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Phagocytosis-Induced Apoptosis in Macrophages Is Mediated by Up-Regulation and Activation of the Bcl-2 Homology Domain 3-Only Protein Bim

Susanne Kirschnek,* Songmin Ying,* Silke F. Fischer,* Hans Häcker,† Andreas Villunger,‡ Hubertus Hochrein,* and Georg Häcker2*

Cell death by apoptosis is important in immune cell homeostasis and in the defense against infectious microorganisms. The physiological event of uptake and intracellular destruction of bacteria is a powerful apoptotic stimulus to macrophages and neutrophil granulocytes. In this study, we provide a molecular analysis of phagocytosis-induced apoptosis. Apoptosis was blocked by Bcl-2 in a mouse macrophage cell line and in primary mouse macrophages. Analysis of the upstream mechanisms revealed that apoptosis was triggered by the Bcl-2 homology domain 3-only protein Bim/Bod. Contact with bacteria or bacterial components induced a strong increase in Bim-expression through TLR and MyD88. Inhibition of the MAPK p38 and JNK reduced both up-regulation of Bim and apoptosis. Phosphorylation of Bim was further observed in mouse macrophages, which appeared to be the result of TLR-dependent phosphatase inhibition. Although TLR-induced Bim was, unlike Bim in resting cells, not bound to the microtubuli cytoskeleton, the up-regulation of Bim was not sufficient to cause apoptosis. A second signal was required that was generated in the process of phagocytosis. Phagocytosis-induced apoptosis was strongly reduced in Bim−/− macrophages. These data provide the molecular context of a form of apoptosis that may serve to dispose of terminally differentiated phagocytes.

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Phagocytosis and intracellular destruction by specialized phagocytes such as granulocytes and macrophages is an important defense mechanism against infections with pyogenic bacteria (such as Strepocolci and Enterobacteriaceae). Phagocytosis can occur upon recognition of conserved bacterial structures, of Abs or of complement components that have been deposited on bacterial surfaces, and phagocytosis is normally followed by the digestion of the phagocytosed bacterium by host cell enzymes. Bacterial destruction is rapid and is concluded within a few hours. During this process, massive phagocyte activation is seen as a result of the binding of bacterial components to specialized receptors on the phagocyte. Various scavenger receptors are involved, but a large share of cell activation events is the result of the triggering of receptors from the TLR family, which play roles in the secretion of cytokines, the enhanced expression of cell surface molecules, and in the process of phagocytosis itself (1–3).

We and others have recently demonstrated that phagocytosis of pyogenic bacteria is also a stimulus for the phagocyte to undergo apoptosis (4, 5). In macrophages, apoptosis was observed at −8−16 h after bacterial ingestion, clearly after the intracellular destruction of the bacteria, which is complete at −4 h postingestion. Although the significance of this form of apoptosis is not fully understood, it bears resemblance to the apoptosis regularly found at the end of a T cell response: rather than developing back into resting cells, the terminally differentiated effector cells are cleared by apoptosis.

Apoptosis is implemented by a set of cellular enzymes referred to as effector caspases, most importantly caspase-3. Caspase-3 can be activated by either caspase-8 (normally upon triggering of death receptors on the cell surface) or by caspase-9 (which is activated in a large signaling complex consecutive to the release of cytochrome c from the cellular mitochondria into the cytosol (6). The initial characterization of “phagocytosis-induced apoptosis” showed that it is indeed classical apoptosis (confirmed by analysis of various parameters such as subG1-staining, annexin V-labeling, nuclear morphology, blockade by caspase inhibition; Ref. 5). This form of apoptosis further involved the activation of caspase-9 but not caspase-8 and it was efficiently blocked by expression of the apoptosis-inhibitor Bcl-2 in the mouse macrophage cell line RAW264.7 (RAW) (5). In the study presented here, we undertook an analysis of the events that trigger this form of cell death.

The release of cytochrome c from mitochondria is regulated by members of the Bcl-2 protein family. Bcl-2 itself has a broad antiapoptotic effect that depends on its ability to bind a number of proapoptotic Bcl-2 family members known as Bcl-2 homology domain 3 (BH3)−only proteins. BH3−only proteins are structurally

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Abbreviations used in this paper: BH3, Bcl-2 homology domain 3; BMDM, bone marrow-derived macrophage; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin; ODN, oligonucleotide; DC, dendritic cell.

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only distantly related to each other and to all other Bcl-2 family members. Unlike Bcl-2 and other relatives, they possess only the so-called Bcl-2 BH3-domain but lack the domains BH1, 2, or 4. Nine BH3-only proteins are known at present, and their activation is the earliest known molecular trigger of apoptosis in most cases (7). Activation of BH3-only proteins can involve de novo gene expression as well as posttranslational modification such as dephosphorylation or proteolytic cleavage. Two BH3-only proteins, Bim and Bmf, are also known to be activated by the release from sites of sequestration at the cytoskeleton, Bim at the microtubuli and Bmf at the actin cytoskeleton.

Because phagocytosis-induced cell death was found to be blocked by Bcl-2, it was likely to involve the activation of BH3-only proteins. Therefore, we directed our attention at the various BH3-only proteins that might transmit this death signal. Because phagocytosis and its downstream events involve massive restructuring of the cytoskeleton, Bim and Bmf were the most obvious candidates. Intriguingly, contact with bacteria or bacterial components caused a strong up-regulation of Bim protein that was mediated by MyD88 and members of the MAPK family. However, the mere up-regulation was not sufficient for the induction of apoptosis, which required a second signal that originated during the process of phagocytosis.

Materials and Methods

Cell lines, bacteria, and stimulation of cells

RAW264.7 mouse macrophages were cultured in Low-Tox Click’s RPMI 1640 (Biochrom) supplemented with 10% FCS (PAN), 50 μM 2-ME, and antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate). Cells were normally grown in nonculture-coated petri dishes and only for experiments seeded into culture-coated plates. Escherichia coli K12 strain DH5α was inoculated from a frozen stock into liquid LB medium and grown overnight at 37°C with shaking. Bacterial cells were collected by centrifugation, passed through a 5.0-μm disposable filter and resuspended in PBS to an OD of 2. For heat inactivation, bacteria were then incubated for 30 min at 65°C, cooled to room temperature, and used for experiments. In some experiments, RAW cells were stimulated with LPS (1 μg/ml; Sigma-Aldrich), CpG oligonucleotide 1668 (1 μM; TIB MolBiol), PamCys, (1 μg/ml; EM Microcollection Tübingen). The broad spectrum caspase inhibitor z-VAD-fmk (Bachem) was used at a concentration of 50 μM. Apoptosis by UV irradiation was induced using a stratalinker 2400 (Stratagene) at an energy dose of 160 mJ/cm². The following kinase/phosphatase inhibitors were used: SB203580 (Alexis), PD98059 (Alexis), SP600125 (Nacalai Tesque), and an elongation factor-1α promoter-driven fusion construct of full-length mouse MyD88 (construct DL11) and the coding sequence of the subunit B of streptomyces glyrase. Stable transfectants were established by limiting dilution in G418-containing medium. The MyD88-based dimerization system has been established by Perlmutter and colleagues (11). Dimerization of GyrB and Gyrf fusion proteins is initiated by the bivalent antibacterial coumermycin A (CM). In the case of MyD88-GyrB, coumermycin A-driven dimerization of MyD88 mimics receptor (TLR)-induced oligomerization of MyD88 and triggers typical TLR-dependent effector functions like MAPK activation and secretion of TNF-α. A detailed analysis of this system and the described MyD88-GyrB RAW cells will be published elsewhere. The cDNA of GyrB was a kind gift from Dr. R. M. Perlmutter.

Coculture of macrophages and bacteria

2 × 10⁶ RAW cells/ml were cocultured with 100 μl of bacterial suspension for 1 h at 37°C. Cells were then washed once with PBS, seeded in 12-well plates in 1 ml complete medium, and cultured for the indicated periods of time. In some experiments, RAW cells were seeded in tissue culture plates the day before (5 × 10⁵ cells/10-cm plate or 2–5 × 10⁶ cells/12-well plate) and stimulated as above. In case of kinase inhibition, cells were pretreated with the corresponding inhibitors for 15 min before further stimulation.

Assays for apoptosis

For assessment of nuclear morphology, cells were stained with Hoechst dye (Sigma-Aldrich), removed from the plate by vigorous pipetting, and scored in UV light under a fluorescence microscope. Assays were done at least in duplicate as indicated and a minimum of 300 cells were counted per sample. Analyses were performed by three investigators in independent experiments, who obtained very similar results. In some experiments cells were labeled with annexin V-FITC (BD Biosciences) according to the manufacturer’s instructions and analyzed by flow cytometry.

For assay of caspase 3 activity (Asp-Glu-Val-Asp cleavage activity), 1% Triton X-100 extracts prepared as described below were diluted 1/10 in reaction buffer (10 mM HEPES-KOH (pH 7), 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 1 mM DTT) supplemented with 100 μg/ml BSA) containing the caspase substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (Ac-DEVD-AMC; Bachem) at a final concentration of 10 μM. Reactions were performed in triplicate in flat-bottom 96-well plates at 37°C for 1 h. Free 7-amino-4-methyl-coumarin was then measured by determining fluorescence at 390 nm (excitation) and 460 nm (emission) in a Cytofluor 96 reader (Millipore). Values were calculated by subtracting background fluorescence.

Western blot analysis

Macrophages were washed once in PBS and harvested in extraction buffer (1% Triton X-100, 50 mM PIPES, 50 mM HEPES, 2 mM MgCl₂, 1 mM EDTA) supplemented with 1 mM DTT and complete protease inhibitor mixture (Roche). Nuclei and cellular debris were pelleted by centrifugation at 2000 × g for 10 min at 4°C. Aliquots of the supernatants were used for determination of protein concentration (Bio-Rad assay). Supernatants were boiled in Laemmli buffer. Equal amounts of protein were loaded onto 12.5% acrylamide gels and resolved by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes. Membranes were probed with...
Abs specific for Bim (Sigma-Adrich), β-actin (Sigma-Adrich), α-tubulin (Sigma-Adrich), FoxO3a (Upstate Biotechnology). Secondary HRP-conjugated anti-rabbit or anti-mouse IgG Abs were obtained from Dianova. Blots were developed using the ECL detection system (PerkinElmer). For treatment with calf intestinal phosphatase, cells were lysed in extraction buffer and then treated with 20 U/50 μl of calf intestinal alkaline phosphatase for 2 h at 37 °C. Controls were incubated in the same buffer but without phosphatase and in the presence of phosphatase inhibitors.

To obtain nuclear extracts, cells were incubated in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM NaF, 1 mM Na3VO4, and complete protease inhibitor mixture) on ice for 15 min, then Nonidet P-40 was added to a final concentration of 0.6%, extracts were vigorously vortexed and centrifuged at 6000 rpm for 2 min at 4°C. The pellet containing the nuclei was washed in buffer A and resuspended in buffer B (10 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, NaF, Na3VO4, and complete protease inhibitor mixture) and further extracted with shaking for 30 min at 4°C. The supernatants obtained after centrifugation at 13,000 rpm for 20 min at 4°C in a microfuge were used as nuclear extracts.

**Subcellular fractionation**

Subcellular fractionation was performed essentially as described (12). Briefly, cells were lysed in 1% Triton X-100 extraction buffer (1% Triton X-100, 50 mM PIPES, 50 mM HEPES, 2 mM MgCl2, 1 mM EDTA) supplemented with 1 mM DTT and complete protease inhibitor mixture (Roche). Lysates were treated with taxol (80 μM; Sigma-Adrich) and an apyrase (10 U/ml; Sigma-Adrich) for 15 min at 37°C. The lysate was then loaded onto a 10% sucrose cushion and centrifuged at 40,000 rpm in a SW41TI rotor in a Beckman ultracentrifuge for 16 h at 4°C. Pellets containing the microtubule fraction were dissolved in Laemmli buffer, supernatants were concentrated by acetone precipitation. Samples were processed by Western blotting as described above.

Sucrose gradient centrifugation was performed by loading the taxol-treated extract on top of a 5–20% discontinuous sucrose gradient followed by ultracentrifugation in a SW41TI rotor at 40,000 rpm for 16 h. The pellet fraction was dissolved in Laemmli buffer, either 1-ml or 2-ml fractions of the supernatant were obtained and acetone precipitated. Fractions were resolved by SDS-PAGE and subjected to Western blotting as described above.

**Results**

**Bcl-2 protects macrophages and granulocytes against phagocytosis-induced apoptosis**

Our previous studies had indicated that Bcl-2 blocked apoptosis when expressed in cells from the macrophage cell line RAW264.7 (RAW cells), suggesting the involvement of the “mitochondrial leg” of the apoptotic pathway (5). To confirm the involvement of the mitochondrial signaling pathway, we generated primary cell cultures from transgenic mice expressing human Bcl-2 throughout the hemopoietic compartment (vav-bcl-2 mice; Ref. 13). In primary BMDM, phagocytosis of *E. coli* led to apoptosis in a large portion of wild-type macrophages but much less in macrophages from vav-bcl-2 mice. Similarly, high-level expression of human Bcl-2 protected RAW cells efficiently against phagocytosis-induced apoptosis (Fig. 1, A and B). Besides macrophages, neutrophil granulocytes play an important role in the phagocytosis and destruction of pyogenic bacteria. Granulocytes freshly isolated from mouse bone marrow were also efficient at taking up *E. coli* bacteria (data not shown). As in macrophages, uptake and digestion of bacteria induced apoptosis in granulocytes (Fig. 1, C and D).

**Expression of the BH3-only protein Bim is up-regulated during contact with bacteria**

Although this is still somewhat controversial, Bcl-2 probably functions on a molecular level by binding and perhaps sequestering proapoptotic BH3-only proteins (7). The efficient inhibition of phagocytosis-induced apoptosis by Bcl-2 therefore suggested that phagocytosis led to the activation of at least one BH3-only protein. The BH3-only proteins Bim and Bmf are, in their inactive state, sequestered to the cytoskeleton. Apoptotic stimuli can induce their...
release from this site, upon which they translocate to the mitochondria and cause the activation of Bax. Cytoskeletal structures undergo massive rearrangements during the process of phagocytosis and the consecutive steps of vesicle transport (1). Bim and Bmf were therefore considered likely candidates for the BH3-only proteins that are activated during phagocytosis-induced apoptosis and were next investigated. Bmf (bound to the actin cytoskeleton in its inactive state) was only weakly expressed in macrophages as judged by Western blotting, and no induction of expression or release from its subcellular localization was observed upon bacterial stimulation (data not shown). Therefore, Bmf does not seem to play a significant role in phagocytosis-induced apoptosis. The expression of Bim by macrophages was next studied by Western blotting. A number of isoforms of Bim have been described, of which BimL and BimEL appear to be the most abundant in most cell types (14). Evidence is accumulating that Bim can be regulated not only by release from the cytoskeleton but also by gene induction (see for instance Refs. 15 and 16). Phosphorylation of Bim has further been described although the significance of this is unclear.

Both BimL and BimEL were easily detectable in resting RAW cells, with BimL being the prominent isoform (Fig. 2). Upon stimulation with bacteria, an increase of Bim-protein expression was detectable after 4–6 h. High protein expression was sustained until 16–20 h (Fig. 2A, and data not shown), at which time many cells had already undergone apoptosis. Analysis of primary BMDM gave similar results (Fig. 2B; the size shift in Bim protein was due to phosphorylation, see below). This up-regulation of Bim expression due to bacterial stimulation correlated with apoptosis induction by live bacteria. However, heat-inactivated bacteria, which are taken up poorly and cause very limited apoptosis (5), were found to have the same potential to enhance Bim expression (Fig. 2A). This suggests that the up-regulation of Bim expression is on its own not sufficient for the efficient induction of apoptosis.

Induction of Bim expression is mediated by TLR signaling via MyD88

A large part of the signaling events that bacterial components elicit in macrophages is mediated through TLR. Various TLR recognize different components of microbial origin. To understand whether TLR were involved in the induction of Bim upon contact with bacteria, we tested the effect of several TLR stimuli on the expression of Bim in RAW cells. LPS (which stimulates TLR4), the bacterial lipopeptide Pam3Cys (TLR2), and CpG-ODN (TLR9) all caused up-regulation of Bim expression (Fig. 3).

The signal originating at TLR can be conveyed into the cells by several adaptor molecules. A large part of this signal is mediated through the adaptor protein MyD88 (17). We found that MyD88 was both necessary and sufficient for the induction of Bim. BMDM from MyD88−/− mice stimulated with LPS showed no discernible up-regulation of Bim (Fig 3C). The shift in Bim caused by phosphorylation was also MyD88-dependent as it did not occur in MyD88−/− cells (Fig. 3C).

The dependency of Bim induction on MyD88 function was further investigated using RAW cells stably expressing a MyD88-gyrase B fusion protein. Addition of the cell-permeable dimeric ligand of gyrase B, coumermycin, causes the dimerization of gyrase B and its fusion partner (18). Coumermycin treatment of RAW cells expressing the MyD88-gyrase B fusion protein causes the known MyD88-dependent signaling events (see also Materials and Methods). This direct activation of MyD88 led to an up-regulation of Bim expression with similar kinetics as seen when using natural TLR ligands (Fig. 3D). MyD88-dependent signals are therefore both necessary and sufficient to cause the up-regulation of Bim in macrophages.

TLR-stimulation is a critical signal in the activation and maturation of DC. Therefore, we studied whether TLR-ligands also caused the up-regulation of Bim in mouse bone marrow-derived DC. As shown in Fig. 3E, a clear up-regulation of Bim was also observed in these cells during TLR-stimulation.

![FIGURE 2](image)

**FIGURE 2.** Expression of the BH3-only protein Bim is up-regulated upon contact with bacteria. A, Detection of Bim by Western blotting in RAW cells treated with live or heat-inactivated E. coli bacteria. RAW cells were seeded in 12-well plates (5 × 10^5/well) overnight and then either incubated with live or heat-inactivated E. coli bacteria for the indicated periods of time or left untreated. Cell extracts were analyzed by Western blotting. B, Untreated control. Similar results were obtained in three independent experiments. B, BMDM from a C57BL/6 mouse were treated with live E. coli and processed as above. Similar results were obtained in three independent experiments.
Bim<sub>EL</sub> and Bim<sub>L</sub> are phosphorylated upon TLR stimulation of RAW cells

A shift of Bim protein to higher m.w. in SDS-PAGE was observed during stimulation with whole bacteria as well as with TLR ligands (Figs. 2 and 3) indicating some form of posttranslational modification. A phosphorylation of Bim has been described earlier (19, 20). To test whether the mobility shift we observed here was due to phosphorylation, extracts from stimulated cells were digested with calf intestinal alkaline phosphatase. The mobility shift could be reversed by phosphatase treatment (Fig. 4A), indicating that the observed modification of Bim was indeed phosphorylation. Analysis of the kinetics showed that phosphorylation of Bim<sub>EL</sub> was rapid and complete while Bim<sub>L</sub> was phosphorylated with similar kinetics but only partially (Fig. 4B). Bim<sub>EL</sub> appeared to become phosphorylated at more than one site as bands of different sizes were observed, while the discrete bands for Bim<sub>L</sub> suggest more limited phosphorylation events (Figs. 2–4).

The MAPKs p38 and JNK are involved in regulation of apoptosis and induction of Bim

TLR triggering causes a number of downstream signaling events, including the activation of MAPKs and the classical NF-κB-pathway. To assess the contribution of these signals to phagoytosis-induced apoptosis, various kinase inhibitors were used. Inhibition of p38 MAPK led to reduction of apoptosis (close to 50% decrease compared with the control) (Fig 5A and B). A weak apoptosis-reducing effect was also seen when JNK was inhibited (Fig. 5A). In contrast, inhibition of the ERK-pathway or PI3K had no noticeable effect on apoptosis (Fig. 5A). Treatment of resting cells with the inhibitors alone had no detectable apoptosis-inducing or cytotoxic effects (data not shown).

To understand the molecular background of this reduction in apoptosis by inhibition of p38 and JNK, the influence of kinase inhibitors on Bim up-regulation and phosphorylation was studied. Intriguingly, inhibition of neither p38 nor JNK blocked TLR-dependent phosphorylation (data not shown) while blockade of ERK-activation had a borderline effect (Fig. 5C). However, inhibition of p38 or (to a smaller extent) JNK but not of ERK-activation reduced the up-regulation of Bim through TLR (Fig. 5D). Inhibitors of S<sub>rc</sub> kinase (PP2) or protein kinase C (staurosporine) affected neither phosphorylation nor induction of Bim (data not shown). Treatment with proteasome inhibitors slightly increased phagocytosis-induced apoptosis without affecting Bim-phosphorylation or induction, making a contribution from NF-κB unlikely (data not shown).

Because none of the tested kinase inhibitors had a pronounced effect on TLR-dependent phosphorylation of Bim, we considered the contribution of other pathways. Several pathways were activated during stimulation with whole bacteria as well as with TLR ligands (data not shown). Inhibition of MAPK P38 or JNK reduces apoptosis and the up-regulation of Bim expression without affecting Bim phosphorylation. RAW cells were pretreated with the indicated kinase inhibitors for 15 min and then stimulated with LPS for 1 h. Cell extracts were analyzed by Western blotting as above. Typical results of three independent experiments are shown.

Inhibition of MAPK P38 or JNK reduces apoptosis and the up-regulation of Bim expression without affecting Bim phosphorylation. RAW cells were pretreated for 15 min with various kinases inhibitors specific for p38 (SB203580), MAPK1 (PD98059), JNK (SP600125), or PI3 kinase (wortmannin), and then coincubated with E. coli for 20 h. Apoptosis was measured by annexin V/propidium iodide staining followed by flow cytometric analysis.

Because none of the tested kinase inhibitors had a pronounced effect on TLR-dependent phosphorylation of Bim, we considered the contribution of other pathways.
the possibility that the effect of TLR-stimulation was not the induction of kinase activity but the reduction of phosphatase activity. We tested this hypothesis by treating resting RAW cells with the broad-spectrum inhibitor of phosphatases calyculin A. As shown in Fig. 5E, this treatment caused a prominent shift of Bim\textsubscript{EL} and, to a lesser extent, of Bim\textsubscript{L} in unstimulated RAW cells. These data point toward the possibility that, in macrophages, Bim is constantly phosphorylated and dephosphorylated by as yet unidentified enzymes.

The induction of Bim through extracellular signals has, in neurons and in T cells, been found to be under the control of the member of the forkhead transcription factor family, FoxO3a. Our observations so far indicate that in RAW cells, FoxO3a translocates into the nucleus upon TLR stimulation (Fig. 5F). This suggests that FoxO3a is also activated upon TLR-signaling and contributes to the induction of Bim also in macrophages.

Bim is activated upon phagocytosis-induced apoptosis

Because Bim\textsubscript{EL} and Bim\textsubscript{L} are normally sequestered to the dynein motor complex on the microtubuli cytoskeleton, transcriptional induction may not be sufficient for its activation but rather sensitize the cell for a second, possibly independent, signal to apoptosis. Indeed, treatment of RAW cells with either heat-killed bacteria or LPS, both of which are only weak inducers of apoptosis under this protocol (Ref. 5, and see below) induced the expression of Bim in a way comparable to live E. coli bacteria. However, phagocytosis of live E. coli is a strong stimulus to undergo apoptosis. This suggested that Bim was induced but not activated by LPS or by heat-killed bacteria. As release of Bim from microtubuli is known to be involved in regulation of apoptosis, we next measured the release of Bim from the microtubuli cytoskeleton in RAW cells that had phagocytosed E. coli and were undergoing apoptosis. The attachment of Bim to microtubuli was assessed by density fractionation over a sucrose gradient. As shown in Fig. 6A (left), the great majority of Bim was found in the pellet fraction in untreated cells, together with the marker of microtubuli, α-tubulin. However, upon phagocytosis of bacteria a significant portion of Bim was found in the fractions of lower m.w. indicating its release from the cytoskeleton (Fig. 6A, right). This shift was much more noticeable for Bim\textsubscript{L} than for Bim\textsubscript{EL}. Similar results were obtained when cell lysates were separated over a 10% sucrose cushion: again Bim\textsubscript{L} but only very little Bim\textsubscript{EL} was found in the supernatant where released Bim is expected (Fig. 6B). Surprisingly, LPS (which failed to induce apoptosis) also caused an accumulation of Bim\textsubscript{L} in the low m.w. fraction (Fig. 6B). These results suggest that, although release of Bim from the cytoskeleton is a step in its activation, additional regulatory mechanisms exist that govern the induction of apoptosis by Bim.

These data suggest that TLR-signaling can increase the levels of Bim protein while a second stimulus is required to activate Bim and to cause apoptosis. To test this model, RAW cells were stimulated with LPS and then subjected to UV-irradiation, a stimulus that is known to activate Bim (at least in human epithelial cells, Ref. 12). Prestimulation with LPS enhanced UV-induced apoptosis in an overadditive manner as predicted (Fig. 6, D and E). Analysis of Bim-release indicated that UV-irradiation caused the release of
at least some Bim-protein in RAW cells (Fig. 6C). LPS-stimulation again up-regulated the expression of Bim, and the combination of LPS and UV increased the proportion of free Bim (Fig. 6C). Taken together, these results indicate that TLR-stimulation leads to the up-regulation of Bim in macrophages, and although this Bim is not firmly attached to the microtubular structure it does not activate the apoptotic pathway until a second signal is provided. As described previously, heat-inactivated bacteria cause up-regulation of Bim in macrophages, and although this Bim is not only of the lymphatic but also of the myeloid blood cell lineage (4, 5). In this study, we provide the molecular context for this form of apoptosis. In macrophages dying by “phagocytosis-induced cell death,” the BH3-only protein Bim is activated and makes a major contribution to this form of apoptosis. At the same time, Bim is up-regulated and phosphorylated via a signal pathway originating from TLR.

Discussion
Phagocytosis and intracellular digestion of bacteria is a principal effector function of cells of the innate immune system (1). We and others have shown that, consecutive to the process of phagocytosis, macrophages and granulocytes die by apoptosis (4, 5). In this study, we provide the molecular context for this form of apoptosis. Macrophages dying by “phagocytosis-induced cell death,” the BH3-only protein Bim is activated and makes a major contribution to this form of apoptosis. At the same time, Bim is up-regulated and phosphorylated via a signal pathway originating from TLR.

TLR have over the past few years been recognized as arguably the most important sensors of microbial presence within the immune system. Upon recognition of conserved molecular structures by TLR, adaptor molecules convey a signal into the cell that causes a broad line of downstream events such as a MAPK activation, activation of NF-kB and the expression of a large number of genes. Evidence has been presented that TLR-signaling can act as a modulator of apoptosis, although the derived models are not entirely consistent with each other. On one hand, TLR-induced NF-kB-activity probably has an antiapoptotic effect (22). On the other hand, TLR2 and TLR4 have been described to have the potent capacity to induce apoptosis, and both adaptor molecules MyD88 and TRIF have been found independently to transmit this signal (23, 24). We found that MyD88-dependent signaling was a powerful stimulus to up-regulate the expression of Bim. The strongest detectable contribution to this increase in Bim expression came from the MAPK p38, and the inhibition of p38 reduced phagocytosis-induced apoptosis. Although the inhibitor used (SB203580) may also, to a smaller degree, affect JNK, an inhibitor with greater specificity toward JNK (SP600125) had a smaller effect, suggesting that p38 is the kinase more important for up-regulation of Bim. It is therefore conceivable that the contribution from p38 consists in the induction of Bim.

BH3-only proteins like Bim are likely to be involved in the majority of instances of apoptosis (7). Bim has been recognized to be responsible for or at least to contribute to apoptosis upon UV-irradiation, taxol treatment, and anoikis (cell death upon loss of intercellular contact) but also to cell death as a consequence of factor withdrawal in neurons and during the contraction phase of a T cell response when Ag-activated T cells die by apoptosis (12, 16, 25–27). Notably, Bim-deficient mice have increased cell numbers not only of the lymphatic but also of the myeloid blood cell lineage (for instance, blood monocyte and spleen granulocyte levels are increased by a factor of ~3 (28), suggesting that Bim is involved in lifespan-defining apoptosis in granulocytes and macrophages. The induction of Bim through TLR in macrophages and DC may therefore be one determinant of the susceptibility to apoptosis in these cells. However, the mere induction is not sufficient to cause apoptosis. This is what one would expect: the straight induction of apoptosis through TLR upon encounter of microbial components would not be productive and indeed does not take place (as evidenced by a host of studies in vitro). Such sensitization of a macrophage or a DC to a later apoptotic stimulus (such as growth factor withdrawal) could well serve to regulate survival in the innate immune system. Little is known about life and death of these cells. In addition to the cellular shifts in Bim−/− mice mentioned, it has been found that Bcl-2 expression enhances the number of

**FIGURE 7.** Bim−/− mice are protected against phagocytosis-induced apoptosis. A, BMDM from wild-type or Bim−/− mice were seeded in 12-well plates (2 x 10⁶ cells/well) and exposed to E. coli for 20 h. Apoptosis was quantified by assessing nuclear morphology. Control-treated; □, E. coli-treated samples. The data shown are representative of four independent experiments that included a total of six wild-type and six Bim−/− mice. B, BMDM were stimulated as above, and apoptosis was quantified by annexin V/propidium iodide staining followed by flow cytometric analysis. Results obtained from one wild-type and one Bim−/− mouse are shown and are representative for a total of four wild-type and four Bim−/− mice analyzed. Numbers give percentages of cells in quadrants.

**Bim−/− mice are protected against phagocytosis-induced apoptosis**

To confirm the role of Bim in phagocytosis-induced apoptosis, BMDM from Bim-deficient mice were tested. Bim−/−-BMDM were as efficient as BMDM from normal mice at phagocyting E. coli bacteria as assessed with GFP-expressing bacteria by flow cytometry (data not shown). However, the induction of apoptosis measured as both nuclear fragmentation and the binding of annexin V/uptake of propidium iodide was strongly reduced in Bim-deficient cells as compared with wild-type cells (Fig. 7). Therefore, it is clear that Bim acts to transmit the apoptotic signal of phagocytosis-induced apoptosis, a mechanism that may act to kill phagocytes after they have fulfilled their function.
DC in vivo (29). Therefore, apoptosis appears to regulate homeostasis in this compartment, and the up-regulation of Bim through TLR may contribute to this.

Originally, the regulation of Bim was described to occur by the release from the microtubuli cytoskeleton upon a trigger from a number of apoptotic stimuli. Several recent studies have suggested that phosphorylation may also be involved but the physiological importance of this is still not clear. Direct phosphorylation of Bim by JNK has been shown in vitro and has been proposed to be a way by which Bim is released (20, 30), and ERK-mediated phosphorylation of BimEL has been suggested to cause its turnover by enhancing proteosomal destruction (31, 32). During MyD88-mediated TLR-signaling, the MAPK p38, JNK and ERK, are all rapidly activated (33, 34). However, only the inhibition of ERK activation had a detectable (albeit small) inhibitory effect on Bim phosphorylation. Intriguingly, the phosphatase inhibitor calyculin A (a broad-spectrum inhibitor with preference for serine/threonine phosphatases) caused the rapid phosphorylation of Bim. This points toward the possibility that, rather than being phosphorylated by kinases activated during TLR-signaling such as MAPK, Bim is constantly phosphorylated and dephosphorylated by constitutively active enzymes. In this scenario, TLR-mediated phosphatase inhibition may be the reason for the appearance of phosphorylated forms of Bim. The biological significance of this balance of modifications remains to be determined but may involve aspects like a change in activity (perhaps by modifying the ability to translocate to mitochondria or to activate Bax) or stability of the protein.

As at least one important step in Bim activation, the protein is released from the microtubuli cytoskeleton (12). This release is probably not a result of direct Bim targeting by the apoptotic process, as the dynein L chain LC8 (which directly binds Bim and attaches it to the dynein motor complex) is also released in the absence of Bim (12). It was surprising that the release of BimL and BimEL appeared different upon phagocytosis, in that a much larger portion of BimL was found in the fraction unbound to microtubuli than of BimEL. Because both isoforms are probably bound via LC8, it is not obvious how their release is differentially regulated. The binding of the two isoforms may lead to an association with different microtubuli substructures, or the LC8-release machinery may be differentially affected by the isoforms. It is also possible that additional as yet unidentified interaction sites of BimL exist with cytoskeletal components.

One observation of our study that is molecularly unexplained is that LPS generates free Bim in a way similar to the phagocytosis of bacteria but is much less active at inducing apoptosis. The simplest model predicts that the release from its site of sequestration is sufficient for Bim to proceed and cause Bax to become activated and to translocate to mitochondria. Although Bim is clearly upstream of Bax and somehow causes its activation, direct association between Bim and Bax is at least uncertain. A number of regulatory steps may exist that govern the activity of Bim even after it has been released, and phagocytosis vs TLR-signaling may differentially impact on these steps. The finding that BimL and BimEL, which have similar affinities to LC8, have different apoptosis-inducing activities upon overexpression (BimL is a more potent killer, Ref. 14), supports the model of such additional regulatory steps. It is further possible that the reduction of apoptosis by p38 inhibition impacts at this step, as p38-activity is known to be involved in microtubular processes. Another possibility is that, during TLR-stimulation in the absence of phagocytosis, the transcriptionally induced Bim does not reach microtubules/LC8, its site of sequestration. Under nonapoptotic conditions, it must somehow be made sure that de novo synthesized Bim is not active while assembling with microtubuli, and TLR-induced Bim may still be at that stage.

The evidence is clear that, upon uptake and subsequent to degradation of bacteria, mouse phagocytes die by apoptosis. The physiological importance of this process has not been determined, but there are at least three aspects that should be taken into account when considering its relevance. First, although especially grana-loocytes are very short lived in any case, activation of the cells upon contact with bacteria is potentially harmful, as production and uncontrolled release of mediators of inflammation by the activated granulocyte may cause unnecessary inflammation that may be prevented by apoptosis and subsequent uptake of the apoptotic cell by other phagocytes. Second, apoptotic cells are rapidly cleared in vivo, at least in part by uptake through DC, and this uptake has been shown to be able to initiate a T cell response to microbial Ag contained within the apoptotic cell (35, 36). Phagocytosis-induced apoptosis may therefore be relevant as a means of conveying bacterial Ag to the adaptive immune system. Third, it appears as a plausible speculation that apoptosis is the “normal” way of disposing of terminally differentiated cells that have fulfilled their function. Numerous studies demonstrate that such is the fate of activated T cells: resting T cells become activated and T effector cells die at the end of an immune response in a Bim-dependent manner (27). Although this to our knowledge has not been explored, it appears plausible that the same principle applies to phagocytes. During and upon phagocytosis, these cells become maximally activated and fulfill their function in the disposal of bacteria. Consecutive to that, the cells could either regress to become resting macrophages or, alternatively, die by apoptosis. The results shown in this study suggest the latter: that activation of phagocytes is a one-way road leading to Bim-dependent apoptosis.

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References


