SI, Materials and Methods

Macrophages and Mice

C57BL/6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice were obtained from Jackson Laboratory. *Casp11*$^{-/-}$ mice were kind gifts from Dr. Vishva Dixit (Genentech)(1). Animals were housed under protocol approved by the Tufts University Medical School Animal Care and Use Committees. Bones from *Casp3*$^{-/-}$, *Casp3*$^{-/-}$*Casp7*$^{-/-}$, *Casp9*$^{-/-}$ were generated and provided by Dr. Anthony Rongvaux(2). Bones from *Gsdmd*$^{-/-}$ mice and *Rip3*$^{-/-}$ mice were gifts from Dr. Kate Fitzgerald, generated by Dr. Vishva Dixit(3). Bones from *Rip3*$^{-/-}$*Casp8*$^{-/-}$ mice were given by Dr. Kate Fitzgerald, generated by Dr. Doug Green(4). Bones from *Ripk1*$^{K45A/K45A}$, *Ripk3*$^{K51A/K51A}$ mice were gifts from Dr. Alexi Degterev and generated by Dr. John Bertin and Dr. Peter J Gough from GlaxoSmithKline (PA, USA)(5, 6). Immortalized WT and *GsdmE*$^{-/-}$ macrophages were a gift from Dr. Emad Alnemri(7). *GsdmE*$^{-/-}$ and *GsdmD*$^{-/-}$*GsdmE*$^{-/-}$ mice were generated by Dr. Feng Shao(8), maintained at the National Institute of Biological Sciences, Beijing, China. Bone marrow-derived macrophages were isolated from mice and propagated for 7 days in RPMI containing 20% FBS, 30% L cell supernatant, 2% Penn/Strep on non-tissue culture treated Petri dishes. Unless otherwise noted, cells were plated at a density of 1x 10^6 cells per cm^2 for experiments in RPMI containing 10% FBS, no antibiotics were used during infection.

Inhibitors and TLR agonists

Lipopolysaccharide (LPS) *S. minnesota* R5 [10ng/ml] and 5z7 [125nM] were purchased from Sigma. For LPS transfections, we used *E. coli LPS* 0111:B4 [2ug/ml] and FugeneHD from Promega according to manufacturer suggestions. zVAD.fmk was purchased from Millipore and used at 50uM. Necrostatin-1 [10uM], Etoposide [150 uM] and Raptinal [10uM] were purchased

from Sigma. IKKβ inhibitors TPCA-1 [50 uM], BMS 345541 [6.25 uM], IMD 0354 [25 uM] and IKK-16 [5 uM]; MAPK inhibitors SB203580 (p38) [10 uM], SP600125 (JNK) [50 uM] and U0126 (MEK1/2) [10 uM] were purchased from Cayman Chemical. LTA [2ug/ml], P3CSK [1ug/ml], PI:C [20ug/ml], IMQ [5ug/ml], CL097 [1ug/ml] and CpG [500 uM] were purchased from InvivoGen. SM-164 [1uM] was purchased from ApexBio.

Human macrophages

De-identified human peripheral blood was obtained from New York Biologics. The use of de-identified human samples followed a protocol approved by the Tufts University School of Medicine Institutional Review Board. Peripheral blood mononuclear cells were isolated via Ficoll gradient. Monocytes were obtained from peripheral blood using the EasySep Direct Monocyte Isolation Kit (STEMCELL technologies). $CD14^{+}CD16^{-}CD68^{-}$ monocytes were extracted and differentiated into $CD14^{+}CD16^{-}CD68^{+}$ macrophages over the course of 7 days in RPMI containing 20% FBS, 200U/ml Penicillin and 200μg/ml Streptomycin. and 100 μg/ml of human monocyte colony stimulating factor (M-CSF; PeproTech)(9). Differentiated macrophages were cultured for 40 hours further in RPMI containing 10% FBS, 200U/ml Penicillin and 200μg/ml Streptomycin, in the absence of M-CSF prior to infection or agonist stimulation.

Time lapse microscopy and kinetic cytotoxicity assay

The Cytation3 automated microscope was used to maintain temperature at 37°C and 5% CO_2 for kinetic imaging of live cell cultures. Cells were seeded in 0.17mm thickness glass bottom imaging plates, in RPMI (Hyclone) media. For kinetic cytotoxicity assays, cells were imaged at 30 minute interviews with 4x magnification to capture 3000-4000 cells per field of view. Propidium iodide (10μg/mL, Life Technologies, P3566) was detected via 535 nm excitation and

617 nm emission, with individual puncta of 4um-10um in size counted as nuclei. For 100% cytotoxicity control, cells were treated with 0.1% Triton X-100, similar to protocols for measuring Lactate Dehydrogenase (Promega). AnnexinV binding requires media controlled at pH 7.2-7.5 (1mM HEPES) and supplemented up to 2mM $CaCl_2$. For kinetic image cytometry in detection of dual AnnexinV and PI positivity, cells were labeled with a total cell stain via Neuro-DiO (ex. 488) or Wheat Germ Agglutinin (ex. 350). AnnexinV was imaged on ex.350 or ex.488, depending on the total cell stain used. Images were taken at 4x magnification, every 2 minutes, and image stacks were analyzed on iVision, where cellular masks were generated via the total cell stain. Signal intensities of AnnexinV and PI were extracted from masked area, and signal intensity was plotted as numerical values to generate kinetic cytometry plots. 20x and 40x magnification images were taken under similar conditions. For Supplemental Figure 4C Live images of cell death of BMDMs were recorded with the PerkinELmer UltraVIEW spinning disk confocal microscopy and processed in the software Volocity. The image data shown were representative of at least four randomly selected fields, as previously described [55]. For Supplemental Figure 4D Cell death were measured by the LDH assay using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit brought from Promega.

Western blotting

At the desired time points post stimulation or infection, media supernatant was collected, and cells were lysed directly in 1X Laemmli Buffer with 5% β-mercaptoethanol, boiled for 10 minutes, and incubated on ice for 10 min prior to loading on SDS PAGE gels. Supernatant proteins were precipitated via methanol/chloroform extraction, and precipitated proteins were resuspended and denatured in 1X Laemmli Buffer with 5% β-mercaptoethanol. Primary

antibodies against caspase-3, caspase-7, caspase-8, caspase-9, CypA, Gapdh, and PARP were purchased from Cell Signaling Technologies. Antibodies to Gasdermins were purchased through Abcam: anti-mouse GsdmD (ab209845), anti-human GsdmD (ab210070) and anti-human/mouse GsdmE (ab215191). Total MLKL antibody was purchased from Millipore (#MABC604). Phospho-MLKL- S345 (#ab196436) antibody purchased from Abcam. Total p38 (9228), phospho-p38 T180/Y182 (4511), total ERK (4696), phospho-ERK T202/Y204 (4370), phospho-IKKα/β Ser176/180 (2697), IKKα (11930), IKKβ (8943), p-NF-κB p65 Ser536 (3033), NF-κB p65 (8242) and IκB-α (4814) were purchased from Cell Signaling Technologies.

ELISA for cytokine secretion

Murine IL-1α, IL-β, TNFα ELISAs, and human IL-1α, IL-1β, TNFα ELISAs were DuoSet ELISA kits purchased from R&D, used according to manufacturer's instructions.

TUNEL Assay

TUNEL staining was performed using the Click-iT™ TUNEL Alexa Fluor™ 488 Imaging Assay Kit (C10245) from Invitrogen. Cells were seeded in 0.17mm thickness glass bottom imaging plates, in RPMI (Hyclone) media. Indicated treatments were added at 10-minute intervals for 220 minutes. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% TritonX-100 in PBS, and stained according to the manufacturer's protocol. Nuclei were stained with Hoescht 33342 (1:10,000). Cells were imaged using the Cytation3 automated microscope with 4x and 20x magnification. The percent of TUNEL (GFP) positive cells were calculated by dividing the number of GFP+nuclei/Total DAPI+ nuclei.

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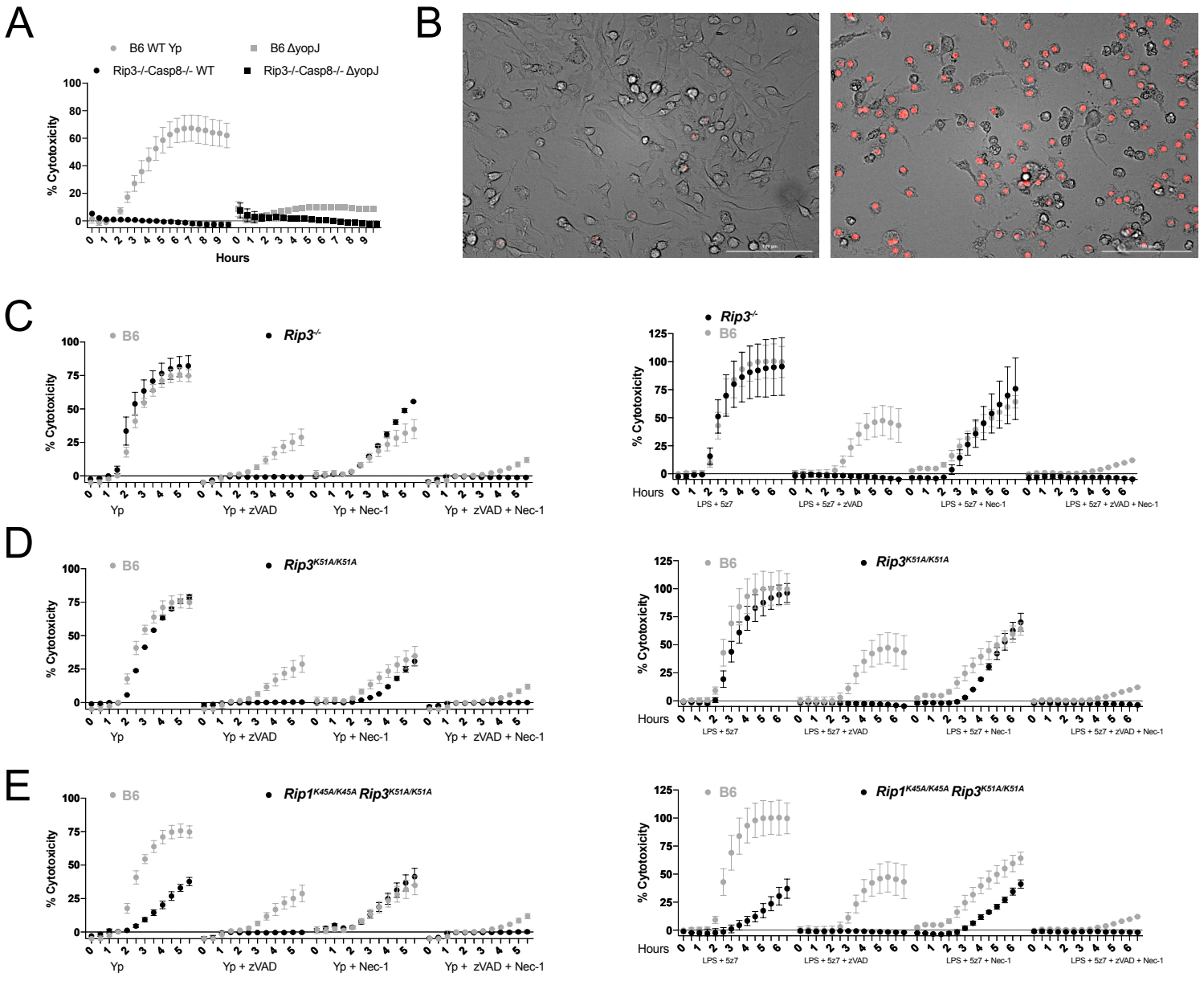
2. Rongvaux A*, et al.* (2014) Apoptotic caspases prevent the induction of type I interferons by mitochondrial DNA. *Cell* 159(7):1563-1577.
3. Newton K, Sun X, & Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 24(4):1464-1469.
4. Oberst A*, et al.* (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 471(7338):363-367.
5. Mandal P*, et al.* (2014) RIP3 induces apoptosis independent of pronecrotic kinase activity. *Mol Cell* 56(4):481-495.
6. Berger SB*, et al.* (2014) Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *J Immunol* 192(12):5476-5480.
7. Rogers C*, et al.* (2017) Cleavage of DFNA5 by caspase-3 during apoptosis mediates progression to secondary necrotic/pyroptotic cell death. *Nat Commun* 8:14128.
8. Wang Y*, et al.* (2017) Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. *Nature* 547(7661):99-103.
9. Jaguin M, Houlbert N, Fardel O, & Lecureur V (2013) Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol* 281(1):51-61.

Legends for Supplemental Videos

Supplemental Video 1, related to Figure 3. AnnexinV and PI time lapse during LPS/zVAD induced necroptosis
Full time course of LPS/zVAD induced necroptosis in B6 BMDMs as shown in Figure 3F. Images were taken with Cytation3 automated microscope, at 4x magnification. Images stacks are processed with iVision (BioVision) software. AnnexinV and PI signal were extracted with iVision and plotted on the Y and X axis, respectively. Each frame of the movie are images taken at 2 minutes apart, up to 5 hours post stimulation.

Supplemental Video 2, related to Figure 3. AnnexinV and PI time lapse during LPS/zVAD induced necroptosis
Full time course of LPS/5z7 induced pyroptosis in B6 BMDMs as shown in Figure 3G. Images were taken with Cytation3 automated microscope, at 4x magnification. Images stacks are processed with iVision (BioVision) software. AnnexinV and PI signal were extracted with iVision and plotted on the Y and X axis, respectively. Each frame of the movie are images taken at 2 minutes apart, up to 5 hours post stimulation.

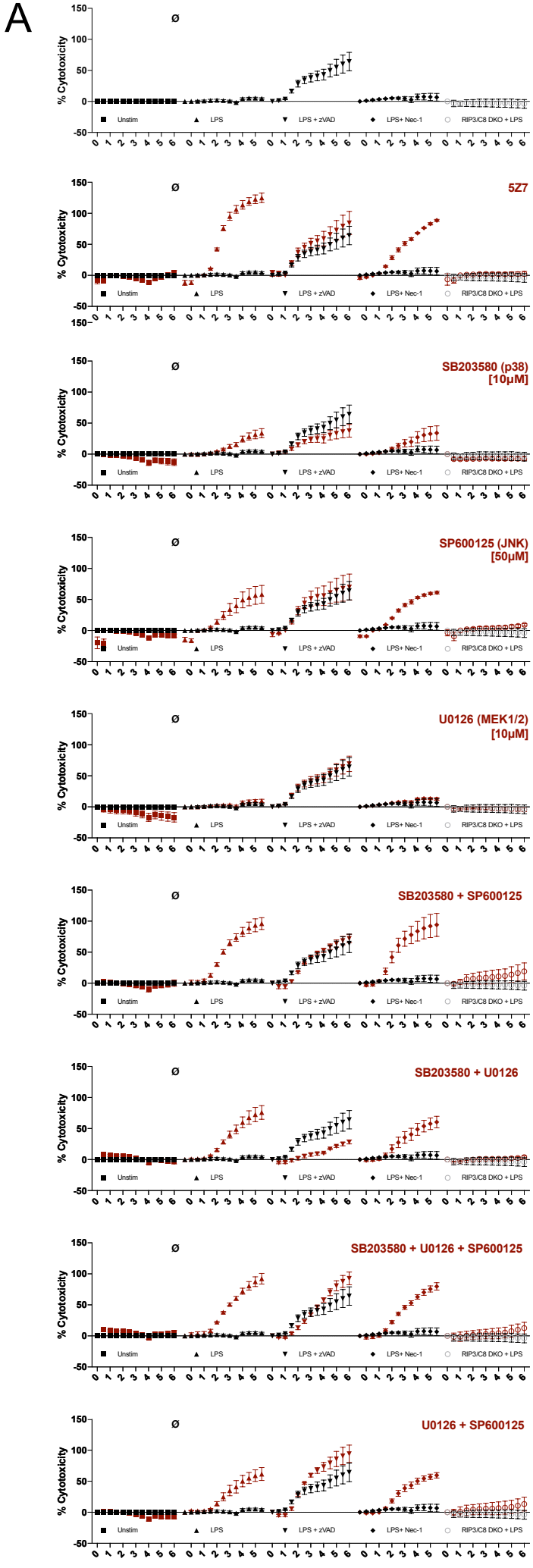


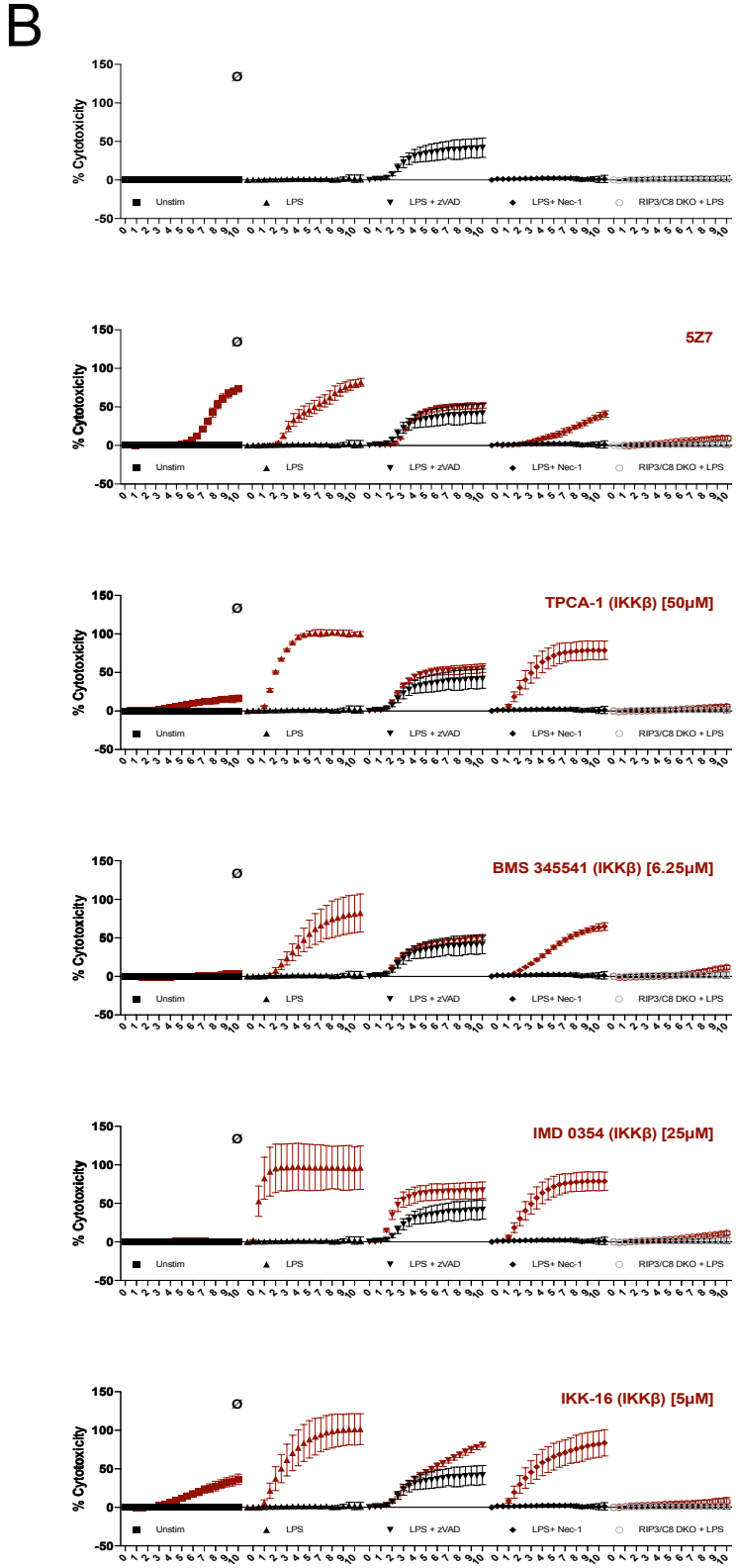
SFig 1A. Related to Figure 1

(A) Bone marrow derived macrophages from C57BL/6 (B6) and *Rip3*$^{-/-}$*Casp8*$^{-/-}$ animals were infected with *Yersinia pseudotuberculosis* IP2666 at MOI of 30. Kinetic cytotoxicity monitored via nuclear incorporation of propidium iodide, images of cell culture taken every 30 minutes.

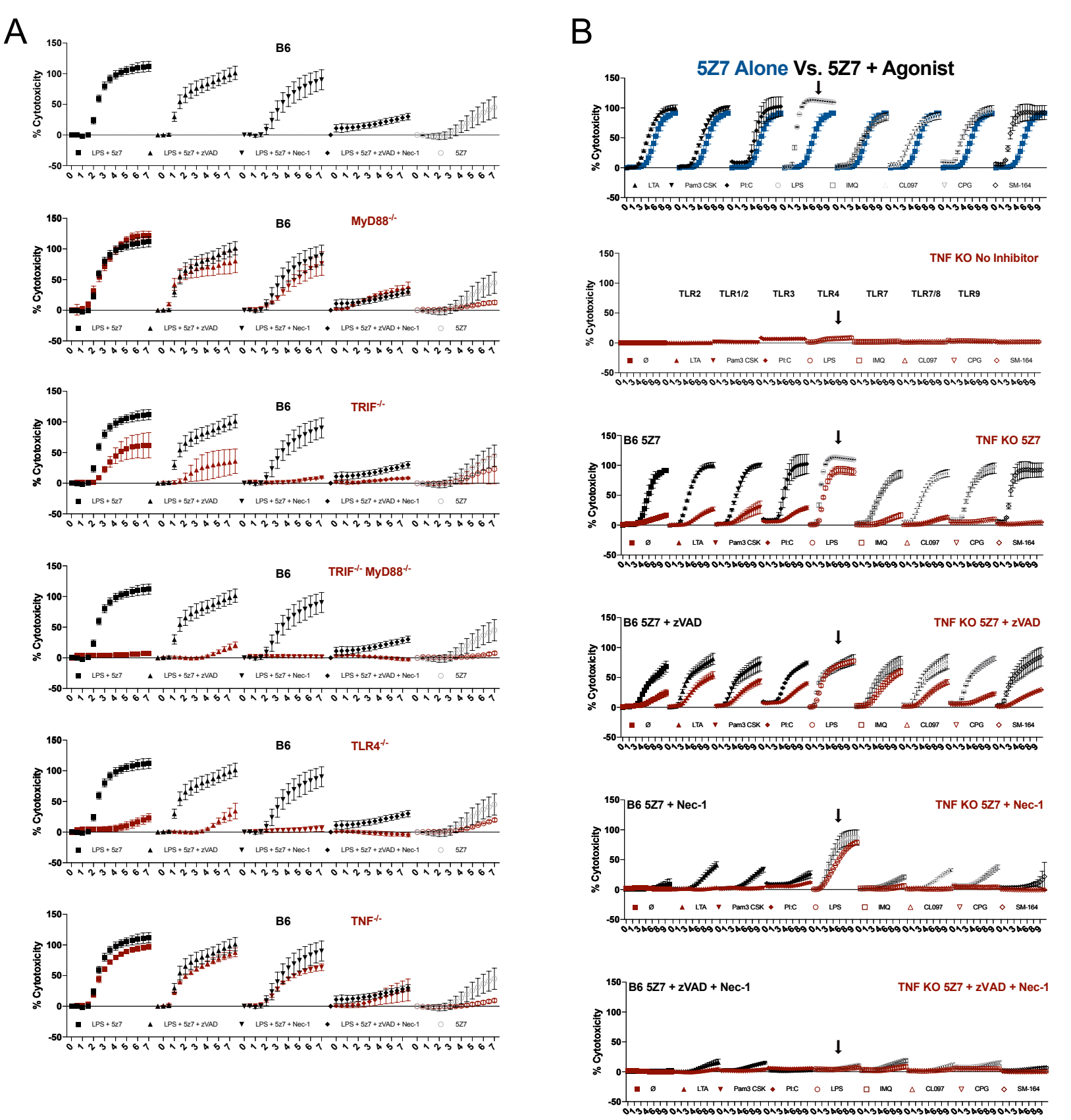
(B) B6 BMDMs are stimulated with LPS [10ng/ml] /5z7 [125nM], representative image of propidium iodide positive cells from unstimulated and 4 hour stimulated cells are shown. Current image at 20x magnification to show cellular morphology and nuclear propidium iodide, scale bar measures 100um.

(C - E) BMDMs from C57BL/6 (B6) and various genetically modified animals were stimulated with *Yersinia pseudotuberculosis* on the left, or LPS [10ng/ml] /5z7 [125nM] on the right. Percentage cytotoxicity was calculated by microscopy via counts of propidium iodide positive nuclei per field of view, normalized to 100% lysis by 0.1% Triton-X. Caspases were inhibited with zVAD treatment [50 uM], and RIP kinase activity was inhibited with Necrostatin-1 (Nec1) [10uM]. All inhibitors were added simultaneously at time zero. All kinetic cytotoxicity data are representative of three or more experiments. See related Figure 1.

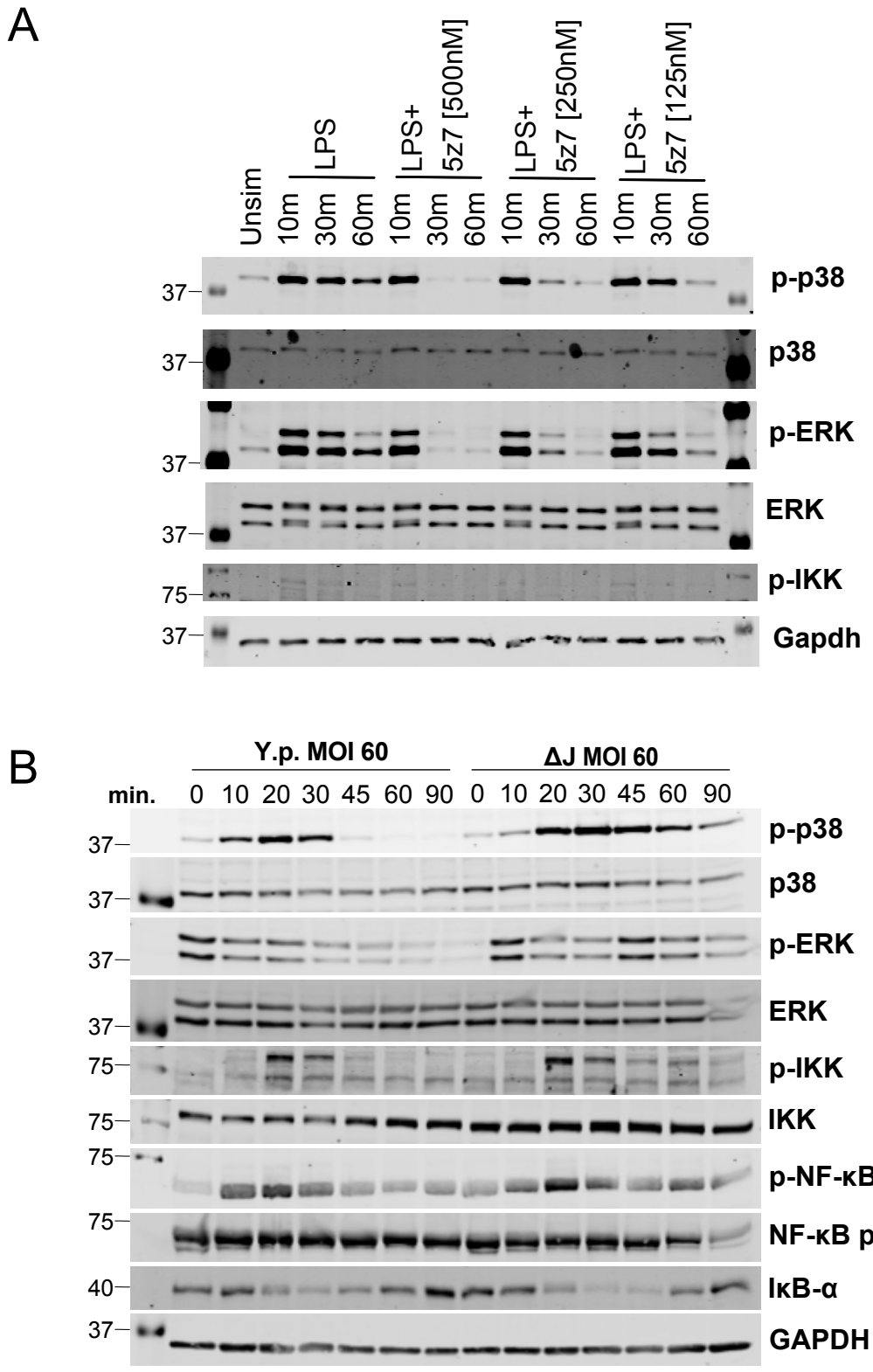




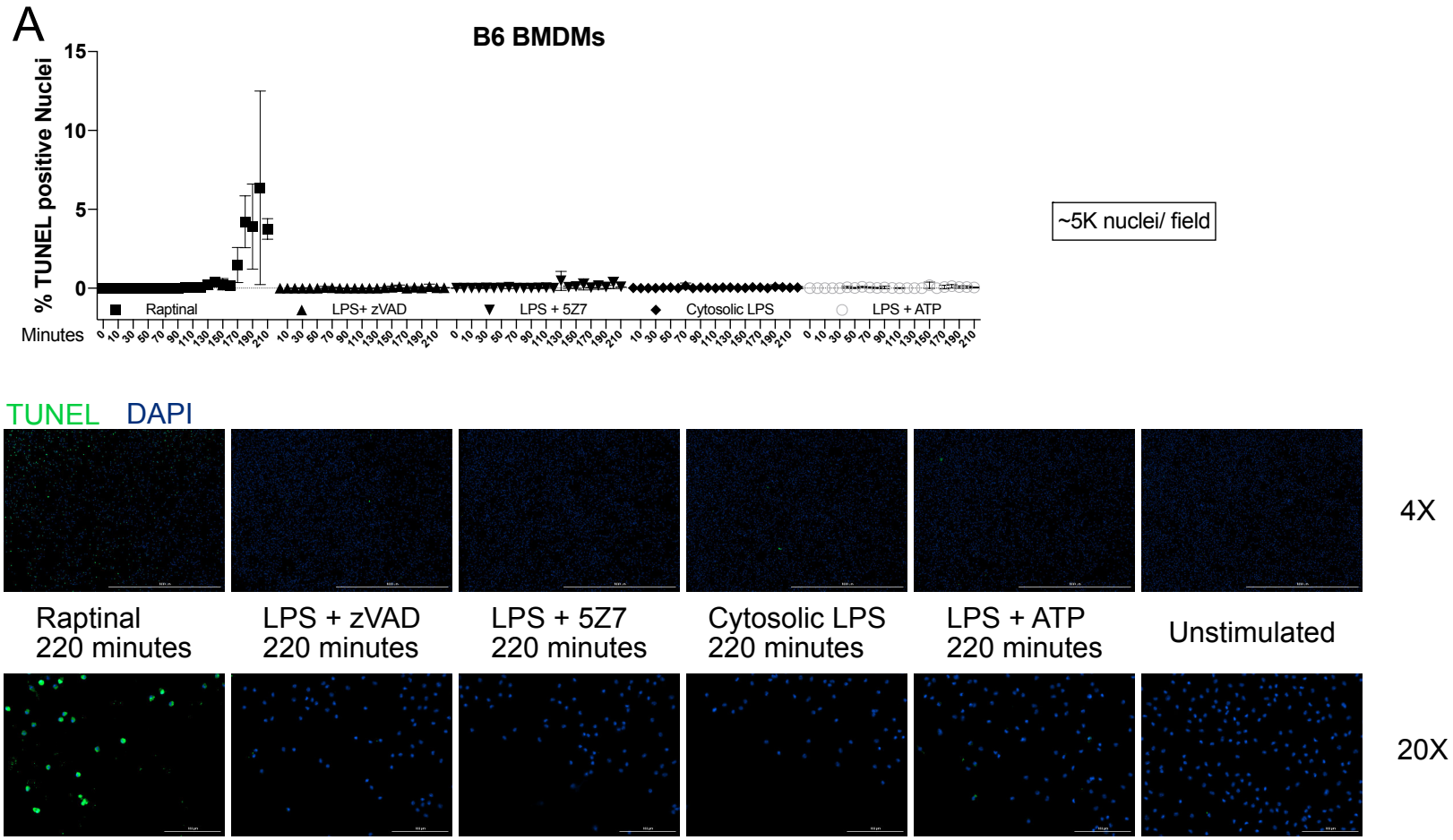
SFig 1.2. Related to Figure 1
C57Bl/6 (B6) and *RIP3*$^{-/-}$*Casp8*$^{-/-}$ bone marrow derived macrophages were treated with LPS, LPS +zVAD or LPS + Nec-1 +/- 5z7 or indicated MAPK (**A**) and IKKβ (**B**) inhibitors or combinations of inhibitors (red). Kinetic cytotoxicity monitored via nuclear incorporation of propidium iodide, images of cell culture taken every 30 minutes.



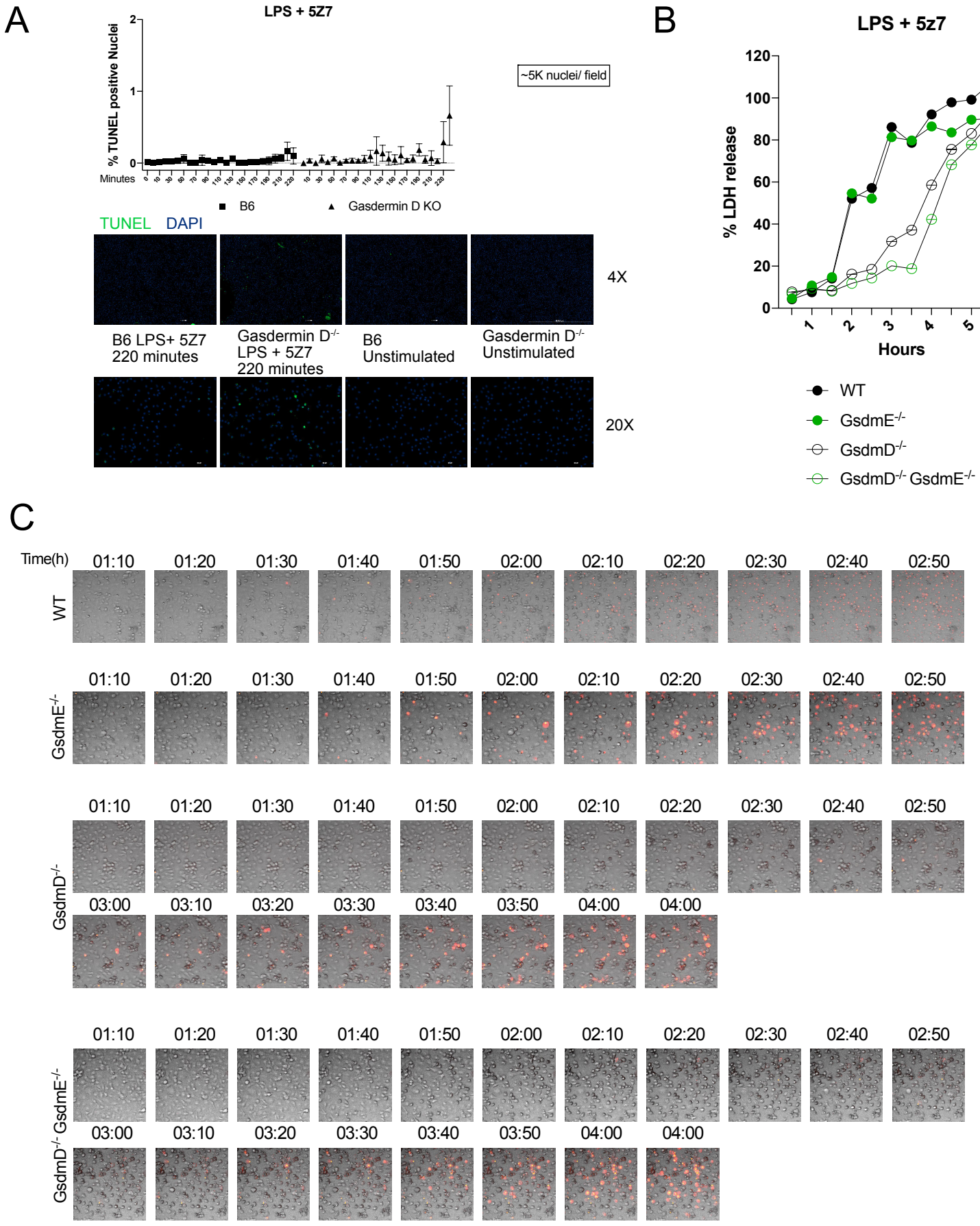
SFig 1.3. Related to Figure 1
(**A**) Bone marrow derived macrophages from C57Bl/6 (B6), *Ticam1*$^{-/-}$ (TRIF), *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$*Myd88*$^{-/-}$, *Tlr4*$^{-/-}$, and *Tnf*$^{-/-}$ were treated with LPS + 5z7, LPS + 5z7 +zVAD, LPS +5z7 + Nec-1, LPS + 5z7 + zVAD + Nec-1 or 5z7. (**B**) C57Bl/6J and *Tnf*$^{-/-}$ (red) BMDMs were treated with no inhibitor, 5z7, 5z7 + zVAD, 5z7 + Nec-1 or 5z7 + zVAD +Nec-1 in the presence of various agonists: LTA (2ug/ml), P3CSK (1ug/ml), PI:C (20ug/ml), LPS (10 ng/ml, indicated by arrow), IMQ (5ug/ml), CL097 (1ug/ml), CpG (500uM), and SM-164 (1uM). Kinetic cytotoxicity monitored via nuclear incorporation of propidium iodide, images of cell culture taken every 30 minutes.



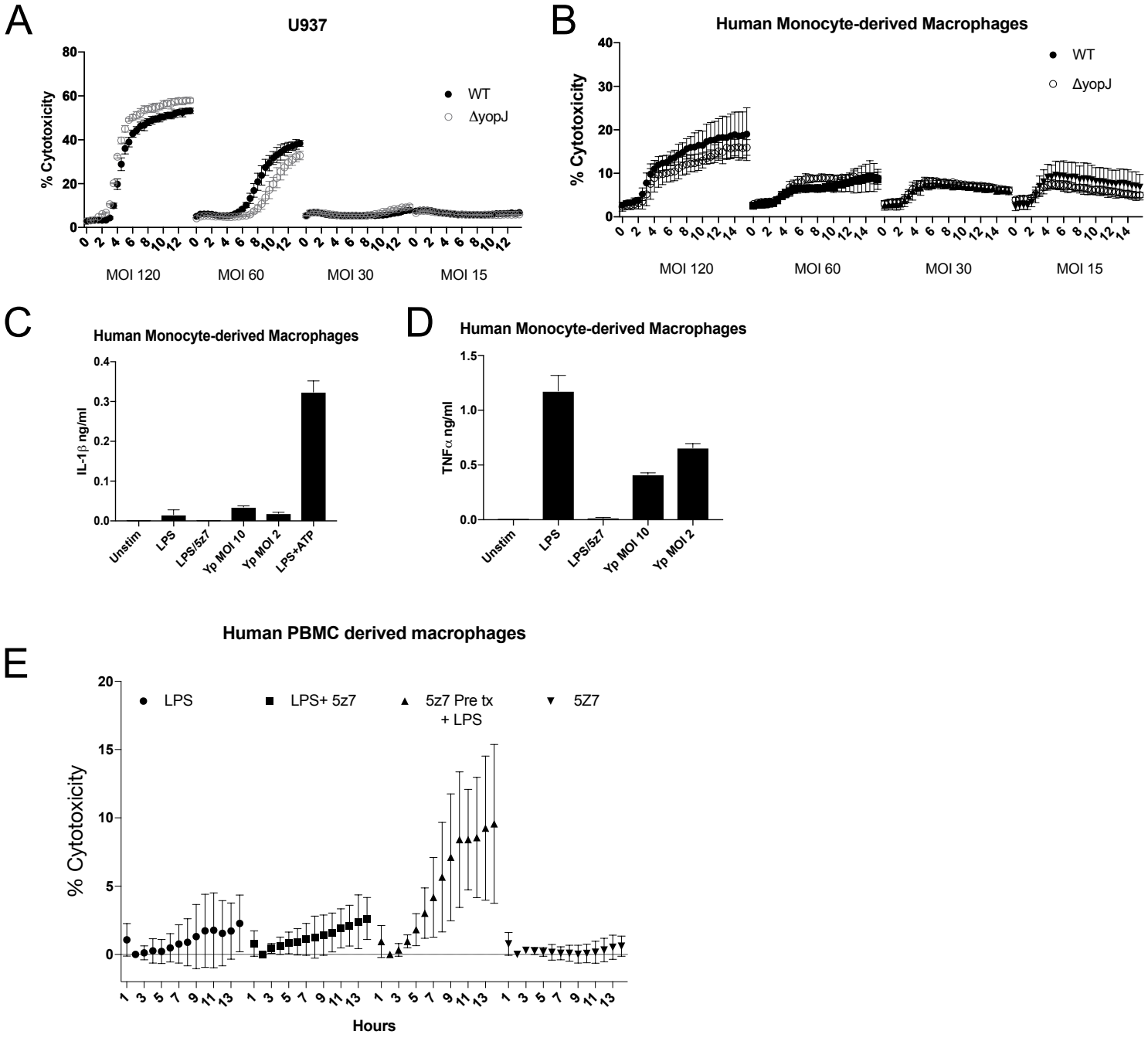
SFig 2. Related to Figure 2
(**A**) B6 BMDMs were stimulated with LPS either with or without indicated concentration of 5z7 and celluar lysates were analyzed at 10, 30 and 60 minutes for indicated proteins by western blotting. (**B**) *RIP3*$^{-/-}$*Casp8*$^{-/-}$ macrophages were stimulated with wild type or Δ*yopJ* (ΔJ) *Yersinia pseudotuberculosis* at an MOI of 60 for indicated time points. Cellular lysates were analyzed by western blot for MAPK (p38, ERK) and NF-κB (IKKα/β, NF-κB p65, IκB-α) pathway components.



SFig 3. Related to Figure 3
(A) C57Bl/6 (B6) BMDMs were simulated with Raptinal, LPS + zVAD, LPS + 5z7, Cytosolic LPS or LPS+ATP at 10 minute intervals for 220 minutes. Quantification of percentage of TUNEL positive nuclei (GFP+/DAPI+) overtime, and representative 4x and 20x images of TUNEL staining at 220 minutes after indicated treatment.



SFig 4. Related to Figure 4
(A) C57Bl/6 (B6) and GsdmD-/- BMDMs were simulated with LPS + 5z7 at 10 minute intervals for 220 minutes. Quantification of percentage of TUNEL positive nuclei (GFP+/DAPI+) overtime, and representative 4x and 20x images of TUNEL staining in unstimulated and 220 min LPS + zVAD stimulated cells. **(B-C)** BMDMs from WT, GsdmD-/-, GsdmE-/-, and GsdmD-/-GsdmE-/- animals were stimulated with LPS + 5z7, extracellular LDH release was quantified over time (**B**), and spinning disk microscopy of propidium iodide cellular incorporation (red) over time (**C**).



SFig 5. Related to Figure 6
(A - B) *Yersinia* induced death of PMA activated U937 cells (**A**) or human peripheral blood mononuclear cell (PBMC) derived macrophages **(B)**. Bacteria were infected at multiple MOI, with no YopJ dependent killing observed. **(C)** Supernatant IL-1β and TNF (**D**) from human monocyte derived macrophages stimulated with LPS/5z7 or Yersinia at MOI that elicit robust IL-1 secretion from murine macrophages. (**E**) Human PBMC derived macrophages were stimulated with indicated conditions and monitored for cytotoxicity over time. The 5z7 pre treatment condition is for 2 hours prior to LPS stimulation.
All kinetic cytotoxicity data, and ELISA are representative of three or more experiments.