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# Supporting Information for

## Rac-mediated hyper-phagocytosis as a cause of immunodeficiency and enhanced CAR-M-mediated cancer cell killing

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**Other supporting materials for this manuscript include the following:**

Movies S1 to S9

**Supplemental Information**

**Materials and Methods**

*Drosophila genetics*

All fly strains in this study were grown on standard cornmeal-yeast medium (https://bdsc.indiana.edu/information/recipes/molassesfood.html). All experimental crosses were maintained at 25°C, 80% humidity and on a 12 hr light/dark cycle unless otherwise stated. Flies were fed dry or wet yeast at 25°C overnight prior to dissection for fixed and live imaging. For generating $Rac^{G12V}$ clones, *UAS- $Rac^{G12V}$* or *UAS-lacZ* lines were crossed with *hsp70-FLP; Acty17bGal4, UAS-moesinGFP* (1) and progeny were heat shocked twice a day for 30 min, about 4h apart, in a 37°C water bath. Flies were then kept at 25°C overnight (on dry/wet yeast) prior to dissection. The following fly lines from Bloomington Drosophila Stock Center were used: *UAS-$Rac1^{G12V}$ (6291), UAS- $Rac1^{T17N}$ (6292), UAS- $Rac1^{WT}$ (28874), UAS-PLCδPHGFP (39693),* and *UAS-lacZ (3956).* The *$drpr^{\Delta 5}$* mutant fly line was kindly provided by Dr. Marc R. Freeman (The Vollum Institute, Oregon Health & Science University, Portland, OR, USA). The stock *slbo-Gal4/CyO; slbo-4XPHEGFP, UMAT-Lyn-tdTomato, ubi-HisRFP* (Montell D.J, stock) was used for live imaging.

*Cell line maintenance*

HL-60 cells used in this study were obtained from Orion Weiner's lab at UCSF. Cells were maintained at 37 °C with 5% $CO_2$ in RPMI 1640 medium (Gibco, # 22400089) with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich #F4135), 1 µg/mL puromycin (Gibco #A1113803). Jurkat cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and 200µg/mL Hygromycin B (ThermoFisher #10687010). HEK-293T cells were grown in Dulbecco's Modified Eagle Medium, high glucose, GlutaMAX™ Supplement, pyruvate (Gibco #10-569-044) with 10% heat-inactivated fetal bovine serum.

*Mouse husbandry*

Mice were maintained in sterile living conditions at the Animal Resource Center, an AAALAC-accredited animal facility, at The University of California, Santa Barbara (UCSB). $Rac2^{+/E62K}$ mice were kindly provided by Amy Hsu (National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health) and backcrossed to C57BL/6 on site. Procedures were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and approved protocols. Ear punch biopsies were used for genotyping by Transetyx, an automated genotyping service. 8-12-week-old female and male mice were used for all experiments.

*Immunostaining of Drosophila samples*

Immunostaining was performed as described previously(2). The antibodies used in the study were Rabbit anti-GFP (G10362, ThermoFisher, 1:1000), E-cad (DCAD2, Developmental Studies Hybridoma Bank, 1:5), Fas3 (7G10, Developmental Studies Hybridoma Bank, 1:10), AlexaFluor 488 and 568 secondary antibodies (ThermoFisher, 1:400) and Phalloidin-Atto 647N [F-actin] (Sigma-Aldrich). Images were acquired on Zeiss LSM780 confocal microscope.

*Lentiviral infection HL60 and Jurkat cells*

$Rac2^{WT}$ and $Rac2^{E62K}$ constructs were obtained from Amy Hsu, NIAID (3). $Rac2^{WT}$-LckGFP or $Rac2^{E62K}$-Lck-GFP was cloned into the pCW57 vector (Addgene, #71782). The construct carrying Lck-GFP sequence without dominant activating Rac2 mutations was used as a control. $Rac2^{G12R}$ was generated from $Rac2^{WT}$ by using Q5 site-directed mutagenesis kit (New England BioLabs)

according to the manufacturer's protocol. pCMV-dR8.2 dvpr (Addgene #8455) and pCMV-VSV-G (Addgene, #8454) were used for lentiviral infection of HL60 cells with control (Lck-GFP only) or $Rac2^{WT}$-Lck-GFP, $Rac2^{G12R}$-Lck-GFP and $Rac2^{E62K}$-Lck-GFP plasmids. HEK293T cells were used for the lentivirus production. The DNA of pCMV-dR8.2 dvpr, pCMV-VSV-G and pCW57-Lck-GFP or pCW57-$Rac2^{E62K}$-Lck-GFP (and other Rac2 mutation constructs) was mixed in 1:1:2 ratio and incubated at RT for 15mins with X-tremeGENE HP DNA transfection reagent (Millipore-Sigma) and Opti-MEM (Gibco, #31985070). This mix was added to the HEK-293T cells. Supernatant containing lentivirus was collected 48h after transfection, filtered using a 0.45µm filter syringe assembly and added directly onto HL60 cells cultured in six-well plates. To enhance the efficiency of lentiviral infection 2 µg/mL Polybrene (Santa Cruz Biotechnology, #sc-134220) was added with the virus. After virus addition, plates were spun at 1500 rpm for 15 min before returning to the incubator. Next day, cells were grown in 1 µg/mL puromycin containing RPMI 1640 medium with 10% heat-inactivated fetal bovine serum. GFP expression was used to confirm the lentiviral infection.
EGFP was replaced with mCherry sequence in the pLenti CMV GFP Hygro (656-4) vector (Addgene, #17446) and lentiviral infection was performed to generate mCherry-expressing Jurkat T cells.

*HL60 in vitro differentiation into macrophage-like cells and engulfment assay*

HL60-LckGFP, HL60-$Rac2^{E62K}$-LckGFP, HL60-$Rac2^{WT}$-LckGFP, or HL60-$Rac2^{G12R}$-LckGFP cells were seeded at a density of $2\times10^5$ cells in HL60 media (as described above) on a coverslip in a 6-well tissue culture treated plate. HL60 cells were differentiated into macrophage-like cells using a protocol modified from Rovera et al., 1979(4). Macrophage differentiation was assessed via flow cytometry using CD11b antibody (BioLegend, #301305). 32nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was added to induce differentiation and 1µg/mL doxycycline was added to induce expression of control (LckGFP), $Rac2^{E62K}$-LckGFP, $Rac2^{WT}$-LckGFP, or $Rac2^{G12R}$-LckGFP. LckGFP was included to label cell membranes. After 48h of differentiation the media was removed and $4\times10^5$ cells mCherry-expressing Jurkat T cells in fresh media were added to the macrophages. After 24h the media was removed and the coverslips were fixed and stained.

*Immunostaining of HL60 cells*

After culturing cells on coverslips, coverslips were removed from the 6-well plate and fixed with 4% paraformaldehyde. Cells were then incubated in BlockAid Blocking Solution (ThermoFisher, #B10710) for 30 min. Primary and secondary antibodies were diluted in blocking solution. Cells were incubated overnight at 4°C with chicken anti-GFP antibody (1:1000, abcam, #ab13970 for phagocytosis assays) or rabbit DCL-1 polyclonal antibody (1:400, Fisher # PIPA599615 for CD302 expression). Next day, the cells on the coverslips were washed 3x with 1X-PBST, incubated with blocking solution for 30 min at RT followed by incubation with AlexaFluor 488 or 568 conjugated secondary antibodies (ThermoFisher,1:400) for 90 min at RT. Coverslips were washed 3x with 1X-PBST and samples were mounted in DAPI-Fluromount-G (Electron Microscopy Sciences, #1798424). Images were acquired on Zeiss LSM780 confocal microscope.

*Quantification of phagocytosis and CD302 expression*

1 µm thick z-sections of the entire sample were captured and HL60 macrophages that engulfed a target were determined by the mCherry (Jurkat) signal inside the GFP-expressing HL60 macrophage. Multiple mCherry+ signals inside a single GFP+ HL60 macrophage were counted as one engulfment event.

For quantification of CD302 expression, sum slices projection method was used to apply a z-projection to a stack of 1 µm thick z-sections. Mean CD302 intensity was calculated from the sum-slices in the ImageJ and normalized to the average of either the control or $Rac2^{E62K}$ for all

the images acquired in an experiment. All the data points collected from three independent experiments were plotted.

*Generation of 3D surface reconstructions*

First, gaussian smoothing was applied to all channels then the surface function was used to segment the individual channels in Imaris (v10).

*Rac-GTP measurements*

G-LISA Rac 1,2,3 Activation Assay Biochem Kit (Cytoskeleton Inc, #BK125) was used to assess the GTP bound Rac in cell lysates. Assays were performed per the manufacturer's instructions.

*Preparation and culture of bone marrow-derived macrophages*

Femurs and tibias were harvested from 8-12-week-old mice and crushed to isolate whole bone marrow (WBM) cells. WBM cells were filtered through 40µm nylon cell strainers and incubated with 1X RBC Lysis solution (Thermo, #50-112-9751) to eliminate red blood cells. Cells were plated with L-929 conditioned media and differentiated for seven days to produce bone marrow-derived macrophages (BMDMs). BMDM differentiation was confirmed by flow cytometry analysis of F4/80 and Mac-1 staining on day seven.

*L929-conditioned media (BMDM media)*

To generate L929-conditioned media, L929 cells were cultured until confluent then passaged and grown for 10 days. On day 10, the media containing M-CSF was collected and filtered through a 40µm nylon cell strainer and aliquots were stored in -20$^{o}$C. BMDMs were subsequently grown in BMDM media: RPMI Glutamax (Gibco, #72400047), 20% MCSF L929-conditioned media, 10% heat-inactivated FBS and 1% Pen-strep.

*Lymphocyte isolation*

Spleens were harvested from 8-12-week-old mice and mashed to generate a single cell suspension. Splenic cells were filtered through 40µm nylon cell strainers and incubated with 1X RBC Lysis solution (Thermo, #50-112-9751) to eliminate red blood cells. T cell lymphocytes were isolated using EasyEights™ EasySep™ Magnet (Stemcell Technologies, #18103) and the EasySep™ Mouse T Cell Isolation Kit (Stemcell Technologies, #19851).

*Phagocytosis assay*

75,000 BMDMs were stimulated with IFN-γ (Biolegend, #50-170-393) for 4hrs on day seven and plated in BMDM media in a 96-well glass bottom plate (Cellvis, #P96-1.5H-N) overnight. On day eight, BMDM media was changed to serum-free media prior to the experiment. After isolation, T cells were stained with CellTrace Far Red (Thermo, #C34564) and pHrodo Red SE (Invitrogen #P36600). 375,000 T cells were added to the wells and co-cultured with the BMDMs overnight. Next day, samples were fixed with 4% PFA and permeabilized with PBS-T (0.1% Triton-X). BMDMs were stained with anti-F4/80 (abcam, #ab6640) and IgG (H+L) Cross-Adsorbed Goat anti-Rat, Alexa Fluor® 488 (Thermo, #A-11008). DAPI was used to visualize DNA. Engulfment was quantified as a CellTrace Far Red or pHrodo signal within a (green) macrophage. Data was acquired on the Leica DMi8 Epifluorescence scope and processed on ImageJ/Fiji for quantification.

*T cell activation assay*

After lymphocyte isolation, T cells were incubated with anti-CD3 (Thermo, #16-0032-82) [1 µg/mL] and anti-CD28 (Thermo, #16-0281-85) [5 µg/mL] in a 6-well tissue culture treated plate for three days prior to the engulfment assay. T cells were harvested, washed then stained with CellTrace Far Red (Thermo, #C34564) and pHrodo Red SE (Thermo, #P36600). Co-cultures were set up as described above in "Phagocytosis assay."

*Mixed phagocytosis assay*

Prior to plating $Rac2^{+/+}$ BMDMs were stained with CellTrace Far Red (Thermo, #C34564) and $Rac2^{+/E62K}$ were stained with CellTrace CFSE (Thermo, #C34554). On day seven BMDMs were stimulated with IFN-γ and plated overnight in a 96-well glass bottom plate. In control wells 75,000 BMDMs were seeded and in the mixed wells 37,500 BMDMs from each genotype were seeded. On day eight, BMDM media was changed to serum-free media prior to the experiment. After isolation, $Rac2^{+/+}$ T cells were stained with CellTrace Yellow (Thermo, #C34567) and pHrodo Red SE (Invitrogen, #P36600). 375,000 T cells were added to the wells then co-cultured with the BMDMs overnight. Next day, samples were fixed with 4% PFA and permeabilized with 0.1% Triton-X. DAPI was used to visualize DNA. Engulfment was quantified as a CellTrace yellow or pHrodo signal within a macrophage. Data was acquired on the Leica SP8 Resonant Scanning Confocal and processed on ImageJ Fiji for quantification.

*BMDM phagocytosis assay time lapse imaging*

Prior to plating, BMDMs were stained with CellTrace CFSE then co-culture assays were set up as described above ("Phagocytosis assay"). 375,000 T cells were added immediately prior to imaging in an Okolabs stage top incubator. Images were collected on a spinning disk confocal microscope (Nikon inverted Ti2-E with a Yokagawa CSU-W1 spinning disk unit and an Orca Fusion BT scMos camera) using a 40 x 0.95 NA Plan Apo air objective. Images were collected every 3 minutes for 10 hours.

*Quantification of macrophage pHrodo Intensity*

Time lapse movies were processed using the Fiji/Image J software. pHrodo intensity was measured at the end of the movies. The composite image was split into the different channels (488, 568, 647) then a threshold was applied to the pHrodo Red SE channel to measure only the pHrodo signal within the macrophages. Values were normalized to the number of macrophages in that field of view. Integrated density (a.u.) was used as the readout for pHrodo intensity.

*Quantification of T cell targets remaining*

Time lapse movies were processed using the Fiji/Image J software. The number of T cells remaining was counted at the end of the movies using the Fiji cell counter plugin. The composite image was split into the different channels (488, 568, 647) and quantifications were done only on the CellTrace Far Red (647) channel.

*Generating CAR-M/P BMDMs*

Bone marrow was harvested from $Rac2^{+/+}$ or $Rac2^{+/E62K}$ C57BL/6 mice. BMDMs were generated by incubating bone marrow with MCSF for 7-10 days as previously described (5). Bone marrow cells were infected with lentivirus encoding the CAR-P-GFP or GFP-CAAX on day 0 of the differentiation. The protocol was confirmed to produce macrophages with >95% purity as measured by CD11b (Biolegend, #101235) and F4/80 (Biolegend, #123117) staining.

*Thymus viability assay*

Thymus were harvested from $Rac2^{+/+}$ or $Rac2^{+/E62K}$ C57BL/6 mice then thymocytes were stained with PE-anti mouse CD3 antibody [1:200] (Biolegend, #100205) and Annexin V-APC (Biolegend, #640919) as per manufacturer's instructions. Flow cytometry was used to analyze samples and assess viability immediately after staining. For live/dead control, thymocytes were heat shocked for three minutes then placed on ice for three minutes and mixed back in with the live cells prior to staining with Annexin V.

*Phagocytosis flow cytometry*

75,000 macrophages infected with either GFP-CAAX or CAR-P-GFP were plated in a well of a 12 well dish. 16 hours later, 375,000 Raji B-cells dyed with CellTrace Violet (Thermo, #C34557) were added to the well. After three hours of co-incubation, the cells were analyzed on an Attune flow cytometer. Infected macrophages were identified by GFP signal, and the amount of internalized Raji was measured by CellTrace Violet signal.

*Whole cell phagocytosis time lapse*

30,000 macrophages were plated in a well of a 96-well glass bottom MatriPlate (Brooks, #MGB096-1-2-LG-L) the day before the experiment. 60,000 Raji B cells expressing mCherry-CAAX were added immediately prior to imaging in an Okolabs stage top incubator. Images were collected on a spinning disk confocal microscope (Nikon inverted Ti2-E with a Yokagawa CSU-W1 spinning disk unit and an Orca Fusion BT scMos camera) using a 40 × 0.95 NA Plan Apo air objective. The microscope was controlled by Nikon Elements.

*RNA-seq sample preparation*

HL60 cells were differentiated into macrophages as described above and $1\times10^6$ cells were freshly frozen and shipped to Genewiz (Azenta Life Sciences) to perform RNA-seq analysis. BMDMs were differentiated as described previously ("Preparation and culture of bone marrow-derived macrophages"). On day 7 fresh stimulation media was added: for M1 activation, BMDM media with 100 ng/ml LPS with 50 ng/ml IFN-γ; for M2 activation, BMDM media with 20 ng/ml IL-4 was used. Macrophage differentiation was confirmed via flow cytometry analysis using antibodies for Mac-1 and F4/80. The next day, stimulated BMDMs were collected by detaching them using ice cold mouse buffer + 5mM EDTA. $1\times10^6$ cells were freshly frozen and shipped to Genewiz (Azenta Life Sciences) to perform RNA-seq analysis.

*RNA-seq data analysis*

Subio Platform was used to process the RNA-Seq FASTQ files. The fastp, HISAT2, Stringtie were installed within the Subio platform and used to trim adapters and filter low-quality reads, align reads on the reference genome, assemble alignments and to evaluate the gene expression levels. After normalization and pre-processing, extraction of differentially expressed genes and enrichment analysis was performed using the Advanced Plug-in within the Subio platform (https://www.subioplatform.com/products/subioplatform/). GO enrichment analysis was performed on differentially expressed upregulated genes with (FC>1.5) or (FC>3) above the significant p-value (p-value <0.05). Pathway enrichment analysis was performed in Enrichr (Ma'ayan laboratory) on upregulated genes with (FC>3).

*CAR-M constructs*

The following design was used for the CAR construct (Morrissey et al. 2018). **Signal peptide:** aa 1–21 CD8 (Uniprot Q96QR6_HUMAN) **Extracellular antibody sequence:** V-L chain: aa 23–130, anti-CD19 CAR (Genbank AMZ04819) -- GS linker: ggtggcggtggctcgggcggtggtgggtcgggtggcggcggatct -- V-H chain: aa 148–267 anti-CD19 CAR (Genbank AMZ04819), **Stalk/Transmembrane:** aa 138–206 CD8 (Uniprot

Q96QR6_HUMAN) **Cytosolic sequence:** aa 19–86 Mouse Fc ERG precursor (Uniprot P20491 (FCERG_MOUSE)) **Fluorophore:** mGFP.
The GFP-caax construct contains an eGFP fused to a C-terminal CAAX targeting sequence: aaaatgtccaaggatggtaagaaaaagaagaagaagtcaaaaaccaagtgtgttatcatg.

*Quantification and statistical analysis*

All quantification methods are detailed in the corresponding method sections and figure legends. Statistical analyses of data were performed using GraphPad Prism, except for the RNAseq analysis (performed in Subio Platform) for which details are provided in the respective methods sections. Statistical parameters (p-values, and statistical tests performed) for all analyses are also reported in their corresponding figure legends.

**Supplementary Figure 1**

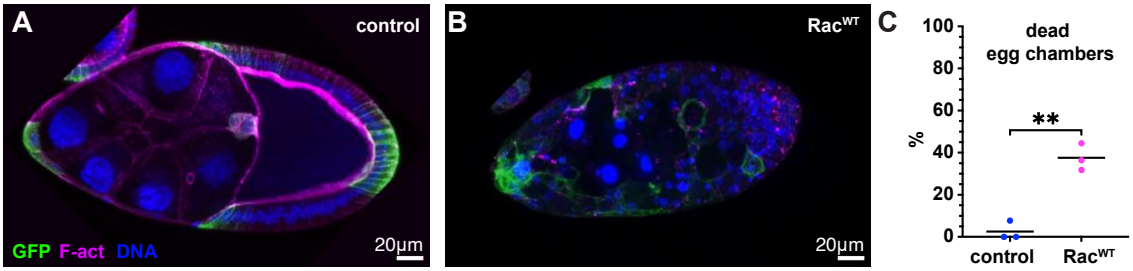


**Fig. S1.** (A) Normal egg chamber morphology in a *slbo-Gal4-UAS- PLCδ1-PH-GFP; UAS-lacZ* fly reared at 25°C. (B) Dead egg chamber from a *slbo-Gal4-UAS- PLCδ1-PH-GFP; UAS- Rac$^{WT}$* fly reared at 25°C. All images oriented anterior on the left. Scale bars are 20 µm. (C) Quantification of egg chamber death. Each dot represents an independent experiment. Data were analyzed using an unpaired t-test. ** indicates p<0.005.

**Supplementary Figure 2**

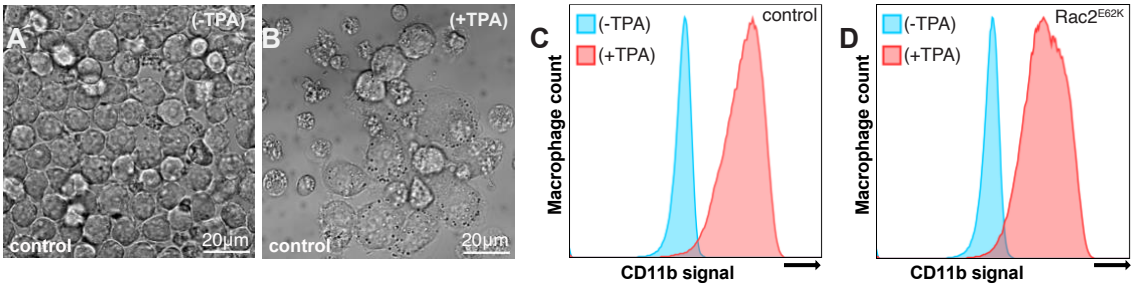


**Fig. S2.** (A) DIC image of control untreated HL60 cells. (B) Control HL60 cells treated with TPA. (C) The histogram shows representative flow cytometry data of CD11b signal of control untreated and TPA treated HL60 cells. (D) The histogram shows representative flow cytometry data of CD11b signal of untreated and TPA treated Rac2$^{E62K}$-expressing HL60 cells. Scale bar denotes 20 µm.

## Supplementary Figure 3

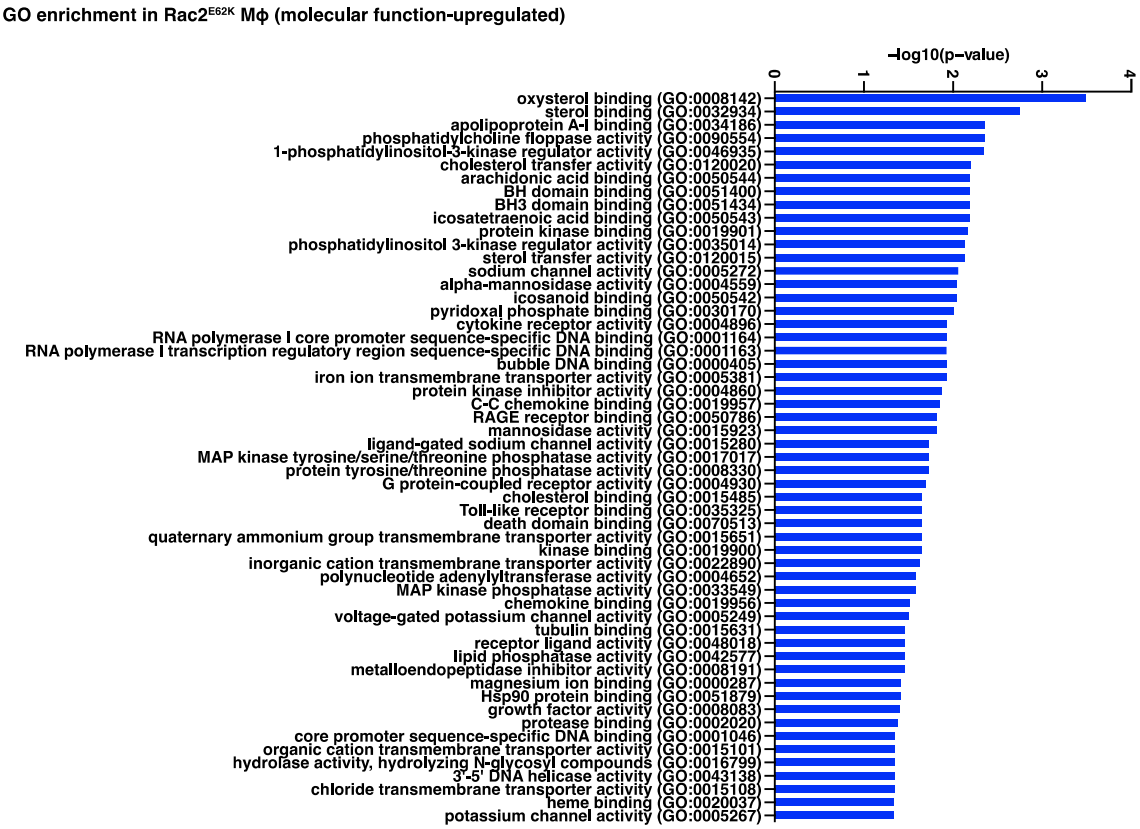


**Fig. S3.** Gene ontology analysis was performed on DEGs in HL60 macrophages expressing Rac2$^{E62K}$ versus control in Enrichr (Ma'ayan laboratory) on upregulated genes with (FC>3) and the GO Molecular Function 2021 output data is plotted.

## Supplementary Figure 4

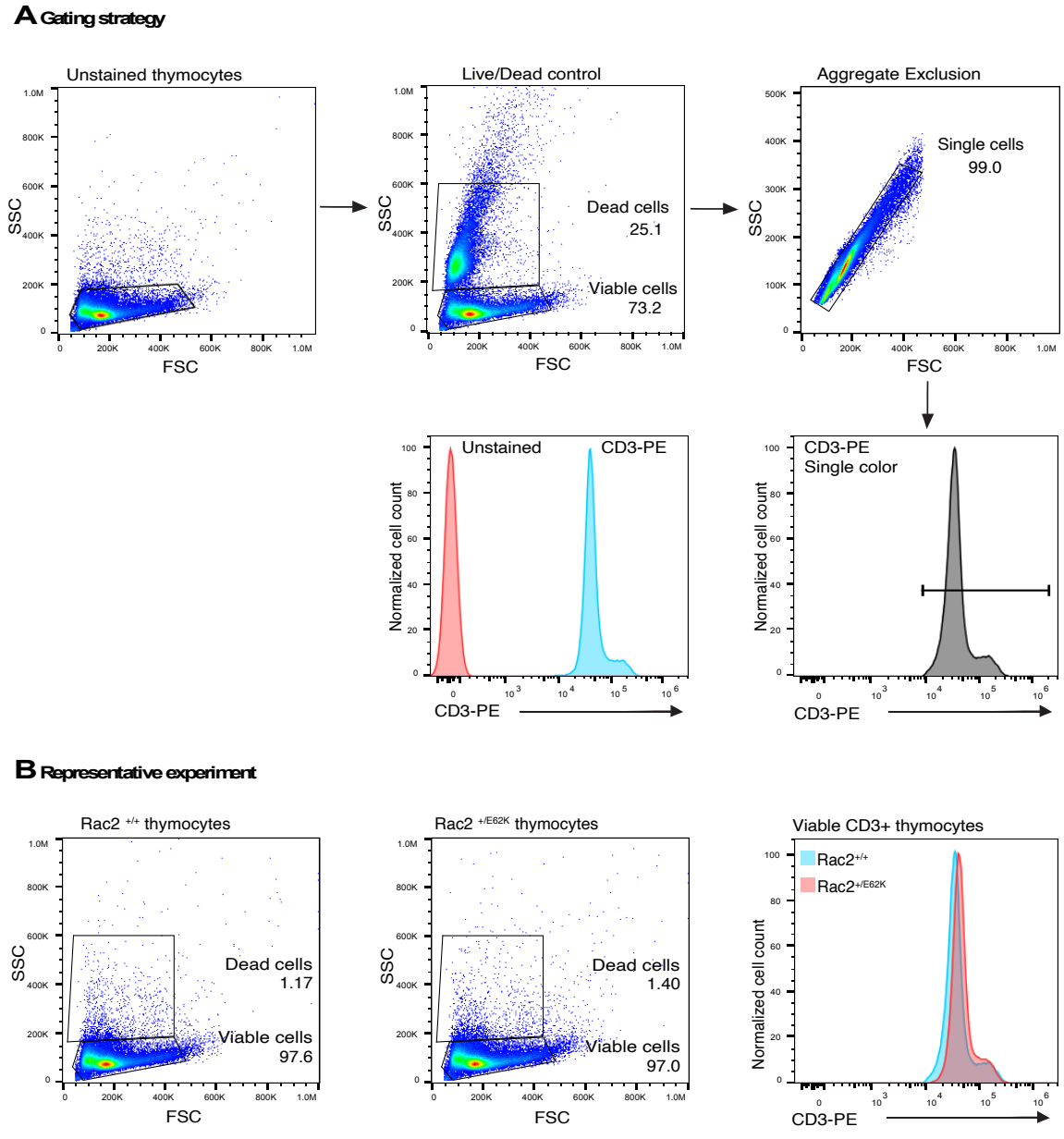


**Fig. S4.** Thymic CD3+ T cell viability in mice was assessed using flow cytometry. A) This gating strategy was used to determine the CD3-PE+ and live/dead populations from thymocytes of $Rac2^{+/+}$ and $Rac2^{+/E62K}$ mice. B) A representative experiment comparing $Rac2^{+/+}$ and $Rac2^{+/E62K}$ thymic CD3+ T cell viability. n=2 biological replicates.

## Supplementary Figure 5

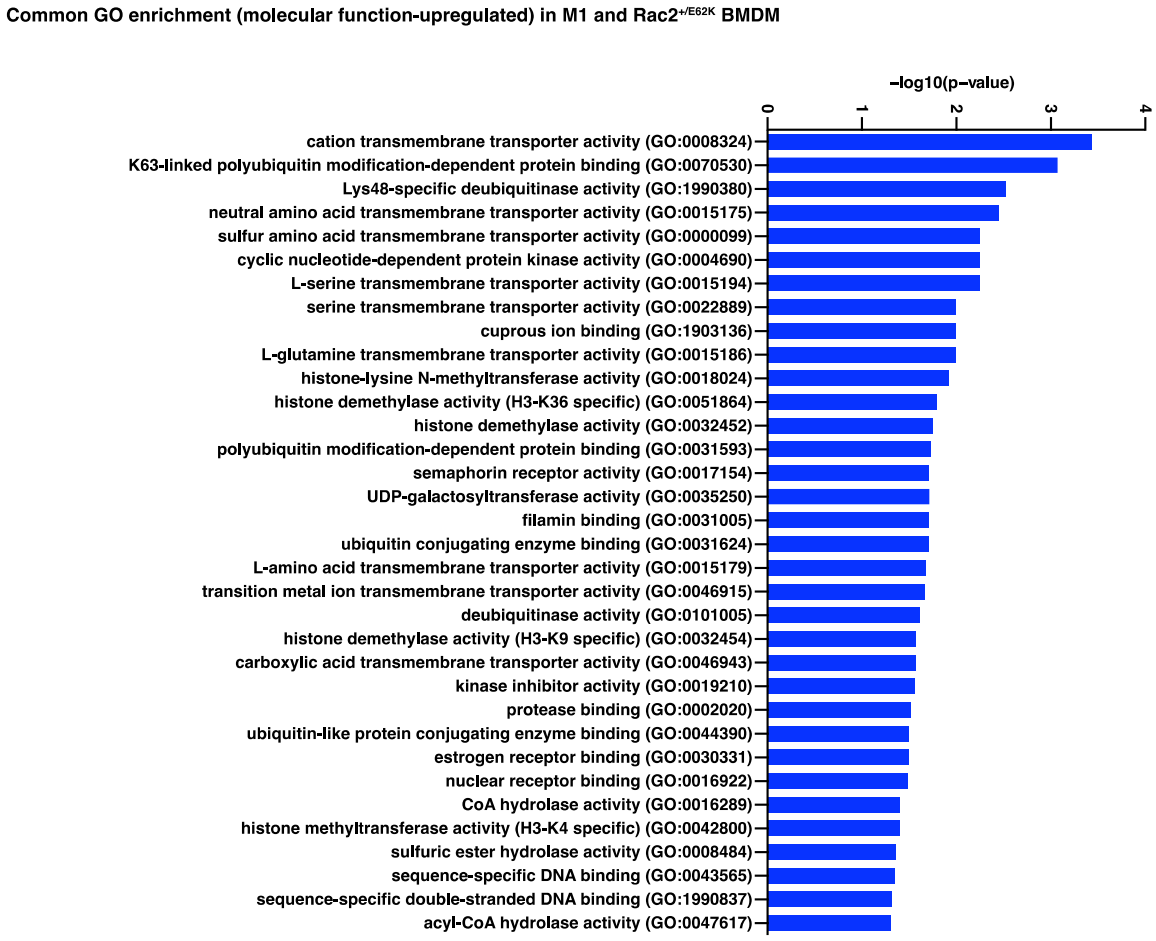


**Fig. S5.** Gene ontology analysis was performed on common DEGs between BMDM of $Rac2^{+/E62K}$ mice and M1 stimulated BMDM in Enrichr (Ma'ayan laboratory) on upregulated genes with (FC>1.5) and the GO Molecular Function 2021 output data is plotted.

**Movie S1.**
Confocal time-lapse imaging of border cell migration.

**Movie S2.**
Movie of 3D surface renderings of Figure 2H and H'.

**Movie S3.**
Movie of 3D surface renderings of Figure 2I and I'. Border cell nucleus is cyan and polar cell nuclei are blue.

**Movie S4.**
$Rac2^{+/+}$ macrophages do not show enhanced engulfment of target cells.
Spinning disc confocal microscopy shows macrophages (green) do not engulf many $Rac2^{+/+}$ T cells (magenta) overtime. Reduced pHrodo signal (yellow) within the macrophages (green)

represents fewer phagocytosis events. Frames were acquired every 3 mins for 10 hours. Scale bar denotes 20 μm.

**Movie S5.**
$Rac2^{+/E62K}$ macrophages hyperphagocytose target cells.
Spinning disc confocal microscopy shows $Rac2^{+/E62K}$ macrophages (green) more rapidly and frequently phagocytosing $Rac2^{+/E62K}$ T cells (magenta). Robust pHrodo signal (yellow) within the macrophages (green) suggests enhanced phagocytosis. Frames were acquired every 3 mins for 10 hours. Scale bar denotes 20 μm.

**Movie S6.**
Unedited $Rac2^{+/+}$ macrophages do not phagocytose whole cancer cells.
Spinning disc confocal microscopy shows BMDMs expressing a membrane tethered GFP (GFP-caax) fail to phagocytose whole Raji B cells (mCherry-CAAX; magenta). Frames were acquired every 5 min for 10 hours. Scale bar denotes 10 μm.

**Movie S7.**
Unedited $Rac2^{+/E62K}$macrophages do not phagocytose many whole cancer cells.
Spinning disc confocal microscopy shows $Rac2^{+/E62K}$ BMDMs expressing GFP-CAAX (green) show little to no engulfment of whole Raji B cells (mCherry-CAAX; magenta). Frames were acquired every 5 min for 10 hours. Scale bar denotes 10 μm.

**Movie S8.**
$Rac2^{+/+}$ CAR-P macrophages phagocytose whole cancer cells.
Spinning disc confocal microscopy shows BMDMs expressing the CAR-P-GFP (green) phagocytose whole Raji B cells (mCherry-CAAX; magenta). Frames were acquired every 5 min for 10 hours. Scale bar denotes 10 um.

**Movie S9.**
$Rac2^{+/E62K}$CAR-P macrophages phagocytose many whole cancer cells.
Spinning disc confocal microscopy shows a $Rac2^{+/E62K}$ BMDM expressing the CAR-P-GFP (green) phagocytose multiple whole Raji B cells (mCherry-CAAX; magenta). Frames were acquired every 5 min for 10 hours. Scale bar denotes 10 um.

**References**


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