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Cell Death Triggered by *Yersinia enterocolitica* Identifies Processing of the Prolinflammatory Signal Adapter MyD88 as a General Event in the Execution of Apoptosis

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Many pathogenic microorganisms have evolved tactics to modulate host cell death or survival pathways for establishing infection. The enteropathogenic bacterium *Yersinia enterocolitica* deactivates TLR-induced signaling pathways, which triggers apoptosis in macrophages. In this article, we show that *Yersinia*-induced apoptosis of human macrophages involves caspase-dependent cleavage of the TLR adapter protein MyD88. MyD88 was also cleaved when apoptosis was mediated by overexpression of the Toll–IL-1R domain–containing adapter inducing IFN-β in epithelial cells. The caspase-processing site was mapped to aspartate-135 in the central region of MyD88. MyD88 is consequently split by caspases in two fragments, one harboring the death domain and the other the Toll–IL-1R domain. Caspase-3 was identified as the protease that conferred the cleavage of MyD88 in in vitro caspase assays. In line with a broad role of caspase-3 in the execution of apoptosis, the processing of MyD88 was not restricted to *Yersinia* infection and to proapoptotic Toll–IL-1R domain–containing adapter inducing IFN-β signaling, but was also triggered by staurosporine treatment. The cleavage of MyD88 therefore seems to be a common event in the advanced stages of apoptosis, when caspase-3 is active. We propose that the processing of MyD88 disrupts its scaffolding function and uncouples the activation of TLR and IL-1Rs from the initiation of proinflammatory signaling events. The disruption of MyD88 may consequently render dying cells less sensitive to proinflammatory stimuli in the execution phase of apoptosis. The cleavage of MyD88 could therefore be a means of conferring immunogenic tolerance to apoptotic cells to ensure silent, noninflammatory cell demise. *The Journal of Immunology*, 2014, 192: 000–000.
downregulates the proinflammatory host cell response. Because these pathways also promote cell survival, the action of YopP/YopJ sensitizes macrophages to undergo apoptosis following *Yersinia* infection (13, 14). The proapoptotic response thereby is accelerated by the activation of TLR4 and TRIF, whereas the MyD88 pathway seems to be dispensable for *Yersinia*-induced cell death (15, 16). TRIF is connected to the cell death machinery by the adapter’s receptor-interacting protein 1 and Fas-associated death domain (DD) to initiate apoptosis through caspase-8 (16–18). The signaling of apoptosis following TLR4 or TLR3 activation seems to be a prominent function of TRIF, which is substantiated by the finding that the overexpression of TRIF significantly compels transfected cells to undergo apoptosis (16–18).

In the current study, we report on a feedback control mechanism that links apoptosis execution with the modulation of proinflammatory signaling. It is shown that human MyD88 is cleaved when apoptosis is induced by *Yersinia* infection or TRIF overexpression. The processing of MyD88 is mediated by caspase-3 and appears to be a general event downstream from effector caspase activation. Caspase-3 cleaves MyD88 within the MyD88 intermediate domain at aspartate-135. This cleaving separates the MyD88 N-terminal DD from the C-terminal TIR domain and disrupts the ability of MyD88 to function as proinflammatory adapter that connects the activation of TLR and IL-1R members to downstream signaling events. The cleavage of MyD88 therefore may help to uncouple the engagement of these transmembrane receptors from the initiation of proinflammatory signaling in apoptotic cells. Several proinflammatory signal transmitters that links apoptosis execution with the modulation of proinflammatory mechanisms.

### Materials and Methods

**Cell culture, stimulation, and infection conditions**

The human embryonic kidney (HEK) 293 cell line was cultured in DMEM cell growth medium containing 10% heat-inactivated FCS (Life Technologies, Darmstadt, Germany). Peripheral blood monocytes were isolated from buffy coats of healthy donors by gradient centrifugation using Ficoll cell separation medium (PAA Laboratories, Pasching, Austria). The cells were resuspended in cell growth medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 5 mM t-glutamine) and differentiated to macrophages by incubation with 10 ng (≥100 U) of M-CSF (PeproTech, Hamburg, Germany) per milliliter for 7 d. Nonadherent, nondifferentiated cells were isolated by centrifugation, MyD88-deficient, macrophages were differentiated by renoverin (23) and were kindly provided by Katherine A. Fitzgerald (Division of Infectious Disease and Immunology, University of Massachusetts Medical School, Worcester, MA). The immortalized macrophages were cultured in DMEM containing 10% heat-inactivated FCS, 5% heat-inactivated horse serum, 1 mM sodium pyruvate, 10 mM Heps, 1% nonessential amino acids, and 2 mM t-glutamine (Life Technologies). Cells were always incubated at 37°C and with 5% CO2 in a humidified atmosphere.

The *Y. enterocolitica* strains used in this study were the serotype O8 wild-type strain WA, and its isogenic yopP knockout mutant WAΔyopp (13). For infection, overnight bacterial cultures grown at 27°C were diluted 1:20 in fresh Luria–Bertani broth and grown for another 2 h at 37°C. 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 infection, bacteria were seeded on the cells by centrifugation at 200 × g for 5 min at a ratio of 20 bacteria per cell. For incubation times >90 min, bacteria were routinely killed by addition of gentamicin (100 μg/ml) after 90 min of infection to prevent bacterial overgrowth. Staurosporine (Sigma-Aldrich, Munich, Germany) was applied to the cells at a concentration of 5 μM. Where indicated, the cells were pretreated with 40 μM of the caspase inhibitor zVAD-fmk (Bachem, Bubendorf, Switzerland) 30 min prior to infection.

**Eukaryotic expression plasmids, cell transfection, and measurement of NF-κB activation**

Transfections of HEK293 cells were performed by the calcium phosphate method using Profection according to the manufacturer’s instructions (Promega, Mannheim, Germany). For the detection of transiently expressed Myc-tagged MyD88 (24), the cells were transfected with 3 μg expression plasmid in 6-cm cell culture dishes. Where indicated, plasmids for TRIF (25), the caspase-8 inhibitor CrmA (26), or IL-1R–associated kinase (IRAK) 4 (27) were included. Expression plasmids for MyD88, IRAK4, TRIF, and CrmA, were kindly provided by Tularik (South San Francisco, CA), Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), and David Vaux (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), respectively. The total amount of DNA was kept constant with empty vector for each transfection condition (6 μg). To insert point mutations into Myc-tagged MyD88, the wild-type *myd88* gene was subjected to site-directed mutagenesis using the QuikChange Mutagenesis Kit, as recommended by the manufacturer. MyD88 expression plasmids immortalized in apoptotic cells. Several proinflammatory signal transmitters were cleaved to be reported as cleaved during apoptosis, such as NF-κB p65, components of the IKK complex, receptor-interacting protein kinases, and TRIF itself (19–22). The degradation and processing of MyD88 could complementarily reduce the sensitivity of apoptotic cells to proinflammatory stimuli and thereby counteract inflammation triggered by the TLR and IL-1R family.

This activity may fine-tune the apoptotic process and preserve apoptosis as a discrete, noninflammatory cell death mechanism.
Alternatively, myc-tagged overexpressed MyD88 was labeled by probing with a monoclonal mouse anti-myc epitope tag Ab (Life Technologies). Immunoreactive bands were visualized using appropriate secondary Abs and ECL detection reagents (GE Healthcare, Bucks, U.K.). When required, the membranes were stripped by successive incubation with 0.1 N NaOH and 0.1 N HCl for 30 s and reused for a second immunoblotting procedure. The stripping method was also applied to recycle the membranes for controlling equal protein loading of the gels with cellular lysates by detecting actin or HSC-70 with appropriate Abs (mouse anti-actin; Merck-Millipore, Darmstadt, Germany; and mouse anti-HSC-70; Santa Cruz Biotechnology, Dallas, TX). Phospho-specific immunoblotting against IRAK4 and TGF-β-activated kinase-1 (TAK1) was performed as described above, using mAbs directed against phosphorylated threonine-345 and serine-346 of IRAK4, or phosphorylated threonine-184 and threonine-187 of TAK1 (Cell Signaling Technology, Danvers, MA). The total cellular pool of transfected IRAK4 was labeled by stripping the membrane and successive immunoblotting with anti-HA (Cell Signaling Technology). Because the recycled membranes showed reduced sensitivity to successive TAK1 immunostaining with the available Abs, the cellular levels of TAK1 were analyzed in parallel control samples by immunoblotting with anti-TAK1 Ab (Santa Cruz Biotechnology, Dallas, TX). The levels of MyD88 or of the phosphorylation of IRAK4 and TAK1 were assessed using antibodies against Escherichia coli as GST-fusion protein. For immunoprecipitation, myc-tagged MyD88 was precipitated with anti-myc Ab from lysates of transfected HEK293 cells 24 h after transfection. The cell lysates were generated by resuspending the transfected cells in caspase cleavage buffer (50 mM HEPES pH 7.2, 0.1% CHAPS, 50 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, 10% Saccharose, 10 mM DTT, 10 μM Leupeptin, 0.3 mM Aprotinin, 1 mM PMSF) and repeated freezing and thawing. The myc complexes were co-precipitated with protein A/G-agarose (Santa Cruz Biotechnology, Heidelberg, Germany), washed five times with caspase cleavage buffer containing 1% Triton X-100, and subjected to in vitro caspase cleavage assay. Accordingly, the immunoprecipitated, purified MyD88 complexes were incubated with 2 U caspase at 37°C for 2–3 h in 80 μl reaction buffer. The reaction was stopped by the addition of SDS sample buffer and by boiling for 5 min. The samples were then subjected to immunoblotting, as described above. In a related approach, the caspase cleavage experiments were conducted on recombinant purified MyD88 proteins. For this issue, cDNAs for human and mouse MyD88 were amplified by PCR and subcloned into plasmid pGEX-4T-1 to express the MyD88 constructs as GST-fusion proteins in E. coli. The GST–MyD88 fusion proteins were purified from E. coli by sonication in caspase cleavage buffer and by incubation of the bacterial lysates with glutathione Sepharose (GE Healthcare, Freiburg, Germany). Proteins bound to the Sepharose beads were pulled down by centrifugation, washed, and subjected to in vitro caspase cleavage, as indicated above. Equal amounts of bacterial lysates were used for GST-fusion protein pull-down and for processing in the caspase cleavage assays. To determine the caspase-3 cleavage site by Edman degradation, human GST–MyD88 was incubated with 10 U caspase-3 at 37°C for 16 h. The sample was then subjected to SDS-PAGE, transferred to PVDF membrane, and stained with Coomassie Blue to detect the cleavage products of MyD88. The protein band that corresponded to the C-terminal fragment of MyD88 was excised from the PVDF membrane, destained with methanol, and subjected to N-terminal Edman sequencing using an automated protein sequencer (473A; Applied Biosystems, Foster City, CA).

Results

MyD88 is processed during the execution of apoptosis

Our previous studies have shown that MyD88 relays the Yersinia-induced, proinflammatory signals downstream from TLR2 and TLR4, but it does not decisively contribute to the proapoptotic response following Yersinia infection (15, 16). In this study, we assessed the protein levels of MyD88 in human monocyte-derived macrophages infected with Yersinia. Infections were performed with the Y. enterocolitica wild-type strain WA, or its isogenic YopP-negative mutant WA-ΔyopP, which is impaired in apoptosis induction (13). The cellular MyD88 levels were analyzed 20 h later. In these experiments, it was noticed that the amount of MyD88 was diminished when cells were infected with the wild-type strain WA (Fig. 1A, lane 2), whereas no significant alteration was observed post infection with the YopP-negative mutant WA-ΔyopP (lane 3). MyD88 disappeared in correlation with the extent of apoptosis triggered by Yersinia and was nearly complete after 20 h, a time point at which > 80% of the cells infected with the wild-type strain were apoptotic (Fig. 1B, lane 6). Importantly, coadministration of the broad-spectrum caspase inhibitor zVAD-fmk substantially stabilized MyD88 levels in the WA-infected cells (Fig. 1A, lane 4). This finding suggests that caspases, activated in the course of Yersinia-induced apoptosis, may target the cellular levels of MyD88. Of interest, the amount of MyD88 was also affected by staurosporine treatment. Staurosporine triggers apoptosis through activation of the intrinsic mitochondrial pathway and conferred 89 ± 6% apoptosis to the macrophages within 20 h. At that time point, MyD88 was substantially decreased (Fig. 1A, right panel), which was comparable to the MyD88 levels in infection with wild-type Yersinia. Thus, the MyD88 levels seemed to be subject to negative regulation by caspases once apoptosis was induced, irrespective of the apoptosis trigger. A more detailed view on the MyD88 levels in macrophages infected with Yersinia at different multiplicities of infection (MOIs) confirmed that the disappearance of MyD88 correlated closely with the extent of apoptosis triggered by the Yersinia wild-type strain. Accordingly, WA infection gradually reduced the levels of MyD88 with increased bacterial loads (Fig. 1C). Apoptosis in the WA-infected macrophages rose from 57 ± 6% to 90 ± 4% at the given MOIs ranging from 5 to 40. WA-ΔyopP less severely affected the viability of the macrophages (12 ± 4% and 30 ± 4% apoptosis at MOIs of 5 and 40, respectively), as expected. Of note, along with the reduction of full-length MyD88 by WA infection, a 17-kDa protein was recognized by the Ab directed against the C terminus of MyD88 (Fig. 1C). A fragment of comparable size was detected also in lysates of HEK293 cells when MyD88 was overexpressed together with TRIF (Fig. 1D). In these experiments, the overexpression of N-terminally myc-tagged MyD88, appearing at a molecular mass of 37 kDa, resulted in the appearance of two additional protein bands upon cotransfection with TRIF (Fig. 1D). The N-terminal fragment was ∼ 20 kDa, as detected by the anti-myc Ab (lane 3), whereas the 17-kDa C-terminal protein band was recognized by the Ab directed against the MyD88 C terminus (lane 7). The overexpression of TRIF triggers 70–90% apoptosis in the transfected cells under these conditions (16–18). This result tempted us to speculate that TRIF-related apoptosis, comparable to apoptosis conferred by Yersinia infection or staurosporine treatment, could be responsible for the fragmentation of MyD88. In line with this idea, the coexpression of CrmA, an inhibitor of caspase-8 that counteracts TRIF-related apoptosis...
(16–18), as well as the caspase inhibitor zVAD-fmk prevented the appearance of the two MyD88 fragments (Fig. 1D). Together, these data suggest that MyD88 is cleaved and processed by caspases in two smaller fragments of 20 kDa (N-terminal) and 17 kDa (C-terminal) during the execution of apoptosis.

**Caspase-3 cleaves human MyD88 at D135**

Caspases are cysteine proteases cleaving their client molecules at aspartate residues within dedicated caspase recognition sequences (30). To pinpoint a potential role of caspases in the fragmentation of MyD88, we mutagenized codons for several aspartate residues
at which the cleavage might occur and replaced them with alanine (Fig. 2A). The mutagenized MyD88 constructs were then compared with wild-type MyD88 to become processed upon cotransfection with the TRIF expression plasmid. Fig. 2B demonstrates that the substitution mutation of aspartate at position 135 prevented the cleavage of MyD88 (lane 5), whereas the other MyD88 mutants were still processed. It may be concluded that D135 represents the caspase cleavage site of MyD88. We subcloned the sequences for amino acids 1–135 and 136–296 of MyD88 into a eukaryotic expression vector in fusion with an N-terminal myc-tag epitope. Comparing the electrophoretic mobilities of these constructs with those of processed, N-terminally myc-tagged wild-type MyD88, we found that the myc-tagged N terminus of MyD88 (ΔMyD88, N-135) displayed exactly the same mobility as the 20-kDa myc-tagged fragment generated upon MyD88 cleavage (Fig. 2C, lanes 3 and 4). The C-terminal part of MyD88 (ΔMyD88, C-136) displayed a higher molecular mass than the original 17 kDa C-terminal fragment because of the fusion of ΔMyD88, C-136 with the myc-tag (data not shown). However, the identical migration pattern of ΔMyD88, N-135 and the N terminus of processed wild-type MyD88 support the idea that MyD88 is cleaved at position D135.

To gain further insights into a potential role of caspases in the fragmentation of MyD88, we subjected immunoprecipitated myc-tagged MyD88 to an in vitro caspase assay using recombinant caspases. The application of caspase-3 thereby led to the generation of a 17-kDa protein band that was recognized by the C-terminal anti-MyD88 Ab (Fig. 3A, lane 2). This protein was slightly visible upon caspase-7 application, and was not detected after treatment with caspase-6 or caspase-8. Consequently, it appears that caspases—in particular, caspase-3—mediate the cleavage of MyD88. Next, wild-type and D135A-mutated MyD88 were analyzed in parallel on their sensitivities to become processed by caspase-3. Only wild-type MyD88, but not the D135A mutant, was cleaved, leading to the formation of the two fragmentation products (Fig. 3B, lanes 5 and 8). This finding substantiates the idea that caspase-3 mediates the processing of MyD88 at D135. D135 is located in the quadripartite sequence AA VD, a motif that is well described as recognized and processed by caspase-3 in vivo (30, 31). Of interest, the AA VD motif of human MyD88 (GenBank accession number Q99836.1) is absent in murine MyD88 (GenBank accession number P22366.3, www.ncbi.nlm.nih.gov/genbank/), in which ARVE replaces the AA VD sequence. In confirmation of the importance of D135 in enabling the processing of human MyD88, we expressed GST-tagged versions of human and murine MyD88 and subjected the purified proteins to cleavage by caspase-3 in vitro. As expected, human, but not murine, MyD88 underwent caspase-3–dependent protein fragmentation (Fig. 3C, compare lanes 2 and 4). We additionally performed Edman sequencing of the C-terminal MyD88 fragment generated by caspase-3 to conclusively determine the MyD88 cleavage site. The Edman degradation reaction sequentially removes one residue at a time from the N terminus of a protein. By this method we mapped a peptide characterized by the sequence X-V-P-R-(T)-A-E-L-(A). The first two amino acids (“X”) could not be identified, and determination of the amino acids in brackets was not unambiguous. However, from the sequence in total it became apparent that the peptide matched amino acids 136–145 of human MyD88 (Fig. 2A). This result confirms that MyD88 is cleaved by caspase-3 immediately after D135. Thus, D135 specifically determines the sensitivity of human MyD88 to caspase-3–related processing.

FIGURE 2. Mutation of D135 prevents the fragmentation of MyD88. (A) Mapping of aspartates in the intermediate region of MyD88. Downstream from the DD, human MyD88 harbors eight aspartates (bold) that may serve as potential caspase-processing sites. The deduced sequence of human MyD88 (GenBank accession No. Q99836.1) is shown between amino acids 111 and 200. The motif identified as being cleaved by caspase-3 is underlined (amino acids 132–135, aavd). (B) Processing of MyD88 aspartate mutants in TRIF-related apoptosis. HEK293 cells were cotransfected with TRIF expression plasmid, and either empty vector control (vector) or vectors encoding wild-type MyD88-myc (WT) or MyD88-myc mutants in which single aspartates were replaced by alanines as indicated. Cellular extracts were prepared 20 h after transfection and subjected to immunoblotting using anti-myc Ab. (C) Comparison of the electrophoretic mobilities of fragmented wild-type MyD88 and ΔMyD88, N-135. HEK293 cells were transfected with expression plasmids for TRIF, MyD88-myc, a combination of both plasmids (TRIF + MyD88), or a MyD88-myc fragment encoding the N-terminal amino acids 1 to 135 (ΔMyD88, N-135). Cell extracts were prepared and processed for anti-myc Ab immunoblotting, as in (B). Equal loading of the gels with cellular lysates was controlled in (B) and (C) by immunoblotting of the membranes against actin. Molecular masses of standard marker proteins are indicated in kilodaltons.

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The processing of MyD88 helps to deregulate proinflammatory signaling events in the execution of apoptosis

The cleavage of MyD88 by caspase-3 in the intermediate domain splits MyD88 into two fragments and separates the MyD88 DD from the TIR domain. This activity may disrupt the ability of MyD88 to couple receptor stimulation to the activation of downstream proinflammatory signaling events. To gain more insight into the functional consequences of the cleavage of human MyD88 for the proinflammatory response, we analyzed the influence of the different MyD88 constructs on NF-κB activation. Wild-type MyD88, D135A-mutagenized MyD88, or the ΔMyD88_N-135 or ΔMyD88_C-136 constructs were cotransfected with a NF-κB luciferase reporter vector into HEK293 cells, and either left untreated (Ø) or subjected to in vitro caspase assay with caspase-3, as described in (A). (C) In vitro processing of recombinant human versus mouse MyD88. Human and mouse MyD88 were purified as recombinant GST-fusion protein and subjected to in vitro caspase assay with caspase-3, as in described in (A). The samples of the caspase assays were separated by SDS-PAGE and processed for immunoblotting using anti-MyD88 Ab (A; left panel; C). In (B), the membrane was additionally stripped and reprobed with anti-myc Ab (right panel).

FIGURE 3. Caspase-3 cleaves human MyD88 at D135. (A) In vitro processing of immunoprecipitated human MyD88 by different caspases. Human MyD88-myc was immunoprecipitated from lysates of transfected HEK293 cells with anti-myc Ab, and either left untreated (Ø) or subjected to in vitro caspase cleavage assay with the indicated caspases at 37˚C for 2 h. (B) In vitro processing of human wild-type MyD88 versus the D135A mutant. Wild-type MyD88 (WT) and the corresponding D135A MyD88 mutant (D135A) were immunoprecipitated from lysates of transfected HEK293 cells, and either left untreated (Ø) or subjected to in vitro cleavage assay with caspase-3, as described in (A). (C) In vitro processing of recombinant human versus mouse MyD88. Human and mouse MyD88 were purified as recombinant GST-fusion protein and subjected to in vitro caspase assay with caspase-3, as in described in (A). The samples of the caspase assays were separated by SDS-PAGE and processed for immunoblotting using anti-MyD88 Ab (A; left panel; C). In (B), the membrane was additionally stripped and reprobed with anti-myc Ab (right panel).
may affect the phosphorylation of IRAK4 within the Myddosome, which assembles when MyD88 is overexpressed together with IRAKs. We cotransfected HEK293 cells with MyD88 and IRAK4 expression plasmids and monitored the phosphorylation of IRAK4 in cell extracts. The used Ab recognizes the phosphorylation of threonine-345 and serine-346, which are two autophosphorylation sites required for IRAK4 activation (33). Fig. 5 shows that the overexpression of both wild-type and D135A-mutagenized MyD88 triggered the phosphorylation of coexpressed IRAK4. (lanes 2 and 3). When apoptosis was induced by treatment with staurosporine, the phosphorylation of IRAK4 was reduced in cells cotransfected with wild-type MyD88 (lane 4), whereas cells expressing D135A-mutagenized MyD88 retained stronger IRAK4 phosphorylation (lane 5). The decrease of IRAK4 phosphorylation correlated with a reduced level of wild-type MyD88, which was not observed, as expected, for the D135A mutant. These experiments indicate that the cleavage of MyD88 in the course of apoptosis can impair the ability of MyD88 to trigger IRAK4 activation. The processing of MyD88 by caspase-3 could therefore be a means that disrupts efficient clustering of MyD88 and concomitant nucleation of proinflammatory signaling complexes. This mechanism may uncouple the activation of proinflammatory cell surface receptors from intracellular signaling.

We next assessed whether the cleavage of MyD88 could also have an effect on the signaling in cells undergoing Yersinia-induced apoptosis. We aimed to perform these experiments in MyD88-negative cells that were reconstituted with either wild-type MyD88 or the noncleavable MyD88-D135A mutant. Because Yersinia triggers apoptosis in macrophages, but not in epithelial cells or monocytic cell types (34), we relied in these experiments on immortalized bone marrow macrophages that were derived from MyD88-deficient mice. The cells were transiently transfected with...
empty control plasmid or expression vectors encoding wild-type or D135A-mutagenized MyD88. Fig. 6 shows that apoptosis induction by the *Yersinia* wild-type strain WA (Fig. 6B) resulted in a time-dependent decrease in the protein levels of wild-type MyD88 (Fig. 6A, lane 6). The levels of MyD88-D135A, in contrast, were less severely destabilized (lane 9), which likely resulted from the inability of caspase-3 to cleave the MyD88-D135A mutant. Longer exposition of the immunoblot enabled detection of the C-terminal cleavage product of wild-type MyD88 (Fig. 6A, lanes 5 and 6). The levels of the MyD88 fragment increased in the course of *Yersinia*-induced apoptosis. Relatively fair amounts of wild-type MyD88 and a long exposure time of the immunoblot were required to detect the MyD88 fragments, which may suggest that the truncated MyD88 proteins were not stable inside the cell. A certain degree of processing of MyD88 became apparent also in cells infected with the YopP-negative mutant WA-ΔyopP (Fig. 6A, lanes 10 and 11). This processing was likely due to a low level of YopP-independent apoptosis that occurred in a fraction of the infected cells (Fig. 6B). It should be noted that the time course of *Yersinia*-mediated apoptosis substantially differs between human (Fig. 1B) and murine macrophages (Fig. 6B), which could result from differences in their origin and maturation (34). We subsequently analyzed whether the MyD88 constructs can reconstitute the MyD88-negative macrophages and confer a functional MyD88-dependent proinflammatory phenotype. The Ab that recognized the phosphorylation of IRAK4 in transfected HEK293 cells (Fig. 5) was, unfortunately, inefficient in detecting a comparable signal on endogenous IRAK4 in macrophages (data not shown). We therefore characterized a second phosphorylation event that occurs downstream from IRAK members on TAK1. TAK1 requires phosphorylation at threonine-184 and threonine-187 to be activated upon TLR or IL-1R stimulation (35). Using an Ab directed against phosphorylated threonine-184 and threonine-187, we analyzed the phosphorylation status of TAK1 in lysates of *Yersinia*-infected cells. It is shown in Fig. 6C that *Yersinia* infection for 2 h initially triggered the phosphorylation of TAK1. This phosphorylation event largely depended on the presence of MyD88 because cells solely transfected with empty control plasmid did not exhibit substantial phosphorylation (Fig. 6C, lower panel). The TAK1 phosphorylation status decreased in the course of *Yersinia*-induced apoptosis triggered by the wild-type strain WA (Fig. 6C, upper panel). This decrease was particularly obvious in macrophages expressing wild-type MyD88, in which the TAK1 phosphorylation status was impaired after 3.5 and 5 h of infection (lanes 4 and 5). TAK1 itself seemed to be destabilized during apoptosis at 5 h (lane 5). Importantly, the phosphorylation of TAK1 was conserved remarkably longer over time in cells expressing the MyD88-D135A mutant, in which the phosphorylation of TAK1 was still obvious after 3.5 h (lane 8). This finding suggests that the stability of MyD88 has influence on the phosphorylation and activation status of TAK1 in cells undergoing apoptosis. The cleavage of MyD88 by caspase-3 may consequently contribute to shutting down the signaling of inflammation through TAK1 in apoptotic cells. In total, these results indicate that the processing of MyD88 disrupts the transmission of intracellular signals induced by the activation of proinflammatory cell surface receptors, which renders the apoptotic cell unable to respond adequately to inflammatory stimuli.

**Discussion**

Apoptosis is a regulated cell death mechanism that ensures the removal of unwanted cells from the multicellular organism without inducing considerable inflammation (36). This form of cell death also plays a role in infectious diseases (37, 38). Apoptosis can mediate the destruction of infected cells and the concomitant elimination of intracellular pathogens. The signaling of apoptosis through TLRs is understood to be part of this host innate immune reaction that enables the silent, noninflammatory clearance of infected cells (5, 16, 18, 39, 40). Apoptosis triggered by TLR4 and TRIF in host immune cells is, furthermore, a physiological response in systemic Gram-negative bacterial infection (41). This pathway is involved in mediating the maturation of dendritic cells and in shaping adaptive immunity. Our previous studies have demonstrated that TLR4 and TRIF also contribute to *Yersinia*-induced apoptosis. *Y. enterocolitica* injects YopP in infected cells to inhibit the anti-apoptotic NF-κB pathway. This activity sensitizes macrophages to undergo apoptosis following activation of TLR4 and TRIF (16). In pursuing the exploration of the life and death signaling circuits in *Yersinia*-infected macrophages, we show in this article that the execution of apoptosis vitally affects the MyD88 pathway. Activation of the apoptotic cascade through TRIF was accompanied by the cleavage of MyD88. Similarly, MyD88 was processed following induction of the intrinsic, mitochondrial apoptotic pathway through staurosporine treatment. Our data provide evidence that MyD88 is cleaved and processed by caspase-3 in the execution phase of apoptosis, which critically affects its activity in the dying cell.

A plethora of cellular proteins have been reported to be cleaved by caspases (19, 30). For most of the proteins, however, the biological function of the cleavage is not defined. Recent studies have indicated that caspases do not dismantle cells by indiscriminate proteolysis, but rather selectively target certain protein complexes and networks (30, 36, 42). Many proteins proteolyzed during apoptosis may, furthermore, yield fragments that correspond to discrete protein domains that could fulfill temporary but specific functions as active or dominant negative effector proteins in the
FIGURE 6. The phosphorylation of TAK1 is influenced by the stability of MyD88 in the course of Yersinia-induced apoptosis. (A) Reconstitution of MyD88-knockout macrophages with wild-type or D135A-mutagenized MyD88 and analysis of the MyD88 protein levels during Yersinia infection. Immortalized MyD88-deficient, murine macrophages were transiently transfected with either empty control vector (vector) or expression plasmids for human wild-type (WT) or D135A-mutagenized (D135A) MyD88-myc. Cells were left untreated (Ø) or infected with wild-type Y. enterocolitica WA or the YopP-negative mutant WA-ΔyopP at an MOI of 20 bacteria per cell for the indicated time points. Then, cell lysates were prepared and subjected to immunoblotting using anti–C-terminal MyD88 Ab and ECL reaction. Short exposure of the immunoblot (upper panel) allowed the assessment of the MyD88 levels in the course of Yersinia infection. The MyD88 protein content was quantified in relation to cellular actin, and the values obtained for untreated cells were set to 100% for WT- as well as D135A-mutagenized MyD88. Results are expressed as percentages thereof. Longer exposure of the immunoblot enabled the detection of the C-terminal MyD88 cleavage product (lower panel). (B) Time course of Yersinia-induced apoptosis. Cells were transfected and infected as in (A). At 2 and 3.5 h after onset of Yersinia infection, the rates of apoptosis were determined by labeling apoptotic cells with annexin V and propidium iodide and counting apoptotic cells by fluorescence microscopy. Results are expressed as mean percentages ± SD from four independent experiments. Apoptosis induction was significant for the wild-type strain compared with noninfected cells at each investigated time point (p < 0.001). (C) MyD88-dependent phosphorylation of TAK1 in the course of Yersinia infection. MyD88-deficient macrophages were transfected with empty control vector (vector), or expression plasmids for wild-type (WT) or D135A-mutagenized (D135A) MyD88-myc, and then either left untreated (Ø) or infected with wild-type WA or YopP-negative WA-ΔyopP at an MOI of 15 bacteria per cell. The differential effects of the MyD88 constructs on the phosphorylation of TAK1 became most apparent at this MOI. At the indicated time points, cell lysates were prepared and subjected to immunoblotting with an Ab (Figure legend continues).
course of apoptosis (31, 43). The targeting of MyD88 by caspase-3 may fit into this concept. By cleaving MyD88, the proteolytic caspase cascade affects a central and essential proinflammatory adapter molecule. Caspase-3 cuts MyD88 into two fragments, each harboring a specific domain that could fulfill a particular function. The C-terminal MyD88 fragment that comprises the TRIF domain can act as dominant negative inhibitor on TLR signaling. The MyD88 N terminus with the DD, in contrast, has some capacity to autonomously recruit binding partners for initiating downstream signaling events. The concerted actions of both MyD88 fragments may fine-tune the apoptotic process, which could govern specific cell death–related events in a temporal or spatial manner. Similar features have been reported for other signal transmitters of the NF-κB pathway that are cleaved by caspases, including TRAF1, receptor-interacting protein 1, IKKβ, IKKγ/NEMO, and NF-κB p65 (19, 20, 22). The processing of these molecules hampers their prosurvival functions and accelerates apoptosis.

These events are apparently precisely controlled over time. The cleavage products of many of these signal transmitters are short lived and shut down in their activities by regulated degradation (44). This activity tends to balance excessive effects of the truncated proteins on the apoptotic cell, to fine-tune the progression of cell death. The MyD88 fragments characterized in our studies could be subjected to a similar regulatory pathway. Fair amounts of the protein and an appropriate time frame were required to detect the MyD88 fragments. This requirement could reflect successive destabilization of the liberated MyD88 domains following caspase-3–dependent cleavage. Removal of the MyD88 cleavage products may ensure a timed and restricted activity of the truncated proteins on the dying cell. Importantly, the cleavage of MyD88 seemed to target predominantly its proinflammatory function, but not the execution of cell death itself. Neither overexpression of the noncleavable MyD88 mutant nor overexpression of the resulting N- or C-terminal MyD88 fragments significantly influenced the initiation or progression of apoptosis in several investigated conditions (data not shown). This finding is in line with previous studies in which TLR-related apoptosis signaling was prominent for the TRIF, but not for the MyD88, pathway (16, 18). Our results suggest, rather, that the processing of MyD88 primarily affects its capacity to signal inflammation. The overexpression of MyD88 under conditions that facilitate formation of the Myddosome triggered the phosphorylation of IRAK4, which was diminished when apoptosis was executed. On the contrary, overexpression of the noncleavable MyD88 mutant remarkably preserved the phosphorylation of IRAK4 in this setting. In the same manner, the phosphorylation of TAK1 was largely controlled by the stability of MyD88 in macrophages that underwent apoptosis in response to Yersinia infection. Thus, the levels of full-length MyD88 can regulate the phosphorylation status of IRAK4 and TAK1 in dying cells. This observation suggests conversely that the cleavage of MyD88 by the apoptosis machinery could specifically impair the initiation of proinflammatory signaling by preventing the phosphorylation and activation of IRAK molecules and of other downstream signal transmitters.

By being fragmented between the death and the TRIF domain, MyD88 loses its adaptor function that connects the activation of TLR and IL-1R members to the initiation of intracellular signaling (4). The proteolysis of MyD88 is consequently predicted to render the dying cell less susceptible to TLR and IL-1R ligands, which disrupts the cellular response to these proinflammatory receptors. This could also be advantageous for an extracellular pathogen, such as Yersinia, that may benefit from impaired TLR signaling of infected host cells. Of interest, the MyD88 caspase-processing site D135 is highly conserved across several vertebral species, including human, dog, zebrafish, zebra finch, clawed frog, and Chinese softshell turtle (Ref. 45 and data not shown). This observation supports the idea that the MyD88 caspase-processing site plays a physiological role in the course of apoptosis. In the mouse MyD88 sequence, D135 is replaced by E135. However, our data indicate that murine MyD88 is also diminished in Yersinia-induced apoptosis (data not shown). Alternative proteases other than caspases activated during apoptosis, such as serine proteases or cathepsins, could be responsible for the processing of mouse MyD88 (46). The removal of MyD88 in apoptosis execution may consequently fulfill a particular, conserved function. It is characteristic of apoptotic cells that plasma membrane integrity is long preserved. This feature ensures that no proinflammatory intracellular constituents are released until the apoptotic cells are internalized by bystander cells (36). As long as the cell membrane of the dying cell is intact, the processing of MyD88 could aid in deactivating proinflammatory signals that could possibly derive from the TLR and IL-1R family in a permissive environment. The cleavage of MyD88 may help to extinguish the proinflammatory responses and properties of dying cells. This cleavage could also be a strategy to counteract oncogenesis. MyD88 has been shown to be implicated in carcinogenesis through the induction of inflammation as well as through cell-autonomous mechanisms of cell transformation (47, 48). The processing of MyD88 could therefore be a regulatory event protecting against diverse disorders of tissue homeostasis that could arise from malfunctions in apoptosis, such as the development of aberrant inflammatory responses or eventually cancer.

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**References**


that recognized TAK1 phosphorylated at T184/187 (upper panel each). Equal loading of the gels with cell lysates was controlled by stripping of the membrane and reprobing with anti-actin Ab (middle panel each). Because the recycled membranes showed reduced sensitivity to successive TAK1 immunostaining with the available Abs, the cellular levels of TAK1 were analyzed in parallel control samples that were treated under the same conditions (lower panel each). The phosphorylation of TAK1 was quantified in relation to actin. The values obtained upon infection with WA-Δypop for 3.5 h were set to 100% for MyD88 WT– and MyD88–D135A–expressing cells. The results are expressed as percentages thereof. Molecular masses of standard marker proteins are indicated in kilodaltons.


