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A Dominant Role of Toll-Like Receptor 4 in the Signaling of Apoptosis in Bacteria-Faced Macrophages

Rudolf Haase,* Carsten J. Kirschning,† Andreas Sing,* Percy Schröttner,* Koichi Fukase,‡ Shoichi Kusumoto,‡ Hermann Wagner,§ Jürgen Heesemann,* and Klaus Ruckdeschel2§

Conserved bacterial components potently activate host immune cells through transmembrane Toll-like receptors (TLRs), which trigger a protective immune response but also may signal apoptosis. In this study, we investigated the roles of TLR2 and TLR4 as inducers of apoptosis in Yersinia enterocolitica-infected macrophages. Yersiniae suppress activation of the antiapoptotic NF-κB signaling pathway in host cells by inhibiting inhibitory κB kinase-β. This leads to macrophage apoptosis under infection conditions. Experiments with mouse macrophages for TLR2, TLR4, or both receptors showed that, although yersiniae could activate signaling through both TLR2 and TLR4, loss of TLR4 solely diminished Yersinia-induced apoptosis. This suggests implication of TLR4, but not of TLR2, as a proapoptotic signal transducer in Yersinia-conferred cell death. In the same manner, agonist-specific activation of TLR4 efficiently mediated macrophage apoptosis in the presence of the proteasome inhibitor MG-132, an effect that was less pronounced for activation through TLR2. Furthermore, the extended stimulation of overexpressed TLR4 elicited cellular death in epithelial cells. A dominant-negative mutant of Fas-associated death domain protein could suppress TLR4-mediated cell death, which indicates that TLR4 may signal apoptosis through a Fas-associated death domain protein-dependent pathway. Together, these data show that TLR4 could act as a potent inducer of apoptosis in macrophages that encounter a bacterial pathogen. The Journal of Immunology, 2003, 171: 4294–4303.

Elicitation of an adequate immune reaction against bacterial pathogens critically depends on appropriate sensing and recognition of the invading organism by host immune effector cells. The family of the transmembrane “Toll-like receptors” (TLRs)3 plays an intriguing role in the cellular identification of common pattern of microbial pathogens. By now, 10 mammalian TLRs have been described (1–5). They can discriminate distinct microbial components. TLR activation provokes rapid onset of immune reactions by the induction of intracellular signaling cascades which lead to the expression of inflammatory response-related genes. A critical mediator of intracellular TLR signaling is transcription factor NF-κB, which regulates the production of various cytokines, adhesion molecules, and antiapoptotic factors (6–10). The up-regulation of synthesis of antiapoptotic proteins through NF-κB activation prevents cellular apoptosis under multiple stress-induced conditions and is also essential for survival of bacteria-faced macrophages. This suggests that bacterial infection could trigger the activation of proapoptotic signals that are hindered in their cytotoxic effects by the antiapoptotic activity of NF-κB (11–13).

In this study, we investigated the roles of TLRs as proapoptotic signal mediators in Yersinia-infected macrophages. Pathogenic Gram-negative Yersinia spp. disrupt the balances of pro- and antiapoptotic signaling in macrophages by down-regulating the activity of NF-κB, which leads to macrophage apoptosis (11). These abilities depend on a specific virulence protein, which is Yersinia outer protein (Yop) P in Yersinia enterocolitica, or its homologue YopJ in Yersinia pseudotuberculosis and Yersinia pestis (14, 15). Y. pestis is the etiological agent of plague, whereas Y. pseudotuberculosis and Y. enterocolitica are enteric pathogens causing gastrointestinal syndromes and lymphadenitis (15). YopP/YopJ is injected by the virulence plasmid-encoded Yersinia type III protein secretion system into the host cell cytoplasm where it binds and inhibits the NF-κB-activating inhibitory κB kinase (IKK)-β, leading to down-regulation of NF-κB activation (14, 15). Our previous studies have shown that proapoptotic signals of innate immunity could synergize with the NF-κB-inhibitory action of YopP/YopJ to mediate macrophage apoptosis (16). This suggests that yersiniae activate a conserved apoptotic pathway that induces cell death when NF-κB activation is suppressed. The sources of the cytotoxic signals potentially generated by Yersinia are hitherto unclear. In this study, we show that signaling through TLR4, but not through TLR2, stimulates apoptosis in Y. enterocolitica-infected macrophages. Apparently, TLR2 and TLR4 differentially induce cytotoxic signals in stimulated cells. Our data indicate that activated TLR4 can potentially signal apoptosis which could lead to the demise of the infected cell.

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, its isogenic yopP-knockout mutant WA-ΔyopP (16), the virulence plasmid-cured strain WA-C, and invasin-negative, virulence plasmid-cured WAΔinv-C (17). Overnight cultures grown at 27°C were

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3 Abbreviations used in this paper: TLR, Toll-like receptor; Yop, Yersinia outer protein; IKK, inhibitory κB kinase; HEK, human embryonic kidney; LTA, lipoteichoic acid; ELAM, endothelial leukocyte adhesion molecule; MyD88, myeloid differentiation factor 88; TIRAP, Toll-IL-1R adapter protein; IRAK, IL-1R-associated kinase; FADD, Fas-associated death domain protein; PKR, dsRNA-dependent protein kinase; Mal, MyD88-adapter-like protein; tBid, truncated Bid; TRIF, TIR domain-containing adapter inducing IFN-β.

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diluted 1/20 in fresh Luria-Bertani broth and grown for another 2 h at 37°C (17). Shift of the growth temperature to 37°C initializes activation of the Yersinia type III secretion machinery for efficient translocation of Yops into the host cell upon cellular contact. To equalize and synchronize expression of Staphylococcus aureus (Refs. 19 and 20; kindly provided by T. Hartung, mouse TNF-

Biochemical Pharmacology, University of Konstanz, Konstanz, Germany), Tnf1a, and TNFR-1 and -2-defective B6.129-Tnf1a/1b mice were obtained, TNFR-1-defective B6.129-purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). For quantitation of TNF-

expression as well as expression of functional TLR4 (24). 129/Sv mice and peritoneal macrophages mice lacking expression of TLR2 and TLR4 were gen-

doubly deficient mice lacking TLR2 ex-

devations (Ref. 27; Dual-Luciferase Reporter System; Promega). The NF-

kappa B-directed firefly luciferase activities were normalized to Renilla lucif-

erase activities to compensate for differences in transfection efficiencies. Data on NF-

kappa B activities are the means ± SD of at least three independent experiments.

Analysis of morphology of transfected cells

To determine the influence of TLR2 and TLR4 on viability of HEK293 cells, the cells were seeded in 24-well cell culture plates and transfected with expression plasmids for CD14 and TLR2 or TLR4 (each 190 ng), MD-2 (19 ng), and pSV-β-galactosidase expression vector (400 ng; Promega). The total amount of DNA was kept constant with empty vector for each transfection (1 μg). Twenty-four hours after transfection, HEK293 cells were serum-starved for 18–20 h, then stimulated as indicated. J774A.1 cells were transfected with 0.33 μg of pSV-β-galactosidase expression vector and 0.66 μg of the plasmid of interest (16). Three hours after transfection, J774A.1 cells were treated with MG-132 and stimulated 30 min later. To identify the transfected cells, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal) at the time points indicated. For assessment of cell death, the morphology of blue transfected cells was determined using light microscopy (7–10, 16). Every single transfected cell was analyzed for an apoptotic appearance. A minim-

um of eight microscopic fields were investigated for each sample. For quantification, the number of apoptotic blue cells was assayed in relation to the total number of transfected cells. Results are expressed as mean percentages ± SD from three independent experiments.

Immunoprecipitations and Western immunoblotting

For the detection of transiently overexpressed TLRs, TLR2 or TLR4 expression plasmids or empty control plasmid (1 μg) were transfected into HEK293 cells seeded in six-well cell culture plates. Total cell lysates were prepared and incubated with anti-Flag mAbs (Sigma-Aldrich) and protein A/G-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) as de-

scribed (28). The immune complexes were washed, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was performed with anti-Flag epitope Abs. To determine cleavage of Bid in LPS- and MG-132-treated J774A.1 macrophages, a total of 105 cells per sample was lysed in 4× Laemmlı sample buffer after stimulation as indicated. The lysates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with poly-

clonal anti-Bid Abs (R&D Systems). Immunoreactive bands were visualized using appropriate secondary Abs and ECL detection reagents (Amer-

sham Pharmacia Biotech, Piscataway, NJ).

Results

TLR4 signaling stimulates apoptosis in Yersinia-infected macrophages

To find out whether TLR signaling could play a role in the mechan-

ism of apoptosis induction by Y. enterocolitica, we investigated apoptosis induced by Yersinia in primary mouse macrophages that are deficient for functional TLR2, TLR4, or both receptors (Fig. 1).
Macrophages with a double deficiency for both TLR2 and TLR4 exhibited the same degree of apoptosis as macrophages with a single TLR4 mutation. Together, these findings suggest that signaling through TLR4, but not through TLR2, plays an important role in Yersinia-conferring apoptosis.

Y. enterocolitica induces cellular signaling via TLR2 and TLR4

Because TLR4 solely appears to be of critical importance in signaling macrophage apoptosis upon Y. enterocolitica infection, we wondered whether yersiniae are impaired in their ability to activate TLR2. The activation of TLRs largely contributes to determine the macrophage TNF-α production in response to bacterial stimuli. Thus, we investigated the roles of TLR2 and TLR4 in Yersinia-induced TNF-α release by peritoneal macrophages isolated from wild-type (C3H/HeN), TLR2-deficient (C3H/HeN TLR2−/−), and TLR4-mutagenized (C3H/HeJ TLR4Δd) mice. Yersinia were grown under the same conditions by which wild-type bacteria efficiently mediate macrophage apoptosis. Wild-type yersiniae (WA-314), which down-regulate NF-κB activities through YopP at the level of IKKβ (14, 16, 37), efficiently suppressed TNF-α production of macrophages from all three mice strains (Fig. 2A), which correlates with previously described effects of YopP (11, 14, 15).

In contrast, the YopP-negative mutant WA-ΔyopP induced a TNF-α release in wild-type macrophages that was remarkably reduced in macrophages deficient for either functional TLR2 or TLR4 (Fig. 2A). This implies that both TLR2 and TLR4 participate in the induction of the macrophage TNF-α response elicited by YopP-negative yersiniae.

To analyze the activation of TLRs by Y. enterocolitica in more detail, we used HEK293 cells for an ectopic transfection model that allows characterization of TLR-specific cell activation by distinct agonists (28). Induction of the cotransfected NF-κB-dependent ELAM-luciferase reporter indicates activation of the NF-κB pathway in response to TLR stimulation. TLR2 and TLR4 were transfected in combinations with the coreceptors CD14 and MD-2 and expression of TLRs was checked by immunoprecipitation and Western blotting with Abs directed against the Flag-epitope tags of TLR2 and TLR4 (Fig. 2B). As expected, the Y. enterocolitica wild-type strain WA-314 did not trigger substantial activation of the NF-κB-dependent reporter gene in any of the transfected cells (Fig. 2C). This potentially results from YopP-mediated IKKβ inhibition (16, 37). In contrast, the YopP-negative mutant WA-ΔyopP mediated prominent NF-κB responses in TLR2- and TLR4-expressing cells (Fig. 2C).

Comparable NF-κB signals were generated by the TLR2-stimulating synthetic lipopeptide P3CSK4 and by TLR4-activating E. coli LPS, respectively. A modest but significant NF-κB signal generated by WA-ΔyopP in control vector-transfected cells was also induced by a virulence plasmid-cured Yersinia strain (WA-C), but not by a mutant additionally negative for the chromosomally encoded invasin gene (WAΔinv-C). Invasin is an outer surface protein that binds to eukaryotic β1 integrins and mediates NF-κB activation (38). Thus, invasin apparently also triggers modest NF-κB activation in HEK293 cells probably through β1 integrins. The TLR2- and TLR4-dependent NF-κB signals induced by the diverse YopP-negative Y. enterocolitica strains indicate that both TLR2 and TLR4 can confer cellular reactivity to Yersinia infection. Use of murine TLR2, TLR4, and MD-2 expression vectors instead of the human constructs produced similar results (data not shown).

TLR4 potently signals agonist-dependent apoptosis

To find out whether the ability of TLR4 to activate apoptosis is restricted to infection of macrophages by Yersinia or a more general phenomenon, we analyzed the apoptosis-conferring abilities of...
specific TLR2 and TLR4 agonists. LPS is a potent activator of TLR4, whereas the synthetic lipopeptide P3CSK4 and highly purified LTA from *S. aureus* mediate activation of TLR2 (1–5, 12, 20, 39). In previous studies, we showed that pretreatment of macrophages with the proteasome inhibitory peptide Z-Leu-Leu-Leu-CHO (MG-132) sensitizes the cells to undergo apoptosis upon stimulation with LPS or YopP-negative yersiniae (11). MG-132 suppresses degradation of the NF-κB inhibitory IκB proteins through the proteasome pathway (40), which substantially inhibits NF-κB activation in macrophages (11). In these conditions, LPS can trigger an apoptotic response. We compared the induction of apoptosis by TLR2 and TLR4 agonists in J774A1 macrophages that were pretreated with MG-132 (Fig. 3A). In correlation with our previous studies, the stimulation with *E. coli* LPS elicited robust apoptosis (11), whereas P3CSK4 and LTA could not provoke substantial cell death (Fig. 3A). To ensure cellular activation in these experiments, the TLR agonists were used at relatively high concentrations (1 μg/ml LPS, 4 μg/ml P3CSK4, 25 μg/ml LTA). In these concentrations, LPS as well as P3CSK4 and LTA elicited a strong TNF-α response in J774A1 macrophages (Fig. 3B), which indicates successful stimulation of the cells by these agents. Similar to *E. coli* LPS, ultra-pure LPS from *S. minnesota* and a chemically synthesized analog of lipid A (18), the component of LPS that harbors the biological activity, efficiently induced macrophage apoptosis in the presence of MG-132 (Fig. 3A). At lower concentrations of 0.1 and 0.01 μg/ml *E. coli* LPS, *S. minnesota* LPS, and synthetic lipid A still mediated macrophage cell death (80–90% and 60–80% apoptotic cells within 4.5 h, respectively), an effect not observed for P3CSK4 (<10% apoptotic cells). These experiments suggest that activation of TLR4 is superior to activation of TLR2 in eliciting an apoptotic response in macrophages upon inhibition of the proteasome pathway.

To elucidate specific involvement of TLR4 in the induction of apoptosis, we performed experiments with peritoneal macrophages isolated from wild-type (C3H/HeN) and TLR4-mutagenized (C3H/HeJ TLR4<sup>ΔΔ</sup>) mice (Fig. 3C). In correlation to the results obtained on J774A1 macrophages, *E. coli* LPS efficiently conferred apoptosis to MG-132-pretreated cells from wild-type mice, whereas P3CSK4 and LTA did not (Fig. 3C). All three stimuli induced significant TNF-α production in wild-type macrophages (data not shown). Interestingly, the apoptosis-inducing capability of LPS was completely abolished in TLR4-defective macrophages from C3H/HeJ TLR4<sup>ΔΔ</sup> mice, which points out that LPS specifically signals apoptosis through TLR4. Furthermore, LPS in combination with MG-132 was fully active on peritoneal macrophages obtained from TNFR-1 and TNFR2-deficient mice, which suggests that apoptosis does not result from TNF-α that is released from the cells upon LPS treatment (data not shown).

Because it has been shown that expression of the *tlr2* gene is up-regulated after NF-κB activation (41, 42), we wondered whether the low rate of apoptosis in TLR2-stimulated and MG-132-pretreated cells could necessarily result from the prevention of TLR2 up-regulation by MG-132-mediated NF-κB inhibition. Thus, we stimulated peritoneal mouse macrophages prepared from

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**FIGURE 2.** Activation of TLR2 and TLR4 by *Y. enterocolitica* infection. *A,* TNF-α production of infected macrophages. Peritoneal macrophages elicited from the indicated mice remained untreated or were infected with wild-type yersiniae (WA-314), or the YopP-negative mutant (WA-<i>Δ</i>yop<i>P</i>). The amount of TNF-α released into the cell culture supernatant was analyzed 20 h after onset of infection. Results are expressed as mean percentages of picograms of TNF-α per milliliter ± SD from three independent experiments. *B,* Analysis of TLR expression. HEK293 cells were transfected with expression vectors for TLR2, TLR4, or empty vector control (vector). Total cell lysates were prepared 48 h later and immunoprecipitated with Abs directed toward the TLR Flag-epitope tags. Immunocomplexes were subjected to SDS-PAGE, immunoblotted with anti-Flag Abs, and visualized with ECL reagents. *C,* Activation of overexpressed TLRs. HEK293 cells were transfected with empty control vector (vector) or expression vectors for TLR2 or TLR4 together with CD14, MD-2, and reporter plasmids. Cells were stimulated as indicated for 18 h and luciferase activities were measured. NF-κB activations were normalized and are expressed as folds of NF-κB activities derived from unstimulated cells solely transfected with the empty vector control.
C3H/HeN mice with mouse TNF-α (40 ng/ml) for 3 or 16 h, which significantly doubled TLR2 expression (43–45), before treatment with MG-132 and P3CSK4. Although the pretreatment with TNF-α nearly doubled macrophage apoptosis in response to P3CSK4 (from 6 ± 2% without TNF-α to 11 ± 3% with TNF-α pretreatment after 8 h P3CSK4 stimulation), the overall degree of apoptosis remained moderate as compared with TLR4 responsive cell death. This implies that the reduced ability of TLR2 in inducing apoptosis does not merely result from impaired TLR2 expression.

To substantiate our observations on the relations between TLR activation and apoptosis, we overexpressed TLR2 and TLR4 in HEK293 cells and analyzed their influences on cellular viability. TLR2 and TLR4 were transfected together with CD14 and MD-2 expression plasmids to obtain functional TLR complexes. For identification of the transfected cells, a β-galactosidase-encoding reporter vector was included (7–10, 16). Staining with X-gal allows the detection of β-galactosidase expression by conferring a blue color to the transfected cells. Apoptosis in these cells is characterized by typical cellular shrinkage and condensation (7–10, 16), which was microscopically evaluated. Fig. 4 shows that the extended stimulation of TLR4 with E. coli LPS for 20–48 h initiated death in transfected HEK293 cells. The cytotoxic effect was less pronounced in cells transfected with TLR2 and stimulated with P3CSK4 (Fig. 4). The dying cells also displayed enhanced membrane binding of the apoptosis marker annexin V, which was determined by cotransfection of the cells with green fluorescent protein-reporter plasmid and labeling with red fluorescent Cy3-annexin V (data not shown). From these data it can be concluded that the overexpression and activation of TLR4 can conspicuously confer death to epithelial cells.

A role of FADD in the TLR4-activated apoptotic pathway

Our data indicate that LPS-stimulated TLR4 can efficiently signal apoptosis when activation of NF-κB is impaired. To identify the signal transducers that are potentially involved in the TLR4-dependent proapoptotic response, we transfected J774A.1 macrophages with different dominant-negative versions of components of the proximal LPS signaling cascade and analyzed the impact of these constructs on LPS-induced NF-κB activation and initiation of apoptosis after MG-132 pretreatment (Fig. 5). Four of the investigated molecules, MyD88, TIRAP/MyD88-adapter-like protein (Mal), PKR, and FADD have been previously shown to play roles in signaling apoptosis upon stimulation with bacteria or bacterial components (46–50). All the constructs analyzed in Fig. 5 were expressed at the expected m.w. sizes in HEK293 cells, which
FIGURE 4. Overexpression and activation of TLR4 induces cell death in HEK293 cells. HEK293 cells were transfected with empty control vector (vector) or expression plasmids for TLR2 or TLR4 together with CD14, MD-2, and p5V-β-galactosidase reporter plasmids. Cells were stimulated with E. coli LPS or P3CSK4 (2 μg/ml each) for 20 and 48 h. Thereafter, cells were stained with X-gal and single transfected blue cells were analyzed for an apoptotic morphology. Results are expressed as mean percentages ± SD of apoptotic vs the total numbers of transfected cells from three independent experiments.
trigger activation of cells through TLR2 as well as TLR4, which reflects the roles of these receptors in the recognition of bacteria. TLR4 confers reactivity to LPS, whereas TLR2 signals in response to bacterial lipopeptides, peptidoglycan, and LTA (1–5, 20, 39). To elucidate involvement of these receptors in activation of apoptosis, we investigated *Yersinia*-conferred cell death in macrophages that were deficient for TLR2 or functional TLR4. Our data showed that TLR4 deficiency potently reduced apoptosis, whereas loss of TLR2 had no significant effects on *Yersinia*-induced cell death. This suggests that signaling through TLR4, but not through TLR2, is of critical importance in *Yersinia*-mediated apoptosis. Our results obtained for cellular challenge with *Yersinia* are in unexpected contrast to the defined proapoptotic function of TLR2. Stimulation of TLR2 by bacterial lipoproteins has been shown to conspicuously induce apoptosis in mononuclear cells, suggesting a dominant role of TLR2 as apoptosis inducer (12, 47). To confirm a relation between TLR4 activation and onset of apoptosis, we compared the effects of specific TLR2 and TLR4 agonists on macrophage viability in the presence of the proteasome inhibitor MG-132 which suppresses NF-κB activation (40). In these experiments, sole stimulation of TLR4 could trigger a pronounced apoptotic response, which indicates that signaling through TLR4 is superior to TLR2 signaling in eliciting macrophage apoptosis in the investigated conditions. However, our data show that TLR2 also could have the capability to evoke apoptosis. The transient overexpression and activation of TLR2 mediated apoptosis in HEK293 cells, although to a lesser extent as compared with TLR4. Previous studies, that were reporting apoptosis through TLR2 stimulation, were conducted predominantly on human monocytic THP-1 cells, THP-1-derived macrophages, and human Schwann cells (12, 47, 60, 61), while our experiments were done on mouse J774A.1 and primary peritoneal macrophages. This suggests that there may exist cell type-specific differences in TLR-responsive apoptosis signaling, which could be related to the distinct engagement of intracellular adapter proteins by TLR2 and TLR4.

TLR2-dependent apoptotic signaling is coupled to the cell death machinery through activation of MyD88 and FADD (47), a cytotoxic pathway that is potentially also involved in *Yersinia*-mediated apoptosis (48, 62). MyD88 binds to the TLR2 intracellular signaling domain and recruits FADD through death domain-death domain interaction (47). Mobilized FADD then initiates the apoptotic cascade by caspase-8 activation. Our experiments using dominant-negative expression constructs suggest that apoptotic signaling via TLR4 could similarly target the MyD88-FADD pathway. A dominant-negative version of FADD (ΔFADD) was highly protective against TLR4-dependent apoptosis, which indicates that apoptotic signaling by TLR4 necessarily goes through FADD. LPS-mediated cell death was furthermore characterized by processing of the caspase-8 substrate Bid, which transmits the apoptotic signal from death receptors to mitochondria (56, 57). The overexpression of Bfl-1/A1, an antiapoptotic Bcl-2 family molecule that inhibits the collaboration of Bid with proapoptotic Bak or
TLR4 activation. The death domain of MyD88 may be more indispensable as a TLR adapter protein, we found that a dominant-negative MyD88 mutant (∆MyD88) could diminish TLR4-mediated apoptosis. This suggests that ∆MyD88 may interrupt recruitment and activation of FADD by TLR4 analogically as by TLR2. Interestingly, a dominant-negative version of TIRAP (∆TIRAP), another adapter of TLR2 and TLR4 (63, 64), was as efficient as ∆MyD88 in reducing TLR4-conferred cell death, which indicates that also TIRAP/Mal could play a role in transducing an apoptotic signal. However, TIRAP/Mal does not possess a death domain (31, 65) and interaction between TIRAP/Mal and FADD has not yet been described, which suggests that the mechanism by which ∆TIRAP reduces apoptosis potentially differs from the inhibitory mechanism of ∆MyD88. Furthermore, it cannot be excluded that overexpression of any dominant-negative TLR adapter blocks activation of downstream pathways by competing with the functional endogenous adapter proteins, which could argue against a specific inhibitory effect of ∆TIRAP or ∆MyD88 in TLR4-dependent apoptosis. In this regard, it would be interesting to investigate the role of a third TLR adapter protein which has been recently identified (66, 67). The Toll-IL-1R-domain-containing adapter inducing IFN-β transduces TLR4- and TLR3-, but not TLR2-, generated signals, which could have implication in the regulation of cellular viability.

Our data show that the death domain of FADD (∆FADD) is much more effective in counteracting TLR4- than TLR2-dependent NF-κB activation in J774A.1 macrophages. This gives indirect indication that the composition of intracellular protein signaling complexes differs for distinct TLRs. The conspicuous inhibition of TLR4-responsive signaling by ∆FADD suggests re-inforced mobilization of FADD by TLR4, which appears to be important for the activation of apoptosis in our experimental conditions. It has been shown that TLR4 but not TLR2 activation results in degradation of the IRAK1 molecule (68, 69). Binding of IRAK1 to the MyD88 death domain is an important step of the TLR-responsive signal relay that leads to NF-κB activation (1–6). The selective degradation of IRAK1 after LPS stimulation, which we also observed in J774A.1 macrophages (data not shown), could give a possible explanation for enhanced recruitment of FADD after TLR4 activation. The death domain of MyD88 may be more accessible to FADD after IRAK1 disappearance, concomitantly leading to increased induction of the apoptotic pathway. IRAK1 degradation upon LPS treatment has been suggested to occur via the proteasome pathway (70), which indicates that the proteasome function could play a role in the regulation of TLR-dependent apoptosis by controlling the turnover of TLR-responsive signal transmitters. Thus, although both TLR2 and TLR4 could potentially engage the FADD pathway to provoke apoptosis, the composition and stability of intracellular adapter protein complexes recruited by the respective TLR may determine the activation of apoptotic signaling and the extent of apoptosis.

It is shown here that TLR4 can efficiently signal apoptosis in bacteria-infected macrophages, which plays an important role in Yersinia-induced apoptosis. A study by Zhang and Bliska (71), which was published while our manuscript was in revision, supports our findings, elucidating TLR4 as the primary source of apoptotic signaling in Yersinia-infected macrophages. Although the authors consider the redundancy of TLR2 in Yersinia-induced apoptosis as a result of a putative inability of TLR2 to recognize yersiniae, our data clearly demonstrate that both TLR2 and TLR4 can confer responsiveness to Yersinia infection, but only TLR4 signals apoptosis. However, TLR4-deficient cells were not completely protected against Yersinia-induced cell death. This indicates that additional cytotoxic pathways are active in the absence of functional TLR4. It has been shown that constitutive NF-κB activation is required for macrophage survival in nonstimulated conditions (72). This suggests the existence of a constitutively active cytotoxic pathway that is balanced by basic NF-κB activation. In fact, the transfection of macrophages with YopP (16) or kinase-inactive IKKβ, as well as extended treatment with MG-132 (data not shown), significantly confers apoptosis, which could support this hypothesis. In this case, TLR4 signaling critically accelerates onset of apoptosis that is otherwise mediated by the NF-κB inhibitory activity of YopP with delayed kinetics. Alternatively, pathways that signal apoptosis independently from TLR4 are activated in Yersinia-infected cells. Our preliminary studies do not indicate Yersinia-conferred activation of other known TLRs besides TLR2 and TLR4 when overexpressed in HEK293 cells. However, additional bacteria-responsive proapoptotic signal transmitters, such as the intracellular nod proteins, which function as cytokine peptidoglycan receptors (73), could be implicated in Yersinia-induced apoptosis. Whether Yersinia as extracellular pathogen induces the nod pathways and whether these pathways cooperate with TLR4 to evoke Yersinia-mediated cell death has not yet been investigated.

The role of apoptotic signaling through TLR4 is still elusive. It could help to limit the lifetime of activated inflammatory cells to protect the organism from developing damage by inflammatory hyperactivation during infection. Furthermore, it could be part of an evolutionary conserved mechanism of innate immune defense. TLRs display some homology to plant disease resistance gene products, which mediate a so-called hypersensitive response when encountering a bacterial pathogen (47, 74, 75). The hypersensitive response resembles apoptosis in animal cells, leading to localized cell death at the site of pathogen invasion which limits dissemination of the microbes (75, 76). Analogously, apoptosis of macrophages could help to prevent spread of pathogenic intracellular bacteria, for instance Francisella tularensis which has been found to inhibit NF-κB activation and to induce macrophage cell death (77, 78). However, under normal circumstances the activation of NF-κB counteracts TLR-dependent apoptosis and macrophage viability is not necessarily restricted during infection. From this observation it appears that some extracellularly pathogenic bacteria, such as yersiniae, could take advantage of the conserved cytotoxic TLR4-signaling pathway to trigger macrophage cell death through the inhibition of NF-κB. This suggests that apoptosis induced by...
TLRs could potentially fulfill diverse roles in the host immune response depending on the invading pathogen and on the challenged cell.

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