Signaling of Apoptosis through TLRs Critically Involves Toll/IL-1 Receptor Domain-Containing Adapter Inducing IFN-β, but Not MyD88, in Bacteria-Infected Murine Macrophages

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The TLRs are important sensors of the innate immune system that serve to identify conserved microbial components to mount a protective immune response. They furthermore control the survival of the challenged cell by governing the induction of pro- and antiapoptotic signaling pathways. Pathogenic Yersinia spp. uncouple the balance of life and death signals in infected macrophages, which compels the macrophage to undergo apoptosis. The initiation of apoptosis by Yersinia infection specifically involves TLR4 signaling, although Yersinia can activate TLR2 and TLR4. In this study we characterized the roles of downstream TLR adapter proteins in the induction of TLR-responsive apoptosis. Experiments using murine macrophages defective for MyD88 or Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF) revealed that deficiency of TRIF, but not of MyD88, provides protection against Yersinia-mediated cell death. Similarly, apoptosis provoked by treatment of macrophages with the TLR4 agonist LPS in the presence of a proteasome inhibitor was inhibited in TRIF-defective, but not in MyD88-negative, cells. The transfection of macrophages with TRIF furthermore potently promoted macrophage apoptosis, a process that involved activation of a Fas-associated death domain- and caspase-8-dependent apoptotic pathway. These data indicate a crucial function of TRIF as pro-apoptotic signal transducer in bacteria-infected murine macrophages, an activity that is not prominent for MyD88. The ability to elicit TRIF-dependent apoptosis was not restricted to TLR4 activation, but was also demonstrated for TLR3 agonists. Together, these results argue for a specific proapoptotic activity of TRIF as part of the host innate immune response to bacterial or viral infection.

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TLRs are important sensors of the innate immune system that serve to identify conserved microbial components to mount a protective immune response. They furthermore control the survival of the challenged cell by governing the induction of pro- and antiapoptotic signaling pathways. Pathogenic Yersinia spp. uncouple the balance of life and death signals in infected macrophages, which compels the macrophage to undergo apoptosis. The initiation of apoptosis by Yersinia infection specifically involves TLR4 signaling, although Yersinia can activate TLR2 and TLR4. In this study we characterized the roles of downstream TLR adapter proteins in the induction of TLR-responsive apoptosis. Experiments using murine macrophages defective for MyD88 or Toll/IL-1R domain-containing adapter inducing IFN-β (TRIF) revealed that deficiency of TRIF, but not of MyD88, provides protection against Yersinia-mediated cell death. Similarly, apoptosis provoked by treatment of macrophages with the TLR4 agonist LPS in the presence of a proteasome inhibitor was inhibited in TRIF-defective, but not in MyD88-negative, cells. The transfection of macrophages with TRIF furthermore potently promoted macrophage apoptosis, a process that involved activation of a Fas-associated death domain- and caspase-8-dependent apoptotic pathway. These data indicate a crucial function of TRIF as pro-apoptotic signal transducer in bacteria-infected murine macrophages, an activity that is not prominent for MyD88. The ability to elicit TRIF-dependent apoptosis was not restricted to TLR4 activation, but was also demonstrated for TLR3 agonists. Together, these results argue for a specific proapoptotic activity of TRIF as part of the host innate immune response to bacterial or viral infection.

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1 Abbreviations used in this paper: Yop, Yersinia outer protein; CrmA, cytokine response modifier A; FADD, Fas-associated death domain protein; IKK, IκB kinase; IκBα, IκB kinase-3, IFN regulatory factor 3; PKR, dsRNA-dependent protein kinase; poly(I:C), polyinosinic-polycytidylic acid; RIP1, receptor-interacting protein-1; TIR, Toll/IL-1R; TRIF, TRIF domain-containing adapter inducing IFN-β.
only TLR4 signaling contributes to induce macrophage apoptosis (12). Also, agonist-specific activation of TLR4, but not TLR2, efficiently elicits cell death under NF-κB inhibitory conditions. These results identify TLR4 as a major inducer of apoptosis in bacteria-infected macrophages (12). This observation is opposed to previous studies that reported an apoptosis-inducing capacity of bacteria-infected macrophages (12). These results identify TLR4 as a major inducer of apoptosis in macrophages (12). Shifting the growth temperature to 37°C abrogated 1/20 in fresh Luria-Bertoni broth and grown for another 2 h at 37°C. 

Several Toll/IL-1R (TIR) domain-containing adapter molecules have been shown to be recruited to the cytoplasmic TIR domains of activated TLRs and to mediate downstream TLR signaling (1–4, 22). These adapters determine the signaling pathways that confer NF-κB activation. There are two major NF-κB-inducing pathways that diverge at the level of the TLR adapter proteins (1–4, 22). One of them depends on MyD88, which is required for NF-κB activation by all known TLRs, with the possible exception of TLR3 (22–26). MyD88-driven NF-κB signaling upon TLR2 and TLR4 stimulation furthermore involves TIR adapter protein, also termed MyD88 adapter-like protein (2, 3, 22). The MyD88-transmitted signal is relayed via IL-1R-associated kinase family members and TRIF receptor-associated factor 6 to the IKK complex, which ultimately mediates NF-κB activation (1–4, 22). The second major pathway emanating from TLRs depends on TIR domain-containing adapter inducible IFN-β (TRIF), also known as TIR-containing adapter molecule-1. TRIF plays the predominant role in TLR3-mediated NF-κB activation and promotes MyD88-independent NF-κB activation in TLR4 signaling (22–26). TLR4-induced TRIF activation furthermore requires the TRIF-related adapter molecule (or TIR-containing adapter molecule-2) (27–29).

The TRIF pathway appears to be specifically induced by activation of TLR3 and TLR4. In addition to NF-κB induction, TRIF signaling facilitates activation of the IFN regulatory factor 3 (IRF-3) transcription factor via IKKε and TRAF family member-associated NF-κB activator-binding kinase 1, followed by the expression of IFN-β genes (22–31).

Numerous reports have shown the involvement of TLR adapters in proapoptotic signaling in stimulated cells (17, 26, 32–37). These studies suggested that both the MyD88- and TRIF-dependent signaling pathways can play roles in monocyte or macrophage signaling of apoptosis upon stimulation with conserved bacterial components, such as bacterial lipopolysaccharides or LPS. In this study we characterized Yersinia-mediated cell death in macrophages deficient for functional MyD88 or TRIF. Our data show that TRIF, but not MyD88, plays a critical role in the induction of apoptosis in Y. enterocolitica-infected macrophages. The ability of TRIF to promote apoptosis is common to treatment of macrophages with TLR4 as well as with TLR3 agonists. This suggests a major role for the TLR4- and TLR3-responsive TRIF pathway in the induction of apoptosis in innate immunity signaling. The initiation of apoptosis through TRIF, which occurs through activation of the death receptor-related Fas-associated death domain (FADD) and caspase-8 apoptotic pathway, may be part of an immune effector mechanism that enables the host to remove infected cells and control infection.

Materials and Methods

Yersinia strains, cell lines, and stimulation conditions

The Y. enterocolitica strains used in this study were the serotype O8 wild-type strain WA-314 and its isogenic yopP-knockout mutant WA-314ΔyopP (18). For infection, overnight cultures grown at 27°C were diluted 1/20 in fresh Luria-Bertoni broth and grown for another 2 h at 37°C (19). Shifting the growth temperature to 37°C initialized activation of the Yersinia type III secretion machinery for efficient translocation of Yops into the host cell upon cellular contact. To equalize and synchronize infection, bacteria were seeded on the cells by centrifugation at 400 × g for 5 min at a ratio of 20 bacteria/cell. For incubation times >30 min, bacteria were killed by addition of gentamicin (100 μg/ml) after 90 min. Murine J774A.1 macrophages were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 5 mM l-glutamine (19). In some experiments, cells were pretreated with the proteasome inhibitory peptide Z-Leu-Leu-Leu-CHO (MG-132; 5 μM, Biomol Research Laboratories, Plymouth Meeting, PA), or with inhibitors for caspase-1 and -5 (z-WEHD-fmk), caspase-2 (z-VDVAD-fmk), caspase-8 (z-IETD-fmk), or caspase-9 (z-LEHD-fmk; all from Calbiochem, La Jolla, CA) at 40 μM for 30 min, then stimulated as indicated. Stimulations were performed with LPS from Escherichia coli O55:B5 (1 μg/ml; Sigma-Aldrich, Munich, Germany), polyinosinic-polycytidylic acid (polyIC; 40 μg/ml; Sigma-Aldrich), or the synthetic bacterial lipoprotein analog N-palmitoyl-L-[2,3-bis(palmitoyl-oxo)-(2R,3S)-propyl]-[(R)-cysteinyl-seryl-(lysyl)3-lysine PSK4] (4 μg/ml; EMD Millipore, Billsberg, Germany) (10). The human embryonic kidney HEK293 cell line was grown in DMEM cell growth medium supplemented with 10% heat-inactivated FCS (12). Jurkat T cells were cultered in RPMI 1640 medium containing 5% heat-inactivated FCS in the absence or the presence of anti-Fas mAb CH11 (Upstate, Charlottesville, VA) for 4 h (38).

Dye Annexin V kit (BD Biosciences, San Jose, CA). 

Expression vectors, transfection, and analysis of quantity and morphology of transfected cells

J774A.1 cells were transfected with the ExGen 500 transfection reagent according to the manufacturer’s instructions (Fermentas, Hanover, MD) (18). HEK293 cells were transfected by the calcium phosphate transfection method (12). Unless indicated otherwise, transfections were conducted cotransfection experiments using 0.2 μg of pSV-β-galactosidase expression vector (Promega, Madison, WI) and 0.8 μg of the plasmids of interest. Mice and peritoneal macrophages

Peritoneal macrophages from C3H/HeN wild-type and TLR4-defective C3H/HeJ TLR4d/d mice (40, 41) were purchased from Charles River (Sulzfeld, Germany). Elicited peritoneal macrophages were obtained from mice 3 days after i.p. inoculation of 10% proteose peptone broth as previously described (12).
of cells undergoing apoptosis (19). The simultaneous application of the DNA stain propidium iodide (Sigma-Aldrich) allowed discrimination of apoptotic from necrotic cells. The rates of cell death were determined by visual scoring of a minimum of 200 cells/sample in a fluorescence microscope. Results are expressed as the mean percentage ± SD of apoptotic cells vs the total number of cells from three independent experiments. To evaluate caspase-8 activity at the single-cell level, unfixed cells were stained with carboxyfluorescein-labeled FAM-LETD-fmk according to the manufacturer’s instructions (ApoFluor caspase activity assay; ICN, Irvine, CA). FAM-LETD-fmk covalently binds to active caspase-8, and the rate of fluorescent cells was analyzed by fluorescence microscopy. The viability and nuclear morphology of cells treated with specific caspase inhibitory peptides were assessed by immunofluorescent labeling with propidium iodide (34). The initiation of apoptosis in J74.4A.1 macrophages that were cotransfected with a GFP reporter plasmid was analyzed by staining with Cy3-labeled annexin V (Sigma-Aldrich), which confers Cy3-dependent red fluorescence to cells undergoing apoptosis (34).

**Measurement of macrophage TNF-α production**

For quantitation of TNF-α production, macrophages were treated as indicated, and cell culture supernatants were removed after a final 20-h incubation. The TNF-α levels in the supernatants were determined by a commercially available capture ELISA using goat anti-mouse TNF-α mAbs as recommended by the manufacturer (R&D Systems, Minneapolis, MN) (12).

**Immunoblotting**

For the detection of transiently overexpressed TRIF and ΔTRIF in HEK293 cells, lysates were prepared from 3 × 10^6 cells/sample 20 h after transfection. The lysis buffer was composed of 50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, and protease and phosphatase inhibitors (Roche). Cellular lysates were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was performed with anti-FLAG (Sigma-Aldrich) or anti-c-Myc (BD Clontech) epitope mouse mAbs to detect the expression of TRIF or ΔTRIF, respectively. To determine caspase-8 processing in TRIF-transfected HEK293 and Fas-activated Jurkat cells, the membranes were probed with mouse monoclonal anti-human caspase-8 Abs (Cell Signaling Technology, Beverly, MA). The Jurkat cell lysates were prepared using a buffer that contained 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Igepal CA-630, and a standard mix of protease inhibitors (Roche). For analysis of cleavage of caspase-8 and Bid in peritoneal macrophages, a total of 2 × 10^6 cells/sample was lysed in 4× Laemmli sample buffer after stimulation as indicated. The lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with rat monoclonal anti-mouse caspase-8 Abs (Alexis Biochemicals, Carlsbad, CA). Immunoreactive bands were visualized using appropriate secondary Abs and ECL detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Thereafter, the membrane was stripped in 62.5 mM Tris (pH 6.7), 0.1 mM 2-ME, and 2% SDS for 30 min at 50°C. The membrane was then reprobed with polyclonal anti-Bid (R&D Systems) and secondary Abs, and developed by chemiluminescence.

**Results**

**TRIF signals apoptosis in Yersinia-infected macrophages**

To identify TLR adapter proteins mediating apoptosis induction by *Y. enterocolitica* downstream from the TLR complexes, we investigated *Yersinia*-conferred cell death in primary murine macrophages deficient for functional MyD88 or TRIF (Fig. 1). MyD88^{−/−} mice harbor a MyD88-null mutation (39), whereas TRIF^{−/−}/ΔTRIF^{−/−} mice bear a frameshift mutation within the C terminus of TRIF that generates a nonfunctional TRIF (26). Apoptosis in peritoneal macrophages obtained from these mice was analyzed in comparison with that in wild-type macrophages. Apoptotic cells were identified by labeling with fluorescein-conjugated annexin V and propidium iodide. Annexin V binds with high affinity to phosphatidylserine exposed on the outer leaflet of cells undergoing apoptosis (19). In these experiments the WA-314 *Y. enterocolitica* wild-type strain induced robust apoptosis in wild-type control cells, which was not observed after infection with the YopP-negative mutant WA-ΔyopP. This confirms that the NF-kB inhibitory activity of YopP is required for efficient execution of apoptosis in

**Yersinia-infected macrophages** (18). Notably, apoptosis in MyD88^{−/−} macrophages was not reduced compared with that in wild-type cells. This shows that the absence of MyD88 does not improve the survival of *Yersinia*-infected macrophages. In contrast, macrophages elicited from TRIF^{−/−}/ΔTRIF^{−/−} mice were considerably protected against *Yersinia*-induced apoptosis. Accordingly, 60–70% of TRIF-mutagenized macrophages survived when >80% of control or MyD88^{−/−} cells were already apoptotic. This indicates that signaling through TRIF, but not through MyD88, plays a role in *Yersinia*-conferred cell death, suggesting a critical function of TRIF as a signal transmitter of apoptosis.

**TLR4 and TLR3 agonists signal apoptosis via TRIF**

To examine whether the ability of TRIF to activate apoptosis is restricted to infection of macrophages by *Yersinia* or may represent a more general phenomenon, we analyzed the apoptosis-confering abilities of specific TLR agonists. LPS is an activator of TLR4, poly(I:C) signals via TLR3, and the synthetic lipopeptide P3CSK4 mediates activation of TLR2 (1–4, 10, 12, 23, 46). In previous studies we showed that pretreatment of macrophages with the proteasome inhibitor peptide Z-Leu-Leu-Leu-CHO (MG-132) sensitizes the cells to undergo apoptosis upon stimulation of TLR4 with LPS (12). MG-132 suppresses degradation of the NF-kB inhibitory IκB proteins through the proteasome pathway (47), which inhibits NF-kB activation in macrophages (48). Treatment with MG-132 could thus mimic the NF-kB inhibitory activity of YopP. In fact, activation of TLR4, but not of TLR2, triggers an efficient apoptotic response in MG-132-treated macrophages (12), which is probably an indication of the roles of these receptors in *Yersinia*-mediated cell death. We compared the induction of apoptosis by the diverse TLR agonists in macrophages prepared from MyD88^{−/−} and TRIF^{−/−}/ΔTRIF^{−/−} mice (Fig. 2A). In correlation with our previous studies, LPS stimulation elicited robust apoptosis of wild-type macrophages in presence of MG-132, whereas P3CSK4 did not (12). Notably, the TLR3 stimulus poly(I:C) could also provoke substantial cell death in MG-132-pretreated cells. This effect was not due to a potential LPS contamination of the poly(I:C) preparation, because poly(I:C), unlike LPS, could also

**FIGURE 1.** The TRIF^{Δyop2Δyop2} mutation provides protection against *Y. enterocolitica*-induced apoptosis. Peritoneal macrophages elicited from C57BL/6 wild-type (WT), MyD88^{−/−}, or TRIF^{Δyop2Δyop2} mice remained untreated or were infected with wild-type yersiniae (WA-314) or the YopP-negative mutant (WA-ΔyopP). Apoptosis was analyzed 7 h after the onset of stimulation by labeling apoptotic cells with fluorescein-conjugated annexin V and propidium iodide. The rate of cell death was determined by visual scoring of a minimum of 200 cells/sample in a fluorescence microscope. Results are expressed as the mean percentage ± SD of apoptotic cells vs the total number of cells from three independent experiments.

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**PROAPOPTOTIC ROLE OF TRIF**

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mediate apoptosis in TLR4-defective C3H/HeJ TLR4<sup>−/−</sup> macrophages (Fig. 2B). C3H/HeJ TLR4<sup>−/−</sup> mice harbor a loss of function mutation within the cytoplasmic portion of TLR4 (40, 41). In C3H/HeN wild-type cells, LPS and poly(I:C) induced a similar degree of apoptosis as in C57BL/6 control macrophages (Fig. 2, B and A, first graphs each). Despite exhibiting distinct effects on cellular viability, all three TLR agonists elicited a significant TNF-α response in wild-type cells (Fig. 2C), which was highest for LPS. This indicates that all agonists can successfully stimulate the cells under the experimental conditions. Interestingly, the apoptosis-inducing capabilities of LPS and poly(I:C) were almost completely abolished in TRIF-defective macrophages (Fig. 2A). In contrast, MyD88 deficiency could not provide protection against agonist-dependent apoptosis. This indicates a crucial involvement of the TRIF signaling pathway in transducing the TLR4- and TLR3-responsive proapoptotic signals. MyD88 apparently is not mandatory for this process. In the absence of MG-132, neither LPS nor poly(I:C) could elicit macrophage apoptosis. Thus, inhibition of the antiapoptotic NF-κB signaling pathway by MG-132 appears to be required for the initiation of apoptosis in response to these stimuli, which correlates well with cell death in <i>Yersinia</i>-infected macrophages that only undergo apoptosis when NF-κB activation is suppressed (18, 48). The data in Fig. 2A furthermore show that a small fraction of TRIF-mutagenized cells could undergo LPS- or poly(I:C)-dependent apoptosis upon inhibition of the proteasome pathway (10–25% apoptotic cells 7 h after onset of stimulation; Fig. 2A, right graph). The rate of apoptosis under these conditions increased to >60% after 20 h of stimulation (data not shown). This effect was not equally observed in LPS-stimulated, TLR4-defective C3H/HeJ TLR4<sup>−/−</sup> cells (5–10% after 7 h of stimulation (Fig. 2B, right graph) and 10–30% after 20 h of stimulation). Thus, TRIF-deficient macrophages were not completely protected against LPS- and poly(I:C)-responsive cell death, which implies that another TLR adapter could confer moderate and delayed apoptosis in the absence of functional TRIF.

**TRIF signals apoptosis through a FADD-caspase-8-dependent pathway**

To strengthen the indications for a proapoptotic function of TRIF, we tested whether TRIF could induce apoptosis when overexpressed in eukaryotic cells. We first transfected murine J774A.1 macrophages with eukaryotic expression vectors encoding either Flag epitope-tagged full-length TRIF or a c-Myc epitope-tagged ΔTRIF version that produces only the TRIF-TIR domain (42). The ΔTRIF construct has been reported to act as a dominant negative inhibitor of TLR-mediated NF-κB activation (42). TRIF and ΔTRIF were expressed in single transfected cells, and this was controlled by immunofluorescence microscopy using anti-Flag or anti-c-Myc epitope Abs, respectively (34) (data not shown). For analysis of apoptosis specifically in transfected cells, a GFP reporter plasmid was cotransfected. The expression of GFP enables the detection of single transfected cells by fluorescence microscopy using anti-GFP Abs (34) (data not shown). To assess TRIF-dependent apoptosis, we cotransfected J774A.1 macrophages with a c-Myc epitope-tagged GFP reporter plasmid and a TRIF construct. The expression of GFP enables the detection of single transfected cells by fluorescence microscopy using anti-GFP Abs (34) (data not shown). The rate of apoptosis in transfected cells was assessed by labeling cells with fluorescein-conjugated annexin V and propidium iodide. Apoptotic cells were analyzed by fluorescence microscopy. Results are expressed as the mean percentage ± SD of apoptotic cells vs the total number of cells from three independent experiments. As shown in Fig. 2A, right graph, TRIF expression in transfected cells significantly increased the rate of apoptosis compared to untransfected cells, indicating that TRIF can induce apoptosis in a FADD-caspase-8-dependent manner. The results presented in Fig. 2 further support the conclusion that TRIF signals apoptosis through a FADD-caspase-8-dependent pathway.

**TRIF transmits apoptotic signals generated by TLR4 and TLR3 agonists.** A. Apoptosis mediated by LPS and poly(I:C) in the presence of the proteasome inhibitor MG-132 depends on functional TRIF. Peritoneal macrophages elicited from C57BL/6 wild-type (WT), MyD88<sup>−/−</sup>, or TRIF<sup>Lps2/Lps2</sup> mice remained untreated or were stimulated with LPS, P3CSK4, or poly(I:C) under conditions with or without pretreatment with the proteasome inhibitor MG-132. Apoptosis in WT and MyD88<sup>−/−</sup> macrophages elicited from C57BL/6 wild-type mice remained untreated or were stimulated with LPS, P3CSK4, or poly(I:C) under conditions with or without pretreatment with the proteasome inhibitor MG-132. Apoptosis in A and B was analyzed 7 h after the onset of stimulation by labeling apoptotic cells with fluorescein-conjugated annexin V and propidium iodide. Apoptotic cells were analyzed by fluorescence microscopy. Results are expressed as the mean percentage ± SD of apoptotic cells vs the total number of cells from three independent experiments. C. TNF-α production in response to LPS, P3CSK4, and poly(I:C) stimulation. Peritoneal macrophages elicited from C57BL/6 wild-type mice remained untreated or were stimulated with LPS, P3CSK4, or poly(I:C). The amount of TNF-α released was analyzed 20 h later. Results are expressed as the mean percentage of picograms of TNF-α per milliliter ± SD from three independent experiments.
microscopy without preceding immunostaining. Simultaneous labeling with Cy3-conjugated annexin V confers a red fluorescence to cells undergoing apoptosis (34). In these experiments, 50–70% of the TRIF-transfected cells displayed enhanced membrane binding of annexin V 8 h after transfection, indicating the onset of apoptosis (Fig. 3A). Apoptosis induction was not detected in cells transfected with the TRIF-TIR domain (ΔTRIF; Fig. 3A). Because expression of the cotransfected GFP decreased during apoptosis, a β-galactosidase-encoding reporter vector was cotransfected for quantification of cell death at later time points. Apoptosis in β-galactosidase-expressing cells, which is characterized by typical cellular shrinkage and condensation, was microscopically evaluated (6–9, 18). Almost all cells transfected with full-length TRIF were apoptotic 18 h after transfection (Fig. 3B, left panel). This pronounced proapoptotic effect was not observed in cells transfected with ΔTRIF or empty vector control. These data show that TRIF overexpression is sufficient to elicit apoptosis in macrophages. This could indicate that the signaling of apoptosis is a prominent function of TRIF. However, because apoptosis in TRIF-transfected cells did not require blockage of NF-κB activation by either *Yersinia* infection or proteasome inhibitor treatment, it cannot be ruled out that TRIF overexpression triggers proapoptotic events that are normally not operative under physiological conditions of TRIF expression.

Our previous studies suggested that *Yersinia*- and TLR4-dependent apoptosis could both involve signaling via FADD and caspase-8. Accordingly, the overexpression of a dominant negative version of FADD (ΔFADD) could provide protection against TLR4- and *Yersinia*-conferred cell death (12, 34). Because TRIF apparently relays the apoptotic signals emanating from TLR4, we examined whether FADD and caspase-8 may also play role in TRIF-mediated apoptosis. In an attempt to obtain indications on involvement of FADD or caspase-8 in TRIF-dependent cell death, we cotransfected the TRIF expression plasmid with vectors encoding ΔFADD (43) or the viral serpin CrmA (44). CrmA acts as specific inhibitor of caspase-1 and caspase-8 (49, 50). Both ΔFADD and CrmA constructs can prevent Fas- and TNF receptor-induced apoptosis through inhibition of caspase-8 (43, 51). The expression of TRIF in the cotransfection experiments displayed in Fig. 3B was verified by immunofluorescence staining with anti-Flag Abs (data not shown). In line with the data previously reported for TLR4-induced apoptosis, transfection of dominant negative ΔFADD remarkably rescued macrophages from cell death resulting from TRIF overexpression (Fig. 3B, right panel). Similarly, CrmA transfection inhibited TRIF-dependent apoptosis of macrophages. These data imply that TRIF engages the FADD-caspase-8 cytotoxic pathway to mediate apoptosis downstream of TLR4. Fig. 3B (right panel) furthermore shows that overexpression of the antiapoptotic molecule Bcl-2 could not prevent apoptosis of TRIF-transfected cells. Under the same conditions, Bcl-2 transfection reduced *J774A.1* cell apoptosis in response to staurosporine treatment; 20–40% of Bcl-2-transfected cells underwent apoptosis 8 h after onset of treatment with 5 μM staurosporine, whereas >90% of control vector-transfected cells were already apoptotic at that time point. This demonstrates that overexpressed Bcl-2 in principle can protect against apoptosis. However, because Bcl-2 predominantly counteracts cell death resulting from activation of intrinsic mitochondrial, but not from extrinsic, death receptor-induced pathways (52, 53), the absence of an inhibitory effect of Bcl-2 reinforces the hypothesis that the death receptor-responsive FADD-caspase-8 pathway plays a critical role in TRIF-conferring apoptosis.

Apoptosis as a consequence of TRIF overexpression was not only confined to macrophages, but became evident also in transfected human embryonic kidney (HEK) 293 cells (Fig. 3C). Cotransfection of ΔFADD and CrmA blocked TRIF-dependent apoptosis in the same manner as in *J774A.1* cells, whereas Bcl-2 had no protective effect. The expression of TRIF and ΔTRIF in the different experimental conditions was analyzed in cellular lysates

**FIGURE 3.** ΔFADD and CrmA inhibit TRIF-promoted apoptosis. A, TRIF-transfected macrophages stain with the apoptotic marker annexin V. *J774A.1* macrophages were cotransfected with GFP reporter plasmid and expression vectors for either full-length TRIF (TRIF) or the TRIF TIR domain (ΔTRIF), and were labeled with Cy3-annexin V (red fluorescence) 8 h after transfection. The micrographs show GFP-producing transfected cells (green fluorescence). B, TRIF transfection mediates *J774A.1* macrophage cell death that is inhibited by ΔFADD and CrmA. *J774A.1* cells were transfected in the left panel with empty expression vector control (vector) or full-length TRIF or ΔTRIF expression plasmids together with a β-galactosidase reporter vector. In the right panel, *J774A.1* macrophages were cotransfected with β-galactosidase reporter vector and either the empty control vector (vector) or the full-length TRIF expression vector (TRIF) together with ΔFADD, CrmA, Bcl-2, or control expression plasmid. C, TRIF-transfected HEK293 undergo apoptosis similar to *J774A.1* macrophages. HEK293 cells were cotransfected with β-galactosidase reporter vector and either the empty control vector (vector), or the full-length TRIF expression vector (TRIF) together with the indicated expression plasmids. Transfected cells in B and C were stained with X-galactosidase 18 h after transfection, and single transfected blue cells were analyzed for an apoptotic morphology. Results are expressed as the mean percentage ± SD of apoptotic vs the total number of transfected cells from three independent experiments. The expression levels of cotransfected TRIF and ΔTRIF in lysates of HEK293 cells were analyzed by Western immunoblotting using anti-Flag or anti-e-c-Myc epitope Abs, respectively (C).
FIGURE 4. TRIF controls LPS-dependent caspase-8 activation. A. TRIF overexpression can mediate processing of caspase-8. HEK293 cells were cotransfected with CD4/TLR4 and either the empty control vector (vector) or the full-length TRIF expression vector (TRIF). Twenty hours after transfection, cellular lysates were prepared, separated by 15% SDS-PAGE, and probed with anti-caspase-8 Abs. Lysates of untreated and anti-Fas Ab-treated Jurkat cells were included as negative and positive controls, respectively. B. LPS-responsive processing of caspase-8 and Bid in MG-132-pretreated macrophages depends on TRIF. Peritoneal macrophages elicited from C57BL/6 wild-type (WT) or TRIFLps2/Lps2 mice remained untreated or were stimulated with LPS in the absence or presence of the proteasome inhibitor MG-132. Cellular lysates were prepared 3 h after LPS addition. Lysates were separated by 15% SDS-PAGE, probed with anti-caspase-8 Abs, and visualized by ECL reaction (upper panel). The arrows in A and B indicate full-length pro-caspase-8 and the processed active p18/20 caspase-8 subunit. B. The intermediate p43 caspase-8 cleavage product was additionally detected. A nonspecific band (n. s.) appearing above caspase-8 p18/20 in B demonstrates equal loading of the gel with cellular lysates. The same membrane was then stripped and reprobed with anti-Bid Abs (B, lower panel). Arrows indicate cleaved, truncated (tBid), and uncleaved forms of Bid (Bid). C. Macrophages treated with LPS- and MG-132 or infected with wild-type Yersinia display caspase-8 activation. Peritoneal macrophages elicited from C57BL/6 wild-type mice remained untreated, were stimulated with LPS in the absence or presence of the proteasome inhibitor MG-132, or were infected with YopP-negative (WA-DyoP) or wild-type (WA-314) yersiniae. The activation of caspase-8 in single cells was assayed 8 h after the onset of stimulation by staining with caspase-8-specific FAM-IETD-fmk. The percentage of fluorescent cells exhibiting caspase-8 activity was determined by fluorescence microscopy.
are characteristics of necrotic cell death (55, 56), suggesting that the dying macrophages undergo necrosis when caspase-8 activity is blocked. This observation correlates with previous reports that describe a FADD-dependent shift from apoptosis to necrosis in cells that are impaired in their caspase activities (55, 56). Together, these data indicate an important role for the TRIF-regulated FADD-caspase-8 signaling pathway in determining the fate of LPS/MG-132- and Yersinia-treated macrophages. No obvious implication of other caspases in the induction of TRIF-related apoptosis could be uncovered by using peptide inhibitors for caspase-1, -2, -5, and -9 (data not shown). These experiments further did not reveal a salient role of one of these caspases in the induction of the TRIF-independent, delayed apoptosis pathway mentioned above (Fig. 2A; data not shown). However, because the caspase-8 inhibitor exerted a 40–50% inhibitory effect also in these conditions, apoptosis that bypasses TRIF may also involve caspase-8 activation.

Discussion
Our previous studies have shown that the bacterial pathogen Y. enterocolitica inhibits antiapoptotic NF-κB signaling and simultaneously engages receptors of the innate immune system to trigger apoptosis in macrophages (18). The induction of apoptosis by Yersinia infection involves signaling through TLR4, but not through TLR2, although both TLRs share the ability to recognize Yersinia (12). To emphasize the implication of single TLR adapter proteins in the transmission of proapoptotic signals downstream from the TLR complexes, we investigated Yersinia-conferring cell death in primary mouse macrophages that were defective for functional MyD88 or TRIF. Both TLR adapter proteins can transduce TLR4-responsive signaling and have been linked to the elicitation of proapoptotic pathways in monocyte or macrophage cells upon challenge with conserved bacterial components (17, 26, 34). Our data show that selectively TRIF, but not MyD88, is involved in the promotion of an apoptotic response in Yersinia-infected macrophages. Furthermore, TRIF plays the major role in conferring cell death that is provoked by the activation of TLR4 through LPS under conditions in which the proteasome pathway is inhibited. Because TRIF is activated by signaling through TLR4, but not through TLR2 (24–26), these results explain the efficient and selective apoptosis-inducing capacity that we previously observed for TLR4. Moreover, the ability of TRIF to confer apoptosis is not restricted to TLR4 activation, but seems to also occur via TLR3. In fact, the TLR3 ligand poly(I:C) could trigger apoptosis under NF-κB inhibitory conditions similar to LPS. These data demonstrate that the transmission of apoptosis signals is a major function of TRIF signal transduction, an activity that is not prominent for MyD88 signaling.

The unapparent role of MyD88 in promoting cell death in our study questions the significance of previous reports that suggested a proapoptotic function of MyD88 (17, 32–36). These conclusions were mainly based on data resulting from the overexpression of dominant negative MyD88, which implies that the observed effects could merely reflect overexpression phenomena. However, most of the studies that have shown apoptotic activities of the TLR2-MyD88 pathway were conducted on human cell lines (10, 13–15, 17, 33), whereas our experiments and those described by Hoebe et al. (26), which first have implicated TRIF as an apoptosis transmitter, were performed on primary mouse cells. This suggests that there could be cell- or species-specific differences in the activation of TLR-dependent proapoptotic pathways. In addition, our data show that TRIF-mutagenized cells could undergo delayed, LPS-dependent apoptosis upon inhibition of the proteasome pathway. This effect may potentially be mediated by another TLR adapter.

Thus, although TRIF clearly plays the major role in transmitting TLR-dependent apoptosis in murine macrophages, alternative TLR signaling pathways could be able to perform limited proapoptotic activities in the absence of functional TRIF. Interestingly, it has recently been shown that activation of dsRNA-dependent protein kinase (PKR) by TLR4 can contribute to Yersinia-induced apoptosis in mouse bone marrow-derived macrophages (57). In these cells, PKR mediates late onset of apoptosis that depends on PKR kinase activity, mediating phosphorylation of elongation initiation factor 2α and concomitant inhibition of protein synthesis. Although this mechanism appears to be of minor importance for TRIF-dependent apoptosis in our studies, because a kinase-inactive PKR mutant did not interfere with with TRIF-related (12) or TRIF-related (data not shown) cell death, PKR could play a role as signal transmitter that confers delayed cell death when expeditious TRIF engagement is impaired.

However, the downstream signal relay that couples apoptosis signaling by TRIF to the cell death machinery is not yet completely understood. Our previous studies have indicated the engagement of FADD and caspase-8 in TLR4- and Yersinia-dependent apoptosis (12, 34), suggesting roles for these molecules in TRIF-mediated cell death. FADD acts as a central adapter molecule in apoptosis signaling by the TNF receptor superfamily (43, 52–54). It activates the apoptotic machinery through induction of caspase-8. In our hands, dominant negative FADD and the caspase-8 inhibitor CrmA could counteract apoptosis elicited by TRIF overexpression. In addition, TRIF controlled the maturation and activation of caspase-8 and its substrate BID in macrophages that were forced to undergo LPS-dependent apoptosis. This demonstrates that TRIF critically participates in the induction of the FADD-caspase-8 pathway downstream from TLR4. However, we were unable to find a direct interaction of TRIF with FADD in coprecipitation experiments, which suggests that TRIF cannot directly activate FADD (data not shown). This is not surprising, because TRIF lacks a death domain that normally mediates interaction of FADD with its upstream activators (22, 25, 42). Interestingly, MyD88 bears such a death domain and can potentially activate FADD in TLR2-dependent apoptosis (17). This appears to be an evolutionary conserved signaling mechanism, because a comparable interaction has been described for dMyD88 and dFADD of Drosophila (58). Whether TRIF may engage MyD88 to confer FADD activation is currently not clear. We did not observe attenuation of TLR4- and TLR3-dependent apoptosis in MyD88-negative macrophages. Furthermore, dominant negative MyD88 could not reduce TRIF-related apoptosis (data not shown), and we were unable to detect an interaction of overexpressed TRIF and MyD88 in coprecipitation experiments (data not shown). These results could argue against a decisive role for MyD88 in TRIF-related cell death. Other molecules that can associate with TRIF include TRAF family member-associated NF-κB activator-binding kinase 1, TNF receptor-associated factor 6, and IRF-3 (30, 31, 42). IRF-3 has been reported to induce apoptosis in a caspase-8-dependent pathway (59), which suggests that IRF-3 could be a downstream signal transmitter of TRIF-directed cell death. However, apoptosis mediated by IRF-3 is supposed to occur through up-regulation of the expression of proapoptotic genes (59). This mechanism appears contrary to TLR4/TLR3- and TRIF-promoted apoptosis, because treatment with the protein synthesis inhibitor cycloheximide does not protect against, but, rather, renders macrophages susceptible to LPS- and poly(I:C)-elicited cell death (16, 60) (data not shown). This implies that the initiation of proapoptotic signaling through TLR4/TLR3 and TRIF does not depend on
de novo protein synthesis, but may be a more direct event of downstream signal transduction. Interestingly, two recent reports identified the kinase receptor-interacting protein-1 (RIP1) as a new TRIF-binding molecule (61, 62). RIP1 relays the TRIF-derived NF-κB activating signal (61, 62), but may also play a role in TRIF-dependent apoptosis signaling (61). RIP1 contains a death domain, which can interact with FADD. This indicates that RIP1 could be an adapter that links TRIF to the cellular apoptosis machinery (61). However, whether RIP1 deficiency can protect against TRIF-related apoptosis has yet to be determined.

The exclusive activation of TRIF by TLR3 and TLR4 indicates evolutionary divergence of the TRIF pathway from the MyD88 pathway to activate a specific cellular response. The induction of IRF-3 by TRIF induces the synthesis of IFN-β, which exerts effective antiviral and immunostimulatory activities (22, 24–31, 42, 63, 64). Activation of TLR3 by virus-derived dsRNA therefore potently inhibits viral replication (63, 64). Similarly, the stimulation of TLR4 can elicit an antiviral response (63, 64), and indeed, a number of studies have implicated TLR4 in viral recognition (65–67). Thus, both TLRs could play a role in conferring viral resistance by activation of IRF-3. However, the potently proapoptotic activity of TRIF argues for an additional function of TRIF in host immunity. Because viruses are obligate intracellular pathogens, they benefit from modulating apoptotic pathways that control the life span of the host cell. Numerous viruses have therefore evolved strategies to manipulate the cellular apoptotic machinery (68–71). In the same manner, several strictly intracellular bacterial pathogens, such as Chlamydia and Rickettsia spp. (72–74), and even facultative intracellular bacteria, i.e., Brucella and Bartonella spp. (74–76), efficiently modify apoptotic host cell signaling. These activities are supposed to prolong the survival of the infected cell, which helps the pathogen to complete its life cycle and to gain time for multiplication (68–76). From this view, the TRIF-related onset of apoptotic signaling during bacterial or viral infection could reflect a defense strategy of the host that counteracts the antipositive activations of the invading pathogen. The execution of apoptosis could finally lead to elimination of the infected cell. Indeed, a number of studies have indicated that intracellular bacteria can concomitantly die during apoptotic death of the host cell, which reinforces the idea that execution of apoptosis could act as an immediate host defense mechanism (77–81). Furthermore, bacteria-infected cells that are driven into apoptosis can serve as sources of bacterial Ag for infected bystander APCs, which facilitates induction of adaptive immunity (82, 83). Thus, the initiation of apoptosis through TRIF could be an important part of the host immune response. In fact, a related mechanism helps plants to control bacterial infection. Plant disease resistance gene products, which share some homology to TLRs, trigger the so-called hypersensitive response when encountering a bacterial pathogen (84, 85). This response resembles apoptosis in animal cells. It limits the spread of microbes through the induction of localized plant cell death at the site of invasion. This parallelism suggests that TLR-responsive signaling of apoptosis through TRIF may represent an evolutionary conserved innate immune effector mechanism that could help to dispatch infected cells and to restrict the replication of intracellular pathogens.

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References


