Supplemental Data

Supplemental Methods

Reagents, flow cytometry and neutrophil isolation

Recombinant murine MIP-2, TNFα (both from PeproTech Inc, NJ) and chemoattractant peptide fMLF (Sigma) were used at the indicated concentrations. Poly-RGD peptides at 20 μg/ml (Sigma), human plasma fibrinogen at 150 μg/ml (ICN Biomedicals) were used to coat plates as previously described (Pereira and Lowell, *J. Immunol.* 2003: **171**:1319-1327). Fluo-4 (Invitrogen) was used at 5μM. Thapsigargin (Sigma) was used at 10 nM and the pan PLC inhibitor U-73122 was used at 3μM. For flow cytometry, single cell suspensions from spleens and bone marrow were prepared and stained as previously described (Scapini, Hu et al. *J Exp Med* 2010: **207**(8): 1757-1773) with anti-mouse mAbs against CD45.1 (A20) or CD45.2 (104), CD11b (M1/70) or CD11a (MEM-25), Ly6G (IA8) all from BD Pharmingen or eBiosciences. Five-color flow cytometry was performed on a modified Becton Dickinson FACScan and data analyzed with FlowJo software (Tree Star, Inc.). Rabbit anti-PLCγ_2 (Santa Cruz, CA), anti-phospho-p40^{phox}, anti-Erk1/2 (both from Cell Signaling), anti-phospho-p47^{phox} (Assay Biotech), mouse anti-PKCα or PKCβ (Becton Dickinson) were used for immunoblotting as described below. Bone marrow-derived neutrophils were isolated as described previously (Pereira and Lowell, *ibid*). Immediately after purification, cells were resuspended in HBSS with 20 mM HEPES pH7.4 (termed HBSS/H) unless otherwise indicated (UCSF).

Immunoprecipitation and blotting

Cells were solubilized in lysis buffer (1% Nonidet P-40, 50mM NaCl, 50mM Tris.HCl [pH 7.4]), containing 5 mM EDTA, 1 mM NaF, leupeptin (1 μg/ml), aprotinin (10 μg/ml), pepstatin A (1 μg/ml), 2 mM sodium orthovanadate, and phenylmethylsulfonyl fluoride (100 μg/ml). Immunoprecipitations were carried out by adding rabbit anti-PLCγ_2, incubating at 4°C overnight followed by addition of protein G- agarose beads. Immune complexes were subjected to SDS-PAGE then transferred onto nitrocellulose membranes. Immunoblots were blocked with 5% milk in PBST (PBS with 0.1% Tween 20) for 1 hr and then incubated overnight with 4G10 anti-phosphotyrosine mAb (Cell Signaling) in 5% BSA PBST. Anti-PKC and anti-phospho-p40^{phox} immunoblotting was done similarly with membrane fractions or whole cell lysates, respectively. Immunoblots were imaged with IR-labeled secondary antibodies using the Licor Odyssey system or with HRP-conjugated secondary antibodies followed by ECL detection (Amersham).

PKCα/β intracellular staining

Neutrophils were plated on pRGD-coated cover slips at 37°C for 10 minutes then fixed in 1% paraformaldehyde for 10 minutes at RT. Cells were washed twice, blocked/permeablized with PBS/10% FCS/0.2% Triton X100 for 30 minutes, and stained with anti-PKCα or PKCβ (Becton Dickinson) antibody (1 μg/ml) in PBS/5% FCS/0.2% Triton X100 at 4°C in the dark overnight. Cells were washed in PBS/5% FCS/0.2% Triton X100 and stained with Alexa488-conjugated secondary Ab (1 μg/ml) at RT in the dark for 1 hour, washed three times and immediately photographed (see below). As controls, secondary Ab staining alone or staining of $PKC\alpha/\beta^{-/-}$ neutrophils were performed.

Subcellular fractionation

Isolated neutrophils (2 X 10^7) were stimulated fMLF (2.5 nM) in 0.2% milk blocked dishes for 3 minutes, cells were collected, pelleted and frozen in liquid nitrogen. Then the cells were resuspended in 250μl relaxation buffer (10 mM HEPES, [pH 7.4], 100 mM KCl, 3 mM NaCl, 3.5mM MgCl2, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) then sonicated for 6 seconds. Nuclei, granules, and unbroken cells were removed by centrifugation at 860 g for 10 mins at 4°C. Supernatants were ultracentrifuged at 100,000 g for 30 minutes at 4°C to separate cytosol and membrane fractions. Supernatants were collected (cytosol), or pellets (membrane) were resuspended in 80 μl 1% N- Octylglucoside (Boehringer Mannheim). Membrane fractions were added to 4X sample buffer and boiled for 10 min. Proteins were subjected to SDS-PAGE and Western blotting with mouse anti-PKCα, PKCβ with rabbit anti-actin as a loading control.

Microscopy

For fluorescence microscopy image acquisition was achieved with an inverted Axiovert 200 M microscope and a ×63/1.4 lens (Carl Zeiss, NJ), equipped with a 175 W xenon highspeed DG4 wavelength selector and a single emission filter wheel (Sutter Instruments, Novato, CA), a PI piezoelectric z-drive (Physik Instrument, Germany), and a cooled-CCD Coolsnap camera (Roper Instruments, NJ). Data were acquired and analyzed with Image J (NIH) analysis software. Light micrographs were taken with a Olympus BX41 microscope (Melville, NY) using 10X and 40X Olympus Apochromat objectives. Images were collected with an Olympus DP21 camera running the Olympus CellSens image acquisition software.

Supplemental Figure Legends

Supplemental Figure 1. WT and *stim1*$^{-/-}$ neutrophils show equal expression of CD11a and CD11b. Neutrophils from WT or *stim1*$^{-/-}$ chimeric mice, either resting or stimulated for 5 mins with the indicated agents (MIP-2 at 10 ng/ml; fMLF at 2.5 nM) at 37°C, were then stained with anti-CD11a or anti-CD11b mAbs and subjected to flow cytometry.

Supplemental Figure 2. *Stim1*$^{-/-}$ neutrophils have defective phagocytosis of Ig opsonized *Staph aureus* bacteria and reduced degranulation of lactoferrin. (A) WT and *stim1*$^{-/-}$ neutrophils (5 X 10^6 in one ml) were incubated with Ig-opsonized Alexa-488 labeled *S. aureus* particles (10^8) in HBSS media, in the presence or absence of fMLF (2.5 nM) or thapsigargin (100 nM) and with or without EGTA (4 mM) in the media, at 37°C with gentle rocking. Samples (200 μl) were removed at the indicated time points and fixed by addition of an equal amount of 2% paraformaldehyde, then kept on ice. Cells were washed twice, suspended in PBS and subjected to flow cytometry. The percentage of neutrophils showing fluorescent *S. aureus* at each time point is plotted. (B) WT and *stim1*$^{-/-}$ neutrophils (2 X 10^5/well) were plated on fibrinogen or RGD peptide-coated microtiter wells in the presence or absence of TNFα (10 ng/ml) or EGTA (4 mM). Cells were incubated for 5 min at 37°C then Ig-opsonized Alexa-488 labeled *S. aureus* beads (10^7) were added to each well and cells were gently rocked at 37°C for 1 hr. Following, an equal volume (200 μl) of cold 2% paraformaldehyde was added to stop the reaction and total fluorescence was measured before and then after 5 gentle washes to remove unphagocytosed beads, using a Spectramax 2e. Data are plotted as the percent of fluorescence after the washes divided by the total, averaged for 6 wells each group (± SD). (C) WT and *stim1*$^{-/-}$ neutrophils were plated on plastic cover slips in 6 well tissue culture dishes and allowed to phagocytose Ig-opsonized Alex-488 labeled *S. aureus* beads as described above. After 1 hour, cells were fixed

with 2% cold paraformaldehyde and stained with rhodamin-pholoidin to visualize polymerized actin (as described in Zhang et al, 2006). Images were acquired in the green and red channels at 100X magnification using a Zeis Axiophot fluorescence microscope. (D) WT and *stim1*$^{-/-}$ neutrophils (2 X 10^5 per well) were plated on uncoated, fibrinogen or RGD peptide-coated microtiter plates in the presence or absence of fMLF (2.5 nM), TNFα (10 ng/ml), thapsigargin (100 nM), EGTA (4 mM) or phorbol 12-myristate 13-acetate (PMA) (1 ng/ml) for 90 minutes at 37°C. Cell supernants (100 μl) were removed and assayed for the secondary granule protein lactoferrin by ELISA assay as described (Mocsai, et al, 1999). Data are mean (± SD n=6 wells each) and are representative of at least 3 independent experiments. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ comparing WT to *stim1*$^{-/-}$ in each group.

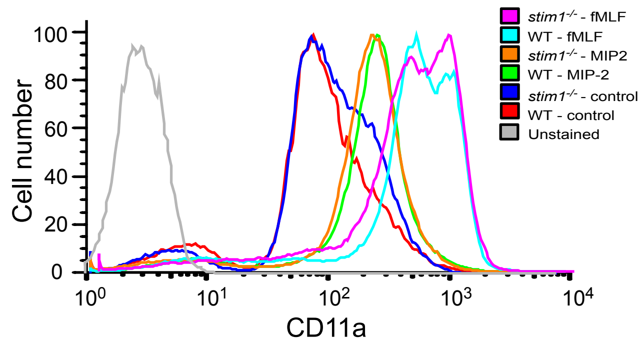
Supplemental Figure 3. Defective activation of the NADPH oxidase in *stim1*$^{-/-}$ can be rescued by PMA treatment. WT neutrophils (2 X 10^5/well) were plated in cytochrome C containing HBSS without Ca^{2+}, or with the indicated concentrations of Ca^{2+} added, in 96 well plates pre-coated with 0.2% milk in the presence of fMLF (2.5 nM) (A) or fibrinogen 10 μg/ml in the presence of TNF-α (B). Reduction of cytochrome C was monitored in a Spectramax plate reader at 37°C over the indicated time period. (C) WT neutrophils were plated on fibrinogen (10 μg/ml) in the presence or absence of TNF-α (10 ng/ml) or fMLF (2.5 nM) and reduction of cytochrome C was monitored. The PLC inhibitor U73122 was added where indicated. WT and *stim1*$^{-/-}$ neutrophils were plated on fibrinogen (10 μg/ml) in the presence or absence of TNF-α (10 ng/ml) (D) or 0.2% milk in the presence of fMLF (2.5 nM) (E) and reduction of cytochrome C was monitored. (F) WT and *stim1*$^{-/-}$ neutrophils were plated in cytochrome C containing HBSS with 3 mM Ca^{2+} and PMA (1 ng/ml), in 96 well plates pre-coated with 2% milk and cytochrome

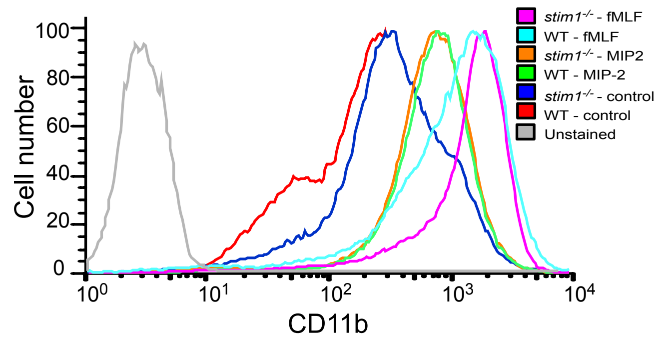
C reduction was monitored. Data are mean (± SD n=3 wells each) and are representative of at least three independent experiments.

Supplemental Figure 4. STIM1-deficient neutrophils migrate normally into the ischemic liver. (A) WT or *stim1*$^{-/-}$ chimeric mice were subjected to liver ischemia/reperfusion injury by ligation of the portal vein and hepatic artery for 30 min followed by 3 hours of reperfusion. Mice were then sacrificed and liver sections were immunostained with anti-Gr-1 to reveal neutrophils in the liver parenchyma. Liver sections from mice not subjected to ischemia/reperfusion injury showed virtually no Gr-1+ cells (not shown).

Supplemental Fig. 1

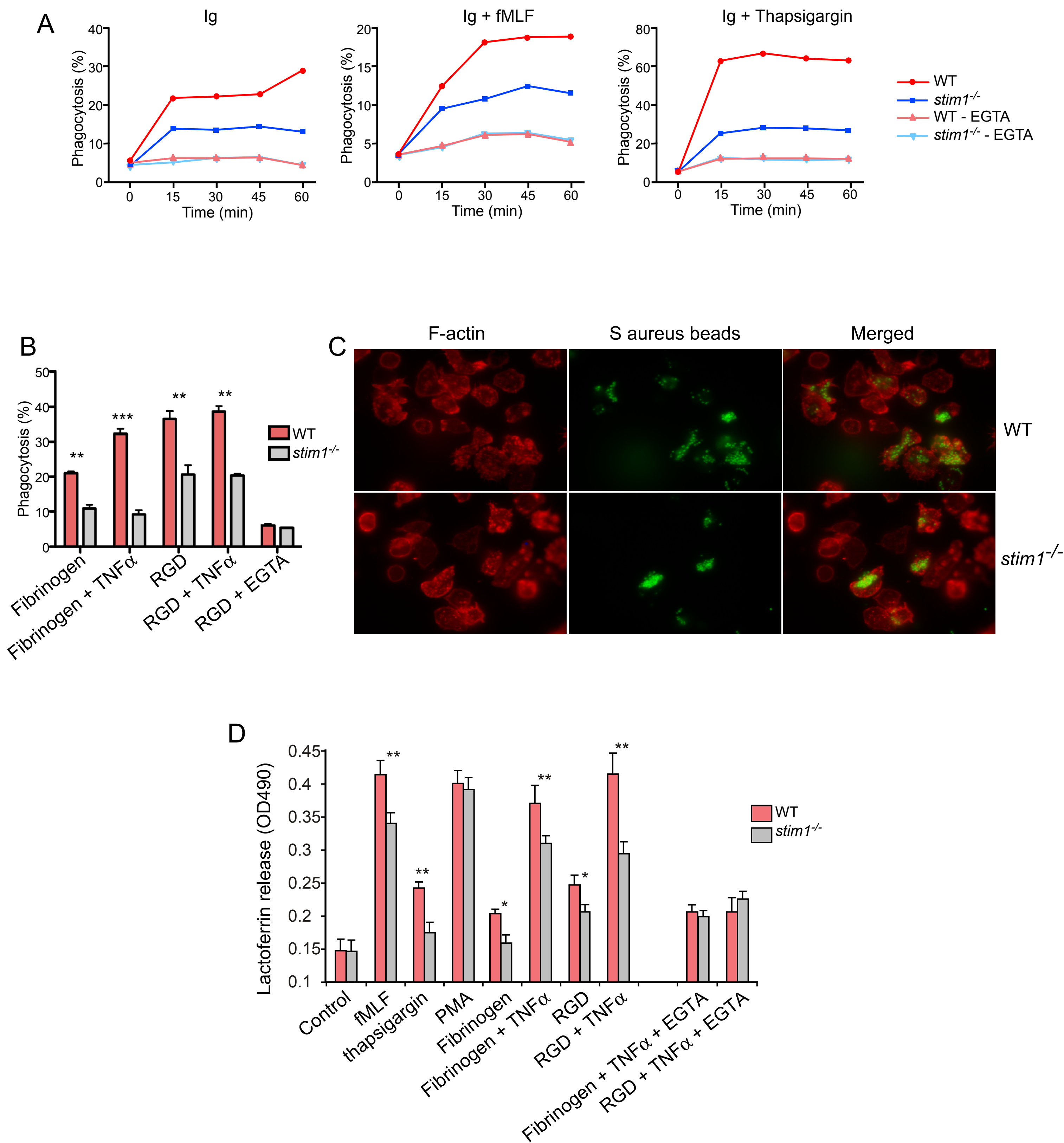
Zhang et al





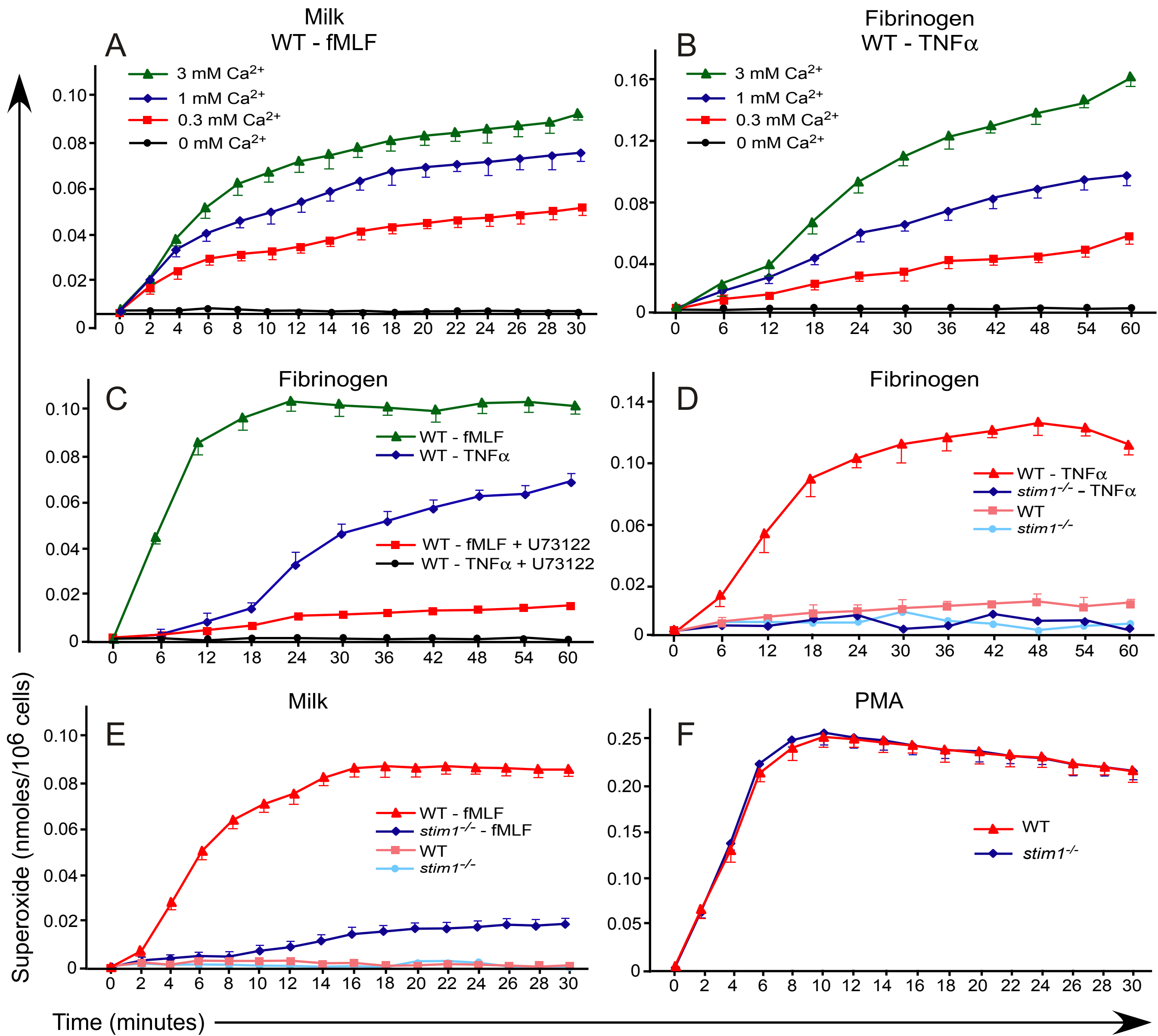
Supplemental Fig. 2

Zhang et al



Supplemental Fig. 3

Zhang et al



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Supplemental Fig. 4

Zhang et al