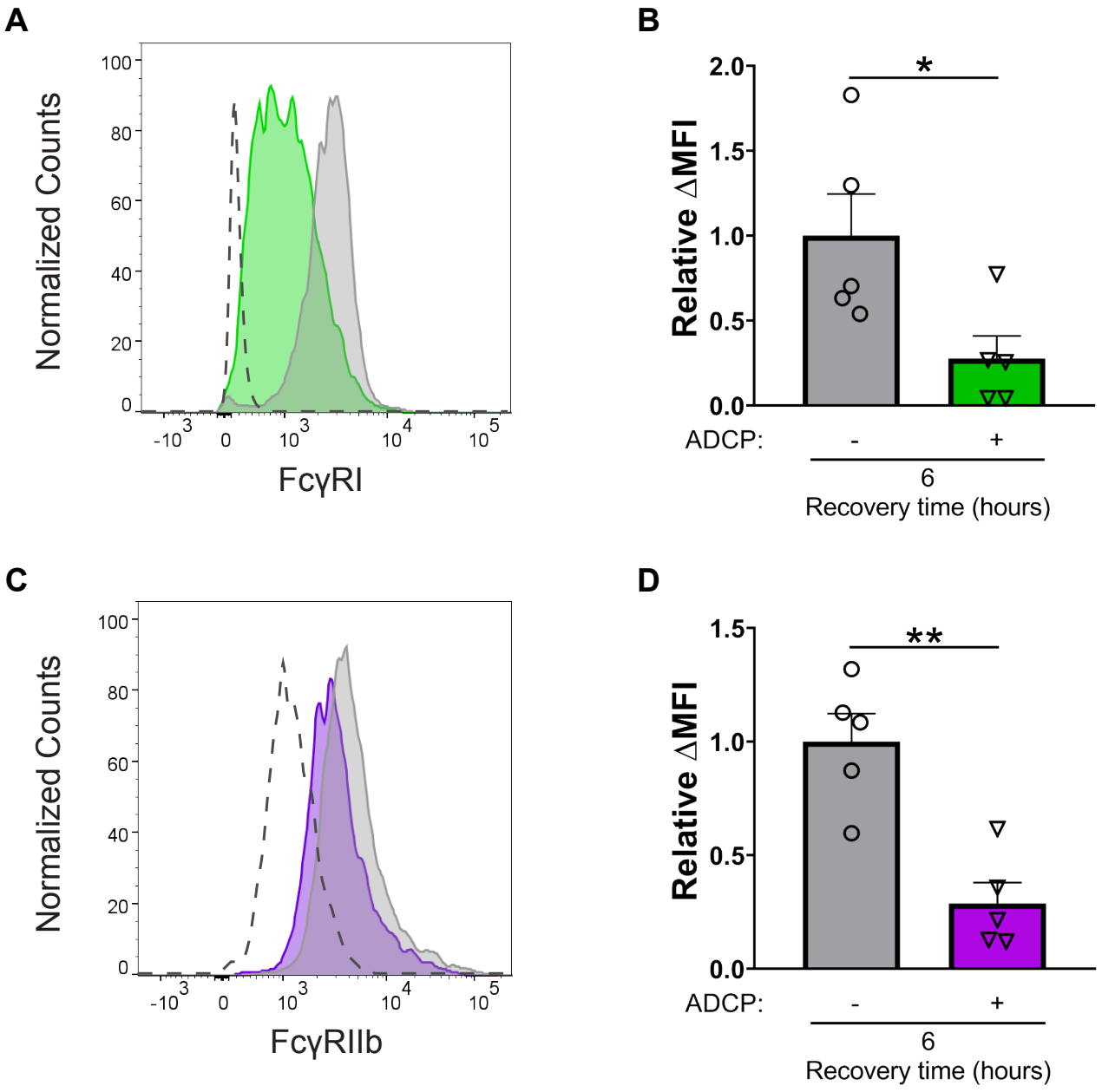
Representative video showing ADCP by hypophagic human MDM, labeled with Cell Tracker Deep Red, upon secondary challenge (2nd challenge) with ALM opsonized CLL cells. Cell Tracker Deep Red channel is shown alone to illustrate formation and processing of "dye voids," which is greatly reduced in hypophagic macrophages. Scale bar, 25μM.

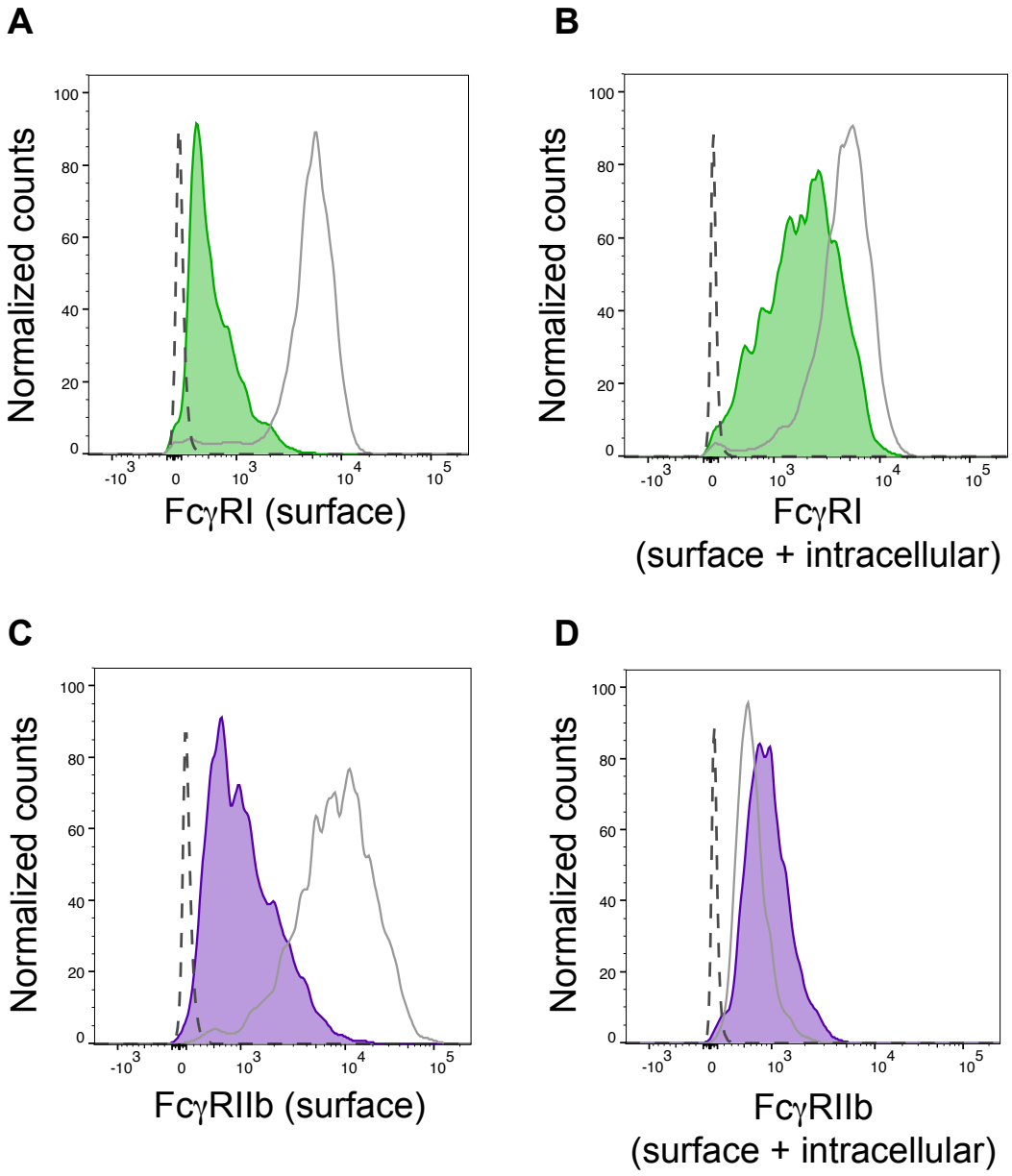

A
0 hr
1 hr
6 hr
BMDM
hMDM
B
CD90 (Voids)
CD90 (Dye)
Phagocytic index
Dye Intensity Index
Hours
C
CD90 (Voids)
CD90 (Targets)
Phagocytic index
% Targets Remaining
Hours
D
Initial:
Re-challenge:
αCD90.2+Thy
αCD90.2
αIgG+Thy
αIgG
No Ab
αCD90.2+Thy
Phagocytic index
Hours
E
BMDM
hMDM

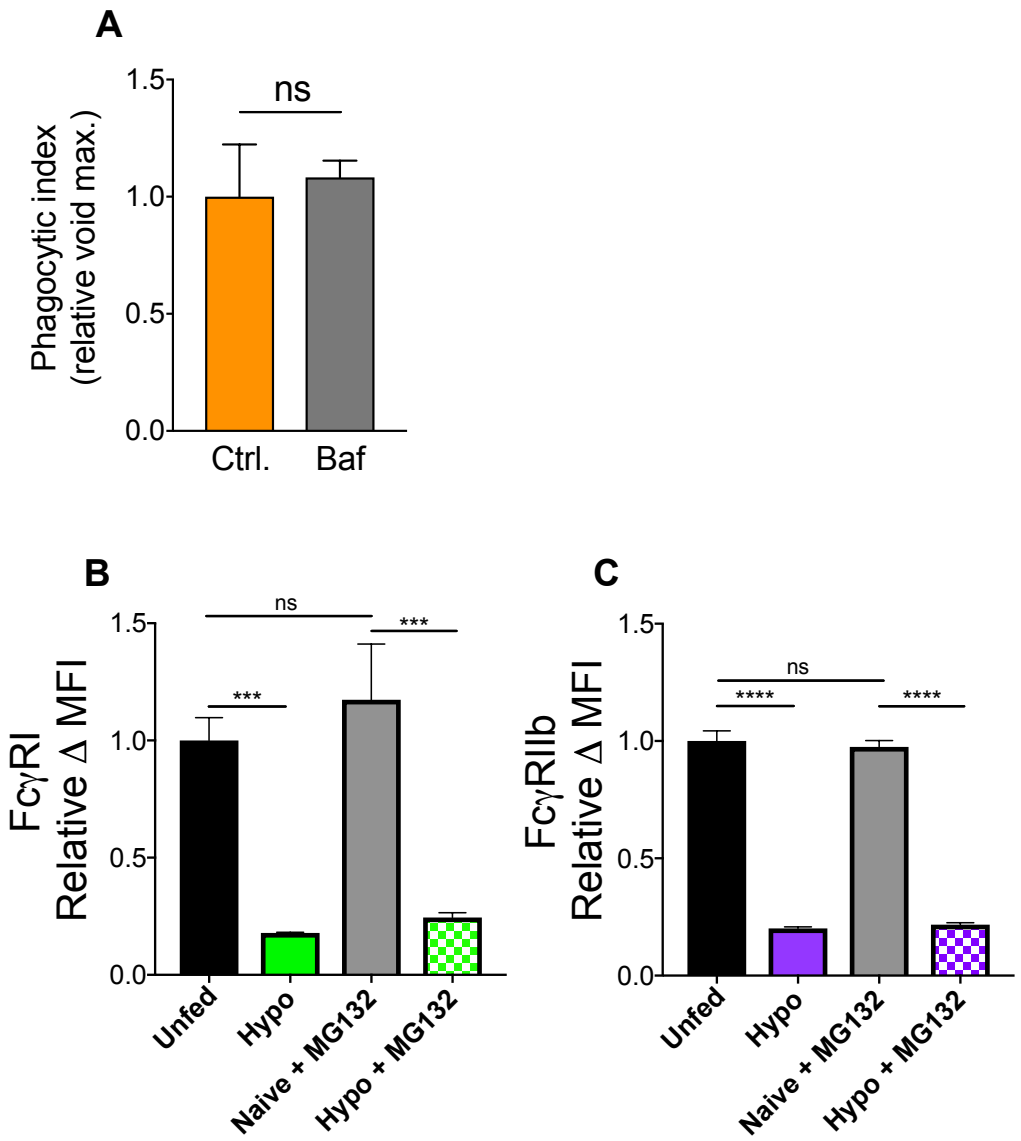

A
Phagocytic index
150
100
50
0
0.0
0.5
1.0
1.5
2.0
Time (hrs)
(-)IgG
(+)IgG
B
Phagocytic index
(relative void max.)
1.5
1.0
0.5
0.0
(-)IgG
(+)IgG
C
Phagocytic index
600
400
200
0
0.0
0.5
1.0
1.5
2.0
Time (hrs)
(-)IgG
(+)IgG
D
Phagocytic index
(relative void max.)
1.5
1.0
0.5
0.0
(-)IgG
(+)IgG

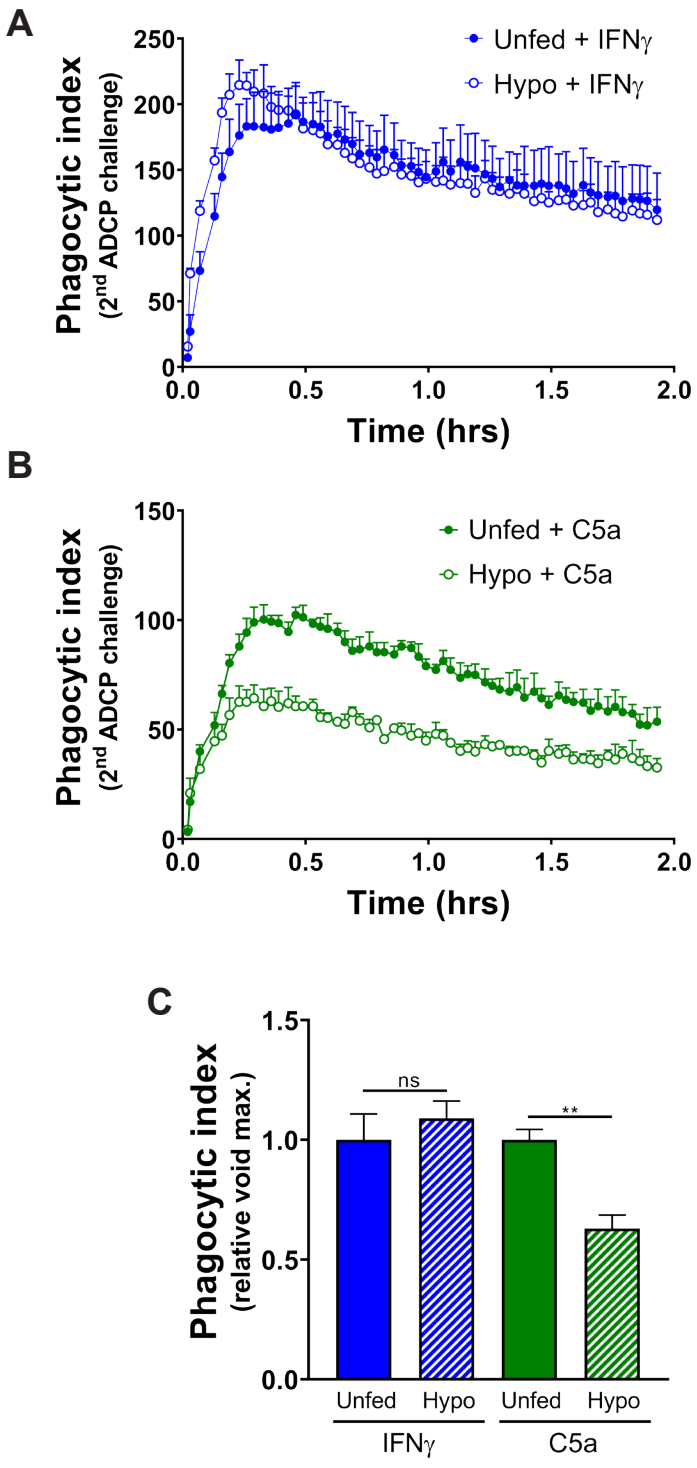

A
Liver
Spleen
Blood
FSC-H
tdTomato
TdT-
89.8
TdT+
9.04
TdT-
7.68
TdT+
85.3
TdT-
40.1
TdT+
54.7
Normalized counts
CD19
CD3e
Ly6C
F4/80
B
Blood
Spleen
Liver
B cells
(% of CD45+ cells)
Untreated
1 hr
6 hr
16 hr
48 hr
C
SSC-A
Annexin V
Apoptotic
42.1
% Annexin V positive
Untreated
Dex Treated


A
Phagocytic index
(2nd ADCP challenge)
300
200
100
0
0
1
2
3
Hours
Fresh media
30 min ADCP supernatant
120 min ADCP supernatant
B
Normalized Counts
FcγRIIa/b
FcγRIIIa
FcγRIIa/b
Relative ΔMFI
FcγRIIIa
Relative Δ MFI
****
Targets:
ALM:
+
-


A
Normalized Counts
FcγRI
B
Relative ΔMFI
ADCP:
Recovery time (hours)
C
Normalized Counts
FcγRIIb
D
Relative ΔMFI
ADCP:
Recovery time (hours)


A
B
C
D
Normalized counts
FcγRI (surface)
FcγRI
(surface + intracellular)
FcγRIIb (surface)
FcγRIIb
(surface + intracellular)


A
Phagocytic index
(relative void max.)
1.5
1.0
0.5
0.0
ns
Ctrl.
Baf
B
FcγRI
Relative Δ MFI
ns
***
***
Unfed
Hypo
Naive + MG132
Hypo + MG132
C
FcγRIIb
Relative Δ MFI
ns
****
****


A
Phagocytic index
(2nd ADCP challenge)
Unfed + IFNγ
Hypo + IFNγ
Time (hrs)
B
Phagocytic index
(2nd ADCP challenge)
Unfed + C5a
Hypo + C5a
Time (hrs)
C
Phagocytic index
(relative void max.)
ns
**
Unfed
Hypo
Unfed
Hypo
IFNγ
C5a