**Supplemental Data**

**Supplemental Methods**

*Antibodies and Reagents*

Recombinant murine MIP-2, TNFα (both from PeproTech Inc, NJ) and chemoattractant peptide fMLF (Sigma Aldrich, St. Louis, MO) were used at the indicated concentrations. For flow cytometry, anti-mouse mAbs against CD11b (M1/70) and Ly6G (IA8) were obtained from BD Bioscience (San Jose, CA). Antibodies used for western blot include β-actin (13E5), HDAC1 (10E2), NFAT2 (D15F1), NF-κBp65 (C22B4), pErk1/2 (E10), pIKKα/β (16A6) (Cell Signaling Technology, Danvers, MA), STIM1, STIM2 (rabbit polyclonals, Sigma Aldrich). Media used for cell isolations and stimulations include Hank's buffered salt solution supplemented with 20mM HEPES (termed HBSS/H, UCSF Cell Culture Facility) and RPMI (Gibco).

*Neutrophil isolation and stimulation*

Neutrophils were isolated by two methods. For most assays, bone marrow neutrophils were obtained as previously described using a single step Percoll gradient[1]. For qPCR, a pure population of neutrophils was obtained by negative selection (MACS, Miltenyi Biotec) per the manufacturer's protocol. A > 95% pure neutrophil population was confirmed by flow cytometry (Ly6G (IA8) and CD11b (M1/70)). For stimulations, isolated neutrophils were resuspended in either HBSS/H with 1mM $CaCl_2$ and 0.5mM $MgCl_2$ or RPMI supplemented with 10% heat-inactivated FBS and penicillin/streptomycin.

*Superoxide measurement*

Neutrophil respiratory burst was measured by a luminol-based chemiluminescent assay as previously described[2]. Briefly, isolated neutrophils were suspended in HBSS/H supplemented with $CaCl_2$ (1mM) and $MgCl_2$ (0.5mM), in the presence of luminol (100 μM) and horseradish peroxidase (8 U/ml, both from Sigma Aldrich). Cells ($2 \times 10^5$) were placed in 96 well plates blocked with milk to inhibit integrin binding and stimulated in the presence of the indicated agonists, fMLF or zymosan (Sigma Aldrich). Chemiluminescence was measured in a Spectramax M5 plate reader at 37°C.

*Phagocytosis Assay*

Isolated neutrophils were incubated with serum-opsonized phRhodo Red *S. aureus* (0.25mg/ml, Molecular Probes) or zymosan particles (0.125mg/ml) for 30 minutes on ice or at 37°C. Reactions were stopped by addition of ice cold PBS and immediately analyzed by flow cytometry. Fluorescence of these particles increases in the lower pH of the phagosome so uptake of bacteria was quantified by percent of PE-positive neutrophils compared with control (ice).

*Degranulation Assays*

Isolated neutrophils ($2 \times 10^5$) were stimulated in 96 well plates for 30 minutes. Supernatants were removed and assayed for lactoferrin release (secondary granules)[3] by ELISA and CD11b upregulation (secretory granules) by flow cytometry.

*Western blotting*

Cell pellets were lysed directly in boiling 1x sample buffer (ThermoFisher) with 10% β-mercaptoethanol and boiled for 7 minutes. Equal cell number samples were separated by SDS-PAGE (Novex-NuPAGE systems) and transferred to PDVF membrane. Membranes were blocked in TBST with 5% BSA (MP Biomedicals) and then incubated in primary antibody overnight at 4°C. Membranes were washed and then incubated with secondary antibody conjugated to HRP (Cell Signaling). Blots were developed using chemiluminescent substrate (Supersignal West Dura, ThermoFisher) and then imaged on a Chemidoc MP imager (Biorad). Images were analyzed using ImageLab software (Biorad).

*Nuclear Fractionation*

Cellular fractions were obtained by sequential lysis of cell pellets ($4 \times 10^6$ cells) using a Subcellular Fractionation Kit (ThermoFisher) per the manufacturer's protocol.

*Real-time Quantitative PCR*

Total RNA was extracted using RNeasy Plus per the manufacturer's instructions with genomic DNA digestion and converted to cDNA (Biorad iScript Supermix). Real time quantitative PCR was performed using the StepOne Plus cycler (Applied Biosystems) and Taqman gene expression assays (Life Technologies). PCR primer efficiencies of target and reference genes were validated to be 100% +/- 5%. Data were normalized to expression of a reference gene (β2-microglobulin or HPRT).

Primer Assay ID:

*Tnfα* - Mm00443258_m1

*Il10* - Mm01288386_m1

*Il6* - Mm00446190_m1

*Cxcl1* - Mm01701838_m1

*Stim1* – Mm01158413_m1

*Stim2* – Mm01223103_m1

*Statistics*

Data shown are expressed as mean +/- SEM of at least 3 independent experiments unless otherwise indicated. Data were graphed using Prism GraphPad software and figures prepared using Adobe Illustrator. Groups were compared using paired or unpaired 2-tailed Student's T-test or 2-way Anova with Tukey's adjustment for multiple comparisons. A p-value was considered to be significant when $p < 0.05$.

**Supplemental Figure 1.** ***Quantifications of SOCE in STIM-deficient neutrophils.*** A) Schematic demonstrating segments of calcium curve used for quantification. Segment 1 – baseline. AUC of this segment represents the relative resting cytosolic calcium concentrations. Segment 2 – ER store release. Segment 3 – initial slope of rise in calcium concentration after addition of calcium. Segment 4 – SOCE, AUC of segment. B) Bone marrow cells loaded with Indo-1 and labeled for Ly6G were stimulated with

the indicated doses of MIP2 or Fcγ-R crosslinking. Cells were stimulated first in calcium-free media to analyze ER $Ca^{2+}$ store release, followed by re-addition of extracellular calcium. C) Analysis of SOCE in neutrophils from WT and *Stim1/2*$^{VavCre}$ mice stimulated with the indicated agonist. D) Quantification of SOCE (AUC of segment after addition of 1mM $Ca^{2+}$) and (E) ER store release (area under the curve (AUC) of cells stimulated in 0mM $Ca^{2+}$); mean +/- SEM from 3-4 independent experiments. (*) $p < 0.05$. F) Quantification of segment 3, slope of rise in calcium concentration. Data represent mean +/- SEM of 3-4 experiments. G) Comparison of the effect of STIM2 deficiency at lower vs higher doses of agonist (Thapsigargin 50nM and 200nM, Ionomycin 15nM and 75nM, fMLF 300nM and 1μM, MIP2 2ng/ml and 50ng/ml, Fcγ-R crosslinking 15μg/ml and 30μg/ml). Data represent mean +/- SEM of 3-4 experiments. (*) $p < 0.05$. H) Calcium flux in cells stimulated with incubated with zymosan on ice in media containing 1mM $Ca^{2+}$ then placed in a 37°C water bath during flow cytometry to induce stimulation. Control cells were incubated on ice then subjected to temperature change alone.

**Supplemental Figure 2.** ***Normal degranulation and migration in STIM2-deficient neutrophils.*** A) Primary granule release was determined by surface staining for CD11b in neutrophils stimulated with zymosan (100μg/ml). B-C) Secondary granule release was assessed by lactoferrin release. Neutrophils from WT, *Stim1*$^{VavCre}$, *Stim2*$^{VavCre}$, and *Stim1/2*$^{VavCre}$ mice were stimulated with fMLF (B) or zymosan (C) at the indicated concentrations and lactoferrin levels in the supernatant were measured by ELISA. D) Calcein AM- labeled bone marrow neutrophils were allowed to migrate through 3μM transwells coated with 10% FCS in response to fMLF or MIP2 at the indicated concentrations. Data are represented as fold increased migration over baseline (media alone). E) TNFα-induced migration. 10ng of TNFα was injected into an airpouch created on the dorsum of WT, *Stim1*$^{VavCre}$, *Stim2*$^{VavCre}$, and *Stim1/2*$^{VavCre}$ mice. The pouch was lavaged at 4 hours and neutrophils identified by staining with Ly6G and CD11b. Mean +/- SEM representative of 2 independent experiments.

**Supplemental Figure 3.** ***Calcium-dependent neutrophil cytokine production.*** A) Neutrophils were stimulated with zymosan (100μg/ml), with or without the calcium chelator BAPTA-AM (10μM), for 4 hours and TNFα levels in the supernatant were determined by ELISA. B) Negative control: cytokine staining of unstimulated cells demonstrating set point for "cytokine +" gate. C) Whole bone marrow cells ($1 \times 10^6$) from WT, *Stim1*$^{VavCre}$, *Stim2*$^{VavCre}$, and *Stim1/2*$^{VavCre}$ mice incubated with low-dose zymosan (20μg/ml) for 5 hours. Neutrophils were identified by staining with Ly-6G and CD11b and

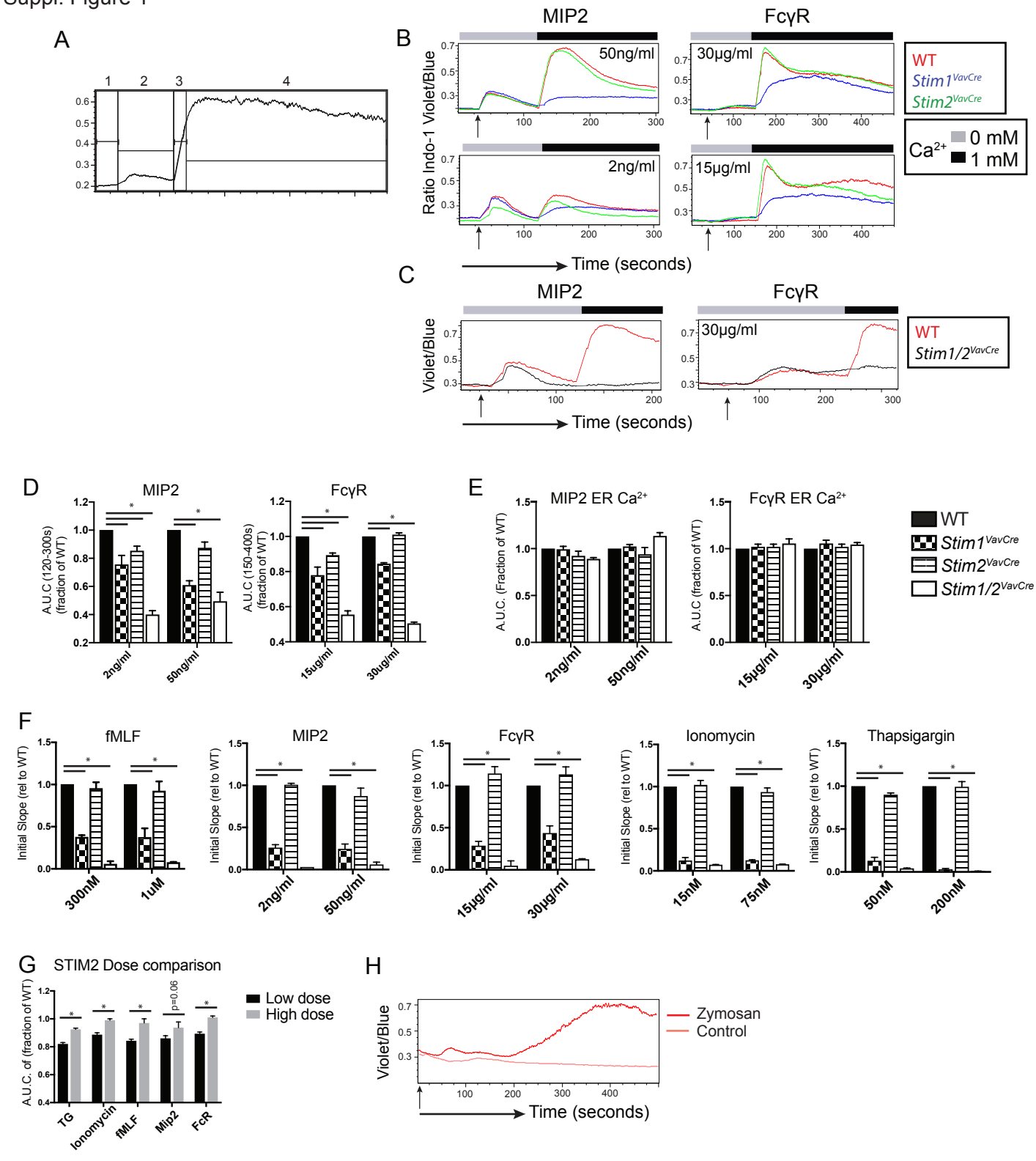
cytokine production measured by intracellular staining for TNFα. D) Percentage of live neutrophils in cytokine stimulation assays as determined by exclusion of a fixable live/dead Aqua stain. Mean +/- SEM compiled from 3 independent experiments.

**Supplemental Figure 4.** ***Phorbol ester PMA rescues ROS production in STIM-deficient neutrophils.*** A-B) Analysis of ROS production in WT, $Stim1^{VavCre}$, $Stim1/2^{VavCre}$ (A) and $Stim2^{VavCre}$ neutrophils (B) stimulated with PMA (100nM).

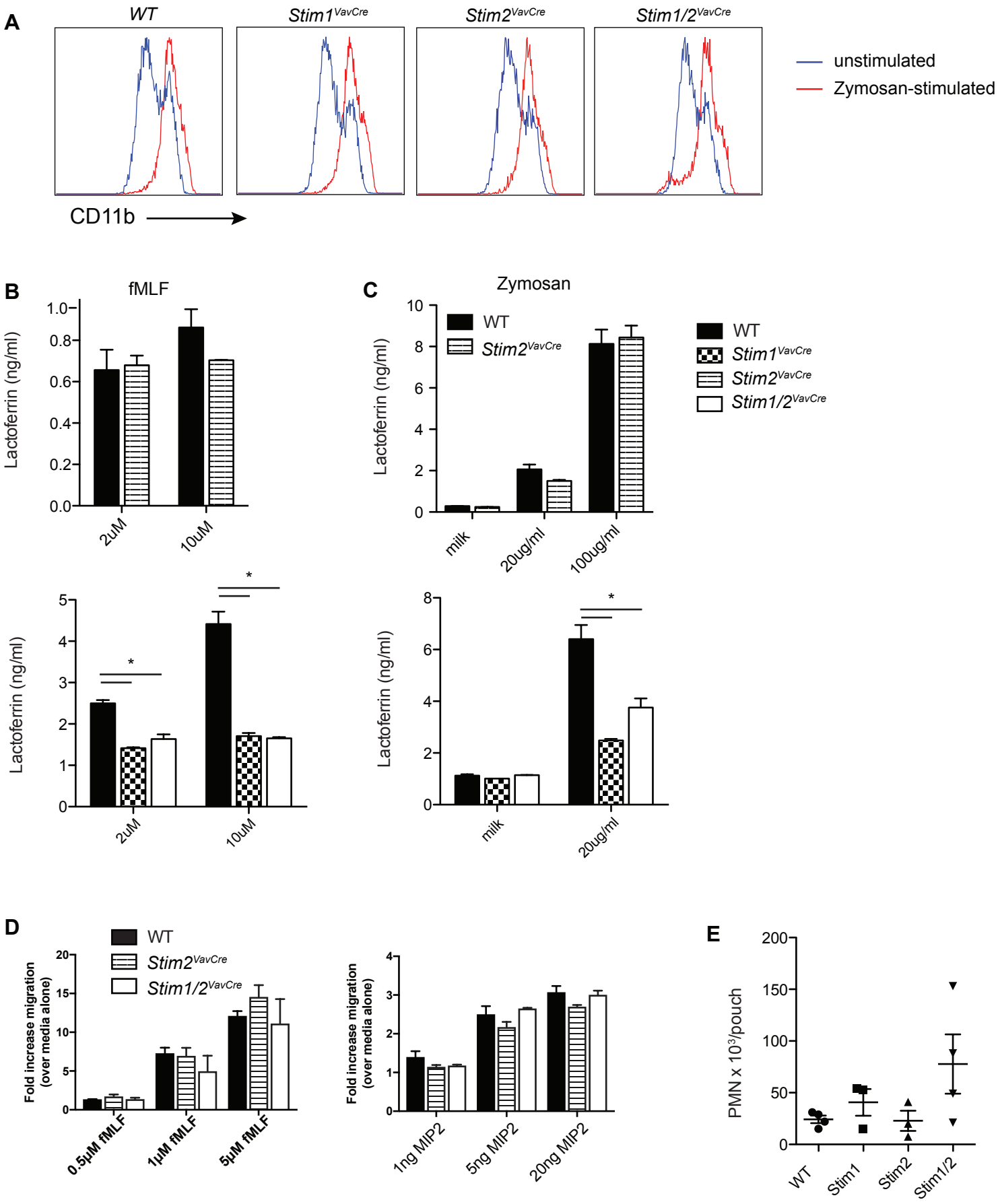
**Supplemental references**


1. Mócsai A, Zhou M, Meng F, Tybulewicz VL, Lowell CA. Syk Is Required for Integrin Signaling in Neutrophils. *Immunity.* 2002;16(4):547-558.
2. Zhang H, Clemens RA, Liu F, et al. STIM1 calcium sensor is required for activation of the phagocyte oxidase during inflammation and host defense. *Blood.* 2014;123(14):2238-2249.
3. Mocsai A, Ligeti E, Lowell CA, Berton G. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J Immunol.* 1999;162(2):1120-1126.
4. Mocsai A, Abram CL, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol.* 2006;7(12):1326-1333.

Suppl. Figure 1



Suppl. Fig 2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2uM
10uM
C
Zymosan
WT
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100ug/ml
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Stim1
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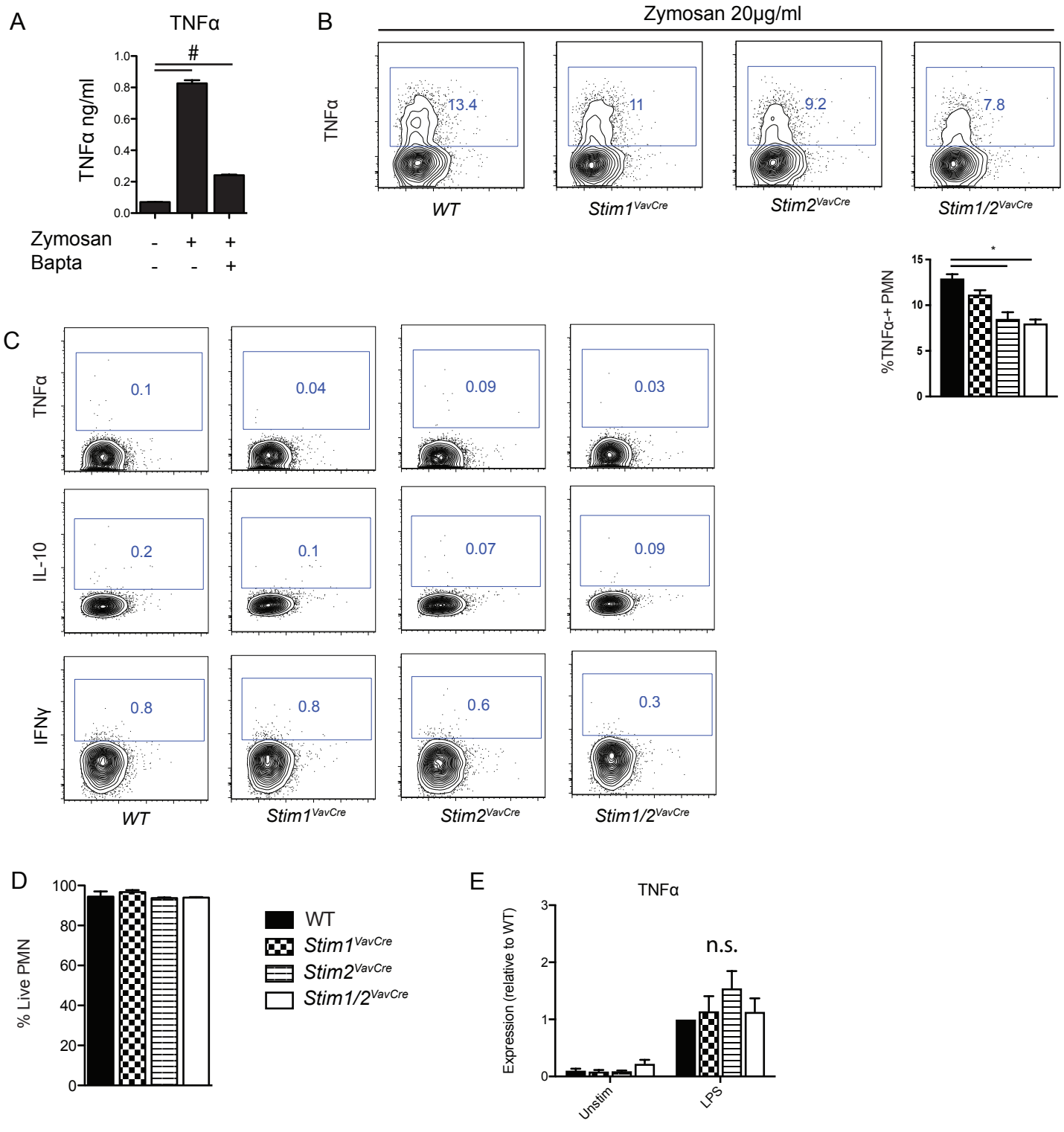
Suppl. Fig 3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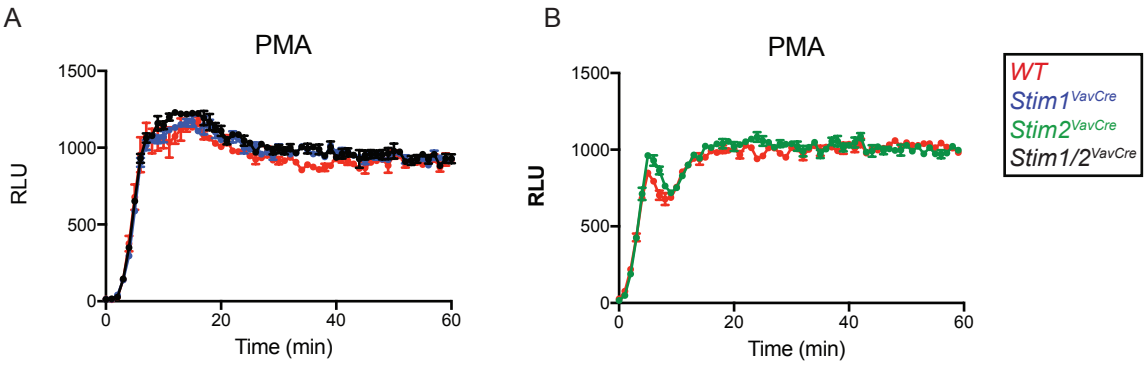
Suppl Fig 4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