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*J Immunol* 2002; 169:3172-3179; doi: 10.4049/jimmunol.169.6.3172

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Caspase-9/-3 Activation and Apoptosis Are Induced in Mouse Macrophages upon Ingestion and Digestion of *Escherichia coli* Bacteria

Hans Häcker, Christine Fürmann, Hermann Wagner, and Georg Häcker

A number of highly virulent, intracellular bacteria are known to induce cell death by apoptosis in infected host cells. In this work we demonstrate that phagocytosis of bacteria from the *Escherichia coli* laboratory strain K12 DH5α is a potent cell death stimulus for mouse macrophages. RAW264.7 mouse macrophages took up bacteria and digested them within 2–4 h as investigated with green fluorescent protein-expressing bacteria. No evidence of apoptosis was seen at 8 h postexposure, but at 24 h ~70% of macrophages displayed an apoptotic phenotype by a series of parameters. Apoptosis was blocked by inhibition of caspases or by forced expression of the apoptosis-inhibiting protein Bcl-2. Processing of caspase-3 and caspase-9 but not caspase-8 was seen suggesting that the mitochondrial branch of the apoptotic pathway was activated. Active effector caspases could be detected in two different assays. Because the adapter molecule myeloid differentiation factor 88 (MyD88) has been implicated in apoptosis, involvement of the Toll-like receptor pathway was investigated. In RAW264.7 cells, heat-treated bacteria were taken up poorly and failed to induce significant apoptosis. However, cell activation was almost identical between live and heat-inactivated bacteria as measured by extracellular signal-regulated kinase activation, generation of free radicals, and TNF secretion. Furthermore, primary bone marrow-derived macrophages from wild-type as well as from MyD88-deficient mice underwent apoptosis upon phagocytosis of bacteria. These results show that uptake and digestion of bacteria leads to MyD88-independent apoptosis in mouse macrophages. This form of cell death might have implications for the generation of the immune response. The *Journal of Immunology*, 2002, 169: 3172–3179.

Phagocytosis of bacteria by macrophages and neutrophil granulocytes is a mechanism used by metazoans to combat bacterial infections. Ingestion by these so-called professional phagocytes destroys most bacterial pathogens, although some bacteria have evolved strategies to evade destruction. The process of phagocytosis itself involves steps of cellular recognition and uptake. A number of receptors and other cellular components involved are known, but the molecular mechanisms of phagocytosis appear to be complex and are incompletely understood (reviewed in Ref. 1).

Although most bacteria are killed by the phagocyte upon ingestion, infection with some of the bacteria that are capable of surviving inside phagocytes eventually leads to the death of the phagocyte; this has been reported for *Listeria, Shigella*, and *Salmonella*, bacteria of relatively high virulence. These bacteria can be qualified as “facultative intracellular”; i.e., a period inside a host cell makes up a significant part of their infectious behavior. Cell death induction by this group of bacteria appears often to occur by apoptotic cell death, although some instances have also been reported where the cell died in the absence of apoptosis in a process commonly called necrosis (reviewed in Ref. 2). Another group of bacteria, such as *Staphylococci, Streptococci*, or *Escherichia coli,* are unable to survive in phagocytes. These bacteria are taken up and killed efficiently by neutrophils and macrophages. However, it has been observed in several studies that ingestion of these bacteria by phagocytes was in some cases followed by the death of the phagocyte. The results of these studies have been partly conflicting. One study described phenotypically nuclear apoptosis in human primary macrophages 6 h after phagocytosis of bacteria; the life span of human neutrophil granulocytes was prolonged upon contact with bacteria (3). Other researchers found that uptake of *E. coli* led to increased apoptosis in human neutrophils (4); this study suggested that an increase in the intracellular production of reactive oxygen species (ROS) might be involved in the induction of apoptosis because inhibitors of ROS production somewhat reduced apoptosis.

Apoptotic cell death has been recognized as an important and physiological part in the life of multicellular organisms. Over the last decade it has become clear that a specialized intracellular pathway exists whose only function it is to kill the cell and to organize its disposal. Activation of this pathway leads to cell death by apoptosis; therefore, apoptosis is an active process (5). The molecular workings of this apoptotic pathway have been extensively studied and some important principles have been worked out, allowing for the probing for defined steps of apoptotic signal transduction.

In this work we describe that cell death is induced by phagocytosis of *E. coli* bacteria. We observed that RAW264.7 (RAW) mouse macrophages are very efficient at taking up and digesting *E. coli*. In the course of this phagocytic process, RAW cells are...
strongly stimulated in a way typical of activation by pathogen-associated molecular patterns. Surprisingly, RAW cells and primary mouse bone marrow-derived macrophages (BMDM) also undergo cell death. Mode and context of phagocytosis-induced cell death were investigated, and the possible relevance of this form of cell death is discussed.

Materials and Methods

Cells and materials

The murine macrophage cell line RAW264.7 (RAW) was cultured in Low-Tox Click’s RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% (v/v) FCS (HyClone Laboratories, Logan, UT), 50 μM 2-ME, and antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate). Cells were normally grown in non-culture-coated petri dishes and only for experiments seeded in culture-coated 12-well plates. *E. coli* K12 strain DH5α was inoculated from a frozen stock or from an agar plate into liquid Luria-Bertani (LB) medium and grown overnight at 37°C with shaking. Cells were collected by centrifugation, passed through a 0.45-μm disposable filter, and resuspended in PBS to OD260. DH5α cells transformed with an expression construct for enhanced green fluorescent protein (GFP) were streaked onto LB plates containing ampicillin. Single colonies were inoculated into liquid LB medium containing ampicillin and processed as above.

Generation of RAW-Bcl-2 cells

RAW cells were transfected by electroporation with an expression plasmid of human Bcl-2 under the control of the elongation factor 2α promoter and a puromycin resistance cassette (6). Cells were selected in puromycin-containing medium under limiting dilution conditions, analyzed by intracellular staining for Bcl-2, and subcloned by limiting dilution. Two subclones from originally independent clones expressing high levels of Bcl-2 were chosen for additional experiments and used in this study.

Generation of mouse BMDM

BMDM were grown according to standard protocols. Briefly, mouse bone marrow was harvested by rinsing the femores of either normal C57BL/6 mice (7) back-crossed onto C57BL/6 for at least six generations and age-matched. Founder mice were kindly provided by Dr. S. Akira (Osaka, Japan), bred in the facilities at our institute, and typed by PCR. Bone marrow cells (2 x 10⁷/10 ml in a non-culture-coated petri dish) were cultured in medium as above supplemented with 10 ng/ml recombinant mouse M-CSF (R&D Systems, Wiesbaden, Germany). On day 3, another 10 ng/ml M-CSF was added. Adherent cells were harvested between day 7 and 9, seeded in 12-well cell culture plates (2 x 10⁶/well), and used after overnight culture for experiments as described for RAW cells.

Coculture of macrophages and bacteria

The day before, 2 x 10⁵ RAW cells per well were seeded into 12-well plates in 1 ml medium. Bacterial suspension (100 μl) containing medium under limiting dilution conditions, analyzed by intracellular staining for Bcl-2, and subcloned by limiting dilution. Two subclones from originally independent clones expressing high levels of Bcl-2 were chosen for additional experiments and used in this study.

Detection of cell death

Cells were normally grown in non-culture-coated petri dishes and only for experiments seeded in culture-coated 12-well plates. *E. coli* K12 strain DH5α was inoculated from a frozen stock or from an agar plate into liquid Luria-Bertani (LB) medium and grown overnight at 37°C with shaking. Cells were collected by centrifugation, passed through a 0.45-μm disposable filter, and resuspended in PBS to OD260. DH5α cells transformed with an expression construct for enhanced green fluorescent protein (GFP) were streaked onto LB plates containing ampicillin. Single colonies were inoculated into liquid LB medium containing ampicillin and processed as above.

Assays for cell death

Assays for nuclear morphology, cells were stained with annexin-V-FITC (BD Biosciences) according to the manufacturer’s instructions and analyzed by flow cytometry.

Assays for caspase activation

Assays for nuclear morphology, cells were stained with annexin-V-FITC (BD Biosciences) according to the manufacturer’s instructions and analyzed by flow cytometry.

Western blotting

Cells were collected by centrifugation, cultured in the presence of caspase inhibitors, and subjected to Western blot analysis. Blots were probed with Abs specific for mouse caspase-3 (BD Biosciences), -8 (StressGen Biotechnologies, Victoria, British Columbia, Canada), -9 (Cell Signaling Technology, Frankfurt am Main, Germany), phospho-extracellular signal-regulated kinase (ERK), or ERK (both from Cell Signaling Technology). Secondary peroxidase-labeled Abs were from Sigma-Aldrich (St. Louis, MO) or Dianova (Hamburg, Germany). Blots were developed using an ECL system (NEN, Boston, MA).

Measurement of TNF levels

Supernatants from cultures were taken at 4 h (starting from the time point when bacteria were washed away) and cytokine levels were determined using a commercially available ELISA kit according to the instructions of...
the manufacturer (R&D Systems). When bacteria were not removed total incubation time was 5 h.

**Measurement of ROS**

Cells were treated as above. After various periods of time cells were labeled by incubation for 15 min with dihydroxyrhodamine 123 (80 μM; Molecular Probes). Azide was then added to 0.2 nM and incubation was continued for 20 min. Cells were then directly analyzed for rhodamine fluorescence by flow cytometry. Fluorescence microscopic inspection confirmed that fluorescence was localized to cellular mitochondria.

**Results**

**Induction of apoptosis upon ingestion of E. coli**

RAW cells were incubated with a suspension of *E. coli* bacteria as described in Materials and Methods. For the experiments shown here, the laboratory strain K12 DH5α was used (a laboratory strain that is also commonly used for cloning purposes and does not express known virulence factors). Bacteria were taken up by the macrophages and digested rapidly (see below). When the cells were further cultured under normal conditions, significant cell death (as detected by measuring membrane integrity) was seen after ~16–24 h (Fig. 2A and data not shown). The cells further displayed clear signs of apoptosis: nuclei assumed the typical condensed and fragmented morphology (Fig. 1A), PI staining of the nuclei revealed a high number of cells that displayed a "sub-G1" staining pattern (9) (Fig. 1B), and the majority of the cells exhibited annexin V binding activity (Fig. 1C). Up to 8 h after exposure to bacteria, no apoptosis was seen (Fig. 2B); between 8 and 24 h, ~60–80% of RAW cells underwent apoptosis as assessed by the criteria of nuclear fragmentation and PI staining (Fig. 2, B and C).

**Uptake and digestion of bacteria**

We next sought to determine how this form of apoptosis correlated with the uptake and degradation of bacteria. RAW cells were incubated with bacteria expressing enhanced GFP, washed, and fixed at time points of up to 6 h. Laser scanning microscopy showed that RAW cells were efficient at taking up bacteria that were visible as fluorescent rods immediately after washing (Fig. 3). Two hours later GFP had already started to assume a vesicular pattern in the cell, and at 4 and 6 h after incubation GFP was largely localized in...
a cellular compartment around the nucleus of the cell, but no bacteria were evident at this stage. This indicates that the macrophages had started to digest the bacteria and, indeed, the number of living bacteria that could be recovered from the cultures declined rapidly (data not shown). Thus, the macrophages were efficient at digesting the internalized bacteria; although this process led to apoptosis, cell death started to occur only after the bacteria had been killed by the phagocyte.

Blockade of bacteria-induced apoptosis by Bcl-2

To derive a clearer picture of this form of cell death we investigated whether the apoptotic phenotype was blocked by expression of the antiapoptotic protein Bcl-2. Bcl-2 is an intracellular membrane-associated protein that can inhibit apoptosis induced by the vast majority of stimuli (10). RAW cells were engineered to overexpress human Bcl-2. Two clones were selected which were found to express high levels of Bcl-2 when analyzed by flow cytometry (Fig. 4A); functional expression was further confirmed by the observation of protection against staurosporine-induced apoptosis (data not shown). These cells were efficient in their uptake of bacteria as analyzed by flow cytometry (Fig. 4B), comparable to wild-type RAW cells (Fig. 6 and data not shown). However, the apoptotic response to bacterial uptake was strongly reduced in both clones: cells from both RAW-Bcl-2 clones investigated showed only little nuclear apoptosis compared with the maternal cells when cells were exposed to bacteria and nuclei were analyzed by microscopy (Fig. 4C).

Participation of caspases and analysis of the apoptotic pathway

The recent progress in the understanding of the cell death pathway allows us to inquire more closely into the activation of the cell death pathway by a given stimulus. Apoptosis involves the activation of caspases, which occurs in two steps: a so-called “initiator” caspase (either caspase-8 or caspase-9) is activated upon adapter-mediated oligomerization, and the active initiator caspase then activates the “effector” caspase caspase-3 (and other effector caspases) by limited proteolysis (11). We investigated the contribution of caspases by analyzing their activation and by blocking caspases with a peptide inhibitor.

When RAW cells were incubated with E. coli bacteria as above and incubated in the presence of the pan-caspase inhibitor z-VAD-fmk the number of cells with an apoptotic phenotype (as measured by analysis of the nuclear morphology) was greatly reduced (Fig. 5A); this indicates that caspase activity was required for phagocytosis-induced apoptosis. Addition of the caspase inhibitor also reduced cell death as measured by assessing membrane integrity (as PI uptake, Fig. 5B). However, this reduction was not as strong as the apoptosis inhibition (Fig. 5A), suggesting that the cells underwent “secondary necrosis” when caspases were inhibited.

Effector caspase activity was next measured with a synthetic specific fluorogenic effector caspase substrate, Ac-DEVD-AMC. Asp-Glu-Val-Asp cleaving activity was detected in lysates from RAW cells 24 h after exposure to E. coli bacteria (but not at 8 h; Fig. 5C). Activation of effector caspases was further detected using a synthetic biotinylated peptide, bio-YVAD-faom. This peptide binds irreversibly and with great preference to activated effector caspase-3 (and other effector caspases) by limited proteolysis (11). We investigated the caspases by analyzing their activation and by blocking caspases with a peptide inhibitor.

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![Figure 3](https://via.placeholder.com/150)

**FIGURE 3.** Uptake and degradation of E. coli by RAW macrophages. RAW cells were seeded in 12-well plates onto coverslips as above. The next day, cells were incubated for 1 h with a suspension of GFP-expressing E. coli bacteria as described in Materials and Methods, washed, and cultured for the indicated periods of time (0 h was directly after washing). Cells were then fixed and analyzed for GFP fluorescence by laser scanning microscopy.

![Figure 4](https://via.placeholder.com/150)

**FIGURE 4.** Bcl-2 inhibits E. coli-induced apoptosis in RAW macrophages. A, Expression of human Bcl-2 in two RAW subclones. RAW wild type (normal lines) and two clones engineered to overexpress human Bcl-2 (see Materials and Methods for details) were stained for intracellular Bcl-2-expression and analyzed by flow cytometry. B, Uptake of bacteria by Bcl-2-expressing RAW cells. Clone RAW-Bcl-2 16/1 was incubated with GFP-expressing E. coli cells as above and washed, and culture was continued. After 6 h, cells were analyzed by flow cytometry for GFP fluorescence (indicating bacterial uptake). Normal line, Cells not exposed to bacteria; bold line, cells exposed to bacteria. C, Induction of apoptosis in RAW wild-type and RAW Bcl-2 cells. Cells were seeded into 12-well plates as above. The next day, aliquot wells were incubated with E. coli bacteria as above. After 24 h, nuclei were Hoechst stained and visually analyzed for apoptosis as above. Values are presented as mean ± SEM of triplicate wells. Filled bars, Maternal RAW cells; hatched bars, RAW-Bcl-2 cells (middle, clone 13/2; right, clone 16/1). These data are representative of three independent experiments.
species can subsequently be detected in an avidin-based affinity blot (12, 13). The biotinylated peptide can be added either to lysates or to intact cells undergoing apoptosis (13). RAW cells were either left untreated or were exposed to bacteria as above. After 24 h, active effector caspases were labeled and detected by affinity blot (see Materials and Methods for details). As shown in Fig. 5D, two labeled proteins were detected in a pattern typical of active effector caspases (13). Labeling was inhibited in the presence of the caspase inhibitor z-VAD-fmk, confirming that the biotinylated proteins indeed were active caspases (Fig. 5D). Thus, caspases are activated in RAW cells upon uptake and digestion of *E. coli* bacteria.

To analyze the activation of individual caspases, caspase processing was investigated by immunoblot. Processing of caspase-3 was clearly detectable by Western blotting (Fig. 5E). When proteolytic processing of the two known initiator caspases was analyzed it was found that caspase-9 but not caspase-8 was processed, indicative of activation (Fig. 5, F and G). These data strongly suggest that upon uptake and digestion of *E. coli* bacteria the caspase-9- and caspase-3-dependent branch of the apoptotic pathway is activated in RAW cells in a Bcl-2-inhibitable manner.

**Activation vs apoptosis**

Contact with bacteria is a strong stimulatory signal for macrophages. An important part in the recognition of (and stimulation by) bacterial components is the engagement of Toll-like receptors (TLRs). In RAW cells, LPS and bacterial (CpG) DNA have been defined as strong stimulators of gene expression (14). We thought it important to investigate the relationship between cell activation and apoptosis in RAW cells that had taken up *E. coli* bacteria. This is especially interesting because TLR appear to have the potential to induce apoptosis upon ligand binding. In particular, TLR2 has been shown to induce apoptosis when expressed and stimulated in
human fibroblasts (15); the death signal transduction likely requires the “adapter” molecule MyD88 (16). We addressed this question from two angles: known TLR stimuli were investigated in parallel with live bacteria for stimulation and apoptosis induction in RAW cells, and macrophages from mice deficient for MyD88 were probed for the susceptibility to apoptosis induced by E. coli bacteria.

Neither LPS nor CpG DNA or a combination of both stimuli were found to be sufficient to induce apoptosis in RAW cells over a period of 48 h, although they are potent TLR stimuli (data not shown). We further noticed that heat-treated bacteria no longer induced apoptosis in RAW cells. Incubation of the bacteria for 30 min at 65°C killed ~99% of the cells as measured by colony formation on agar plates (data not shown). Heat-inactivated GFP-expressing bacteria were almost indistinguishable from normal E. coli by Gram stain (data not shown) or GFP expression (Fig. 6A). However, uptake by RAW cells was strongly reduced following heat treatment, suggesting that some surface structure on the bacterial cells had been damaged (Fig. 6C). The capacity to induce apoptosis was reduced on a similar scale (Fig. 6B).

Conversely, when compared for their stimulatory capacity for RAW cells, heat-treated bacteria were found to stimulate the macrophages to a level comparable to that of non-heat-treated E. coli in the assays used (we focused on the investigation of events that are known to be triggered by TLR): activation of the mitogen-activated protein kinases ERK1/2 was very similar in the macrophages, regardless of whether normal or heat-inactivated bacteria were used for stimulation (Fig. 7A). TNF production by RAW cells upon 4 h of incubation with either live or heat-killed bacteria was also almost the same (Fig. 7B); for this assay, we used a slightly different protocol in that bacteria were not washed away after 1 h but were left on the RAW cells for the entire period. Heat-killed bacteria still do not induce apoptosis when left on the macrophages for 24 h (Fig. 7C). Another effector function in macrophages that can be elicited in a TLR-dependent fashion is the generation of reactive oxygen species (ROS).

**FIGURE 6.** Comparison of untreated and heat-inactivated E. coli bacteria. A, GFP expression of bacteria. Normal (normal line) or heat-inactivated GFP-expressing (at 65°C for 30 min; bold line) bacteria were analyzed for GFP fluorescence by flow cytometry. B, Apoptosis-inducing potential. RAW cells were seeded into 12-well plates. The next day, some wells were incubated with either normal or heat-inactivated bacteria, washed, and cultured as above. Apoptosis was assessed after 24 h by Hoechst staining and counting under a fluorescence microscope as above. Values are mean ± SEM of three wells. Similar results were obtained in three separate experiments. C, Uptake of bacteria. RAW cells were incubated with GFP-expressing bacteria, washed, and cultured as above. Uptake was assessed as GFP fluorescence of RAW cells after 6 h. Normal line, RAW cells not exposed to bacteria; bold line, RAW cells exposed to normal bacteria (left panel) or heat-inactivated bacteria (right panel).

**FIGURE 7.** TLR-dependent stimulation by untreated and heat-inactivated E. coli bacteria. A, ERK1/2 phosphorylation in RAW cells. RAW cells were seeded in 12-well plates as above. The next day, either untreated or heat-inactivated bacteria were added at various time points as indicated. All cells were lysed at the same time, and total ERK1/2 and phosphorylated ERK1/2 were detected by immunoblotting. Upper panel, Filled arrowhead indicates phospho-ERK. Lower panel, Open arrowhead indicates total ERK. B, TNF secretion induced by bacteria. RAW cells were exposed to bacteria as above with the exception that bacteria were not removed after 1 h but were left in the cultures (to avoid changing supernatants, which would affect cytokine concentrations). After a 4-h incubation supernatants were taken and TNF content was measured by ELISA. Values are mean ± SEM of three wells. Similar results were obtained in three separate experiments. C, Induction of apoptosis. RAW cells were left untreated or incubated with bacteria. In some wells, bacteria were added and washed away after 1 h as above (as indicated). To some wells, bacteria were added and not removed (Left-on). After 24 h apoptosis was measured by Hoechst staining and assessment of nuclear morphology as above. D, ROS induction by E. coli bacteria in RAW cells. Cells were exposed to bacteria, washed, and incubated as above. Six hours later, cells were incubated with 2,4-dihydroxyrhodamine and analyzed by flow cytometry (see Materials and Methods for details). Similar results were obtained in three separate experiments at various times (2–6 h) of incubation.
and analyzed for apoptosis by PI staining and analysis of sub-G_1 nuclei as obtained for a total of bar in each group is from the same mouse, etc.). Very similar results were described above for RAW cells; replicates were further incubated with LPS between RAW cells stimulated with live bacteria and those stimulated after the bacteria had been destroyed by the macrophages and induced and almost no apoptosis was observed. In contrast, cell activation appeared to be the same in both situations. In this respect, we focused on the investigation of a number of activation events that are known to occur as a consequence of TLR stimulation. Therefore, it appears that TLR signaling was similar for both live and heat-killed bacteria.

Although a potential to induce apoptosis has been demonstrated for TLR2, we think it unlikely for three reasons that TLR signaling was involved in apoptosis induction upon phagocytosis of bacteria in RAW cells. First, ligands that deliver strong TLR signals into RAW cells, such as LPS, CpG DNA, or heat-killed bacteria, did not induce apoptosis in the cells and under the conditions used in this study; although several reports show that LPS has the potential to induce apoptosis, especially in IFN-γ-prestimulated macrophages (see Refs. 18 and 19), even high concentrations of LPS (10 μg/ml) did not induce any detectable apoptosis in our RAW cells or in BMDM (10 ng/ml already induces maximal TNF release in our RAW cells; data not shown). This difference may be the result of a different level of cellular activation. Second, work in fibroblasts transfected to express TLR2 has suggested that cell death induction occurs via MyD88-, Fas-associated death domain protein-, and caspase-8 dependent signal transduction (16) (perhaps with the caveat that these results were largely obtained using transfections to express dominant negative proteins). Similarly, apoptosis induced by the Gram-negative bacterium Yersinia enterocolitica in J774 mouse macrophages appears to be transmitted by MyD88, Fas-associated death domain protein, and caspase-8 (20); however, no caspase-8 activation was seen in phagocytosis-induced cell death in RAW cells. Third, BMDM from MyD88-deficient mice underwent apoptosis as efficiently as BMDM from wild-type animals. This suggests that, if TLR are involved in this form of cell death, their contribution is indirect: TLR could elicit cellular signaling, which then causes caspase-9 activation, probably via the release of mitochondrial cytochrome c. The notion that cytochrome c release is involved is also supported by the finding that Bcl-2 efficiently inhibits this form of apoptosis: the main action of Bcl-2 is probably to prevent the release of cytochrome c (21).

We have not investigated which mechanisms are involved in upstream processes. At present, the most likely scenario is that when cells are killed by Bcl-2 involves the action of one of several so-called BH3-only proteins (distant relatives of Bcl-2; Ref. 22) that then activates the proapoptotic Bcl-2-like proteins Bax and/or Bak (23). We are following up this line at present.

In summary, these results show that phagocytosis of pyogenic bacteria by macrophages (which is the normal way of disposing of these bacteria) activates the apoptotic pathway in these phagocytes, resulting in classical apoptosis. What could be the purpose (if any) of this process? There are many possible interpretations. It

**FIGURE 8.** Induction of apoptosis by E. coli bacteria in primary BMDM from wild-type and MyD88-deficient mice. BMDM from wild-type (filled bars) or MyD88^+/− (open bars) mice were seeded in 12-well culture dishes (3 × 10^5/well) and incubated overnight. Cells were then either left untreated or incubated with bacteria for 1 h and washed as described above for RAW cells; replicates were further incubated with LPS (10 μg/ml). Twenty hours later, cells were harvested, fixed with ethanol, and analyzed for apoptosis by PI staining and analysis of sub-G_1 nuclei as in Figs. 1B and 2B. Data are given as means ± SEM of triplicate wells; each bar represents cells from one mouse in the same experiment (i.e., left bar in each group is from the same mouse, etc.). Very similar results were obtained for a total of five wild-type mice and three MyD88^+/− mice.

ROS (15). Because ROS have been further implicated in the induction of apoptosis in granulocytes (4) we compared ROS generation upon incubation of RAW cells with either normal or heat-inactivated E. coli bacteria. As shown in Fig. 7D, untreated bacteria were slightly more efficient in inducing ROS in RAW cells. Although the difference was marginal, a contribution from ROS to apoptosis induction cannot be ruled out. These data show that at least the activation markers investigated were very similar between RAW cells stimulated with live bacteria and those stimulated with heat-killed bacteria, but only the former induced apoptosis. The fact that the uptake of heat-treated bacteria is strongly reduced suggests that uptake is necessary for the induction of apoptosis but not the activation of the cells.

Because of this constellation we asked whether phagocytosis of inert particles was sufficient for cell death induction. RAW cells were exposed to fluorescence-labeled polystyrene beads, and uptake and apoptosis were monitored. Beads were taken up efficiently and stored inside the cells; the pictures looked very similar after 4 and 24 h (data not shown). However, there was no apoptosis seen after 24 h (data not shown). Thus, the mere act of phagocytosis is not sufficient to induce apoptosis in RAW macrophages.

As already discussed, MyD88 has been implicated in the transduction of the apoptotic signal from TLR, and MyD88^−/− mice were used in this study to test the involvement of this molecule. It is first important to note that primary BMDM show the same behavior as RAW cells in that they undergo apoptosis upon phagocytosis of whole bacteria but not upon treatment with LPS (up to 10 μg/ml; Fig. 8, filled bars). The same is the case for BMDM from MyD88^−/− mice; if anything, cells from these mice may die a little more efficiently (Fig. 8, open bars). Therefore, it appears that MyD88 is not critically involved in the transduction of the apoptotic signal upon uptake and digestion of E. coli bacteria in mouse macrophages.

**Discussion**

In this study we describe and investigate apoptosis induced by phagocytosis of low-virulence E. coli bacteria. Apoptosis occurred after the bacteria had been destroyed by the macrophages and involved typical features of apoptosis and the activation of the classical apoptotic pathway. These results describe mechanistic processes of apoptosis induction and raise the question about implications for the development of an immune response.

When RAW cells were exposed to E. coli, the bacteria were taken up and digested rapidly. Apoptosis occurred as a consequence of this process, but only consecutively; it is very unlikely that intracellular growth of the bacteria was involved in the death of the cell. Interaction of macrophages with bacteria induces the uptake of bacteria and the activation of the phagocyte. Although some receptors are known, the precise regulation and orchestration of phagocytosis are uncertain (reviewed in Ref. 1). Activation is probably mainly achieved by the engagement of members of the TLR family of receptors (17). When RAW cells were exposed to live bacteria, the bacteria were taken up and apoptosis occurred; when bacteria were heat inactivated, uptake was significantly reduced and almost no apoptosis was observed. In contrast, cell activation appeared to be the same in both situations. In this respect, we focused on the investigation of a number of activation events that are known to occur as a consequence of TLR stimulation. Therefore, it appears that TLR signaling was similar for both live and heat-killed bacteria.

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We have not investigated which mechanisms are involved in upstream processes. At present, the most likely scenario is that when cells are killed by Bcl-2 involves the action of one of several so-called BH3-only proteins (distant relatives of Bcl-2; Ref. 22) that then activates the proapoptotic Bcl-2-like proteins Bax and/or Bak (23). We are following up this line at present.

In summary, these results show that phagocytosis of pyogenic bacteria by macrophages (which is the normal way of disposing of these bacteria) activates the apoptotic pathway in these phagocytes, resulting in classical apoptosis. What could be the purpose (if any) of this process? There are many possible interpretations. It
appears unlikely that the bacteria benefit from the cell death induced because apoptosis occurs only after they have been killed. In contrast, the death of the phagocytes is likely to affect the immune system in some way: apoptotic cells are cleared by cells from the innate immune system, probably largely macrophages and dendritic cells, and they are not inert particles. We propose the hypothesis that phagocytosis-induced cell death is used to deliver bacterial Ag to where it can be presented to T cells. Dendritic cells, the main professional APCs, are very efficient at taking up both bacteria and apoptotic cells; the Ag contained in these will efficiently be presented to T cells. There is a body of evidence suggesting that the uptake of apoptotic cells deactivates the uptaking cell, probably by binding to a phosphatidyserine receptor (24, 25); dendritic cells that have taken up apoptotic cells then are able to tolerate rather than activate T cells specific for Ag from the apoptotic cell (review in Ref. 26). However, it has also been shown, in a system where Salmonella bacteria were present in macrophages, that bacterial Ag could be recovered from an apoptotic macrophage and consecutively presented to T cells in a stimulatory fashion by a dendritic cell (27). Therefore, we speculate that apoptotic cells can induce a productive T cell response provided that a strong DC stimulus, such as LPS or other pathogen-associated molecular patterns, is in the system, and this would certainly be the case for macrophages that ingest bacteria and undergo apoptosis. Phagocytosis of microbial agents is found in single-celled organisms, while the phagocytosis of apoptotic cells probably evolved early in multicellular organisms (the nematode Caenorhabditis has a sophisticated apparatus that organizes this step). In the more advanced mammalian immune system, phagocytosis of bacteria and phagocytosis of apoptotic cells appear to be preferentially assigned to different cell types. Thus, it is conceivable that the old mechanism of uptake of apoptotic cells has in this study been put to use making microbial Ag available to the adaptive immune system.

Acknowledgments
We thank Dr. S. Akira for the generous gift of MyD88-deficient mice and Dr. Susanne Kirschnek for critical comments on the manuscript.

References