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Cell-Extrinsic TNF Collaborates with TRIF Signaling To Promote *Yersinia*-Induced Apoptosis

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Innate immune responses that are crucial for control of infection are often targeted by microbial pathogens. Blockade of NF-κB and MAPK signaling by the *Yersinia* virulence factor YopJ inhibits cytokine production by innate immune cells but also triggers cell death. This cell death requires RIPK1 kinase activity and caspase-8, which are engaged by TLR4 and the adaptor protein TRIF. Nevertheless, TLR4- and TRIF-deficient cells undergo significant apoptosis, implicating TLR4/TRIF-independent pathways in the death of *Yersinia*-infected cells. In this article, we report a key role for TNF/TNFR1 in *Yersinia*-induced cell death of murine macrophages, which occurs despite the blockade of NF-κB and MAPK signaling imposed by *Yersinia* on infected cells. Intriguingly, direct analysis of YopJ injection revealed a heterogeneous population of injection-high and injection-low cells, and demonstrated that TNF expression came from the injection-low population. Moreover, TNF production by this subpopulation was necessary for maximal apoptosis in the population of highly injected cells, and TNFR-deficient mice displayed enhanced susceptibility to *Yersinia* infection. These data demonstrate an important role for collaboration between TNF and pattern recognition receptor signals in promoting maximal apoptosis during bacterial infection, and demonstrate that heterogeneity in virulence factor injection and cellular responses play an important role in promoting anti-*Yersinia* immune defense. *The Journal of Immunology*, 2016, 197: 4110–4117.

Many microbial pathogens have evolved mechanisms to inhibit innate immune signaling pathways, thereby limiting the ability of infected cells to propagate inflammatory cues such as cytokine secretion (1, 2). Of the signaling pathways frequently targeted by pathogens, NF-κB and MAPK pathways elicit key host-protective antimicrobial defenses (3). However, these signaling pathways are also coupled to prosurvival signals that limit cell death pathways activated by microbial pattern recognition and cytokine receptors (3). Inhibition of innate immune signaling can, therefore, not only result in a block in cytokine and antimicrobial effector production but also trigger cell death. This induction of cell death may be an evolutionarily ancient response to pathogen virulence factors.

The YopJ protein of pathogenic yersiniae is an acyl-transferase that belongs to a family of secreted virulence factors injected into host cells by bacterial pathogens that infect plants, insects, and higher eukaryotes (4–6). The activity of YopJ blocks MAPK and NF-κB signaling to interfere with the production of inflammatory cytokines (7–9). In the absence of YopJ, the virulence of *Yersinia pseudotuberculosis* is attenuated in vivo following oral infection (10). However, in addition to inhibiting cytokine production, YopJ-induced blockade of NF-κB and MAPK signaling also triggers cell death downstream of TLR4-dependent TRIF signaling (7, 11–16).

TLR4/TRIF-dependent cell death induced by YopJ requires the components of the extrinsic apoptosis pathway, specifically RIPK1, Fas-associated death domain (FADD), and caspase-8 (17–19). Interestingly, although absence of RIPK1 or caspase-8 abrogates YopJ-induced cell death, TLR4- and TRIF-deficient cells still exhibit significant, although reduced, death (13–15, 18, 19), implying that an additional TLR4/TRIF-independent signal contributes to *Yersinia*-induced apoptosis. However, such a pathway has not previously been described.

The TNFR superfamily comprises a number of cell surface receptors that are coupled to induction of both transcriptional and apoptotic responses (20). Notably, TNFR1 can induce extrinsic apoptosis through RIPK1 and caspase-8 in a variety of immune and parenchymal tissues (21). This response can have protective roles in the context of tumorigenesis but is pathologic in the setting of liver and intestinal damage (21, 22). In the context of infection, however, TNF is thought to act primarily as a mediator of inflammation and activator of innate immune and endothelial cells. YopJ blockade of NF-κB and MAPK activation virtually eliminates the production of TNF and other inflammatory cytokines by *Yersinia*-infected cells (7–9). Thus, the potential contribution of TNF signaling to *Yersinia*-induced cell death has never been examined.

In this article, we identify a key role for TNF in promoting *Y. pseudotuberculosis*–induced cell death and demonstrate that despite the greatly reduced TNF production that accompanies

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FasL, Fas ligand; LDH, lactate dehydrogenase; MOI, multiplicity of infection; Nec-1, necrostatin-1; PI, propidium iodide; TACE, TNF-α–converting enzyme; Wt, wild-type.

infection with virulent *Y. pseudotuberculosis*, TNF/TNFR1 is responsible for TRIF-independent apoptosis of infected cells. Intriguingly, cells undergoing apoptosis and cells producing TNF represented distinct populations. Unexpectedly, TNF-producing cells were injected with *Yersinia* YopJ, although to a significantly lower level than apoptotic cells. Thus, in a phenotypically heterogeneous population of infected cells, TNF production by cells that are injected but remain uninhibited by YopJ synergized with TRIF to promote maximal apoptosis in response to *Yersinia* infection. Finally, oral infection of TNFR1-deficient mice demonstrated a protective function for TNFR1 signaling in vivo. These findings establish an unanticipated role for cytokine responses in directing apoptosis of *Yersinia*-infected cells and a potential role for TNFR1-dependent apoptosis in promoting host protection against *Yersinia* infection.

Materials and Methods

Cell culture and infections

Bone marrow–derived macrophages (BMDMs) from C57BL/6J (Jackson), *Tnfrsf1a*$^{-/-}$ (23) (Jackson), *Trif*$^{-/-}$ (24), and *Trif*$^{-/-}$*Tnfr1*$^{-/-}$ mice (25) were cultured and infected as previously described (19). *Y. pseudotuberculosis* strain IP2666 and isogenic *yopJ* mutant bacteria were grown overnight with aeration in 2×YT broth at 26°C. *Y. pseudotuberculosis* were diluted into inducing media (2×YT containing 20 mM sodium oxalate and 20 mM $MgCl_2$) and grown with aeration for 1 h at 26°C followed by 2 h at 37°C. BMDMs were infected at a multiplicity of infection (MOI) of 20:1, unless otherwise noted. Cells were incubated at 37°C, and gentamicin (100 μg/ ml) was added 1 h post infection. The following were added 1 h before infection, where indicated: 100 μM zVAD-fmk (zVAD; SM Biochemicals), 60 μM necrostatin-1 (Nec-1; Calbiochem), 3 μM GSK2399872A (GSK'872; GlaxoSmithKline), 50 μM TAPI-2 (Sigma-Aldrich), and 80 μM dynasore (Sigma-Aldrich).

Cell death

Lactate dehydrogenase (LDH) release was measured from cell supernatants and quantified using the Cytotox96 Assay Kit (Promega) according to the manufacturer's instructions and as previously described (19). For flow cytometry, cells were stained with the Zombie Yellow Fixable Viability Kit (BioLegend) and with CD45.2 and CD45.1 Abs (BioLegend) prior to fixation and permeabilization (BD Cytofix/Cytoperm Kit). Cells were stained for intracellular TNF (BioLegend) and cleaved caspase-3 (#9661; Cell Signaling). Flow cytometry samples were analyzed on the LSR II or LSRFortessa (BD).

Western blotting and ELISA

Cell lysates were harvested in lysis/sample buffer and run on 4–12% NuPAGE gels (Invitrogen). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and blotted for caspase-8 (1G12; Enzo Life Sciences), caspase-3 (#9662; Cell Signaling), caspase-1 (sc-514; Santa Cruz), and β-actin (Sigma-Aldrich). Cytokine release was measured by ELISA on cell supernatants, using capture and detection Abs against TNF (430902; BioLegend) and CCL5 (500-P118 and 500-P118Bt; PeproTech).

CCF4-AM injection assay

BMDMs were infected with YopJ-deficient bacteria complemented with β-lactamase–linked YopJ or GST control expressing plasmid (pACYC). At 1 h post infection, cells were loaded with CCF4-AM (LiveBLAzer FRET-B/G Loading Kit; Invitrogen) according to the manufacturer's instructions, including the addition of probenecid and with the modification of diluting Solution C 4-fold in HBSS. Cells were returned to 37°C and harvested for cell staining as above for TNF and cleaved caspase-3 at 2 h post infection.

Animal infections

Mice were fasted for 12–16 h and inoculated by gastric gavage with 2×10^8 CFU wild-type (Wt) *Y. pseudotuberculosis* (32777) from overnight culture in 2×YT containing irgasan. Tissues were collected, bead homogenized (MP Biomedical), and plated at 10-fold dilutions on LB plates containing irgasan to determine bacterial burdens (CFU per gram tissue). All animal studies were performed in accordance with University of Pennsylvania Institutional Animal Care and Use Committee approved protocols.

Video microscopy

Immortalized B6 macrophages were plated on 35-mm polymer coverslip dishes (Ibidi) 16 h prior to infection. Propidium iodide (PI) (Calbiochem) was added to the media and cells were infected with *Yersinia* as described above. Gentamicin was added 1 h post infection at the microscope. Cells were imaged on a Yokagawa spinning disk confocal microscope at ×63 magnification, 37°C and 5% CO_2 over 4 h.

Results

Yersinia *YopJ promotes TRIF-dependent and -independent apoptosis through RIPK1 kinase activity*

YopJ promotes caspase-8–dependent apoptosis of innate immune cells through the inhibition of NF-κB– and MAPK-dependent prosurvival signaling (7, 12, 16–19). Although this response has been attributed to TLR4/TRIF (13–15), TRIF-deficient BMDMs still undergo significant *Yersinia*-induced death (14, 15, 18, 19). This finding implies a role for additional pathways in the induction of cell death. Both TRIF and death receptor signaling can promote survival, caspase-8–dependent apoptosis, or RIPK3-dependent programmed necrosis, depending on the nature of the genetic and environmental conditions present (26). We therefore sought to determine whether TRIF-independent death is also mediated by caspase-8–dependent apoptosis, or by an alternate death pathway, such as programmed necrosis. Similarly to Wt BMDMs, *Trif*$^{-/-}$ BMDMs undergo YopJ-dependent cleavage of apoptotic caspases, -8 and -3, following infection with Wt *Y. pseudotuberculosis* (Fig. 1A). As expected (19), the pancaspase inhibitor zVAD prevented the cleavage of caspase-8 and -3 but did not protect cells from death, suggesting that caspase inhibition induces a switch from *Y. pseudotuberculosis*–induced apoptosis to RIPK3-dependent programmed necrosis in both Wt and *Trif*$^{-/-}$ BMDMs (Fig. 1A, 1B, Supplemental Fig. 1). Indeed, the RIPK3 kinase inhibitor GSK'872 inhibited death of both zVAD-treated Wt and *Trif*$^{-/-}$ BMDMs infected with *Y. pseudotuberculosis* (Fig. 1B). Importantly, single deficiency of RIPK3 (18, 19) or GSK'872 treatment alone (Fig. 1B) has no effect on *Yersinia*-induced cell death of BMDMs. These data indicated that *Y. pseudotuberculosis*–induced cell death in both Wt and *Trif*$^{-/-}$ BMDMs is mediated by caspase-8–dependent apoptosis and is independent of RIPK3-programmed necrosis.

Whereas YopJ-induced apoptosis in Wt BMDMs requires the signaling molecule RIPK1 (18, 19), caspase-8 can mediate apoptosis via RIPK1 kinase–dependent or –independent pathways (22). Whether TRIF-independent cell death depends on RIPK1 activity is unknown. Importantly, Nec-1, a specific inhibitor of RIPK1 kinase activity, reduced YopJ-induced apoptosis of both Wt and *Trif*$^{-/-}$ BMDMs (Fig. 1C). Altogether, these data demonstrate that both the TRIF-dependent cell death and TRIF-independent cell death induced by *Y. pseudotuberculosis* involve RIPK1 kinase–dependent apoptosis.

TNFR1 mediates TRIF-independent apoptosis and caspase-1 cleavage during Yersinia *infection*

The TNFR superfamily mediates extrinsic apoptosis through recruitment and activation of FADD, RIPK1, and caspase-8 (21, 22). Given the requirement for all three of these factors in *Yersinia*-induced apoptosis (17–19), we therefore tested the possibility that TNF might play a role in *Y. pseudotuberculosis*–induced cell death. Surprisingly, despite the known blockade of TNF production by YopJ (7–9), TNFR1-deficient (*Tnfr1*$^{-/-}$) BMDMs displayed significantly decreased cell death and caspase-3 cleavage, compared with Wt BMDMs (Fig. 2A, Supplemental Fig. 2A). Strikingly, macrophages deficient for both TRIF and TNFR1 (*Trif*$^{-/-}$*Tnfr1*$^{-/-}$) were also completely protected from *Y. pseudotuberculosis*–induced

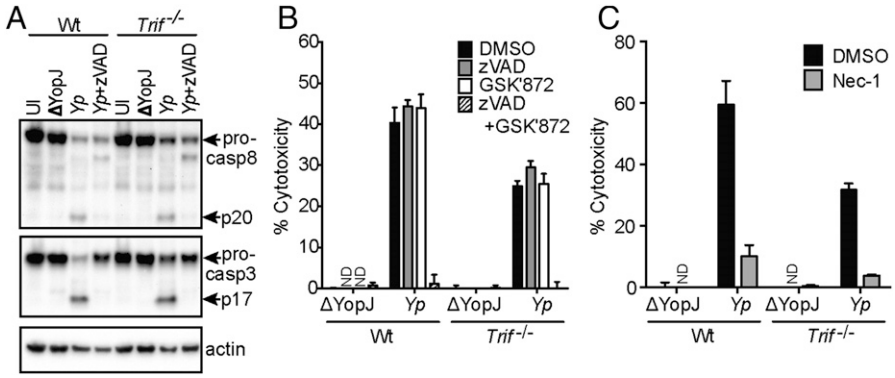

FIGURE 1. *Yersinia* YopJ promotes TRIF-dependent and -independent apoptosis through RIPK1 kinase activity. (**A**) Wt or TRIF-deficient ($Trif^{-/-}$) BMDMs were left uninfected (UI) or infected with Wt *Y. pseudotuberculosis* (*Yp*) or YopJ-deficient *Y. pseudotuberculosis* (ΔYopJ) for 4 h. Cell lysates were probed for caspase-8 and caspase-3 cleavage. Results are representative of two independent experiments. (**B** and **C**) Percentage of cytotoxicity was measured by LDH release from B6 and $Trif^{-/-}$ BMDMs infected with ΔYopJ or *Yp* for 4 h. The following were added 1 h before infection, where indicated: 100 μM zVAD-fmk (zVAD), 300 nM GSK'872, and 60 μM Nec-1. Error bars indicate mean ± SD of triplicates and are representative of three or more independent experiments. ND, not detected.

cell death, suggesting that in the absence of TRIF, the remaining cell death is caused by the TNFR pathway (Fig. 2A). BMDMs lacking both TNFR1 and TRIF signaling were completely protected from *Y. pseudotuberculosis*–induced death up to 8 h post infection, by which time both $Tnfr1^{-/-}$ and $Trif^{-/-}$ macrophages exhibit levels of death similar to that in WT cells (Supplemental Fig. 2B). Consistently, cleavage of caspase-8 and -3 was abrogated in the double-knockout cells but still present in either TRIF or TNFR single-deficient cells (Fig. 2B). Critically, TNF-deficient BMDMs phenocopied $Tnfr1^{-/-}$ cells, in that they were partially protected from cell death in response to *Y. pseudotuberculosis* infection, indicating that the role of TNFR1 is mediated by TNF itself (Fig. 2C, Supplemental Fig. 1).

In addition to the activation of apoptotic caspases, *Yersinia* infection of unprimed BMDMs induces caspase-1 activation through a RIPK1, caspase-8, and FADD pathway (18, 19, 27, 28). This pathway of inflammasome-independent caspase-1 activation occurs downstream of TLR4/TRIF signaling during *Yersinia* infection (18, 19). Interestingly, we observed that either $Trif^{-/-}$ or $Tnfr1^{-/-}$ BMDMs still showed residual, albeit reduced, processing of caspase-1 following *Y. pseudotuberculosis* infection (Fig. 2D), suggesting that both pathways could independently mediate caspase-8–dependent caspase-1 processing. Indeed, only $Trif^{-/-}Tnfr1^{-/-}$ cells did not undergo caspase-1 cleavage following *Y. pseudotuberculosis* infection, definitively demonstrating that caspase-1 processing in $Trif^{-/-}$ BMDMs was mediated by TNFR1 (Fig. 2D).

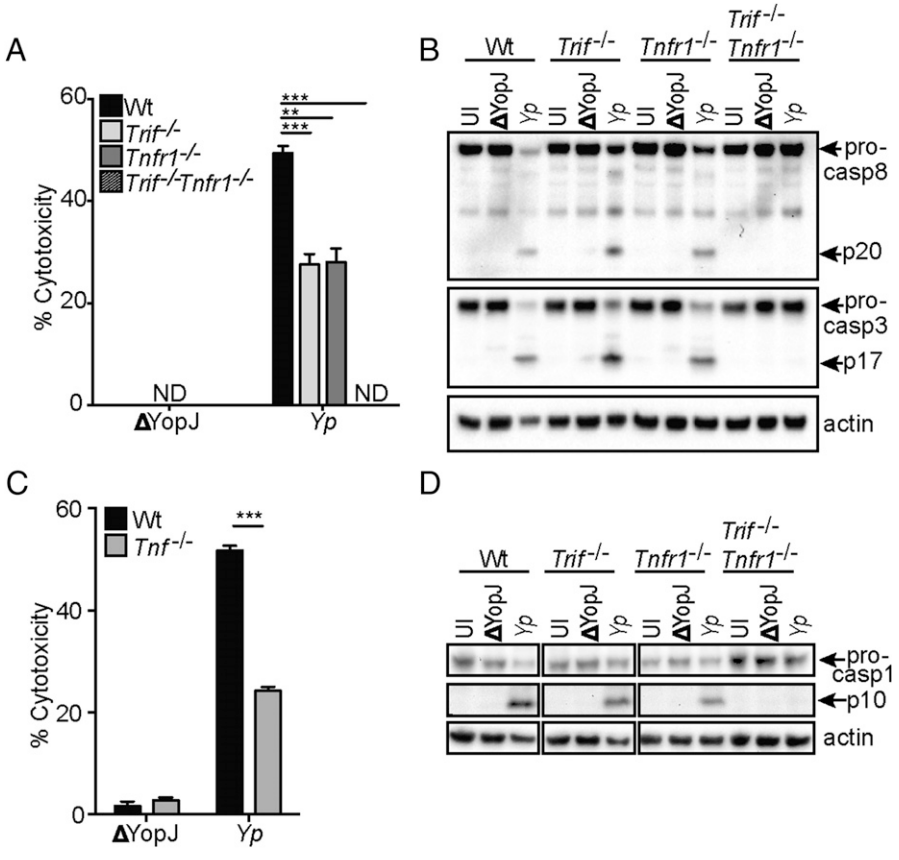

FIGURE 2. TNFR1 mediates TRIF-independent apoptosis and caspase-1 cleavage during *Yersinia* infection. (**A**) Cytotoxicity was measured by LDH release from Wt, $Trif^{-/-}$, TNFR1-deficient ($Tnfr1^{-/-}$), and TRIF/TNFR1-deficient ($Trif^{-/-}Tnfr1^{-/-}$) BMDMs infected with YopJ-deficient *Y. pseudotuberculosis* (ΔYopJ) or Wt *Y. pseudotuberculosis* (*Yp*) for 4 h. (**B**) Cell lysates were probed for caspase-8 and caspase-3 by Western analysis. (**C**) Cytotoxicity was measured by LDH release from Wt and TNF-deficient ($Tnf^{-/-}$) BMDMs, as in (A). (**D**) Western analysis for caspase-1, as in (B). Error bars indicate mean ± SD of triplicates. Representative of three or more independent experiments. $^{**}p < 0.001$, $^{***}p < 0.0001$. ND, not detected; UI, uninfected.

Interestingly, although most BMDMs infected with *Yersinia* exhibited a profound blebbing of cellular membranes prior to taking up the membrane-impermeant dye PI, indicative of apoptosis (Supplemental Video 1), some cells appeared to lyse and release their cytoplasm concomitant with becoming PI positive, suggestive of pyroptosis or programmed necrosis-like morphology and kinetics (Supplemental Video 2). This observation is consistent with our previous findings that caspase-1 contributes to *Yersinia*-induced cell death, but is not absolutely required (19), suggesting that at least some fraction of the cells undergo pyroptosis in response to *Y. pseudotuberculosis* infection. Overall, these findings provide the first direct evidence, to our knowledge, that TNF/TNFR1 signaling contributes to *Yersinia*-induced cell death and caspase-1 processing.

Cell-extrinsic TNF promotes Yersinia*-induced apoptosis*

Given the well-established impact of YopJ on limiting NF-κB and MAPK signaling, which blocks production of cytokines, including TNF (7–9), we sought to determine the source of the TNF that was responsible for the *Y. pseudotuberculosis*–induced cell death. As expected, *Y. pseudotuberculosis*–infected cells secreted very low but detectable amounts of TNF compared with BMDMs infected with YopJ-deficient bacteria (Fig. 3A). *Trif*$^{-/-}$ cells secreted even less TNF compared with Wt cells (Fig. 3A). Single-cell analysis of TNF expression by flow cytometry demonstrated that, indeed, the vast majority of *Y. pseudotuberculosis*–infected cells produced little to no TNF and overlapped with uninfected cells; however, we observed a small but significant population of *Y. pseudotuberculosis*–infected cells that produced low levels of TNF in response to *Y. pseudotuberculosis* infection (Fig. 3B). Intriguingly, this TNF-producing population was entirely distinct from the cells undergoing apoptosis, as determined by cleaved caspase-3 staining (Fig. 3C). These data imply that a subset of cells within *Y. pseudotuberculosis*–infected cultures serves as the source of TNF that promotes YopJ-induced cell death in nonproducing cells. TNF could be absent in the cleaved caspase-3–positive cells because they are dying or have already released TNF owing to loss of membrane integrity at the time of the analysis. This explanation is unlikely, however, as the cells were gated on live cells that had an intact plasma membrane. Importantly, *Tnfr1*$^{-/-}$ cells exhibited no difference in their ability to produce TNF but had greatly reduced levels of cleaved caspase-3, demonstrating a key role for TNFR signaling in promoting *Y. pseudotuberculosis*–induced apoptosis (Fig. 3C).

Heterogeneous injection of YopJ leads to TNF production by a subset of infected cells

The blockade of inflammatory innate signaling and cytokine production by YopJ limits, but does not completely abrogate, production of TNF by infected cells. These TNF producers could be uninfected cells, cells that escaped injection of YopJ, or cells that are somehow resistant to the effects of YopJ. To distinguish between these possibilities, we used a YopJ–β-lactamase (BlaM) injection reporter system (29) to determine whether cells that were injected by YopJ were capable of producing TNF. This reporter system takes advantage of a compound fluorophore, CCF4-AM, which contains a β-lactam ring, and whose fluorescence properties are therefore altered in the presence of β-lactamase (29). YopJ-deficient *Y. pseudotuberculosis* were complemented with YopJ protein fused to β-lactamase (YopJ-Bla), which reconstituted the ability to inhibit cytokine expression and to induce apoptosis (Supplemental Fig. 3). Detection of CCF4-AM cleavage thus provided us with a way to quantitate cells specifically injected with functional YopJ protein because it was fused directly to β-lactamase. Intriguingly, we observed that under these infection conditions, all of the BMDMs in culture were injected by YopJ, but that a subset of the cells exhibited low injection (Fig. 4A). Importantly, all of these low-injection cells were negative for cleaved caspase-3 (Fig. 4B) but made up the entirety of the TNF-producing population of cells (Fig. 4C). Notably, all of the cells containing cleaved caspase-3 were highly injected, suggesting that apoptosis requires a high level of YopJ injection (Fig. 4B). Interestingly, lack of TNFR1 did not significantly alter the relative abundance of low-injection versus high-injection cells, but significantly reduced the frequency of cleaved caspase-3–positive cells (Fig. 4B). This finding suggested that TNFR1 signaling increased the likelihood that a YopJ-injected cell would undergo apoptosis.

The preferential production of TNF by low-injection cells could be due to levels of injected YopJ in these cells being below a threshold necessary to efficiently block cell signaling and cytokine production. To test the relationship between infection dose, cell death, and TNF production, we titrated the dose of *Y. pseudotuberculosis* infection down from 20 to 2.5. As expected, this resulted in a corresponding increase in the frequency of low-injection cells (Fig. 4D). There was a corresponding increase in the frequency of TNF-producing cells and a decrease in the frequency of apoptotic cells (Fig. 4D–F). Thus, although the number of TNF-producing cells rises as the MOI decreases, cell death

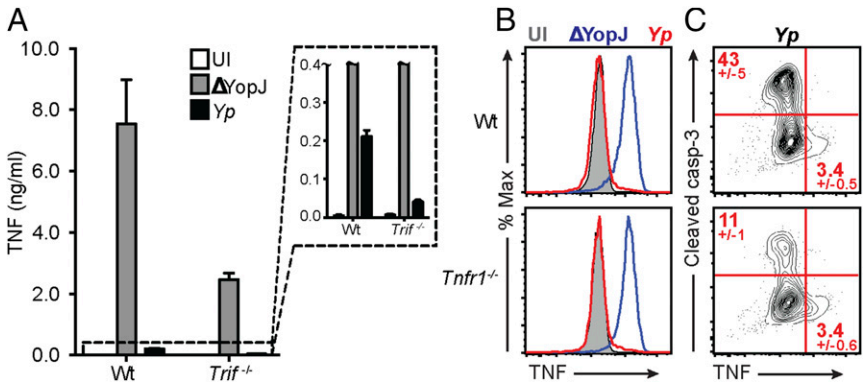

FIGURE 3. Cell-extrinsic TNF promotes *Yersinia*-induced apoptosis. (**A**) Soluble TNF measured by ELISA from supernatants of Wt and *Trif*$^{-/-}$ BMDMs left uninfected or infected with YopJ-deficient *Y. pseudotuberculosis* (ΔYopJ) or Wt *Y. pseudotuberculosis* (*Yp*). Inset indicates TNF production by *Yp*-infected cells. Error bars indicate mean ± SD of triplicates. (**B**) Wt and *Tnfr1*$^{-/-}$ BMDMs were left uninfected (gray-filled histogram) or infected with ΔYopJ (blue histogram) or *Yp* (red histogram). Two hours post infection, cells were stained and analyzed by flow cytometry (gated on singlet live cells). (**C**) Distinct populations of cells are positive for intracellular TNF or cleaved caspase-3 (casp-3). Gate frequencies represent mean ± SD of triplicates. Data are representative of three or more independent experiments. UI, uninfected.

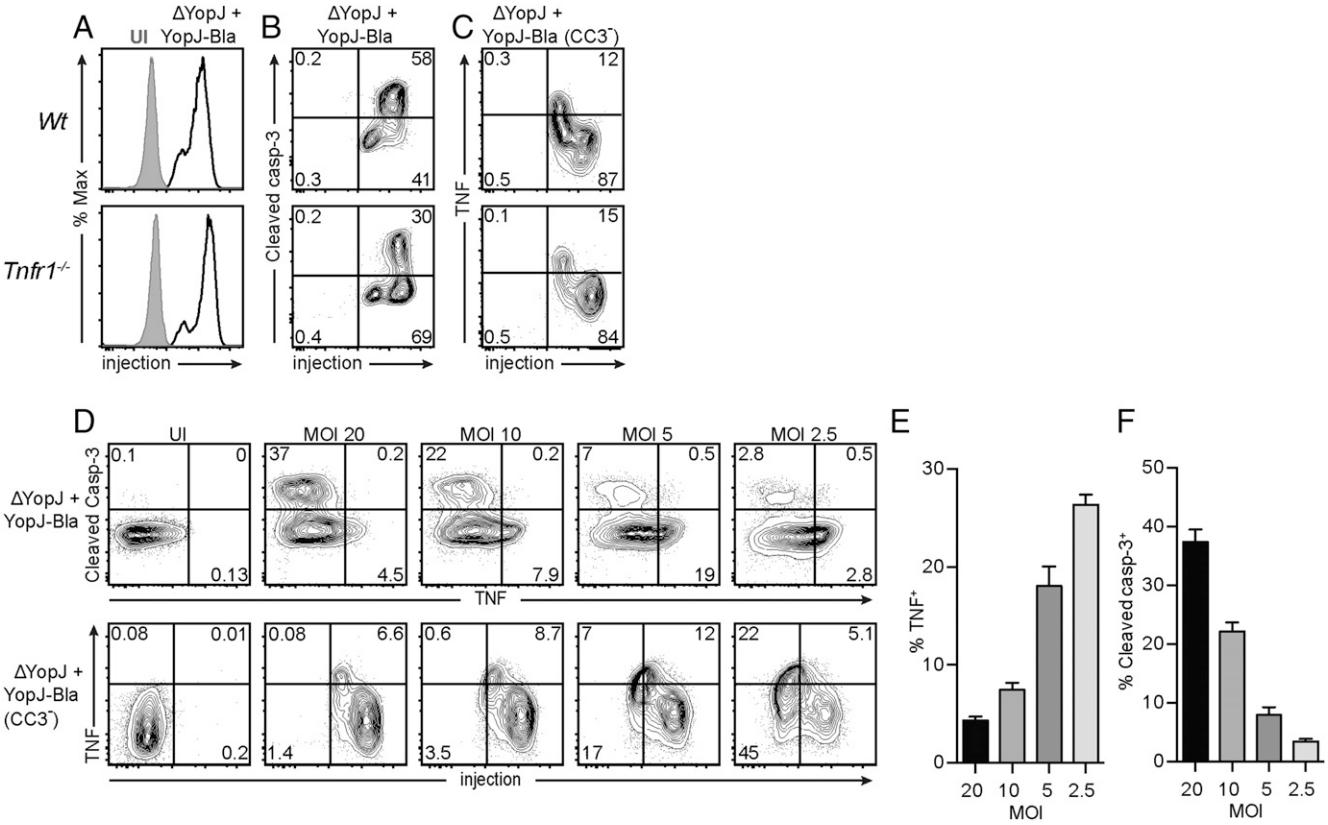

FIGURE 4. Heterogeneous injection of YopJ leads to TNF production by a subset of infected cells. (**A**–**D**) Wt or Tnfr1$^{-/-}$ BMDMs were left uninfected (UI) or infected at MOI 20 with YopJ-deficient *Y. pseudotuberculosis* (ΔYopJ) complemented with plasmid containing β-lactamase–linked YopJ (ΔYopJ + YopJ-Bla). (A) YopJ injection was measured by CCF4-AM cleavage and compared with (B) cleaved caspase-3 in total live cells or with (C) TNF expression in cleaved caspase-3-negative (CC3$^-$) cells. (D) MOI was titrated from 20 to 2.5, and YopJ injection, cleaved caspase-3, and TNF were measured as above. (**E** and **F**) Frequency of TNF$^+$ and cleaved caspase-3$^+$ cells from (D). Frequencies represent mean ± SD of triplicates. Data are representative of two independent experiments. UI, uninfected.

likely decreases owing to a combination of reduced TLR stimulation at lower MOIs, together with levels of injected YopJ falling below a threshold necessary for the induction of apoptosis. Altogether, these studies demonstrate that heterogeneity in the extent of injection is present within a seemingly uniform population of cells, and that differences in injection efficiency account for the ability of a subset of cells to produce TNF, resulting in enhanced apoptosis of highly injected cells.

Membrane TNF and endocytic trafficking promote TRIF-independent apoptosis during Yersinia *infection*

TNF secretion is regulated transcriptionally as well as at a posttranslational step, in which trimeric TNF is assembled on the cell surface and subsequently released as a soluble cytokine by the metalloprotease TNF-α–converting enzyme (TACE) (30, 31). Of interest, both membrane-bound and soluble TNF are able to mediate many of the inflammatory effects of TNF (32–34). We therefore used a specific TACE inhibitor to test whether secreted TNF was required or whether membrane-bound TNF was sufficient to promote *Y. pseudotuberculosis*–induced apoptosis. As expected, TACE inhibitor treatment dramatically reduced the amount of TNF released into the cell supernatant (Fig. 5A). Surprisingly however, TACE inhibitor treatment had no impact on *Y. pseudotuberculosis*–induced cell death in Wt or *Trif*$^{-/-}$ cells, suggesting that surface TNF is sufficient to provide proapoptotic signals in the context of YopJ (Fig. 5B).

Although TNFR1-mediated signaling through NF-κB and MAPK pathways is initiated upon receptor ligation at the plasma membrane, internalization is thought to be required for the formation of the death-inducing signaling complex (DISC) that initiates apoptosis (35–37). Notably, activation of TRIF-dependent transcriptional responses downstream of TLR4, including the induction of type-I IFN and certain other chemokines such as CCL5, also require TLR4 endocytosis, which is mediated by dynamin (38). This is in contrast to MyD88-dependent TLR4 signaling, which occurs from the plasma membrane. As expected, blocking dynamin-mediated endocytosis potently inhibited the production of the TRIF-dependent cytokine CCL5 by Wt cells (Fig. 5C). Interestingly, this blocking also completely prevented *Y. pseudotuberculosis*–induced apoptosis in Wt, TRIF-deficient, and TNFR-deficient BMDMs (Fig. 5D). These data implicate dynamin-dependent endocytosis in both TNFR1- and TRIF-dependent *Y. pseudotuberculosis*–induced apoptosis.

Cell-extrinsic soluble or membrane-bound TNF promotes Y. pseudotuberculosis*–induced apoptosis*

The TACE inhibitor studies indicated that membrane TNF was sufficient to promote *Y. pseudotuberculosis*–induced cell death, but did not exclude the possibility that soluble TNF mediates this effect under normal circumstances. Indeed, addition of soluble TNF to *Tnf*$^{-/-}$ BMDMs fully restored *Y. pseudotuberculosis*–induced apoptosis to Wt levels (Fig. 6A, 6B). The significantly reduced levels of TNF produced by TRIF-deficient cells could potentially also account for the reduced level of apoptosis in these cells. However, although TNF addition slightly increased *Y. pseudotuberculosis*–induced apoptosis of *Trif*$^{-/-}$ BMDMs, it was not fully restored to Wt levels, demonstrating that TRIF and TNF play

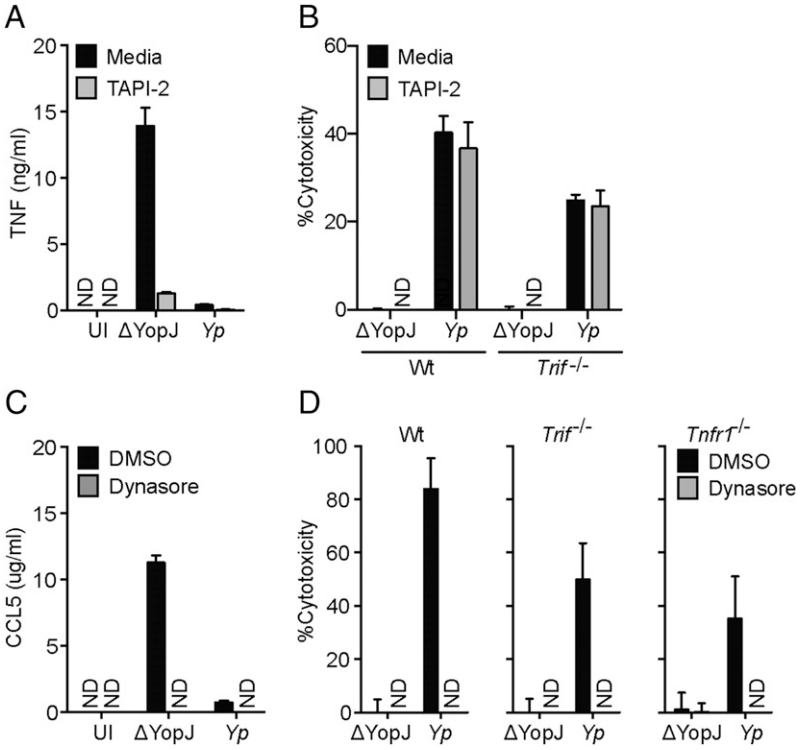

FIGURE 5. Membrane TNF and endocytic trafficking promote TRIF-independent apoptosis during *Yersinia* infection. (**A**) Secreted TNF was measured by ELISA of supernatants of Wt BMDMs left untreated or treated with TAPI-2 and left uninfected or infected with YopJ-deficient *Y. pseudotuberculosis* (ΔYopJ) or Wt *Y. pseudotuberculosis* (*Yp*). (**B**) LDH release from Wt and $Trif^{-/-}$ BMDMs treated with TAPI-2. (**C**) CCL5 measured by ELISA and LDH release (**D**) from B6 BMDMs treated with Dynasore or vehicle control, as indicated. Error bars indicate mean ± SD of triplicates and are representative of three or more independent experiments. ND, not detected; UI, uninfected.

distinct, non-redundant roles in mediating *Y. pseudotuberculosis*–induced death (Fig. 6B).

To further test whether cell-extrinsic TNF promotes *Y. pseudotuberculosis*–induced apoptosis, we infected mixed cultures of BMDMs derived from TNF-deficient and Wt congenically marked bone marrow. When mixed at a 19:1 ratio of $Tnf^{-/-}$/Wt cells, just 5% of Wt cells capable of producing TNF were sufficient to mediate maximal levels of apoptosis in $Tnf^{-/-}$ cells, demonstrating that minimal levels of TNF are required to induce cell death (Fig. 6C). Intriguingly, treatment of the 19:1 cultures with TACE inhibitor reduced apoptosis of $Tnf^{-/-}$ cells in the mixed cultures to nearly that of $Tnf^{-/-}$ cells alone (Fig. 6C). However, at a $Tnf^{-/-}$/Wt ratio of 1:1, TACE inhibition had no detectable impact on cell death in $Tnf^{-/-}$ cells, similar to what we observed in cultures of Wt and $Trif^{-/-}$ BMDMs alone following TAPI treatment (Supplemental Fig. 4, Fig. 5B). This finding suggests that when cell–cell contacts are limiting, TNF secreted by Wt cells plays a key role in promoting *Y. pseudotuberculosis*–induced apoptosis. However, when cell–cell contact between TNF-producing and nonproducing cells is not limiting, cell surface TNF is sufficient to mediate maximal apoptosis. Collectively these studies indicate that both cell surface and soluble TNF are capable of promoting *Y. pseudotuberculosis*–induced apoptosis and likely have a common function during infection.

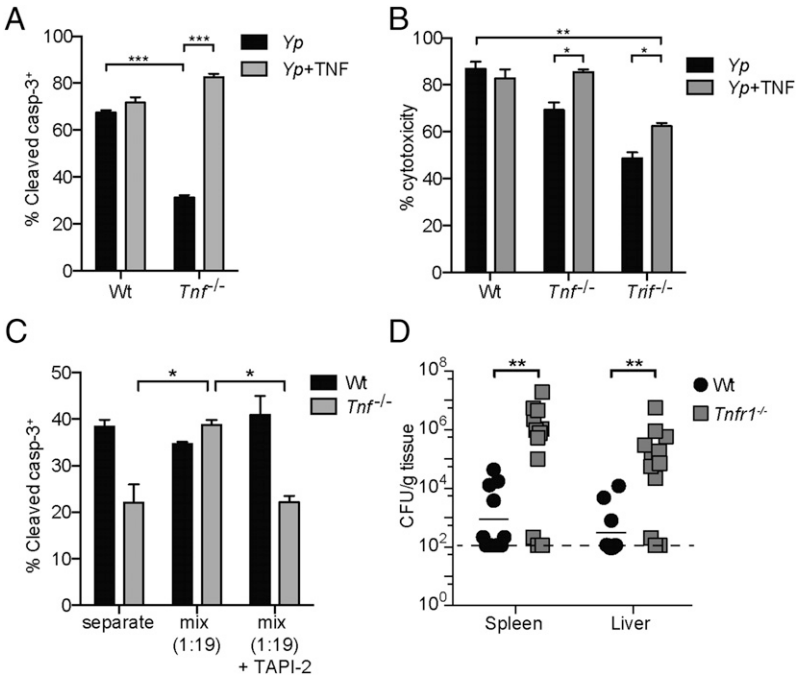

FIGURE 6. Cell-extrinsic TNF promotes *Yersinia*-induced apoptosis and in vivo antibacterial immunity. (**A** and **B**) Wt, $Tnf^{-/-}$, and $Trif^{-/-}$ BMDMs were infected with Wt *Y. pseudotuberculosis* (*Yp*), treated with 1 ng/ml rTNF at 30 min post infection, and harvested for flow cytometric analysis of cleaved caspase-3 (casp-3) (A) or measured for LDH release (B). (**C**) $Tnf^{-/-}$ (CD45.2) and congenically marked Wt (B6.SJL, CD45.1) BMDMs were plated separately or mixed at a 19:1 ($Tnf^{-/-}$/Wt) ratio and infected with Wt *Yp* for 2 h. Mixed cultures were analyzed by flow cytometry for cleaved caspase-3, using CD45 staining to differentiate Wt and $Tnf^{-/-}$ cells. Cells were treated with TAPI-2 or control media 1 h prior to infection, where indicated. Error bars indicate mean ± SD of triplicates. Data are representative of two or more independent experiments. (**D**) Wt and $Tnfr1^{-/-}$ mice were infected orally with 2×10^8 CFU *Yp*, and tissue bacterial burdens were measured at day 5 post infection. Line represents geometric mean of data pooled from two experiments; total n = 10–12 mice. Dashed line indicates limit of detection. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Finally, we sought to determine whether TNFR1-dependent apoptosis significantly affected control of *Y. pseudotuberculosis* during in vivo infection. Compared with Wt mice, *Tnfr1*$^{-/-}$ mice had increased bacterial burdens in both the spleen and liver 5 d after oral infection. This indicates an important in vivo role for TNF signaling in host defense that may depend on the induction of TNFR1-dependent apoptosis (Fig. 6D). Altogether, these results reveal a previously unappreciated role for cell-extrinsic TNF in macrophage killing during *Yersinia* infection and suggest that this function contributes to antibacterial immune defense.

Discussion

Cell death is a prominent feature of infection by many pathogens, including *Yersinia* (7, 10, 11). During *Yersinia* infection, cell death is triggered by the activity of YopJ, which promotes bacterial dissemination from mucosal tissues (10). However, *Yersinia*-induced cell death may also provide a means for infected cells to elicit inflammatory signals or induce phagocytosis of cell-associated bacteria to overcome YopJ-dependent signaling inhibition (39–41).

Although TLR4/TRIF contribute to apoptosis during *Yersinia* infection, significant cell death occurs in *Yersinia*-infected cells even in the absence of TLR4/TRIF-dependent signals (13–15). These data suggest that an additional, as-yet-undefined, pathway contributes to *Yersinia*-induced cell death. In this article, we show a key role for TNF signaling in promoting *Yersinia*-induced apoptosis, implicating innate cytokine signaling in promoting host cell death during infection. To our knowledge, this is the first direct evidence that TNF signaling contributes to *Yersinia*-induced macrophage apoptosis, and supports a model in which cell-extrinsic cytokine signals play an important role in mediating the death of *Yersinia*-infected cells.

The role of TNF in *Yersinia*-induced apoptosis may be particularly important because at 37°C, *Yersinia* alters its LPS to a form that is poorly detected by TLR4 (42–44). This feature likely limits both antibacterial cytokine responses and TLR4-dependent induction of apoptosis in vivo. Thus, additional pathways to promote apoptosis may play critical roles in host defense. Indeed, TRIF deficiency alone does not significantly affect control of *Yersinia pseudotuberculosis* in an in vivo oral infection model (45), and Fas/Fas ligand (FasL) contribute to apoptosis of *Y. pestis*–infected cells in the lung and promote control of infection (46). Our studies now demonstrate an important role for TNFR1 in control of oral *Y. pseudotuberculosis* infection, which contrasts both with TRIF deficiency and with previous investigations of the contribution of TNFR1 to control of *Yersinia* during systemic infection (45, 47). Prior studies with *Y. enterocolitica* found that TNFR1 played a pathologic role and that *Tnfr1*$^{-/-}$ mice were more resistant to infection (47). These differences may be due to either the *Yersinia* species or the route of infection.

Furthermore, although *Y. pseudotuberculosis* inhibits TNF production by cells that it encounters at sites of infection, the recruitment of additional effector cells offers an abundant source of TNF for inducing apoptosis and potential clearance of cells inhibited during earlier stages of infection. Newly recruited cells would likely not have been exposed to the inhibitory effects of *Yersinia*-injected virulence factors and may even be primed by inflammatory cues to produce TNF while entering the infected tissue. Notably, primed macrophages and monocytes respond differently when encountering *Yersinia* than do naive macrophages, particularly with respect to their mode of cell death (48). Although cytotoxic lymphocytes, including NK cells and adaptive T cells, contribute to the killing of *Yersinia*-infected cells later in infection (41), our findings suggest that innate immune cells could play an early role in inducing apoptotic cell death through cytokine-dependent signaling.

Our data revealed that heterogeneity in the level of injected YopJ and bacterial inhibition of cell signaling results in a subset of cells being able to produce TNF, despite the block in NF-κB signaling imposed by YopJ. Titrating the infection of BMDMs results in both increased TNF production and decreased apoptosis, implying that a high threshold of injected YopJ is needed to prevent cytokine production and to potentiate TNF-induced apoptosis in infected cells. In mixed cultures, only a very small fraction of TNF-producing cells is sufficient to induce maximal cell death of TNF-deficient cells, consistent with the finding that exogenous TNF also restores cell death to *Tnf*$^{-/-}$ cells. Intriguingly, titrating the ratio of TNF-sufficient and TNF-deficient cells while blocking TNF secretion revealed that limiting cell–cell contact between TNF producers and nonproducers increased the importance of secreted TNF in cell death. Thus, our data demonstrate that cell surface TNF is capable of providing the apoptotic signal if secretion is inhibited.

Our data also demonstrated a requirement for dynamin-dependent endocytosis in the induction of both TLR4/TRIF- and TNFR1-induced apoptosis. This requirement is presumably dependent on the formation of an intracellular DISC, raising the question of how extrinsic TNF might mediate its apoptotic effect when membrane bound. TNF that cannot be cleaved from the surface of cells owing to mutation of the TACE cleavage site is also capable of inducing apoptosis in TNFR-expressing cells (33). Notably, similar findings have been reported for other surface protein members of the TNF family, particularly FasL (49). FasL is expressed on activated NK cells and CD8$^+$ T cells, and contributes to their ability to induce apoptosis of infected cells (50–52). Importantly, membrane-bound FasL is sufficient to induce Fas internalization, formation of a DISC, and apoptosis (49). Thus, the requirement for endocytosis may apply only to a receptor signaling complex, independent of death receptor ligand, for the induction of apoptosis.

Whether cell surface TNF is a primary driver of apoptosis when large amounts of TNF are being secreted, and whether it is sufficient to mediate the full extent of protection from bacterial infection in vivo remains to be determined. Overall, our results provide new insight into mechanisms governing pathogen-induced apoptosis, and highlight a previously unappreciated synergy between TLR and TNFR that regulates the response to bacterial infection.

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Disclosures

J.B. and P.J.G. are employees of GlaxoSmithKline.

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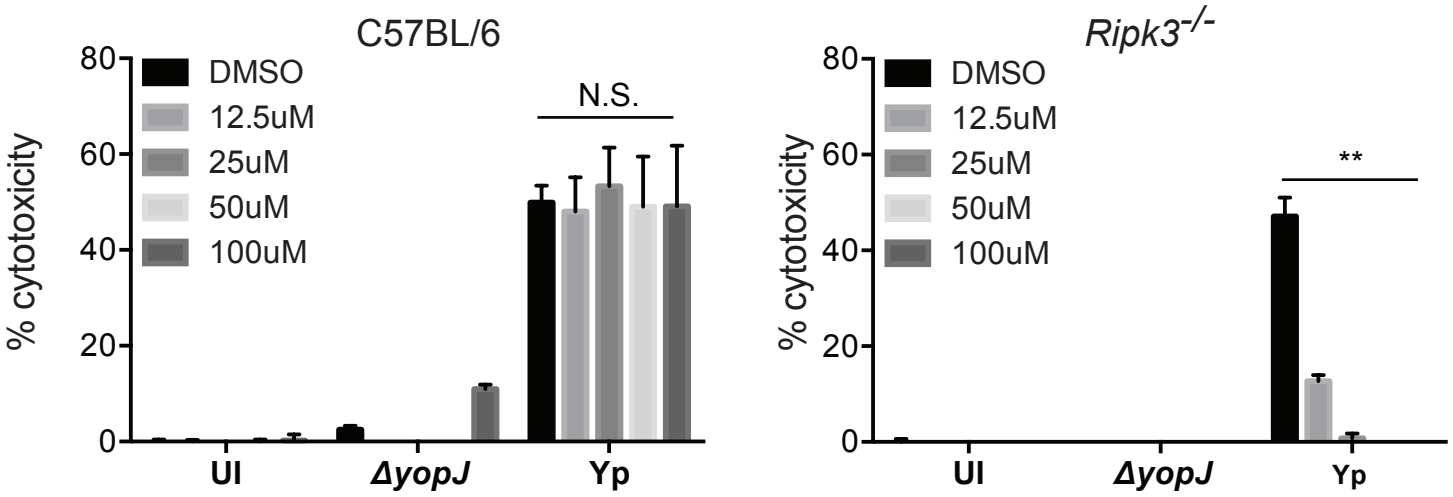
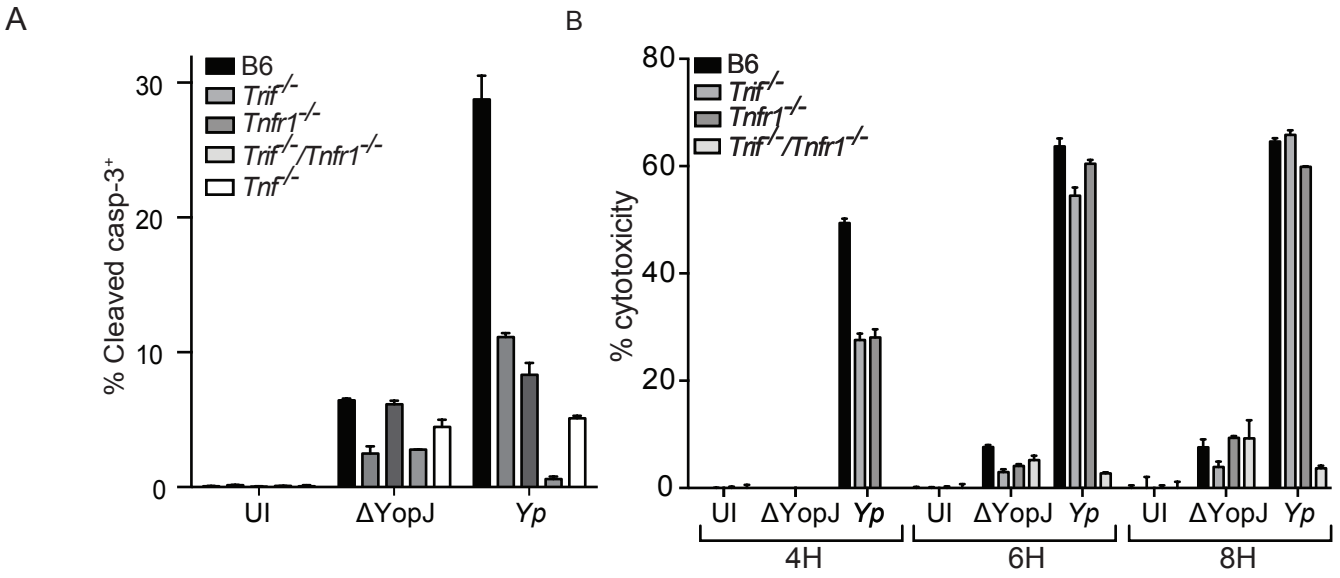
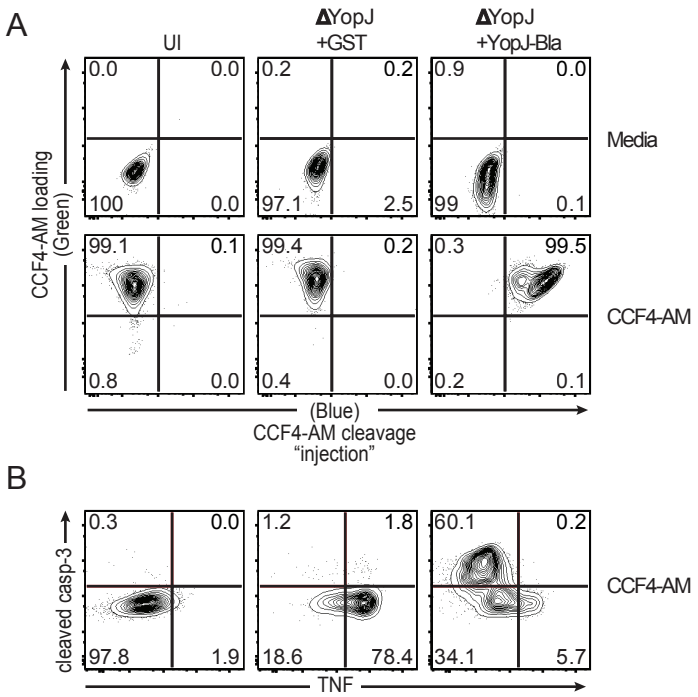


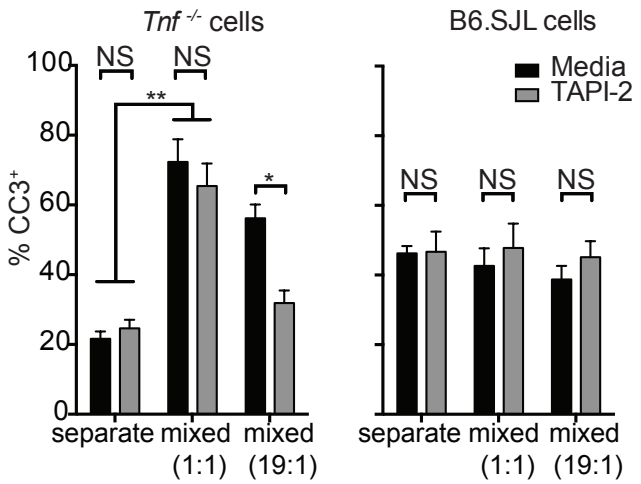
Supplemental Figure 1. zVAD potentiates programmed necrosis in Yp-infected WT cells and prevents apoptosis in *Ripk3*$^{-/-}$ cells across arange of concentrations. zVAD-fmk was added to cells at indicated concentrations one hour prior to infection with YopJ-deficient or WT Yersinia (Yp). 4 hours post-infection, cytotoxicity was measured by assaying release of lactate dehydrogenase (LDH) into the supernatant. zVAD protects *Ripk3-/-* cells from *Yp*-induced cell death at all concentrations tested, in contrast to WT cells, where zVAD potentiated programmed necrosis in response to Yp infection, at all concentrations of zVAD tested.



Supplemental Figure 2. TRIF-independent apoptosis during *Yersinia* infection is mediated by TNFR1 signaling. A. Indicated genotypes of BMDMs were left uninfected or infected with ΔYopJ or *Yp*. At 2 hours post-infection, cells were stained and analyzed by flow cytometry for frequency of cleaved caspase-3 (Casp3)-positive cells (gated on singlets and live cells). Results are representative of two independent experiments. **B.** Indicated genotypes were left uninfected or infected with ΔYopJ or *Yp* and monitored for cytotoxicity as described in Materials and Methods over a timecourse of 4, 6, and 8 hours.



Supplemental Figure 3. *Yersinia* injection as measured by CCF4-AM cleaveage. Wt BMDMs were left uninfected (UI) or infected at MOI 20 with YopJ-deficient *Yp* complemented with pACYC plasmid expressing either control GST (ΔYopJ+GST) or beta-lactamase linked YopJ (ΔYopJ+YopJ-Bla). At 1 hour post-infection, cells were loaded with CCF4-AM or control media. At 2 hours post-infection, cells were harvested and analyzed by flow cytometry for (**A**) CCF4-AM loading (488 excitation;515/20 emission) and CCF4-AM cleavage by injected YopJ-Bla (405 excitation; 450/50 emission) and for (**B**) cleaved caspase-3 and TNF staining.



Supplemental Figure 4. Mixed BMDM cultures restore maximal *Yersinia*-induced apoptosis in TNF-deficient cells. *Tnf*$^{-/-}$ (CD45.2) and congenically marked Wt (B6.SJL, CD45.1) BMDMs were plated separately or mixed at a 1:1 or 19:1 (*Tnf*$^{-/-}$:Wt) ratio and infected with *Yp* for 2 h. Mixed cultures were analyzed by flow cytometry for cleaved caspase-3, using CD45 staining to differentiate Wt and *Tnf*$^{-/-}$ cells. Cells treated with TAPI-2 or control media 1 hour prior to infection where indicated. Error bars indicate mean +/- SD of triplicates. *Tnf*$^{-/-}$ cells bar graph refers to percent of CD45.2 *Tnf*$^{-/-}$ cells in separate or mixed cultures (as indicated) containing cleaved Casp3; B6.SJL cells refers to % of CD45.1 B6.SJL cells in corresponding cultures containing cleaved Casp3.

Supplemental Video Legends

Supplemental Video 1. *Yersinia*-infected macrophages exhibit morphological changes associated with apoptosis. Immortalized B6 macrophages were plated on 35 mm polymer coverslip dishes (Ibidi) 16 hrs prior to infection. Propidium iodide (Calbiochem) was added to the media and cells were infected with *Yersinia* as described in Materials and Methods. Cells were imaged on a heated stage over 4 hours with frames taken every 15 minutes.

Supplemental Video 2. *Yersinia*-infected macrophages exhibit morphological changes associated with pyroptosis. Immortalized B6 macrophages were plated on 35 mm polymer coverslip dishes (Ibidi) 16 hrs prior to infection. Propidium iodide (Calbiochem) was added to the media and cells were infected with *Yersinia* as described in Materials and Methods. Cells were imaged on a heated stage over 4 hours with frames taken every 15 minutes.