Divergence of Apoptosis-Inducing and Preventing Signals in Bacteria-Faced Macrophages Through Myeloid Differentiation Factor 88 and IL-1 Receptor-Associated Kinase Members

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The induction of apoptosis in host cells is a common strategy by which pathogenic bacteria interfere with the host immune response. The *Yersinia enterocolitica* outer protein P (YopP) inhibits activation of transcription factor NF-κB in macrophages, which suppresses NF-κB-dependent antiapoptotic activities. The simultaneous initiation of proapoptotic signaling by yersiniae infection or LPS treatment results in macrophage apoptosis. In this study, we used YopP as a tool to dissect survival- and death-inducing pathways in bacteria-faced macrophages. We cotransfected J774A.1 macrophages with expression plasmids for YopP and dominant-negative mutants of signal transmitters of the NF-κB cascade downstream from the LPS receptor complex. Dominant-negative myeloid differentiation factor 88 (MyD88) or IL-1R-associated kinase (IRAK) 2 diminished LPS-induced apoptosis in YopP-transfected macrophages, suggesting implication of MyD88 and IRAK2 in signaling cell death. In contrast, dominant-negative IRAK1 and TNFR-associated factor 6 (TRAF6) did not provide protection, but augmented LPS-mediated apoptosis in the absence of YopP, which indicates roles of IRAK1 and TRAF6 in the antiapoptotic signal relay of the NF-κB cascade. The distinct functions of IRAK members in macrophage survival were reflected by opposing effects of dominant-negative IRAK1 and IRAK2 on *Y. enterocolitica*-mediated apoptosis. Yersiniae- and LPS-dependent cell death were substantially attenuated by a specific caspase-8 inhibitory peptide or by dominant negative Fas-associated death domain protein (FADD). This suggests, that *Yersinia*-induced apoptosis involves a proapoptotic signal relay through MyD88 and IRAK2, which potentially targets the Fas-associated death domain protein/caspase-8 apoptotic pathway, whereas IRAK1 and TRAF6 counteract the bacteria-induced cytotoxic response by signaling macrophage survival. *The Journal of Immunology*, 2002, 168: 4601–4611.

The induction of apoptosis in host cells is part of the strategies by which pathogenic bacteria modulate the immune response of the host (1). Despite the common apoptotic end, the mechanisms of apoptosis induction largely differ between diverse bacterial pathogens. The Gram-negative bacterium *Yersinia* evolved a strategy that selectively mediates apoptosis in macrophages (2), which facilitates the spread of the bacteria in the host lymphoid tissue and enables the establishment of a systemic infection (3). Apoptosis due to yersiniae infection involves a plasmid-encoded type III protein secretion system that translocates *Yersinia* virulence proteins, the so-called *Yersinia enterocolitica* outer proteins (Yops), inside the host cell cytoplasm after cell contact (4). This type III secretion mechanism is shared by pathogenic *Yersinia* spp., including *Yersinia pestis*, the etiological agent of plague, and *Y. enterocolitica* and *Yersinia pseudotuberculosis*, which cause gastrointestinal syndromes, lymphadenitis, and septicemia (4). In the host cell, the Yops interfere with key cellular processes of the primary immune reaction. One specific Yop, YopP in *Y. enterocolitica* or its homologue YopJ in *Y. pseudotuberculosis* and *Y. pestis*, exerts a number of distinct effects on the host cell. It suppresses the production of inflammatory cytokines, such as TNF-α and IL-8, and mediates macrophage apoptosis (5–9). YopP/YopJ inhibits the activities of several parallel host cell signaling cascades. It targets and blocks the NF-κB-activating IkB kinase IKKβ, resulting in impairment of activation of transcription factor NF-κB (10). In addition, YopP/YopJ binds and inhibits members of the mitogen-activated protein kinase (MAPK) kinase family, which leads to disruption of MAPK pathways (10). It was recently proposed that YopP/YopJ possesses a ubiquitin-like protein protease activity that may contribute to these suppressive effects by disturbing the processing of intracellular signaling complexes (11). As the MAPK and NF-κB signaling cascades synergistically control the synthesis of host inflammatory cytokines, the inversion of these pathways by *Yersinia* spp. accounts for the cytokine suppressive effect (12, 13). *Yersinia*-induced apoptosis also crucially relies on inhibition of the NF-κB signaling pathway by YopP/YopJ (10, 13, 14). NF-κB mediates survival of eukaryotic cells by the prevention of apoptosis (15–22). It transcriptionally up-regulates the synthesis of antiapoptotic proteins, such as inhibitors of apoptosis and Bcl-2 family members (19–22). These proteins counteract proapoptotic signals elicited by diverse extracellular stimuli, which consequently preserves cellular viability (16–22). Several studies demonstrated that the antiapoptotic function of NF-κB is essential for the mediation of self-defense and survival of macrophages when encountered with bacteria or LPS (13, 14, 23, 24). LPS simultaneously activates...
pro- and antiapoptotic signaling pathways in macrophages, and the rapid activation of NF-κB provides protection against the cytoxic response. Consequently, the inhibition of NF-κB activation sensitizes macrophages to undergo apoptosis upon LPS treatment (13, 14, 23). Our previous study showed that Y. enterocolitica exploits this mechanism to compel the macrophage to undergo apoptosis (14). We demonstrated that Y. enterocolitica YopP disrupts the NF-κB survival pathway in J774A.1 macrophages at the level of IKKβ (14). IKK-β phosphorylates the NF-κB inhibitory IκB proteins, which sequester nonactivated NF-κB in the cytoplasm (15, 16). Phosphorylated IκBs are degraded through the proteasome pathway, which mediates release and nuclear translocation of NF-κB upon cellular activation. The transfection of J774A.1 macrophages with a YopP expression vector mediates moderate apoptosis, but this effect is substantially enhanced by the additional initiation of LPS signaling (14). This reveals a synergism between LPS-induced signal transduction and inhibition of NF-κB by YopP, leading to severe apoptosis in macrophages. Overexpression of the transcriptionally active p65 NF-κB subunit reverses Y. enterocolitica- and YopP- plus LPS-mediated cell death. Thus, the disruption of the antiapoptotic functions of NF-κB by YopP along with the activation of LPS signaling critically determines the fate of the Y. enterocolitica-infected macrophage (14). As LPS by itself mediates macrophage NF-κB activation, which is blocked by YopP at the IKKβ level, the proapoptotic LPS signal must occur upstream from IKKβ.

In this study, we used YopP as a tool to seek for signaling molecules that are potentially involved in the yersiniae- and LPS-induced proapoptotic response. In macrophages and monococytes, LPS initiates a signaling cascade that involves the transmembrane Toll-like receptors (TLR) 2 and 4, the surface receptor glycoprotein CD14, and the helper molecule MD-2 (25). Toll-like receptors (TLR) 2 and 4, the surface receptor glycoprotein CD14, and the helper molecule MD-2 (25) mediate TLR4-dependent signals. Combination of TLR4-responsive NF-κB activation, which is mediated by YopP, and the proapoptotic LPS signal must occur upstream from IKKβ.

Three IRAK molecules, IRAK1, IRAK2, and IRAK-M (33–36), can relay the NF-κB-activating signal via TNFR-associated factor 6 (TRAF6) and TGF-β-activated kinase 1 to the IKK complex, which ultimately mediates NF-κB activation (25–29). Recently, another adapter, designated MyD88-adapter-like (Mal) or Toll-IL-1 receptor domain-containing adapter protein (TIRAP), was identified which signals TLR4-responsive NF-κB activation independently from MyD88 (37, 38).

We transfected J774A.1 macrophages with dominant negative constructs of components of the NF-κB signaling cascade for LPS. Our data point out differential roles of these signaling intermediates in determining the fate of the macrophage that faces a bacterial pathogen. The proapoptotic signal relay exploited by Y. enterocolitica YopP potentially involves MyD88 and IRAK2, which appear to target the Fas-associated death domain protein (FADD)/ caspase-8 apoptotic pathway. Inversely, IRAK1 and TRAF6 are mediators of macrophage survival upon LPS treatment. This indicates that bifurcation of death-inducing and -preventing signals in bacteria-challenged macrophages depends on MyD88 and IRAK family members.

Materials and Methods

Bacterial strains, cell culture, and stimulation conditions

The Y. enterocolitica wild-type (WT) strain WA-314, serotype O8, and the respective yopP knockout mutant WA-314ΔyopP were grown as described previously (14). The murine macrophage cell line J774A.1 was cultured as described elsewhere (14), treated with 10 ng/ml LPS from Escherichia coli O55:B5 (Sigma-Aldrich, Munich, Germany), or infected as indicated. Infections were performed at a ratio of 50 bacteria per cell. For incubations times longer than 90 min, bacteria were killed by addition of gentamicin (100 μg/ml) after 90 min of infection. In some experiments, the cells were pretreated with the proteasome inhibitor peptide Z-Leu-Leu-Leu-CHO (Mg-132, 2.5 μM; Biomol, Plymouth Meeting, PA), or with inhibitors for caspase-2 (z-VDVAD-fmk), caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk; all from Calbiochem, La Jolla, CA), caspase-10 (z-AEVD-fmk; Biovision, Palo Alto, CA), or with broad-spectrum caspase inhibitor (z-VAD-fmk; Bachem, Heidelberg, Germany) at 40 μM for 1 h, then stimulus as indicated. Elicited peritoneal macrophages were obtained from male C57/HeN mice 3 days after i.p. inoculation of 1 ml of 10% protease peptone broth (13). Peritoneal exudate cells were washed and cultured at 37°C in cell culture growth medium. After 3 h, nonadherent cells were removed by repeated washing, and remanent macrophages were treated as indicated. Expression vectors, J774A.1 cell transfection, and analysis of quantity and morphology in infected macrophages

A total of 5 × 10⁶ J774A.1 cells/well was transfected with 4 μl ExGen 500 according to the manufacturer’s instructions (Fermentas, Hanover, MD). ExGen 500 is a nonliposomal gene delivery reagent consisting of linear polyethyleneimine molecules that share high cationic charge-density potential (39). Its gene transfer activity is related to its capacity for condensing DNA, interacting with anionic proteoglycans of the cell membrane, entering cells by endocytosis, and protecting DNA from lysosomal degradation. ExGen 500 appeared appropriate for our experimental approach because of low toxicity on J774A.1 cells. The interexperimental transfection efficiencies varied between 1% and 10%, providing around 500–5000 transfected cells/sample. Unless indicated otherwise, transfections were conducted as cotransfection experiments using 1 μg of plasmid DNA. In dependence on the experiment, 0.33 μg of pSvB-β-galactosidase expression vector (Promega, Madison, WI) was combined with 0.66 μg of the plasmid of interest, or 0.2 μg of pSvB-β-galactosidase expression vector were combined with 0.4 μg of two different plasmids of interest (14). β-Galactosidase expression correlation with expression of proteins encoded by cotransfected plasmids in the transfected cells (14). Most of the expression plasmids used in this study have been characterized and described before. They included expression vectors for YopP (14); dominant-negative ΔTRAF6 and WT-TRAF6 (Ref. 40; kindly provided by J. Inoue, Institute of Medical Science, University of Tokyo, Tokyo, Japan); dominant-negative ΔMyD88 (152–296), ΔIRAK1 (1–215), ΔIRAK2 (1–96) (Refs. 35 and 41; kindly provided by M. Muzio, Department of Immunology and Cell Biology, Mario Negri Institute, Milan, Italy); dominant-negative ΔIRAK-M (1–161) Ref. 36; kindly provided by Z. Cao and H. Wescbe, Tularik, South San Francisco, CA; dominant-negative ΔPADD (42; kindly provided by C. Vincenz, Department for Pathology, University of Michigan Medical School, Ann Arbor, MI); WT- and dominant-negative (39). Its gene transfer activity is related to its capacity for condensing DNA, interacting with anionic proteoglycans of the cell membrane, entering cells by endocytosis, and protecting DNA from lysosomal degradation. ExGen 500 appeared appropriate for our experimental approach because of low toxicity on J774A.1 cells. The interexperimental transfection efficiencies varied between 1% and 10%, providing around 500–5000 transfected cells/sample. Unless indicated otherwise, transfections were conducted as cotransfection experiments using 1 μg of plasmid DNA. 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Wescbe, Tularik, South San Francisco, CA; dominant-negative ΔPADD (42; kindly provided by C. Vincenz, Department for Pathology, University of Michigan Medical School, Ann Arbor, MI); WT- and dominant-negative ΔPADD (42; kindly provided by P. C. Vincenz, Department for Pathology, University of Michigan Medical School, Ann Arbor, MI). pcDNA3.1 was used as expression plasmid for yopP. The yopP gene was amplified from Y. enterocolitica WA-314 and cloned into the multicloning site of plasmid pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). Empty expression vectors containing no inserts were used as negative controls. For expression of green fluorescent protein (GFP)-labeled YopP, the yopP gene was amplified from Y. enterocolitica WA-314 and cloned into the multicloning site of plasmid pEGFP-C1 (Clontech Laboratories). The resulting plasmid produce YopP in fusion to the C terminus of enhanced GFP under control of the CMV promoter. Eighteen hours after transfection, cells were treated with LPS or infected with yersiniae as indicated. To identify transfected macrophages, cells were immunofluorescently labeled or stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) after certain points of time. For assessment of cell death in cotransfected cells, blue transfected cells were counted and the morphology of the transfected cells was determined using light microscopy (14, 17–20). Every single transfected cell was analyzed for an apoptotic appearance. A minimum of eight microscopic fields was investigated for each sample. For quantification, the number of apoptotic blue cells was assessed in relation to the total number of transfected cells. Results are expressed as mean percentages ± SD from three independent experiments. For accurate comparison of the different dominant-negative IRAK members, the cDNA for ΔIRAK-M was amplified and inserted into the multicloning site of plasmid pcDNA3.1 (Invitrogen, San Diego, CA). pcDNA3.1 was used as expression plasmid for ΔIRAK1 and ΔIRAK2 (41). All PCR products were checked for sequence accuracy by sequencing.

Fluorescent labeling of transfected and apoptotic cells

To quantify cell death in J774A.1 and mouse peritoneal macrophages in response to Yersinia infection, apoptotic cells were labeled with fluorescein-conjugated annexin V (Boehringer Mannheim, Mannheim, Germany)
as described previously (2). The cells transfected with the GFP plasmids were stained with Cy3-labeled annexin V (Sigma-Aldrich). Annexin V binds with high affinity to phosphatidylserine exposed on the outer leaflet of apoptotic cells and confers fluorescein- or Cy3-dependent fluorescence to the cells undergoing apoptosis. Viability of cells after treatment with specific caspase inhibitory peptides and Yersinia was assessed by exclusion of the DNA stain propidium iodide (2). The rate of cell death was determined by visual scoring of a minimum of 200 cells per sample in a fluorescence microscope. Results are expressed as mean percentages ± SD of apoptotic/dead fluorescent cells vs the total number of cells from three independent experiments.

For immunofluorescent labeling of transfected cells, the cells were stimulated as indicated, washed twice with PBS, fixed with 3.7% paraformaldehyde, permeabilized with 0.02% Nonidet P-40, and blocked with goat serum (14). To specifically label single proteins, the following primary IgG Abs were used: goat polyclonal anti-p65 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-AU-1 (Covance Research Products, Richmond, CA; toward ΔIRAK2 and ΔMyD88), mouse monoclonal anti-HA (Cell Signaling Technology, Beverly, MA; toward WT-MKK1), rabbit polyclonal and mouse monoclonal anti-FLAG (Sigma-Aldrich; toward ΔTRAF6, WT-TRAF6, and ΔIRAK-M), mouse monoclonal anti-c-myc (Clontech Laboratories; toward IRAK1), and rabbit polyclonal anti-β-galactosidase (Clontech Laboratories). Primary Abs were stained with appropriate fluorescein- or rhodamine-conjugated secondary anti-IgG Abs (Sigma-Aldrich).

**Statistical analysis**

Data were expressed as mean percentages ± SD. Statistical analysis was performed using Epi Info (version 6, Center for Disease Control, Atlanta, GA). Statistical significances of differences were determined by the χ2 test with a p value <0.01 considered to be significant (44).

**Results**

**Microbial stimulation confers apoptosis in J774A.1 and primary mouse peritoneal macrophages upon inhibition of the proteasome pathway**

*Y. enterocolitica* efficiently suppresses NF-κB activation and triggers apoptosis in the murine J774A.1 macrophage cell line (13). Identical effects have been demonstrated in primary mouse peritoneal macrophages, in which the NF-κB inhibitory action of *Y. enterocolitica* similarly correlates with the induction of apoptosis (13). Survival of LPS-challenged macrophages depends on de novo protein synthesis (45, 46), and these observations suggest, that onset of apoptosis is related to inhibition of the antipapoptotic NF-κB pathway by *Yersinia* infection. A link between artificial suppression of NF-κB activation and the induction of cell death by bacterial infection has been demonstrated in several macrophage cell lines (13, 23). In our hands, pretreatment of J774A.1 macrophages with the proteasome inhibitory peptide Z-Leu-Leu-Leu-CHO (MG-132) sensitizes the cells to undergo apoptosis upon subsequent stimulation with LPS or nonviral, yopP-negative yersiniae (13). MG-132 suppresses degradation of the NF-κB inhibitory IκB proteins through the proteasome pathway (47), which substantially inhibits NF-κB activation in J774A.1 macrophages (13). To investigate whether a similar effects occurs in primary macrophages, we treated elicited mouse peritoneal macrophages with MG-132 and subsequently stimulated with *Yersinia* strains or LPS. The WT *Y. enterocolitica* strain WA-314 efficiently conferred cell death in both J774A.1 and peritoneal macrophages (Fig. 1). Similarly, 60–75% of the peritoneal macrophages significantly underwent apoptosis upon challenge with LPS or the yopP-negative mutant WA-ΔyopP when the cells were pretreated with MG-132. These effects were similar to those observed in J774A.1 cells. These results confirm a potential antipapoptotic role of NF-κB in bacterial infection of macrophages and suggest that the observed effects in J774A.1 cells closely reflect the situation in primary macrophages.

**MyD88 but not IRAK1 or TRAF6 can signal apoptosis in LPS-stimulated macrophages**

Stimulation with LPS efficiently triggers apoptosis in J774A.1 macrophages that were transiently transfected with a YopP expression plasmid (14). To investigate the roles of well-known LPS signal transducers in this process, we cotransfected dominant-negative constructs of components of the NF-κB signaling cascade for LPS along with the YopP expression plasmid. For identification of the transfected cells, the cells were cotransfected with a β-galactosidase-encoding reporter vector (14, 17–20). Staining with X-gal allows the detection of β-galactosidase expression by conferring a blue color to transfected cells. The number of transfected cells and their morphology were scored by microscopy. Apoptotic cell death is characterized by typical cellular shrinkage and condensation (14, 17–20). Cells that were transfected with the empty expression vector control and treated with LPS exhibited blue color and a normal cellular shape (Fig. 2A, top). These cells apparently remained viable. In contrast, LPS challenge of YopP-transfected macrophages conferred a characteristic apoptotic phenotype, the majority of the cells being reduced in size and condensed as a result of apoptotic destruction (Fig. 2A, bottom). The dying cells have been shown to display apoptotic DNA fragmentation (14). We additionally analyzed phosphatidylserine exposure in these cells using Cy3-conjugated annexin V. Cy3-annexin V binds with high affinity to phosphatidylserine in the outer leaflet of apoptotic cells and confers red fluorescence. For identification of the transfected cells, YopP was expressed as fusion protein with GFP, which mediates green fluorescence. In correlation with the results obtained by β-galactosidase coexpression, cells transfected with the empty GFP vector control remained viable upon LPS treatment and did not stain with Cy3-annexin V (Fig. 2B, top). A single apoptotic cell characterized by red fluorescence is included in Fig. 2B (top). In

**FIGURE 1.** Inhibition of the proteasome pathway renders J774A.1 and primary macrophages susceptible to bacteria-induced apoptosis. J774A.1 and primary mouse peritoneal macrophages remained untreated or were stimulated with WT *yersinia* (WA-314), the YopP-negative mutant (WA-314ΔyopP), or with LPS under conditions with or without pretreatment with the proteasome inhibitor MG-132 for 1 h (2.5 μM). Apoptosis was analyzed 5 h after onset of stimulation by labeling apoptotic cells with fluorescein-conjugated annexin V. Fluorescein staining was scored visually by fluorescence microscopy. Results are expressed as mean percentages ± SD of apoptotic cells vs the total number of cells from three independent experiments. *, Compared with MG-132-treated, nonstimulated cells, exposure to WA-314ΔyopP or LPS significantly conferred apoptosis (*p < 0.01).*
contrast, YopP-transfected and LPS-treated cells became Cy3-annexin V positive, which indicates occurrence of apoptosis (Fig. 2B, bottom). Interestingly, YopP-GFP expression and Cy3-annexin V labeling behaved antagonistically. Strong expression of GFP-YopP correlated with weak Cy3-annexin V staining and vice versa (Fig. 2B, bottom). Thus, the expression of YopP-GFP was reduced in the later stages of apoptosis, which are characterized by enhanced phosphatidylserine exposure. Accordingly, Fig. 2B (bottom) displays the apoptotic body of a fragmented, YopP-transfected cell exhibiting weak green GFP fluorescence, but substantial red annexin V staining. This suggests that YopP-GFP is degraded during the apoptotic process. Since β-galactosidase expression appeared more stable than expression of GFP in YopP-transfected cells, we considered β-galactosidase expression as the more suitable reporter for the cotransfection experiments with YopP.

Initially, we analyzed the most upstream LPS signaling intermediates distal from the receptor complex, namely, ΔMyD88, ΔIRAK1, and ΔTRAF6. To quantify the degree of apoptosis in the cotransfected cells, the number of β-galactosidase-expressing apoptotic cells was determined in relation to the total number of transfected cells (Fig. 2C). Cells cotransfected with YopP and the empty vector control efficiently underwent apoptosis upon exposure to LPS (Fig. 2C, YopP plus vector: 85 ± 6% apoptosis). Treatment of the cells with the broad-spectrum caspase inhibitor z-VAD-fmk (40 μM), which prevents activation of caspase cysteine proteases during the apoptotic response, substantially suppressed cell death and only 20–30% of the cells underwent apoptosis. A dominant-negative mutant version of the adapter protein MyD88 (ΔMyD88), which abrogates LPS-induced NF-κB activation (31, 32), provided partial protection against LPS-promoted apoptosis: Hence, ΔMyD88 significantly reduced LPS-enhanced apoptosis in YopP-transfected cells (Fig. 2C, YopP plus ΔMyD88: 54 ± 6% apoptosis). This indicates that ΔMyD88 is able to block a proapoptotic signal that is elicited by LPS stimulation. However, both dominant-negative ΔIRAK1 and ΔTRAF6, mutant constructs of signaling molecules downstream from MyD88, did not suppress YopP plus LPS-mediated cell death (Fig. 2C, YopP plus ΔIRAK1: 96 ± 3% apoptosis; YopP plus ΔTRAF6: 92 ± 6% apoptosis). ΔIRAK1 and ΔTRAF6 slightly augmented apoptosis in YopP-transfected cells, which may point out a synergistic action of ΔIRAK1 and ΔTRAF6 with YopP in impairing cellular viability. In expectation that a dominant-negative version of an apoptosis-inducing signaling molecule reduces cell death, these results rule out implication of IRAK1 and TRAF6 in signaling a death-triggering response. Inversely, these data indicate, that relay of the LPS-dependent proapoptotic signal depends on MyD88, but not on IRAK1 and TRAF6.
IRAK2 potentially confers NF-κB activation and cell death in infected macrophages

The IRAK family includes two other members beside IRAK1, which are IRAK2 and IRAK-M (34–36). Both IRAK2 and IRAK-M share structural as well as functional similarities with IRAK1 and can relay a MyD88-dependent NF-κB-activating signal upon LPS treatment (36). We investigated involvement of these molecules in the macrophage apoptotic response evoked by *Y. enterocolitica* and Yop/P/LPS treatment. Surprisingly, dominant-negative ΔIRAK2 produced different results in cotransfection experiments with Yop/P as compared with ΔIRAK1 (Fig. 3A). In relation to YopP and control vector-transfected cells (Fig. 3A, YopP plus vector; 82 ± 7% apoptosis), cells transfected with YopP and ΔIRAK2 significantly survived subsequent treatment with LPS (Fig. 3A, YopP plus ΔIRAK2: 44 ± 6% apoptosis). Thus, unlike ΔIRAK1, ΔIRAK2 apparently exerts a protective effect against enhancement of apoptosis through LPS stimulation. The dominant-negative ΔIRAK-M construct provided slight protection. Another set of experiments revealed that ΔIRAK2 not only attenuated YopP/LPS-mediated apoptosis, but also cell death elicited by *yersinia* infection (Fig. 3B). Whereas WT *Y. enterocolitica* efficiently triggered apoptosis in cells transfected with the empty control vector or with ΔIRAK1, apoptosis was significantly blocked in ΔIRAK2-transfected cells. Thus, ΔIRAK2 obviously disrupts the transmission of an apoptotic signal that is activated by LPS treatment or *yersinia* infection. This suggests that IRAK2 may participate besides MyD88 in relaying the apoptotic signal.

To rule out that these differences were simply a result of distinct effects of ΔIRAK1 and ΔIRAK2 on the NF-κB survival pathway, we analyzed the impacts of overexpression of dominant-negative ΔIRAK1 and ΔIRAK2 on LPS-induced NF-κB activation. NF-κB activation was investigated at the single cell level by fluorescence microscopy. To preclude nonspecific fluorescent labeling, we cotransfected ΔIRAK1, ΔIRAK2, and the empty vector control with the β-galactosidase reporter plasmid. The samples were simultaneously probed with anti-β-galactosidase (red fluorescence) and anti-c-myc or anti-AU-1 (green fluorescence) Abs to detect β-galactosidase and ΔIRAK1 (anti-c-myc) or ΔIRAK2 (anti-AU-1) in the transfected cells. As displayed in Fig. 3C, cells transfected with the β-galactosidase and the empty control vector selectively exhibited red fluorescence (staining of β-galactosidase; Fig. 3C, top), whereas ΔIRAK2-transfected cells were additionally characterized by yellow fluorescence, resulting from superimposition of red and green fluorescent signals (staining of ΔIRAK2; Fig. 3C, bottom). Similar results were obtained for ΔIRAK1 (data not shown). Cross-processing of ΔIRAK1-transfected cells with anti-AU-1 and ΔIRAK2-transfected cells with anti-c-myc Abs did not produce any conspicuous fluorescence signals (data not shown). These results indicate that the mouse monoclonal anti-AU-1 and anti-c-myc Abs used in these studies specifically label overexpressed constructs bearing the respective Ab epitope tags. Furthermore, cotransfection with the β-galactosidase plasmid seems to be an appropriate approach to identify transfected cells.

We first analyzed nuclear translocation of NF-κB using goat polyclonal anti-NF-κB p65 Abs (Fig. 3D, green fluorescence). The samples were additionally probed with the anti-c-myc or anti-AU-1 Abs to detect ΔIRAK1 or ΔIRAK2 expression in the transfected cells (Fig. 3D, red fluorescence). Overexpressed ΔIRAKs apparently accumulated in the perinuclear and nuclear region, which agrees with previous observations (48). In nonstimulated cells, the major amount of NF-κB was localized in the cytoplasm (Fig. 3D, top). No NF-κB-dependent fluorescent signal was detectable after processing the samples with secondary fluorescein-conjugated anti-rabbit instead of anti-goat Abs. The secondary Ab alone, without appropriate primary Ab, did not produce any conspicuous fluorescence signals (data not shown). Upon treatment with LPS, NF-κB moved to the nucleus and exhibited a nuclear staining pattern in the nontransfected cells, which indicates NF-κB activation (Fig. 3D, bottom). In contrast, overexpression of dominant-negative ΔIRAK2 obviously impeded NF-κB in its ability to move to the nucleus and NF-κB was predominantly detected in the cytoplasm of transfected cells (Fig. 3D, bottom). The same held true for overexpression of dominant-negative ΔIRAK1 (data not shown). To substantiate a potential inhibitory action of ΔIRAKs on induction of the NF-κB pathway, we additionally monitored NF-κB activation using a GFP-NF-κB reporter vector (pNF-κB-d2EGFP). Use of this plasmid allows real time analysis of binding of endogenous NF-κB to the κ enhancer in the promoter region of the vector, resulting in transcription of the gfp gene. The NF-κB reporter vector was cotransfected with ΔIRAK1, ΔIRAK2, WT-TRAF6, or WT-MKK1. The cells were processed with Abs against the epitope tags of the respective overexpressed proteins for identification of the transfected constructs by fluorescence microscopy (red fluorescence). Expression of gfp in dependence on NF-κB activation was simultaneously assayed (green fluorescence). The percentage of green and red fluorescent cells, indicating transfected cells with activated NF-κB, was determined in relation to the total number of transfected cells (cells with red or red and green fluorescence; Fig. 3E). Although a multitude of the transfected cells exhibited non-specific NF-κB activation in the absence of stimulation (30–50%), cells transfected with WT-TRAF6 and WT-MKK1 significantly responded to treatment with LPS by additional NF-κB induction, resulting in increased numbers of green and red fluorescent cells (Fig. 3E). In contrast, NF-κB activation in cells overexpressing ΔIRAK1 or ΔIRAK2 was not significantly enhanced upon LPS treatment. These results confirm an inhibitory effect of the dominant-negative ΔIRAK constructs on the NF-κB pathway, which was demonstrated in previous publications (31, 32, 35, 36, 41). Thus, although both dominant-negative ΔIRAK1 and ΔIRAK2 share the capacity to suppress NF-κB activation, the outcomes on cellular survival and apoptosis are obviously different. ΔIRAK2 rather exerts a protective effect on *yersinia*- and Yop/P/LPS-mediated cell death, in contrast to ΔIRAK1. This implies that a pro-apoptotic signal elicited by bacterial infection may bifurcate at the IRAK level by involving IRAK2.

IRAK1 and TRAF6 potentially mediate survival of macrophages upon LPS treatment

The data provided here suggest a role of MyD88 and IRAK2 in signaling a LPS-dependent apoptotic response under conditions when NF-κB activation is inhibited by YopP. On the other hand, MyD88 and IRAK2, as well as IRAK1, IRAK-M, and TRAF6, are known to participate in transmitting the NF-κB-dependent survival signal (31, 36), which suggests that the transfection of dominant-negative versions of these molecules alone should influence survival of LPS-treated macrophages. To check this, we transfected J774A.1 macrophages with the respective dominant-negative constructs and assessed cellular viability upon LPS treatment in the absence of YopP or *yersinia* (Fig. 4A). *ΔMyD88* did not considerably affect viability of LPS-treated macrophages, indicating that the cytotoxic pathways remain silent and that potential inhibition of NF-κB activation does not lead to apoptosis. In the same manner, overexpression of dominant-negative ΔIRAK2 only marginally provoked cell death, although it impairs the NF-κB pathway (Fig. 4A). In contrast, subversion of NF-κB activation by overexpression of ΔIRAK1 significantly triggered apoptosis in LPS-challenged macrophages (Fig. 4A, 76 ± 6% apoptosis). Similar results
FIGURE 3. Distinct effects of ΔIRAK members on YopP/LPS- and Y. enterocolitica-mediated apoptosis. A, Inhibition of YopP- and LPS-mediated apoptosis by ΔIRAK2, but not ΔIRAK1. J774A.1 cells were cotransfected with β-galactosidase reporter vector and either the empty expression vector control alone (vector) or the YopP expression vector (YopP) along with ΔIRAK1, ΔIRAK2, ΔIRAK-M, or control expression plasmid. Transfected cells were left untreated or exposed to LPS for 8 h. Thereafter, cells were stained with X-gal and single transfected cells were analyzed for an apoptotic morphology. Results are expressed as mean percentages ± SD of apoptotic vs the total number of transfected cells from three independent experiments. *, Compared with the vector control, cotransfection of YopP with ΔIRAK2 significantly attenuated death in LPS-treated cells (p < 0.01). B, Inhibition of Y. enterocolitica-induced apoptosis by ΔIRAK2. J774A.1 cells were transfected with β-galactosidase reporter vector and either empty expression vector or expression vectors for ΔIRAK1 or ΔIRAK2. Cells were infected with YopP-negative (WA-314ΔyopP) or WT (WA-314) yersiniae and stained with X-gal after 8 h. The number of apoptotic blue cells was determined. Results are expressed as mean percentages ± SD of apoptotic vs the total number of transfected cells from three independent experiments. *, Compared with the vector control, transfection with ΔIRAK2 significantly diminished death in WA-314-infected cells (p < 0.01). C, Immunofluorescent staining of ΔIRAK2 specifically labels transfected cells. J774A.1 cells were cotransfected with β-galactosidase reporter vector (β-Gal) and either empty expression vector (vector) or ΔIRAK2 expression vector (ΔIRAK2). After 18 h, cells were processed for double immunofluorescence staining using rabbit anti-β-galactosidase and rhodamine-conjugated secondary Abs to detect β-galactosidase expression (red fluorescence), and mouse monoclonal anti-AU-1 and fluorescein-conjugated secondary Abs to detect ΔIRAK2 expression in transfected cells (green fluorescence). Yellow fluorescence results from superimposition of red and green fluorescent signals. The arrows indicate β-galactosidase-positive transfected cells. D, ΔIRAK2 inhibits nuclear translocation of endogenous NF-κB p65 in LPS-treated J774A.1 macrophages. Cells were transfected with ΔIRAK2 expression vector, left untreated, or exposed to LPS for 60 min, then processed for double immunofluorescence staining using goat anti-p65 and fluorescein-conjugated secondary Abs, to detect location of endogenous p65, and mouse monoclonal anti-AU-1 and rhodamine-conjugated secondary Abs to detect ΔIRAK2-transfected cells. The arrows indicate ΔIRAK2-transfected cells (red fluorescence) with obvious cytoplasmic NF-κB p65 staining pattern (green fluorescence). Yellow fluorescence due to superimposition of red and green fluorescent signals may result from locally enhanced green fluorescent background staining in stimulated and nonstimulated cells. E, ΔIRAK1 and ΔIRAK2 inhibit induction of NF-κB-responsive GFP expression.
were obtained for ΔTRAF6, acting downstream from the IRAKs in the NF-κB cascade (Fig. 4A, 71 ± 4% apoptosis). Apoptosis in ΔIRAK1- and ΔTRAF6-transfected and LPS-stimulated cells was considerably suppressed by pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk (28 ± 3% and 29 ± 7% apoptotic cells, respectively). ΔIRAK-M exerted an intermediate effect on macrophage viability as compared with ΔIRAK1 and ΔIRAK2. Immunofluorescence labeling with Abs toward the epitope tags of the overexpressed proteins revealed that ΔMyD88, ΔTRAF6, and ΔIRAK-M were expressed in the transfected cells (data not shown). Furthermore, these dominant-negative LPS signaling constructs were similarly to ΔIRAK1 and ΔIRAK2 able to impair induction of the cotransfected GFP-NF-κB reporter (pNF-κB-d2EGFP) upon LPS stimulation. Accordingly, the NF-κB-dependent increase in fluorescent cell numbers was <30% in stimulated vs nonstimulated cells, in contrast to 80–90% for WT-MKK1. These data indicate that disruption of the NF-κB pathway by ΔIRAK1 and ΔIRAK2, but not by ΔMyD88 or ΔIRAK2, actively supports cell death in LPS-treated macrophages. These effects resemble the action of YopP, enabling LPS to mediate apoptosis in macrophages when NF-κB is inhibited. Apparently during suppression of the NF-κB survival signal by ΔIRAK1, ΔTRAF6, or YopP, upstream death-triggering signals still proceed and subsequently mediate macrophage apoptosis. On the contrary, ΔMyD88 and ΔIRAK2 apparently also block the death-promot-

FIGURE 4. Dominant-negative LPS-signaling intermediates differentially influence viability of LPS-treated macrophages. A, ΔIRAK1 and ΔTRAF6 confer apoptosis in LPS-treated J774A.1 cells. Cells were cotransfected with β-galactosidase reporter vector and either empty expression vector or expression vectors for ΔMyD88, ΔIRAK1, ΔIRAK2, ΔIRAK-M, or ΔTRAF6. Transfected cells were left untreated or exposed to LPS for 8 h. Thereafter, cells were stained with X-gal and single transfected cells were analyzed for an apoptotic morphology. B, ΔMyD88 and ΔIRAK2 counteract ΔIRAK1-conferred apoptosis in LPS-treated J774A.1 cells. Cells were cotransfected with β-galactosidase reporter and ΔIRAK1 expression vector, and either ΔMyD88 or ΔIRAK2 or empty expression plasmid. Transfected cells were left untreated or exposed to LPS for 8 h, then stained with X-gal. Single transfected cells were analyzed for an apoptotic morphology. Data in A and B show mean percentages ± SD of apoptotic vs total number of transfected cells from three independent experiments. A, * p < 0.01. Compared with the vector control, transfection with ΔIRAK1 and ΔTRAF6 significantly mediated death in LPS-treated cells (p < 0.01). B, * p < 0.01. Compared to the vector control, cotransfection of ΔIRAK1 with ΔMyD88 and ΔIRAK2 significantly attenuated death in LPS-treated cells (p < 0.01).

Y. enterocolitica-induced apoptosis involves the FADD/caspase-8 signaling complex

We attempted to characterize a potential downstream apoptotic signal relay transmitted through MyD88 and IRAK2. The execution of apoptosis in eukaryotic cells depends on the activation of caspases, a family of cysteine proteases that accomplish the apoptotic response in a proteolytic cascade (49). To characterize the roles of single caspases in Y. enterocolitica- and YopP-mediated apoptosis, we used specific caspase inhibitory peptides. J774A.1

in LPS-treated cells. J774A.1 cells were cotransfected with the NF-κB reporter vector pNF-κB-d2EGFP (0.66 μg) and either ΔIRAK1, ΔIRAK2, WT-TRAF6, or WT-MKK1 (0.33 μg). The cells were stimulated with LPS for 2.5 h and processed with Abs against the epitope tags of the respective overexpressed proteins for identification of the transfected cells (red fluorescence). Production of GFP in dependence on NF-κB activation was simultaneously assayed (green fluorescence). The percentage of green and red fluorescent cells, indicating transfected cells with activated NF-κB, was determined in relation to the total number of transfected cells (cells with red fluorescence). Results are expressed as mean percentages ± SD of transfected cells displaying NF-κB induction vs total number of transfected cells from three independent experiments. *, Compared with untreated cells, LPS stimulation mediated significant NF-κB induction in WT-MKK1 and WT-TRAF6, but not in ΔIRAK1- or ΔIRAK2-transfected cells (p < 0.01). C and D, Results from one representative experiment of two and three performed, respectively.
macrophages were pretreated with the caspase inhibitors and subsequently infected with yersiniae (Fig. 5A). In accordance with previous studies, the broad-spectrum caspase inhibitor z-VAD-fmk (lane designated ΔΣcaspases, Fig. 5A) efficiently prevented loss of viability in yersiniae-infected macrophages (2). Furthermore, the inhibitors of caspase-8 and 9 provided at least partial protection against yersiniae-induced cell death (Fig. 5A, 27 ± 3% and 39 ± 5% apoptosis vs 66 ± 9% apoptosis in untreated cells), in contrast to inhibitors of caspase-2 and 10. This points out particular involvement of caspase-8 as well as caspase-9 in apoptosis triggered by Y. enterocolitica. The FADD is a crucial mediator of caspase-8 activation (50, 51). To substantiate a connection to the FADD/ caspase-8 apoptotic pathway, we cotransfected J774A.1 cells with activation (50, 51). To substantiate a connection to the FADD/ death in LPS-treated cells (p/H11021/H11569, Compared with the untreated vector control, cotransfection of YopP with H9004/H9018 caspases) or the exposed to LPS for 8 h under conditions with or without pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk (26/H9004/H9018) point out particular involvement of caspase-8 as well as caspase-9 in apoptosis triggered by Y. enterocolitica. The FADD is a crucial mediator of caspase-8 activation (50, 51). To substantiate a connection to the FADD/caspase-8 apoptotic pathway, we cotransfected J774A.1 cells with dominant-negative ΔFADD and YopP expression plasmids or treated the cells with z-VAD-fmk or the caspase-8 inhibitory peptide. Fig. 5B shows that ΔFADD (37 ± 6% apoptosis) and the caspase-8 inhibitor (44 ± 3% apoptosis) significantly reduced cell death mediated by YopP and LPS stimulation, although to a lesser extent as compared with z-VAD-fmk (26 ± 4% apoptosis). This suggests that FADD and caspase-8 may indeed be involved in the yersiniae- and LPS-activated proapoptotic response.

Discussion

The activation of NF-κB rescues macrophages from apoptosis mediated by bacterial infection or LPS treatment (13, 14, 23, 24). Y. enterocolitica takes advantage of this mechanism to trigger apoptosis in macrophages (14). Translocated YopP down-regulates the activities of the NF-κB signaling cascade by blockage of IKKβ, which is the major LPS-responsive NF-κB-activating kinase in macrophages (52). The subsequent initiation of proapoptotic LPS signaling upstream from IKKβ compels the macrophage to undergo apoptosis. In this study, we used YopP as a tool to investigate the signal relay that mediates apoptosis of infected macrophages under conditions when NF-κB activation is suppressed. The innate immune system engages a number of TLRs that recognize diverse conserved microbial products, such as LPS, peptidoglycan, bacterial lipopolysaccharides, or flagellin (25–29, 53). All of these TLRs signal through the adaptor protein MyD88, which activates an signaling cascade that ultimately leads to nuclear translocation of NF-κB.

To identify potential proapoptotic LPS-responsive signal transducers in Yersinia- and YopP-mediated apoptosis, we cotransfected J774A.1 macrophages with dominant-negative constructs of proximal LPS signaling molecules along with a YopP expression plasmid. We analyzed occurrence of apoptosis in transfected cells upon LPS stimulation. In our hands, a dominant-negative version of MyD88 provided partial protection against LPS-induced apoptosis in YopP-transfected cells. This suggests a role of MyD88 in transmitting a LPS-dependent proapoptotic signal. Dominant-negative versions of IRAK1 and TRAF6, signaling molecules downstream from MyD88, failed to suppress LPS-enhanced apoptosis. On the contrary, ΔIRAK1 and ΔTRAF6 conferred macrophage cell death after LPS stimulation in the absence of YopP. This indicates that inhibition of the NF-κB cascade by ΔIRAK1 or ΔTRAF6 can replace the NF-κB-suppressive effect of YopP, which sensitizes J774A.1 macrophages to LPS-induced apoptosis. Thus, IRAK1 and TRAF6 appear to play important roles in the survival-mediating branch of the LPS-signaling networks, but do not play a role in mediating LPS-induced apoptosis. 

FIGURE 5. Inhibition of the FADD/caspase-8 apoptotic pathway attenuates Y. enterocolitica- and YopP/LPS-mediated apoptosis. A, Inhibitors of caspase-8 and caspase-9 reduce Y. enterocolitica-triggered cell death. J774A.1 cells were left untreated (Ø) or pretreated with broad-spectrum caspase inhibitor z-VAD-fmk (ΔΣcaspases) or specific inhibitors for caspase-2 (z-VDVAD-fmk), caspase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), or caspase-10 (z-AEVD-fmk) for 1 h. Cells were infected with YopP-negative (WA-314ΔyopP) or WT (WA-314) yersiniae. Six hours after onset of infection, cellular viability was investigated by labeling apoptotic cells with the DNA stain propidium iodide. Dye uptake was scored visually by fluorescence microscopy. Results are expressed as mean percentages ± SD of apoptotic/dead cells vs the total number of cells from three independent experiments. *, Compared with untreated cells, z-VAD-fmk, z-IETD-fmk, and z-LEHD-fmk significantly inhibited apoptosis in WA-314-infected cells (p < 0.01). B, z-VAD-fmk, z-IETD-fmk, and ΔFADD inhibit YopP/LPS-mediated apoptosis. Cells were cotransfected with β-galactosidase reporter vector and either empty expression vector (vector) or YopP expression vector (YopP) along with ΔFADD or empty control expression plasmid. Transfected cells remained untreated or were exposed to LPS for 8 h under conditions with or without pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk (ΔΣcaspases) or the caspase-8-specific inhibitor z-IETD-fmk (Δcaspase-8). Thereafter, cells were stained with X-gal and single transfected cells were analyzed for an apoptotic morphology. Results are expressed as mean percentages ± SD of apoptotic vs the total number of transfected cells from three independent experiments. *, Compared with the untreated vector control, cotransfection of YopP with ΔFADD or application of z-VAD-fmk or z-IETD-fmk significantly attenuated death in LPS-treated cells (p < 0.01).
not signal LPS-responsive apoptosis. In contrast, a dominant-negative version of IRAK2 diminished YopP- plus LPS- and also Yersinia-mediated cell death. Furthermore, ΔIRAK2 as well as ΔMyD88, only marginally evoked cell death in LPS-treated macrophages in the absence of YopP. ΔIRAK2 and ΔMyD88 on the contrary attenuated ΔIRAK1-mediated apoptosis in cotransfected cells. These results suggest implication of IRAK2 besides MyD88 as a proapoptotic signal transducer in LPS signaling. This seems to be a specific role of IRAK2, since the NF-κB suppressive effect of ΔIRAK2 in LPS-stimulated J774A.1 macrophages was comparable to that of ΔIRAK1, confirming common functional properties of IRAK1 and IRAK2 in NF-κB activation. The third IRAK member, IRAK-M, displayed intermediate effects on cellular viability, as compared with those of IRAK1 and IRAK2.

Alignment of the amino acid sequences of IRAK1, IRAK2, and IRAK-M revealed 31–38% similarity in between the single IRAK members (36). Selectively, IRAK1 possesses a kinase activity that induces potent autophosphorylation (34, 36). In contrast, IRAK2 lacks key residues in the conserved protein kinase subdomains, making it catalytically inactive (35, 36, 54, 55). A weak kinase activity was reported for IRAK-M (36). These distinct features of the IRAK members imply that they may differentially influence cellular signaling processes. A unique role of IRAK1 was demonstrated in the control of p38 MAPK activation and spreading of macrophages upon LPS treatment (56). This process requires the IRAK1 kinase activity, whereas the kinase activity is dispensable for NF-κB activation (36, 54, 55, 57). IRAK1 has been shown to mediate phosphorylation of the Toll-interacting protein (Tollip) (58). Tollip forms a complex with IRAK members in resting cells, thereby preventing NF-κB activation. Cellular stimulation mediates recruitment of Tollip-IRAK complexes to the cytoplasmic receptor, which leads to phosphorylation of IRAK1 and Tollip and dissociation of the receptor-associated complex (58–60). This event may be required for transmission or termination of activating signals. The data provided in our study indicate opposing effects of IRAK1 and IRAK2 on macrophage cytotoxic pathways in response to bacterial infection. This suggests that the bifurcation of apoptosis-promoting and -preventing signals depends on IRAK family members. A unique role in cellular signaling was also reported for IRAK2. The MyD88-related adapter protein Mal/TIRAP activates NF-κB selectively through IRAK2, but not through IRAK1 (37, 38). This confirms distinct functions of IRAK molecules in the cellular signaling networks in response to bacterial infection and tempts to ask for the roles of Mal/TIRAP and Tollip in the preservation of cellular viability, which were not yet investigated.

The apoptosis-preventing effect of the most upstream dominant-negative signaling intermediate investigated, ΔMyD88, was less pronounced than that of ΔIRAK2. This may support a model in that MyD88, as common upstream activator of both IRAK1 and IRAK2 (35, 61), participates in the regulation of both divergent pathways. A report from Aliprantis et al. (62) indicates implication of MyD88 in proapoptotic signaling in monocytic cells that were treated with bacterial lipoproteins. They found that MyD88 is coupled to the apoptotic machinery through binding and engagement of the death receptor adaptor protein FADD. A corresponding pathway was recently identified in Drosophila (63). FADD mediates the onset of apoptosis by activating the initiator caspase-8 (50, 51). A study on endothelial cells suggests that also bacterial LPS is able to engage a FADD-dependent apoptotic pathway (64). In an attempt to characterize downstream apoptotic processes in our infection model, we investigated the impact of specific caspase inhibitory peptides on Y. enterocolitica-induced cell death. Inhibitors of caspase-8 and caspase-9 provided substantial protection against apoptosis due to yersinia infection. In addition, a dominant-negative version of the caspase-8 activator FADD considerably suppressed YopP/LPS-induced apoptosis. These results suggest implication of the FADD/caspase-8 module as well as of caspase-9 in the Y. enterocolitica- and YopP/LPS-induced apoptotic response. Aliprantis et al. (62) demonstrated binding of MyD88 to FADD through death domain interactions. Since IRAK members also harbor a death domain, we speculated that IRAK2 may similarly interact with FADD. However, preliminary immunoprecipitation experiments with overexpressed FADD and IRAK2 rule out direct in vivo interaction in between the two molecules (data not shown). A study by Denecker et al. (65) revealed that Y. enterocolitica induces cleavage of the proapoptotic Bcl-2 family member Bid. Truncated Bid bridges death receptor signaling to the mitochondrial apoptotic pathway, resulting in the release of cytochrome c from the mitochondrion and in the activation of caspase-9. Since upstream processing of Bid is efficiently mediated by caspase-8 (50, 51), these findings may coincide with our results showing involvement of the FADD-caspase-8 complex and of caspase-9 in the bacteria-induced proapoptotic response. Together, our study indicates that bacterial infection of macrophages results in activation of a MyD88- and IRAK2-dependent apoptotic signal relay, which potentially targets the FADD/caspase-8 death signaling pathway. IRAK1 and TRAF6 on the contrary signal macrophage survival to counteract the cytotoxic response. Interestingly, the proposed functions of MyD88 and IRAK1 seem to differ in endothelial cells. Banerman et al. (66) recently found involvement of both MyD88 and IRAK1 in signaling endothelial apoptosis upon LPS treatment. This cytotoxic pathway does apparently not engage FADD, although FADD was also reported to confer LPS-induced endothelial apoptosis (64). This suggests that there are differences in the regulation of macrophage and endothelial cell survival in response to bacterial infection, which may be related to the distinct functions of the two cell types within the host immune response. Alternatively, the signals that regulate entry to apoptosis downstream from MyD88 and IRAK1 are less prominent in LPS-stimulated macrophages than in endothelial cells. Fig. 6 gives an overview on the postulated model, how Y. enterocolitica exploits the signaling networks of innate immunity in macrophages to mediate macrophage apoptosis.
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