Materials

Luminol, isoluminol, superoxide dismutase (SOD), horse radish peroxidise (HRP), dynasore, and human serum were from Sigma-Aldrich. Murine and human TNFα was from R&D Systems. All other cytokines were from Peprotech. Carboxyl polymer beads were from Bang Laboratories. Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+} was from Sigma-Aldrich. Tissue culture reagents were from Invitrogen. All buffer components were from Sigma-Aldrich and were endotoxin-free or low-endotoxin, as available.

Mouse strains

$p40^{phox-/-}$ [1], and $p40^{phoxR58A/R58A}$ [2] mice, on a mixed 129/Sv, C57BL/6J (WT) background, have been described previously. Generation of Rac1 conditional knockdown mice on both a WT (C57BL/6J) or $Rac2^{-/-}$ background[3] and $Rab27a^{-/-}$ on a C57BL/6J (WT) background,[4] were as previously described. For mouse bone marrow reconstitution experiments, donor mice were WT C57BL/6J mice, $p40^{phox-/-}$ mice or $p40^{phoxR58A/R58A}$ mice. Recipient mice were C57BL/6J mice for C57BL/6J donors or $Rag2^{0}IL2rg^{0}$ mice[5] for mixed background donors. In all experiments, mice were compared with appropriate age and strain matched wild-type controls. All animals were housed in the small animal barrier unit (SABU) at the Babraham Institute under specific pathogen-free conditions, as appropriate. During mouse bone marrow

reconstitution experiments, animals were housed in individually ventilated cages (IVC) in the IVC facility in the small animal unit (SAU). This work was approved by Home Office Project License PPL 80/1875 and PPL 80/2335. All procedures were carried out under Home Office Personal Individual License PIL 80/10089.

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers with written consent (REC Approval number 06/Q0108/165), and mature mouse neutrophils isolated from bone marrow, as described previously[6]. Purity of mouse neutrophils was determined by FACS-analysis of their distinct FSC/SSC properties or by cytospin and REASTAIN Quick-Diff (Reagena) staining, and were at least 95% (human) or 70-85% (mouse) pure. After washing, neutrophils were resuspended in Dulbecco's PBS with Ca^{2+} and Mg^{2+}, 1g/l glucose, 4mM sodium bicarbonate (DPBS+), and primed, where indicated, with 1000U/ml mouse TNFα and 100ng/ml GM-CSF for 1hr at 37°C (mouse) or 200U/ml human TNFα for 30min at 37°C (human) with occasional, gentle mixing.

Preparation of particles

Staphylococcus aureus (*S.aureus*) Wood 46 bacteria was grown, opsonised with DPBS+/10% mouse serum (isolated as described previously[2]), and prepared as described previously[6]. Serum-opsonised zymozan (Molecular Probes) was prepared as previously described[7]. For superoxide assays, 10µl of sheep red blood cells (SRBC) in Alsevers (TCS Biosciences) were washed (1500xg, 4min) and resuspended in 1ml DPBS+ containing 0.1% fatty acid- and endotoxin-free BSA (DPBS++). SRBC were opsonised with 1/1000 dilution of rabbit anti-SRBC IgG (MP Biomedicals), rotating end-over-end at room temperature (RT) for 20min. SRBC were washed twice and resuspended in 800µl of DPBS++. For phagocytosis assays

and live imaging, 20µl of washed SRBC were opsonised with a sub-agglutinating concentration (1/600 dilution) of rabbit anti-SRBC IgG for 30min, RT. IgG-SRBC were washed twice in DPBS++, resuspended in 100µl DPBS++ and kept on ice until use. 3µm carboxyl beads were coated with methylated-BSA via EDAC-mediated coupling and opsonised (or left unopsonised) with 1/250 mouse IgG α-BSA, as previously described[1].

Preparation of IgG-BSA immune complexes

Immune complexes were immobilized by coating wells (luminometer superoxide assay) or coverslips (fixed cells) overnight at 4ºC with PBS containing BSA (100µg/ml). After washing in PBS, wells were blocked with 1% fat-free milk in PBS for 45min at RT. Immune complexes were formed by addition of 1/10000 dilution of mouse anti-BSA antibody (Sigma-Aldrich) in DPBS+ for 1hr at RT, followed by washing to remove unbound antibodies. Neutrophils were added to immune complexes and superoxide generation measured as described above. For fixed cells, neutrophils were allowed to interact (adhere and spread) with immune complexes for 45min at 37°C, prior to fixing and processing for NBT or immunofluorescence as described below.

Nitroblue tetrazolium (NBT)/formazan assays

For fixed single cell assays, primed murine BMN or human PMN were incubated with 0.2mg/ml nitroblue tetrazolium (NBT, Sigma) for 10min at 37ºC. Where appropriate, cells were also pre-incubated with 80µM dynasore or 375U/ml SOD (or vehicle control) for 10min prior to performing phagocytosis assays or interaction with immobilised immune complexes described above and Materials and Methods. Non-phagocytosed *S.aureus* were lysed by addition of 5U/ml lysostaphin (Sigma) for a further 10min. Samples were cytospun or allowed to adhere onto glass coverslips, fixed, and mounted as described for phagocytosis

assays. Dark formazan deposition was detected by DIC imaging on a Zeiss LSM 510 META point-scanning confocal microscope with an integrated camera, using a Plan/Apochromat 63x/1.4 oil objective.

For colorimetric cell population NBT Assays, formazan deposits were quantitated by fixing samples in MeOH, dissolving deposits in 2M KOH and DMSO, and measuring OD_{655} (Biorad Microplate Reader 680) as previously described[9]. NADPH oxidase specific components of measurements were calculated by subtracting values determined in the presence of DPI (3µM). Briefly, primed BMN were pre-incubated with 0.2mg/ml NBT in the presence or absence of SOD (375U/ml) or DPI (3µM), for 10min prior to the assay. Optimal BMN density and incubation time was determined, and $2x10^6$ primed BMN were allowed to interact with immune complexes in 24 well dishes for 30min at 37°C. Serum-*S.aureus*, IgG-SRBC, and IgG-bead assays were performed in suspension at 37°C, employing $6x10^5$, $1x10^6$, and $2x10^6$ primed BMN, respectively, and incubating for 10, 20 and 20 min respectively. For assays using human neutrophils, $6x10^5$ cells and 20 min incubations were used for both IgG-SRBC and bead assays.

Western blotting

$5x10^5$ neutrophils were sonicated into 1x SDS-loading buffer, subjected to SDS-PAGE, transferred, and immunoblotted for Rac1 (Upstate/Millipore), $p40^{phox}$ (Millipore), $p47^{phox}$ (Millipore), or $p67^{phox}$ (Millipore or BD Transduction Laboratories). Signal was detected by ECL and quantified using Aida Image Analyser 2.2.

Cell culture

Platinum-E (Plat-E) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 4500mg/l glucose, L-glutamine and pyruvate, and supplemented with 10% Foetal

Bovine Serum (FBS) and 1% penicillin/streptomycin. Foetal liver (FL) cells were cultured at a concentration of $5x10^5$/ml in StemPro-34 serum-free medium supplemented with the provided StemPro-Nutrient supplement, 1% Glutamax and 1% penicillin/streptomycin/amphotericin B. In addition, a cocktail of cytokines was used to support haematopoietic stem cell (HSC) and progenitor cell proliferation: 100ng/ml mSCF, 100ng/ml TPO, 10ng/ml human IL-6, 6ng/ml murine IL-3 and 20ng/ml Flt3-Ligand (Martin Turner, Babraham Institute, personal communication).

Retroviral vectors

The bicistronic retroviral transfer vector pMIGR1 has been described previously[10]. The GFP-tagged wild type (WT) isolated *phox* homology domain (iPX) from $p40^{phox}$ was cloned into pMIGR1, lacking the IRES-GFP sequence. Full length murine $p67^{phox}$ and $p40^{phox}$ IMAGE cDNA clones, were obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH and verified by sequencing. Full length $p67^{phox}$ was amplified from the IMAGE clone and subcloned into pMIGR1 lacking IRES-GFP via pEGFP-N1 (Clontech) to generate pMIGR1-p67-GFP. The point mutant $p67^{phox}$-H69E was created by site-directed mutagenesis. Full length $p40^{phox}$ was amplified from IMAGE clone and subcloned into pMIGR1-GFP (generated by excising-iPX($p40^{phox}$) from pMIGR1-GFP-iPX($p40^{phox}$) to create pMIGR1-GFP-$p40^{phox}$.

Production of retroviral vectors

Retroviral vectors were produced by transient transfection of the packaging cell line Plat-E, using the Lipofectamine 2000 method, with some modifications to the protocol as listed

below. Plat-E were derived from HEK 293T and stably transfected with the packaging plasmid pGag-pol-IRES-bs^r, as well as the ecotropic envelope plasmid pEnv-IRES-puro^r, as previously described[11]. Briefly, retroviral transfer vector DNA and Lipofectamine were combined in Optimem media in a ratio of 1:3 (w/v) for 20min at RT. Optimem/DNA/Lipofectamine were then added to cells suspended in Plat-E culture media lacking antibiotics. Per 1.2x10^7 cells, 16.8μg of DNA and 50.4μl Lipofectamine was used. Cells were plated on 14cm tissue culture plates. Twenty-four hours after transfection, the medium containing the DNA-Lipofectamine 2000 complexes was removed and replaced by StemPro-34 serum-free medium supplemented with the provided StemPro-Nutrient supplement and 1% penicillin/streptomycin/amphotericin B. Forty-eight hrs post-transfection, the virus-containing supernatant was harvested and centrifuged at 800xg for 5 min at 4˚C to pellet cell debris. The supernatant was removed and filtered through a 0.45μm polyamide filter (Sartorius). The filtered supernatant was directly used for transduction of FL cells.

Foetal Liver (FL) reconstitution

FL cells were harvested from E14.5 day old embryos. To induce proliferation, cells were cultured in the presence of cytokines for a period of 24hrs. Cells were then transduced with retroviral vectors. 1ml viral supernatant was used per 5x10^5 cells. Cells were transduced on non-tissue culture treated plates coated with 12.5ng/ml retronectin (Takara Bio Inc). All transductions were performed in the presence of the cell-specific cytokine cocktails described previously. Seventeen hours post-transduction, cells were washed in HBSS, resuspended in PBS/10% FBS and 1-2x10^6 FL cells injected in 300μl into 8-week old recipient mice which had been lethally irradiated (employing two doses of 5 Gy from a ^{137}Cs source, separated by

3hrs) the day before injection. A small proportion of the cells were kept in culture for FACS-analysis of GFP or mCherry marker gene expression 72hrs post- transduction.

Live imaging

Neutrophils were isolated and primed (or left unprimed) as described. After priming, where appropriate, neutrophils were kept on ice until use. To prevent adhesion of neutrophils to glass, and thus to increase the membrane area available for phagocytosis, which is of importance for efficient uptake of large particles (unpublished observation), coverslips (∅ 22 mm) were blocked with FBS for at least 2hrs at room temperature. Coverslips were washed twice in DPBS++, and placed in imaging chambers. 16µl of IgG-SRBC were added to 75µl of neutrophils, after which they were centrifuged for 1min at 200xg at 4°C. Neutrophils and IgG-SRBC were gently mixed and immediately added to imaging chambers containing 400µl of DPBS++ and that were briefly pre-warmed to 37°C. The ratio of IgG-SRBC:neutrophils was approximately 20:1. Mouse serum-opsonised *S.aureus* were added at a similar ratio, however, $1x10^6$ neutrophils were added to either FBS-blocked or non-coated borosilicate glass coverslips in imaging chambers in a volume of 1.5ml, allowed to settle for 5min at 37°C and *S.aureus* were added in a volume of 500µl (for a ratio of bacteria:neutrophils of 20:1, 40µl of bacteria at a concentration of $5x10^8$/ml were diluted to 500µl in DPBS+). For widefield imaging, GFP- and DIC-images were recorded at an exposure time of 750msec, and 4sec intervals. The length of each movie was 15min. Images were recorded using an Olympus CellR epifluorescence microscope equipped with an Olympus 1x2-UCB camera, using a Plan/Apochromat 60x/1.4 oil objective.

Quantification of the mean fluorescence intensity (MFI) around the cup and/or phagosome was performed as follows using ImageJ software. For imaging of phagocytosis of *S.aureus* bacteria, individual frames were taken from movies showing neutrophils prior to phagocytosis and containing phagocytosed bacteria. The MFI in the cytosol prior to phagocytosis was measured as an average of the MFI in 4 random ROIs (Figure S9A). A region of interest (ROI) was manually drawn around the S.aureus containing phagosome, while excluding the intraphagosomal space (Figure S9B), after which the MFI within this ROI was quantified. The MFI in the cytosol was measured in 3 random ROIs (Figure S9B), after which the MFI around the phagosome was normalised against cytosolic fluorescence by dividing the phagosomal fluorescence intensity by the average of the three measured cytosolic fluorescence intensities.

For imaging of phagocytosis of IgG-SRBC, the MFI in the cytosol before the phagocytic event took place (Figure S9C), the intensity around the site of attachment to the particle (Figure S9D), around the phagocytic cup (Figure S9E), around the phagosome (Figure S9F), and the mean cytosolic fluorescence after the phagocytic event took place (Figure S9G) was quantified. The MFI at a random location in the cytosol, the site of attachment of the neutrophil to the particle, around the phagocytic cup, or around the phagosome was normalised against the average cytosolic fluorescence of three random ROIs.

For 3D live imaging, a series of z-stacks were recorded at 0.5 μm intervals. To encompass the whole cell, a set of 20-22 z-stacks was acquired for each time-frame. Typically, the exposure was 50-100msec depending on the intensity of intracellular fluorescence. The time-interval between each frame was minimum 2.78sec. Images were acquired on a spinning Nipkow disk Nikon-Eclipse confocal microscope equipped with an ultrasensitive EM-CCD camera (Andor

iXon 897) allowing very fast image aquisition, using a Plan/Apo 100x oil objective (NA 1.4). 3D reconstruction was performed using Volocity software. AVI movies of 3D-reconstructed, unprocessed z-stacks were created using Volocity and ImageJ software.

Protein expression and purification

Constructs pOPT-His-iTPR(p67^{phox})-WY containing the TPR domain from human p67^{phox} (kind gift from Michael Wilson, MRC Laboratory of Molecular Biology, Cambridge, UK) and pOPT-His-iTPR(p67^{phox})-H69E, generated using site-directed mutagenesis, were expressed in BL21(DE3)pLysS competent *E. coli* (Stratagene), with protein expression induced with 1mM isopropyl-β-D-thiogalactoside (IPTG) for 4hrs. Cells were centrifuged for 20mins at 1500g and bacterial pellets resuspended in resuspension buffer, consisting of 20mM Tris-HCl pH 8.0, 4°C, 0.1M NaCl and 1mM DTT, and supplemented with the following cocktail of protease inhibitors: 0.2mM PMSF; 10mg/ml of each antipain, aprotinin, pepstatin A and leupeptin. Bacteria were lysed by sonication following incubation with detergent 10% Triton X-100. Lysates were cleared by centrifugation for 45mins at 40000rpm, 4°C, in a Beckman-Coulter Optima L-XP ultracentrifuge, using the 70Ti rotor. Supernatants were added to 2ml Talon resins (Clontech) that were equilibrated in resuspension buffer. Samples were mixed end-over-end for 30mins on ice. Samples were then loaded onto columns and resins were allowed to settle at 4°C. Columns were washed in wash buffer 1, consisting of 20mM Tris-HCl pH 8.0 4°C, 0.1M NaCl, 1mM DTT and 1% Triton X-100, followed by wash buffer 2, consisting of 20mM Tris-HCl pH 8.0 4°C, 0.1M NaCl, 1mM DTT and 20mM imidazole. Samples were eluted with elution buffer, consisting of 20mM Tris-HCl pH 8.0 4°C, 0.1M NaCl, 1mM DTT and 300mM imidazole. Eight consecutive fractions were collected for each of the proteins, and protein containing fractions of which the concentrations were measured using a Bradford protein assay were pooled. Pooled fractions

were then loaded onto PD-10 columns which were previously equilibrated with gel filtration/Biacore buffer consisting of 20mM Tris-HCl pH 7.4 RT, 100mM NaCl and 5mM DTT. Proteins were eluted with gel filtration buffer containing the standard cocktail of protease inhibitors. Seven fractions were collected, protein concentrations were measured as before, and protein-containing fractions were pooled. Concentrations of the purified proteins were determined by SDS-PAGE against BSA standards followed by coomassie staining.

Surface plasmon resonance assays

These assays were performed using a Biacore 3000. An M5 sensor chip (Biacore) cross-linked with streptavidin was coated with biotinylated GTPγS-loaded Rac1 containing a glutamine (Gln, Q) 61 to leucine (Leu, L) substitution to inhibit its intrinsic GTPase activity (a kind gift from Michael Wilson, MRC Laboratory of Molecular Biology, Cambridge, UK). To coat the chip, 5µl of 2.3µM protein solution in gel filtration/Biacore buffer was loaded onto the chip. As a negative control for non-specific binding of proteins to the chip, non-coated chips were used. 50nM of His-iTPR(p67^{phox})-WT or -H69E were then added to the assay and interaction measured in response units (RU) over time.

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Figure S1. Effects of p40^{phox} R58A mutation on superoxide generation in unprimed mouse neutrophils

Unprimed mouse bone marrow derived neutropihls from wild type (WT, black squares), or p40$^{phoxR58A/R58A}$ (R58A, grey diamonds) animals were prepared, pre-incubated with luminol/HRP (B) or NBT (C) as described in Materials and Methods and Supplementary methods respectively, prior to addition to either serum-opsonised *S.aureus* (B,Ci) or IgG-opsonised SRBC (B,Cii), prepared as described in Supplementary methods. The effect of priming agents themselves on ROS production over the priming period is shown in (A). (A,B) Chemiluminesence was recorded on a 96 well plate using a Berthold Microlumat Plus luminometer. All incubations were performed in at least duplicate. Shown are data (mean ± range) from one experiment representative of at least three, expressed as relative light units/sec (RLU/sec), as well as accumulated light emission (ROS production) over 20min for a combination of ≥ 3 experiments (mean ± SEM) (B), expressed as a percentage of responses in primed WT mouse neutrophils(see Figs3,4). *p<0.0001, paired Students T-test compared to WT response. (C). Non-phagocytosed serum-*S.aureus* or IgG-SRBC were lysed prior to processing. Samples were cytospun (i) or allowed to adhere (ii) onto glass coverslips, fixed in 4% paraformaldehyde, washed, mounted, and dark formazan deposition visualised as described in Fig1. Shown are representative DIC images, including an enlarged section of formazan deposition in WT cells.

Figure S2. Expression of GFP-tagged *phox* components in primary mouse neutrophils

(A) Expression of p40^{phox} (top), p67^{phox} (middle) and p47^{phox} (bottom) in BMN isolated from p40$^{phox-/-}$ mice heterologously expressing GFP or GFP-p40^{phox} (GFP-p40), or WT mice. The dashed line indicates grouping of images obtained from different parts of the same Western blot. (B) Expression of p67^{phox} in BMN isolated from WT mice, or WT mice heterologously expressing p67^{phox}-GFP (p67 WT-GFP), or p67^{phox} H69E-GFP (p67 H69E-GFP). BMNs were sonicated into SDS sample buffer, subjected to SDS-PAGE (4x10^5 cells/lane) and immunoblotted for p40^{phox}, p67^{phox} and p47^{phox} as described in Supplementary Materials and Methods.

Figure S3. Localisation of GFP-p40^{phox} expressed in a p40$^{phox-/-}$ background during *S.aureus* and IgG-SRBC phagocytosis

Neutrophils expressing GFP-p40^{phox} on a p40$^{phox-/-}$ background were prepared and used for live, widefield imaging as described in Fig 3C, 4C and Materials and Methods. Cells were incubated with *S.aureus* bacteria (A) or IgG-SRBC (B) and images were recorded for 15 minutes in the GFP (and DIC, not shown) channel or Texas-Red (and DIC, not shown) channel at 4 second intervals, and at an exposure time of 750 msec. (A) Individual time-frames are shown before (1) and after/during phagocytosis of *S. aureus* (2). (B) Individual time-frames are shown before phagocytosis (1), at the time of attachment of IgG-SRBC to neutrophil (2), at the time of formation of a phagocytic cup (3), at the time of phagosome formation and GFP-p40^{phox} localisation to the phagosome (4) and after phagocytosis (5). The indicated time points in individual frames are in seconds, with t=0 representing the time of attachment of particle to neutrophil. Imaging experiments were performed with neutrophils from at least 2 different individual mice, on at least 2 separate days. *S. aureus* phagosomes are indicated by white arrows. IgG-SRBC phagosomes are indicated by stars and pre-

phagosomal structures containing GFP-p40^{phox} are indicated by arrows. Quantification of fluorescence intensities before and after/during phagocytosis was performed and is presented as described in Fig3C.

Figure S4. The iPX domain interferes with targeting of p67^{phox} during phagocytosis

(A) Images of neutrophils expressing mCherry-iPX-WT (left) or p67^{phox}-GFP (right) undergoing serum-opsonised *S.aureus* phagocytosis were captured over time on a Nikon-Eclipse spinning disk confocal microscope equipped with an ultrasensitive EM-CCD camera. Shown is a single confocal plane through an *S.aureus* containing phagosome (indicated by a yellow arrow), indicating lack of typical p67^{phox}-GFP accumulation on the phagosome. (B) Primed BMN from C57BL/6J mice or C57BL/6J mice expressing mCherry-iPX were pre-incubated with NBT prior to incubation with IgG-SRBC, prepared as described in Supplementary Materials and Methods. Cells were allowed to adhere on glass for 3 min at 37°C, prior to fixing and mounting as described in Materials and Methods. Confocal images of mCherry fluorescence were obtained by imaging on a Zeiss LSM Meta confocal microscope. Dark formazan deposition was detected by DIC imaging. Shown are representative images, illustrating loss of formazan deposition in cells expressing mCherry-iPX.

Figure S5. Effect of knockdown of Rac1 and Rac2 on neutrophil ROS production

(A,C) Expression of Rac1 in BMN isolated from two cohorts of mice subjected to conditional knockdown of Rac1 on either WT or Rac2 knockout background, that exhibited greater (A) or lesser (C) Rac1 knockdown. $1x10^6$ BMN isolated from wild type (WT), Rac1 knockdown on a WT background (Rac1KD), Rac2 knockout (Rac2KO) and Rac1 knockdown on a Rac2KO background (Rac1KD/Rac2KO) were pelleted, and resuspended by sonication into SDS-sample buffer, subjected to SDS-PAGE, and immunoblotted for Rac1 as described in Materials and Methods. Quantitation of Rac1 expression as a percentage of WT neutrophil expression is also shown. Shown is one representative blot of three (A, top panel). To accurately quantitate severe knockdown of Rac1 in (A) total samples (100%) in Rac1KD and Rac1KD/Rac2KO cells were run alongside 10% of higher expressing WT and Rac2KO samples (A, bottom panel). (B) Primed BMN from WT (black), Rac1KD (dark grey), Rac2KO (light grey) and Rac1KD/2KO (open bars) genotypes were incubated with serum-opsonised *S.aureus* at 37°C for 7 min in suspension. Samples were cytospun to glass coverslips, fixed and mounted, and viewed using a Zeiss LSM 510 META point-scanning microscope, as described in Materials and Methods. Number of *S.aureus* phagocytosed/cell for each genotype in at least 30 cells from each condition were counted and expressed as mean ± SEM as a percentage of number of phagocytic events in WT BMN. (D) Primed BMN from WT (black squares/bar), Rac1KD (dark grey diamonds/bar), Rac2KO (light grey triangles/bar) and Rac1KD/2KO (open circles/bar) genotypes, with Rac1 expression levels from (C), were incubated with *S.aureus* in the presence of luminol/HRP as described in Materials and Methods, with ROS responses measured as described in Figure 3A. Shown are data (mean ± range) from one representative experiment performed in duplicate as relative light units/sec (RLU/s), as well as accumulated light emission over 20 min (mean ± SEM, $n \geq 3$), expressed as a percentage of the response in WT mouse neutrophils. *$p<0.001$, one-way Anova compared to WT expression/responses as appropriate.

Figure S6. H69E mutation disrupts interaction between p67^{phox} and Rac1

His-tagged isolated wild type (His-iTPR-WT) or H69E mutated (His-iTPR-H69E) TPR domain of human p67^{phox} were expressed and isolated as described in Supplementary Materials and Methods. Binding of His-iTPR-WT and His-iTPR-H69E to biotinylated GTPγS-loaded Rac1-Q61L was assessed by SPR analysis as described in Materials and Methods. A representative SPR sensorgram showing measurements of interaction (in response units (RU)) between His-iTPR(p67^{phox})-WT and GTPγS-loaded Rac1-Q61L (WT), His-iTPR(p67^{phox})-H69E and GTPγS-loaded Rac1-Q61L (H69E), or His-iTPR(p67^{phox})-WT with the uncoated chip (WT, no Rac) is presented. This experiment was performed in duplicate.

Figure S7. Inhibition of clathrin-mediated endocytosis does not affect neutrophil superoxide generation in response to IgG-opsonised SRBC or latex beads

(A) Primed mouse BMN were pre-incubated in suspension with 80μM dynasore or vehicle control (DMSO) prior to addition of Texas-Red labelled transferrin (Molecular Probes). Cells were incubated with transferrin for 15min at 37°C, then allowed to adhere onto glass coverslips, fixed in 4% paraformaldehyde, washed and mounted as described in Materials and Methods. Samples were imaged on a Zeiss LSM 510 META point-scanning confocal microscope. Shown are representative images. (B,C) Mouse neutrophils were pre-incubated with NBT in the presence of 80μM Dynasore or vehicle control (DMSO), and the absence (B,C) or presence (C) of SOD, prior to addition of IgG-opsonised SRBC (i), or IgG-beads

(ii) as described in Supplementary Materials and Methods. Non-phagocytosed SRBC were lysed prior to processing. (B) Samples were allowed to adhere onto glass coverslips, processed and visualised as described in Fig1. Shown are representative DIC images, with IgG-SRBC phagosomes indicated by a white star. (C) Formazan deposits in cell populations were quantitated by dissolving in KOH and DMSO, and measuring resultant OD_{655} of samples as described in Supplementary Materials and Methods. Results shown are mean ± SEM for at least two experiments performed in triplicate, and expressed as a percentage of DMSO treated response in the absence of SOD. (D) Mouse neutrophils were pre-incubated with dynosore or DMSO control, prior to addition of IgG-beads. Samples were prepared, fixed and stained and visualised for $p67^{phox}$ as described in Materials and Methods. Show are representative images.

Figure S8. Rac2 deficiency results in increased sensitivity of ROS responses induced by *S. aureus* to wortmannin

Primed BMN from WT (black squares) and Rac2KO (grey triangles) mice were pre-incubated for 10 min with wortmannin (0.01-300nM as indicated) or DMSO vehicle control, in the presence of luminal/HRP as described in Materials and Methods. Cells were added to serum-*S.aureus*, and ROS responses measured over 20min, as described in Fig3A. Data (mean ± SEM, $n \geq 6$) are accumulated light emission, expressed as a percentage of response in the absence of inhibitor (DMSO control). *$p<0.0001$, paired Student's T-test between WT and Rac2KO responses at indicated wortmannin concentrations.

Figure S9. Method for quantification of fluorescence intensity in live, widefield imaging experiments

(A,B) Quantification of cytosolic and phagosomal fluorescence in single frames from a movie of a BMN expressing GFP-p40^{phox} before phagocytosis (A) or containing an ingested *S.aureus* bacterium (B) was performed using ImageJ software as described in Materials and Methods. (A) Cytosolic fluorescence intensities before phagocytosis in four different ROIs. (B) A region of interest (ROI) was manually drawn around the phagosome and the intra-phagosomal space was excluded. Three different ROIs were drawn to calculate the average cytosolic fluorescence intensity. The *S. aureus* bacterium is indicated by an arrow. (C-G) Single frames taken from a movie of a BMN expressing p67^{phox}-GFP during phagocytosis of IgG-SRBC. (C) Cytosolic fluorescence intensities before phagocytosis in four different ROIs. (D) Fluorescence intensity in the area around the site of attachment of the neutrophil to the IgG-SRBC. (E) Fluorescence intensity in the phagocytic cup, as well as cytosolic fluorescence intensities in three different ROIs. As in (B), the intra-phagosomal space was excluded. (F) Fluorescence intensity in the phagosome as well as cytosolic fluorescence intensities in three different ROIs. (G) Fluorescence intensities in the cytosol after the phagocytic event had taken place in four different ROIs. The position of the IgG-SRBC is indicated by a star and the position of the pre-phagosomal structure is indicated by an arrow.

Figure S1

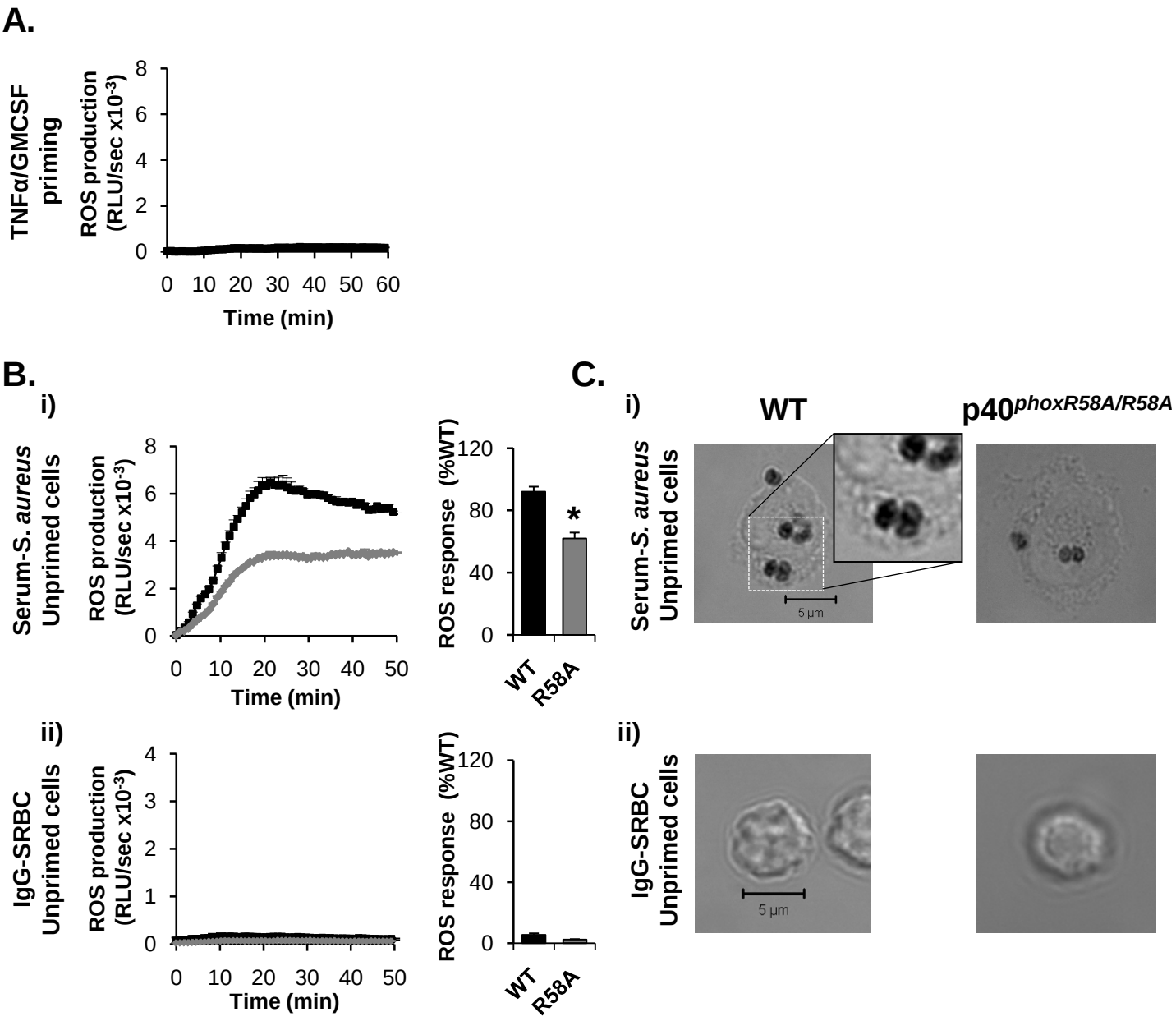

A.
TNFα/GMCSF priming
ROS production (RLU/sec x10-3)
Time (min)
B.
i)
Serum-S. aureus Unprimed cells
ROS production (RLU/sec x10-3)
Time (min)
ROS response (%WT)
WT
R58A
ii)
IgG-SRBC Unprimed cells
ROS production (RLU/sec x10-3)
Time (min)
ROS response (%WT)
WT
R58A
C.
i)
WT
p40phoxR58A/R58A
Serum-S. aureus Unprimed cells
5 µm
ii)
IgG-SRBC Unprimed cells
5 µm

Figure S2

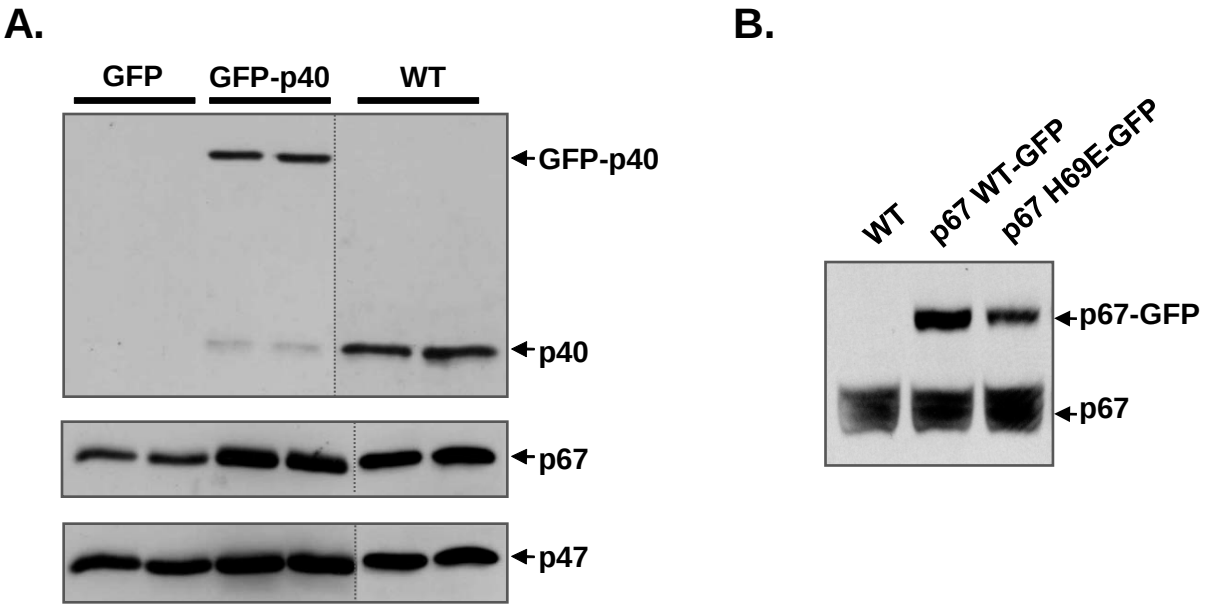

A.
GFP
GFP-p40
WT
GFP-p40
p40
p67
p47
B.
WT
p67 WT-GFP
p67 H69E-GFP
p67-GFP
p67

Figure S3

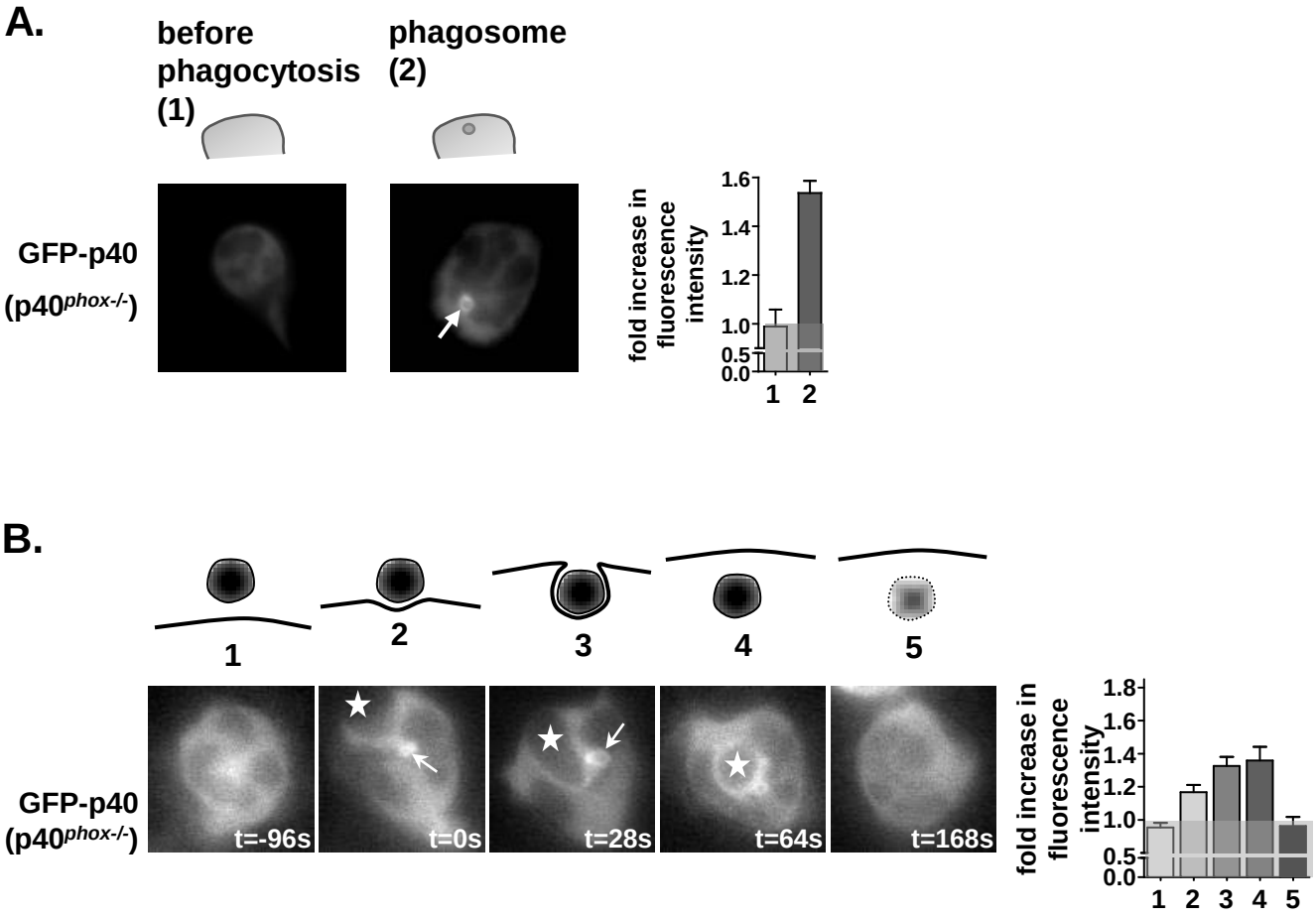
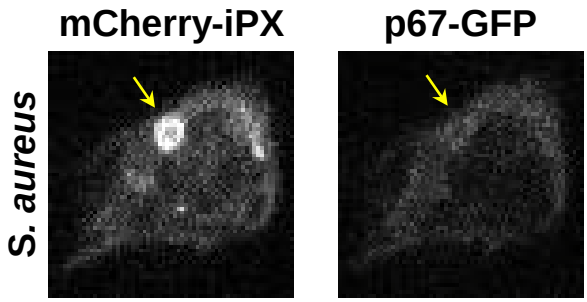

A.
before phagocytosis (1)
phagosome (2)
GFP-p40
(p40phox-/-)
fold increase in fluorescence intensity
1.6
1.4
1.2
1.0
0.5
0.0
1
2
B.
1
2
3
4
5
GFP-p40
(p40phox-/-)
t=-96s
t=0s
t=28s
t=64s
t=168s
fold increase in fluorescence intensity
1.8
1.6
1.4
1.2
1.0
0.5
0.0
1
2
3
4
5

Figure S4

A.


mCherry-iPX
p67-GFP
S. aureus

B.

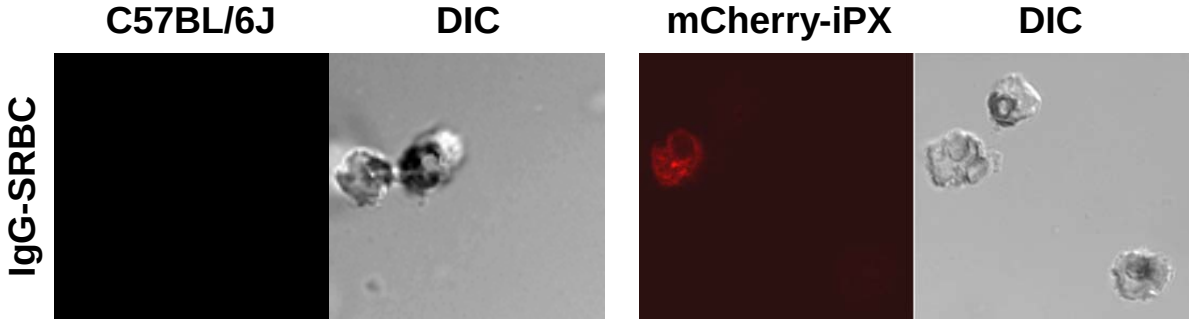

C57BL/6J
DIC
mCherry-iPX
DIC
IgG-SRBC

Figure S5

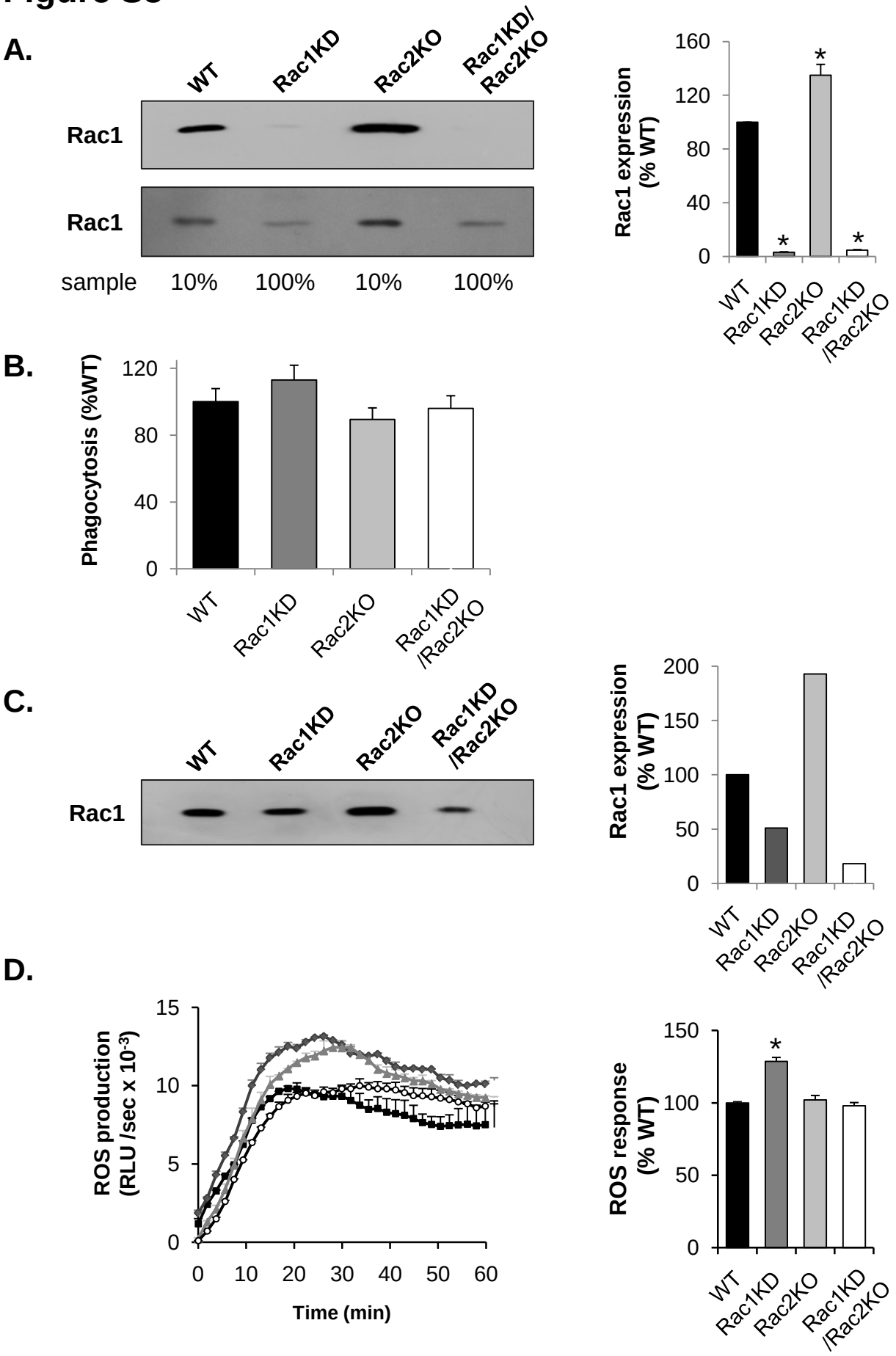

A.
WT
Rac1KD
Rac2KO
Rac1KD/
Rac2KO
Rac1
Rac1
sample
10%
100%
10%
100%
Rac1 expression
(% WT)
160
120
80
40
0
*
*
*
WT
Rac1KD
Rac2KO
Rac1KD
/Rac2KO
B.
Phagocytosis (%WT)
120
80
40
0
WT
Rac1KD
Rac2KO
Rac1KD
/Rac2KO
C.
WT
Rac1KD
Rac2KO
Rac1KD
/Rac2KO
Rac1
Rac1 expression
(% WT)
200
150
100
50
0
WT
Rac1KD
Rac2KO
Rac1KD
/Rac2KO
D.
ROS production
(RLU /sec x 10-3)
15
10
5
0
0
10
20
30
40
50
60
Time (min)
ROS response
(% WT)
150
100
50
0
*
WT
Rac1KD
Rac2KO
Rac1KD
/Rac2KO

Figure S6

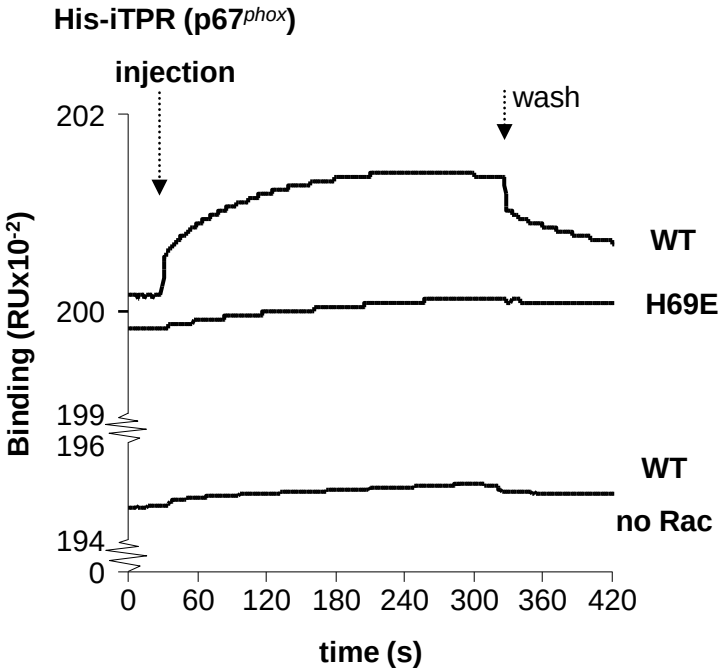

His-iTPR (p67phox)
injection
wash
202
200
199
196
194
0
Binding (RUx10-2)
WT
H69E
WT
no Rac
0
60
120
180
240
300
360
420
time (s)

Figure S7

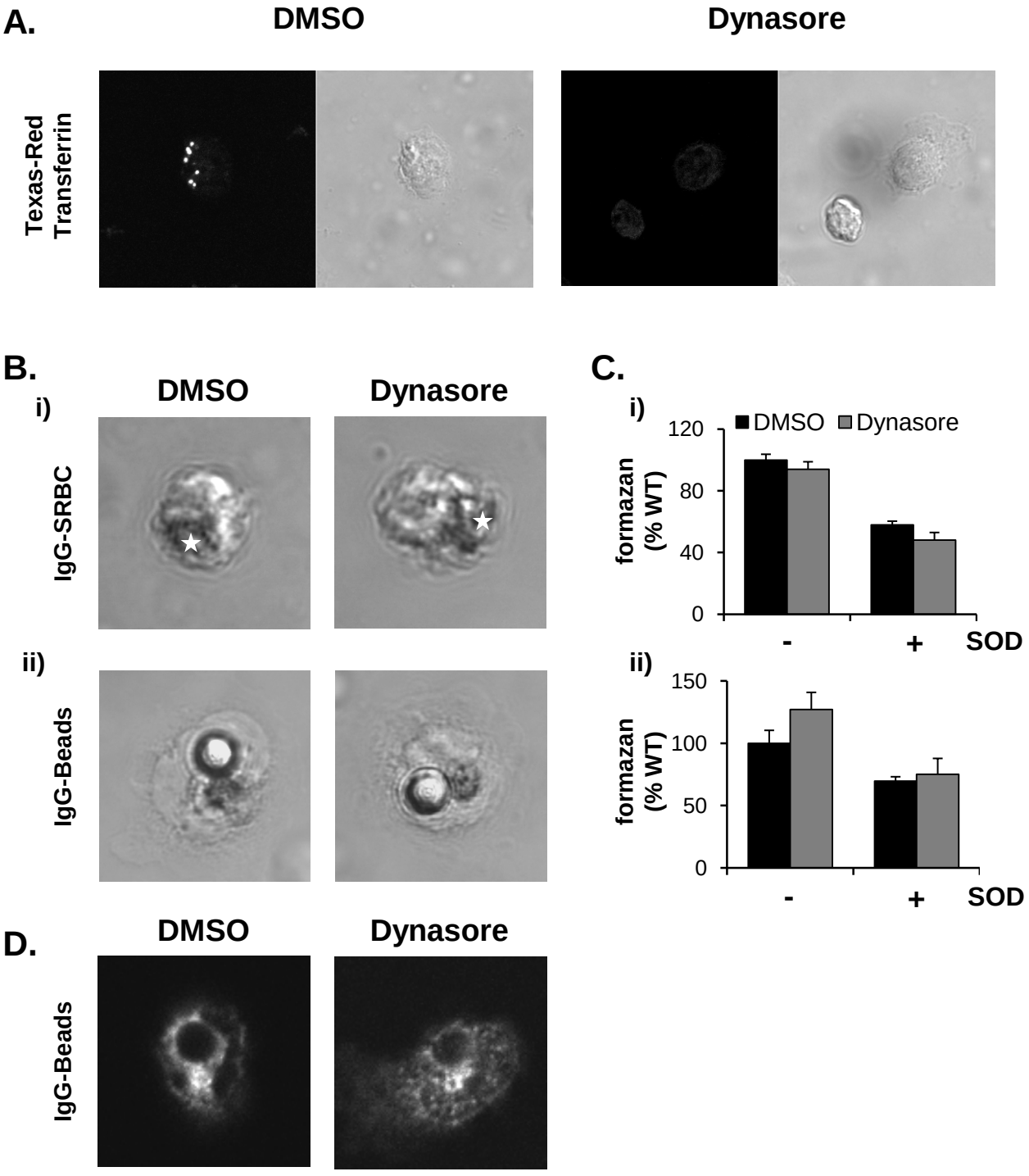

A.
DMSO
Dynasore
Texas-Red Transferrin
B.
DMSO
Dynasore
i)
IgG-SRBC
ii)
IgG-Beads
C.
i)
DMSO
Dynasore
120
80
40
0
formazan (% WT)
-
+
SOD
ii)
150
100
50
0
formazan (% WT)
-
+
SOD
D.
DMSO
Dynasore
IgG-Beads

Figure S8

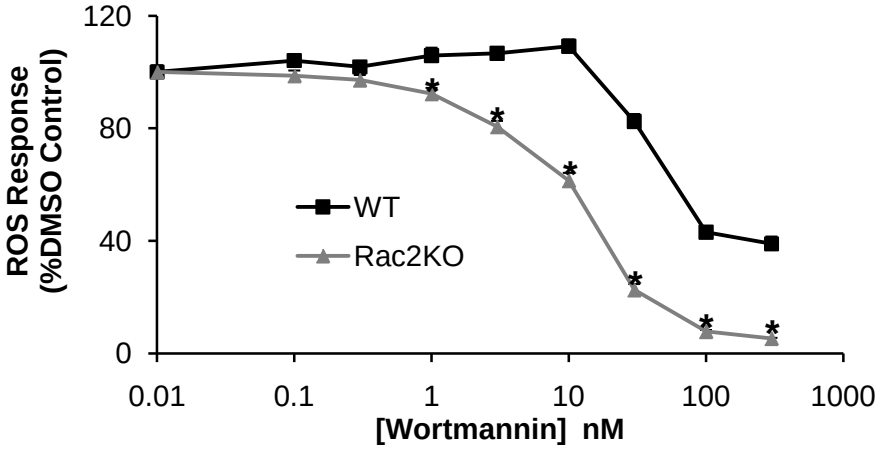
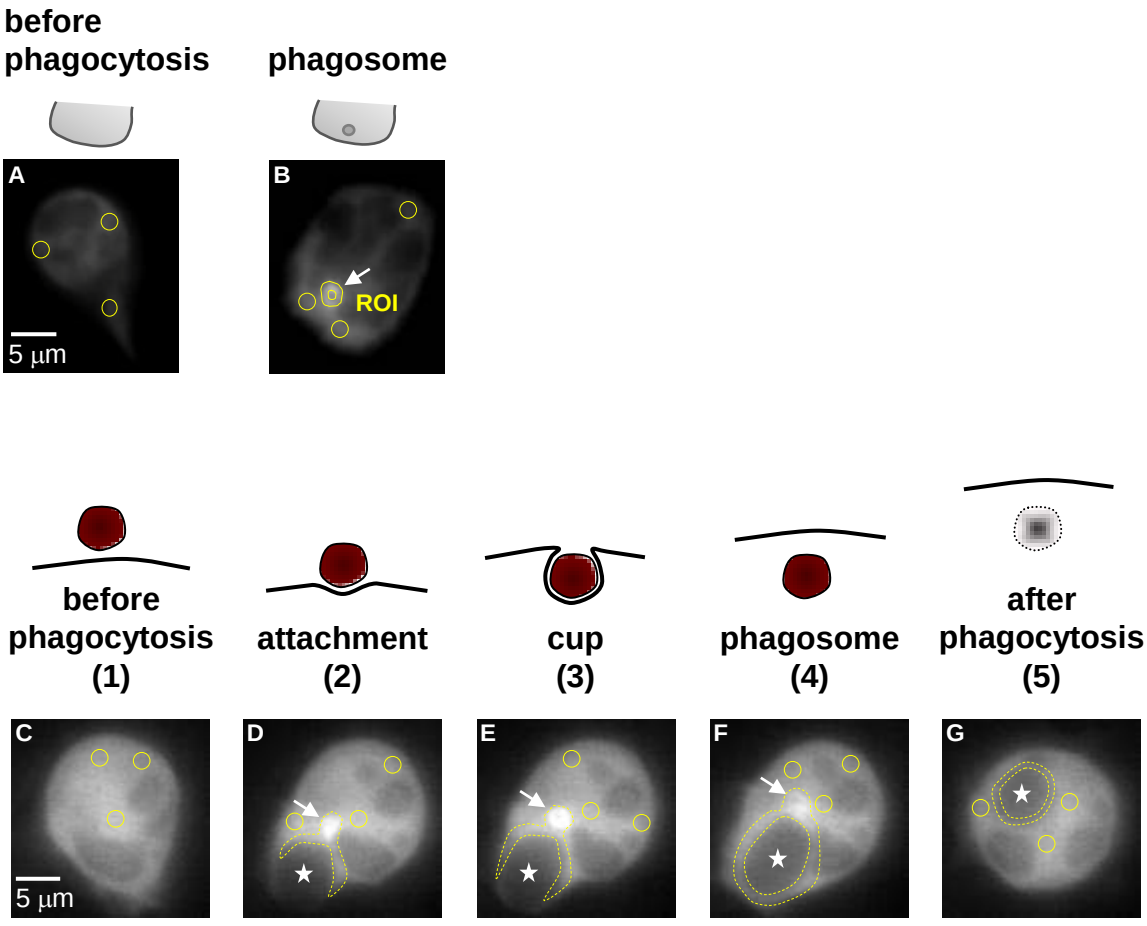

120
80
40
0
ROS Response
(%DMSO Control)
WT
Rac2KO
*
*
*
*
*
0.01
0.1
1
10
100
1000
[Wortmannin] nM

Figure S9


before phagocytosis
phagosome
A
B
ROI
5 μm
before phagocytosis (1)
attachment (2)
cup (3)
phagosome (4)
after phagocytosis (5)
C
D
E
F
G
5 μm