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A Novel Mechanism Underlying the Basic Defensive Response of Macrophages against Mycobacterium Infection

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Following inhalation of Mycobacterium tuberculosis, including bacillus Calmette–Gue´rin (BCG), pathogens enter and grow inside macrophages by taking advantage of their phagocytic mechanisms. Macrophages often fail to eliminate intracellular M. tuberculosis, leading to the induction of host macrophage death. Despite accumulating evidence, the molecular mechanisms underlying M. tuberculosis infection–induced cell death remain controversial. In this study, we show the involvement of two distinct pathways triggered by TLR2 and β2 integrin in BCG infection–induced macrophage apoptosis. First, BCG infection induced activation of ERK1/2, which in turn caused phosphorylation/activation of the proapoptotic protein Bim in mouse macrophage-like Raw 264.7 cells. BCG-infected Raw cells treated with U0126, an MEK/ERK inhibitor, led to the suppression of Bim phosphorylation alongside a remarkable increase in the number of viable macrophages. Small interfering RNA–mediated knockdown of Bim rescued the macrophages from the apoptotic cell death induced by BCG infection. Stimulation with Pam3CSK, a TLR2 agonist, induced macrophage apoptosis with a concomitant increase in the phosphorylation/activation of MEK/ERK and Bim. These observations indicate the important role of the TLR2/MEK/ERK/Bim pathway in BCG infection–induced macrophage apoptosis. Second, we used the β2 integrin agonists C3bi and fibronectin to show that the β2 integrin–derived signal was involved in BCG infection–induced apoptosis, independent of MEK/ERK activation. Interestingly, latex beads coated with Pam3CSK and C3bi were able to induce apoptosis in macrophages to the same extent and specificity as that induced by BCG. Taken together, two distinct pattern-recognition membrane receptors, TLR2 and β2 integrin, acted as triggers in BCG infection–induced macrophage apoptosis, in which MEK/ERK activation played a crucial role following the engagement of TLR2. The Journal of Immunology, 2014, 192: 4254–4262.

The induction of both apoptotic and necrotic cell death has been reported during the onset of tuberculosis in M. tuberculosis–infected macrophages. Necrotic cell death causes dissemination of intracellular pathogens, resulting in expansion of the site of infection. In contrast, apoptotic cell death is accompanied by complete packaging of the pathogens within apoptotic bodies, which are immediately engulfed by neighboring phagocytes. Because macrophages reportedly kill and digest M. tuberculosis packed in apoptotic bodies (3), and because several reports mention the potential of virulent M. tuberculosis strains to induce necrotic cell death (4–7), apoptotic cell death in macrophages with bacterial infection is presumed to be a kind of host defense mechanism.

Several reports have been published on the mechanisms of M. tuberculosis infection–induced cell death. Keane et al. (8) showed that macrophages undergo apoptosis when infected with an avirulent strain of M. tuberculosis at a low multiplicity of infection (MOI), whereas virulent strains avoid causing macrophage apoptosis at a low MOI. It has also been reported that virulent M. tuberculosis can inhibit macrophage apoptosis by the upregulation of antiapoptotic Bcl-2 family protein expression (9–11). In contrast, several reports have shown a role for MyD88-dependent TNF-α secretion in the induction of apoptotic cell death in host macrophages (12). Conversely, however, another report indicated that TNF-α is incapable of inducing apoptosis in M. tuberculosis–infected macrophages (13). Thus, there is no established consensus on the mechanism regulating cell death in M. tuberculosis–infected macrophages. To understand the pathology of tuberculosis, elucidating the basic responses of macrophages against Mycobacterium infection is important.
Intracellular infection with *M. tuberculosis* is initiated by the pathogens binding to host phagocytes. The mammalian innate immune system recognizes invading microorganisms by phagocytic receptors, such as TLRs and β2 integrin (14–18). In fact, a role for TLRs in *M. tuberculosis* infection–induced macrophage death has been reported (19–25). However, the signaling pathway transmitting TLR activation to macrophage death remains unclear. Likewise, there is little information available on the participation of β2 integrins in macrophage apoptosis, although a role for β2 integrin in the induction of neutrophil apoptosis has been reported (26).

In the current study, we demonstrated that new signaling pathways, which were triggered mainly by TLR2 and ancillary by β2 integrin, are responsible for the induction of apoptosis in macrophages infected with *Mycobacterium*. Mechanistic analysis revealed that TLR2 ligation triggered activation of the MEK/ERK pathway, in turn induced Bim phosphorylation and its mitochondrial translocation and deactivation of the antiapoptotic protein Bcl-2, leading macrophages to apoptosis. In contrast, apoptotic signaling via β2 integrin was independent of the MEK/ERK pathway. The MEK/ERK dependency of both Bim activation and apoptosis induction was also observed when bacillus Calmette–Guérin (BCG) was replaced by nontuberculosis mycobacteria or when Raw 264.7 cells were replaced by thioglycollate-elicited mouse peritoneal macrophages (TGC-macrophages), suggesting a crucial role for TLR2/MEK/ERK/Bim signaling in macrophage apoptosis. Altogether, these results might offer a molecular basis for understanding macrophage responses during *Mycobacterium* infectious diseases including tuberculosis.

### Materials and Methods

#### Reagents

MEK inhibitor U0126 was purchased from Merck-Millipore. Ab against phospho-ERK1/2 (#9106) was obtained from Cell Signaling Technology (CST) and against ERK1/2 was purchased from Santa Cruz Biotechnology. Anti-Bim Ab (#8219), which was used in both Western blotting and confocal microscopic analysis, was purchased from CST. Anti–phospho-THR Ab (#9381) was also obtained from CST, and phospho-Ser Ab (P-S747) was obtained from Sigma-Aldrich. Anti–Bcl-2 Ab (sc-7382) was purchased from Santa Cruz Biotechnology. Anti–TLR2 Ab (clone T2.5) was obtained from Hycult Biotech (#HM1054), and anti–β2 integrin Ab was obtained from Merck-Millipore and Sigma-Aldrich, respectively. TLR2 agonist Pam3CSK4 was obtained from Alexis, and TLR3 agonist polyinosinic-polycytidylic acid (Poly (I:C)) was obtained from Sigma-Aldrich. Latex beads (2-μm diameter) were also obtained from Sigma-Aldrich.

#### Cell and bacterial cultures

Raw 264.7 cells were cultured in complete growth medium (DMEM) supplemented with 10% heat-inactivated FBS and antibiotics. BCGs were grown in Mycobroth (Kyokuto). *Mycobacterium sp.* No. 2, which was isolated from soil in an urban area in Tokyo and has a high degree of relatedness to *M. gilvum* PYR-GCK (99% identity in 16S rDNA sequence), was kindly gifted from Dr. Mineki (Tokyo University of Science, Noda, Japan) and used as one of the nontuberculosis *Mycobacterium* (NTM), which were defined as an acid-fast bacteria by acid-alcohol staining. To establish the BCG infection, Raw 264.7 cells were cocultured with BCG at an MOI of 10 for the indicated time in the complete growth medium. After BCG infection, unengulfed extracellular BCGs were washed out with PBS three times, and then infected cells were applied to various examinations.

#### Water soluble tetrazolium salt assay

Cells (2.0 × 10^4 cells/well) were seeded on a 96-well culture plate in complete culture medium with BCG and various reagents. The relative number of viable cells was evaluated by the water soluble tetrazolium salt (WST) assay with a Cell Counting Kit-8 (Dojindo) according to the manufacturer’s instructions. Absorbance at 450 nm was measured using a microplate reader (ARVO-MX 1420; PerkinElmer).

#### Sucrose-gradient fractionation

Subcellular fractionation was performed as described previously (27). Briefly, Raw 264.7 cells, with or without BCG infection, were lysed in extraction buffer (1% Triton X-100, 50 mM PIPES, 50 mM HEPES, 2 mM MgCl2, 1 mM EDTA, and 1 mM DTT) containing protease inhibitors. Lysates were taxol and apyrase for 15 min at 37°C. These samples were gently loaded onto an equal volume of 10% sucrose and centrifuged at 40,000 rpm in a swing rotor for 16 h at 4°C. Pellets were dissolved in Laemmli buffer. Supernatants were concentrated by acetone precipitation, and then precipitants were dissolved in Laemmli buffer. These samples were subjected to Western blotting analysis as described previously (28).

#### Confocal microscopic analysis

Cells were seeded on coverslips, which were coated with fibronectin (0.5 mg/ml), and cultured in complete growth medium. For mitochondrial staining, cells were loaded with 1 mM MitoTracker Red (Molecular Probes) at 37°C for 1 h. After fixation with cold methanol, cells were then permeabilized with 0.2% digitonin. After a blocking step using 2% BSA, cells were stained with primary Abs followed by FITC- or Alexa 633–conjugated secondary Ab. These coverslips were then mounted on slide glass and analyzed by confocal microscopy (Fluoview FV1000; Olympus) using an oil immersion objective lens (Olympus; original magnification ×60, numerical aperture 1.35).

#### Immunoprecipitation

Cells were seeded on a six-well plate and cultured for 4 h with BCG or reagents. The cells were then washed with PBS and lysed with 10 mM Tris-HCl (7.4) containing 150 mM NaCl, 1% Nonidet P-40, and inhibitors for proteinases and phosphatases. These lysates were incubated with anti-Bim Ab or anti–Bcl-2 Ab for 2 h at 4°C, followed by incubation with Protein G-Sepharose beads (GE Healthcare) for 1 h at 4°C. Immunoprecipitants were then denatured with Laemmli buffer and subjected to Western blotting as described above.

#### Small RNA interference

Cells (1.0 × 10^5 cells) were transfected with Bim small interfering RNA (siRNA; targeting all three isoforms of Bim: BimEL, L, and S; Sigma-Genosys) or control random siRNA duplex (Sigma-Genosys) using Nano Juice (Novagen) according to the manufacturer’s instructions and then seeded on 12-well plates. Forty-eight hours after transfection, cells were subjected to Western blot analysis or WST assay.

#### TUNEL assay

To detect apoptotic cell death, TUNEL reaction was performed using the In Situ Cell Death Detection Kit (Roche Applied Science). Briefly, cells were seeded on coverglass and incubated with BCG or various reagents. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.03% Triton X-100 in PBS. After washing with PBS, these cells were subjected to the TUNEL assay according to the manufacturer’s instructions. These coverglasses were then mounted on slide glass and analyzed by confocal microscopy.

#### Flow cytometry analysis

To detect apoptotic cell death by another method, phosphatidylinerine exposure on the surface of macrophages was detected using the Annexin V–FITC apoptosis detection kit (Sigma-Aldrich) following the manufacturer’s instructions. After BCG infection, adherent macrophages were detached by incubation with ice-cold 2% EDTA for 10 min and collected. After washing three times with PBS, these cells were stained with Annexin V–FITC and propidium iodide and analyzed by flow cytometry (FACSArray; BD Biosciences).

#### Statistical analysis

Results are expressed as means ± SD. Differences between experimental groups were analyzed using one-way factorial ANOVA (one-factor ANOVA) and a post hoc test. When a p value was <0.05, the difference was considered statistically significant.

#### Results

**Role of MEK/ERK signaling in BCG infection–induced apoptosis in Raw 264.7 cells**

To investigate the molecular mechanisms of macrophage death induced by *Mycobacterium* infection, we employed the nontuberculous *M. tuberculosis* strain BCG-Tokyo. As a model of *M. tuberculosis* infectious diseases including tuberculosis...
infection–induced cell death, mouse macrophage-like cells, Raw 264.7 cells, were cocultured with BCG at an MOI of 10, and the number of viable macrophages was then evaluated using the WST assay. The number of viable cells increased continuously in Raw 264.7 cells without BCG infection up to at least day 3, whereas in BCG-engulfed macrophages, their growth stopped on day 2 and then decreased (Fig. 1A). This decrease in the number of viable cells seemed to be due to apoptosis because Annexin V– and TUNEL-positive cells were increased in macrophages following infection with BCG (Fig. 1B, 1C). Of note, BCG infection induced apoptosis in macrophages, and the resulting decrease in the number of viable cells was negated by U0126, an MEK/ERK inhibitor. Conversely, SB203580, a p38 inhibitor, and SP600125, a JNK inhibitor, showed no effect on macrophage apoptosis (Supplemental Fig. 1). These results suggested that apoptosis in macrophages following infection with BCG is mediated through the MEK/ERK signaling pathway.

**Participation of TLR2 in the BCG-induced apoptosis of Raw 264.7 cells**

We next examined the role of TLRs in BCG-induced macrophage apoptosis. In this study, the synthetic TLR agonist Pam3CSK for TLR2, Poly (I:C) for TLR3, and LPS for TLR4 were employed, and their ability to decrease the number of viable cells was evaluated by the WST assay. Pam3CSK decreased the number of viable cells (Fig. 2A), whereas neither Poly (I:C) nor LPS had any effect (Supplemental Fig. 2A, 2B). These results are consistent with the fact that BCG is a Gram-positive microorganism and with previous reports that have described the importance of TLR2 in the host defense against *Mycobacterium* (11, 12). This decrease in the number of viable cells also seems to be due to apoptosis because Annexin V– and TUNEL-positive cells were increased in macrophages following stimulation with Pam3CSK (Fig. 2B). The Pam3CSK-induced decrease in the number of viable cells was completely inhibited by U0126 (Fig. 2A). Furthermore, both the decrease in the number of viable cells and the increase in DNA fragmentation induced by infection with BCG were abrogated when the cells were pretreated with an anti-TLR2 function-blocking mAb (Fig. 2C, 2D). The anti-TLR2–mediated rescue of macrophages from BCG infection–induced apoptosis was accompanied by the suppressed phosphorylation of ERK (Fig. 2E). Under the experimental conditions used, the anti-TLR2 Ab showed no significant effects on the infection efficiency of BCG (Supplemental Fig. 2C). These results suggest that BCG-induced apoptosis in macrophages is triggered by TLR2 recognition of BCG and the subsequent activation of the MEK/ERK pathway.

**Role of MEK/ERK-mediated phosphorylation of BimEL in BCG-induced apoptosis**

One member of the Bcl-2 family, Bim, has been shown to be a major physiological antagonist for prosurvival proteins in immune cells (29, 30). Moreover, the involvement of Bim-mediated signaling in macrophage apoptosis has been reported previously (27). Therefore, we examined the participation of Bim in our experimental model. When cell lysates from BCG-infected macrophages were subjected to immunoblot analysis with an anti-Bim pAb, Bim was detected as a double band of ∼26 and ∼23 kDa (Fig. 3A). No bands indicating BimL (∼15 kDa) or BimS (∼12 kDa) were detected in each experimental condition (Supplemental Fig. 2E), although this pAb has been verified by the manufacturer to detect all Bim isoforms. The upper ∼26-kDa band disappeared completely when the cell lysates were treated with calf intestinal alkaline phosphatase (Fig. 3B), suggesting that it was due to the phosphorylation of BimEL. The ∼26-kDa band of phosphorylated BimEL became more intense postinfection with BCG (Fig. 3A), but was abolished completely by treatment with U0126, which was consistent with the restoration of cell viability. Similar results were obtained when the macrophages were stimulated with Pam3CSK instead of BCG infection (Fig. 3A). The contribution of TLR2 to BCG-induced BimEL phosphorylation was also confirmed by the observation that the anti-TLR2 Ab could suppress BCG infection–induced BimEL phosphorylation (Fig. 3C). Under these experimental conditions, an immunoprecipitation study demonstrated that phosphorylation of the serine residue in BimEL was enhanced in BCG-infected macrophages and significantly suppressed by U0126 (Fig. 3D, top panel), whereas phosphorylation of the threonine residue was not observed even when the cells were infected with BCG (Fig. 3D, bottom panel).

Because several groups have reported on the pivotal role of MEK/ERK-mediated phosphorylation of the serine residue of BimEL in the regulation of its proteasomal degradation, we next focused on the degradation of BimEL under our experimental conditions. When uninfected Raw 264.7 cells were treated with the proteasome inhibitor MG132, the ∼23-kDa form of BimEL, probably representing nonphosphorylated BimEL, accumulated (Fig. 3E). Conversely, in the case of Raw 264.7 cells with BCG infection, no enhancement in the intensity of the ∼26-kDa phosphorylated BimEL band was detected in MG132-treated cells.

**FIGURE 1.** U0126 rescues Raw 264.7 from apoptotic cell death induced by BCG (MOI 10). (A) Raw 264.7 cells (2 × 10⁴ cells) were incubated for indicated days, and cell growth was measured by WST assay. (B) Raw 264.7 cells, harvested by EDTA at 2 d after coculturing with BCG, were stained with Annexin V–FITC and subjected to FACS analysis. (C) Raw 264.7 cells were seeded on coverslip and incubated with BCG for 3 d. Then these cells were applied for TUNEL staining. Percentage of TUNEL-positive cell number divided by total cell number was shown in bar graph. Random three fields in each experimental condition were selected and calculated. *p < 0.05.
although the nonphosphorylated BimEL band increased in intensity (Fig. 3E). These results indicate that the ∼26-kDa phosphorylated BimEL observed in this study might not be directed to proteasomal degradation.

Finally, we confirmed the importance of BimEL in BCG-induced apoptosis using an siRNA-mediated knockdown technique. As a result, DNA fragmentation, induced by BCG infection, was suppressed when BimEL expression was downregulated in macrophages by BimEL siRNA (Fig. 3F). Taken together, serine phosphorylation of BimEL, initiated in response to the recognition of BCG by TLR2, is induced in an MEK/ERK-dependent manner and responsible for the subsequent induction of apoptosis.

**BimEL localization in Raw 264.7 cells harboring intracellular BCG**

The translocation of Bim is a key event in the induction of apoptosis (30, 31). The release of Bim from microtubules initiates apoptotic signaling by trapping antiapoptotic proteins, including Bcl-2, on mitochondrial membranes. To investigate the subcellular localization of BimEL after BCG stimulation, we first evaluated the release of BimEL from microtubules in macrophages postinfection with BCG. Using sucrose-density fractionation, we divided the cell lysate into two fractions: tubulin-rich and nontubulin fractions (Fig. 4A, right panel). Immunoblot analysis of the nontubulin fractions revealed the presence of phosphorylated BimEL in the cells after BCG infection, whereas there was no detectable band in the nontubulin fraction from control cells (Fig. 4A, left panel). We also employed confocal microscopy analysis by immunostaining with an anti-Bim pAb and anti-tubulin mAb. Bim and tubulin appeared as a merged yellow image (i.e., colocalized) in untreated macrophages (Fig. 4B, uninfected panel), whereas they showed entirely different localization in BCG-infected macrophages (Fig. 4B, BCG infected panel). A merged yellow image was again obtained when the cells were treated with U0126 (Fig. 4B, BCG infected + U0126 panel).

Confocal microscopy using the anti-Bim pAb and MitoTracker Red showed that the colocalization of Bim with mitochondria was induced by infection with BCG (Fig. 4C). Furthermore, the released BimEL was shown to form a complex with Bcl-2 in BCG-infected macrophages (Fig. 4D). U0126 completely suppressed the mitochondrial colocalization of BimEL and its complex formation with Bcl-2. These results suggest that infection with BCG induces the release of BimEL from microtubules through phosphorylation in an MEK/ERK-dependent manner and that the released BimEL binds to Bcl-2, subsequently leading to apoptosis.

**Role of β2 integrin–derived signaling in BCG infection–induced apoptosis of Raw 264.7 cells**

Because complete rescue from BCG infection–induced apoptosis was not achieved using the anti-TLR2 Ab (Fig. 2C), it was speculated that an additional mechanism existed for the induction of apoptosis following BCG infection. Therefore, we next ascertained whether β2 integrin participates in BCG infection–induced macrophage apoptosis. We employed C3bi as a β2 integrin agonist...
in this study. As shown in Fig. 5A, this agonist was capable of significantly decreasing the number of viable macrophages. Because C3bi induced DNA fragmentation (Fig. 5B), the reduced number of viable macrophages was thought to be due to apoptosis. Another $\beta_2$ integrin agonist, fibrinogen, was also able to decrease the number of viable macrophages (Supplemental Fig. 4A). The suggestion that $\beta_2$ integrin was associated with BCG-induced macrophage apoptosis was supported by results showing the ability of an anti–$\beta_2$ integrin Ab to attenuate the decrease in the number of viable macrophages postinfection with BCG (Fig. 5C). Under this experimental condition, no significant effects on the infection efficiency of BCG were shown by the addition of the anti–$\beta_2$ integrin Ab (Supplemental Fig. 4B). Thus, the signaling mediated by $\beta_2$ integrin engagement also leads to the apoptosis of macrophages, although its intracellular signaling pathway remains to be addressed (Supplemental Fig. 4C).

Crucial role of TLR2- and $\beta_2$ integrin–derived signals in Mycobacterium infection–induced macrophage apoptosis

To verify further the contribution of TLR2 and $\beta_2$ integrin to BCG-induced macrophage apoptosis, anti-TLR2 and anti–$\beta_2$ integrin function-blocking Abs were used in a WST assay. The decrease in the number of viable macrophages induced by BCG infection was completely diminished by pretreatment with the anti-TLR2 Ab combined with the anti–$\beta_2$ integrin Ab (Fig. 6A). On the basis of these results, 2-μm–diameter latex beads (almost the same size as BCG bacteria) were coated with Pam3CSK and C3bi, agonists for TLR2 and $\beta_2$ integrin, respectively, and the ability of these BCG-like beads to affect macrophage viability was evaluated. When macrophages were cocultured with the BCG-like beads, a decrease in the number of viable cells occurred to a similar level as that induced by BCG (Fig. 6B). Macrophages incubated with uncoated control beads showed no significant change in the number of viable cells, although the efficiency of latex bead engulfment was independent of the Pam3CSK and C3bi coating. The BCG-like beads also induced phosphorylation of Bim and its translocation to the mitochondria (Supplemental Fig. 4D, 4E).

Recognition of microorganisms through TLR2 and $\beta_2$ integrin is not specific to M. tuberculosis engulfment. A decrease in the number of viable cells was observed even when macrophages were exposed to nontuberculosis Mycobacterium sp. No. 2. (Fig. 7A). This bacterium is a subspecies of M. gilvum PYR-GCK.
Defined as an acid-fast bacterium by acid-alcohol staining. The decrease in the number of viable cells induced by non-tuberculosis Mycobacterium sp. No. 2 was also shown to be dependent on the activation of MEK/ERK, as judged by an inhibition study using U0126 (Fig. 7A, Supplemental Fig. 4F). Finally, we examined whether the MEK/ERK-dependent apoptosis signaling pathway is also functional in primary macrophages infected with BCG using TGC-macrophages. As shown in Fig. 7B and Supplemental Fig. 4G, the MEK/ERK dependency of Bim phosphorylation and apoptosis induction was observed in BCG-infected TGC-macrophages. Taken together, the proposed signaling pathway including TLR2/MEK/ERK/Bim might act as a common route that is widely responsible for the induction of apoptosis in macrophages infected not only with M. tuberculosis but also with NTM.

**Discussion**

In this study, we demonstrated the importance of TLR2- and β2 integrin-derived signals in macrophage apoptosis induced by BCG infection. Consistent with our observations, the involvement of TLR2 following infection with M. tuberculosis and the ensuing responses of macrophages have been reported previously (21, 22, 24, 25). Because BCG infection–induced ERK phosphorylation was significantly blocked by cytochalasin B or D (Supplemental Fig. 2D), simple binding of Mycobacterium through TLR2 ligation on the cell surface seemed to be insufficient for activating the proapoptotic pathway observed in this study. The ligation of TLR2 during phagocytic uptake of the microorganism might be important for BCG infection–induced apoptosis under our experimental conditions.

Meanwhile, several reports have also mentioned the importance of TLR4 (19, 20, 23), despite the fact that M. tuberculosis is a Gram-positive microorganism. Thus, controversy remains over which TLRs contribute predominantly to the M. tuberculosis infection–induced apoptosis of macrophages. In our study, there was no significant decrease in the number of viable macrophages observed on stimulation with a TLR4 agonist, whereas a significant decrease resulted from stimulation with a TLR2 agonist (Fig. 2A, 2B).

**FIGURE 4.** Localization change of Bim in macrophage after BCG engulfment. (A) Whole-cell lysates from Raw 264.7 cells cocultured with BCG for 4 h were divided into tubulin-rich and nontubulin fractions by sucrose-gradient fractionation. Successful fractionation was confirmed by Western blotting analysis using anti-tubulin Ab (right panel), and then collected nontubulin fraction was subjected to Western blotting analysis using anti-Bim Ab (left panel). (B and C) Raw264.7 cells were seeded on coverglass and incubated with BCG for 4 h. (B) Immunofluorostaining using anti-Bim (green) and anti-tubulin (red) Abs was performed, and these samples were analyzed by confocal microscopy using an oil immersion objective lens (original magnification ×60, numerical aperture 1.35). Representative images from three independent experiments are shown. Individual single-channel images are shown in Supplemental Fig. 3A. (C) Immunofluorostaining was performed with anti-Bim Ab (green) and MitoTracker Red (red). Representative images from three independent experiments are shown. Individual single-channel images are shown in Supplemental Fig. 3B. (D) Bcl-2 binding proteins were immunoprecipitated (LP) by anti–Bcl-2 Ab as described in Materials and Methods. Bim protein in immunoprecipitated samples was detected by Western blotting.

**FIGURE 5.** Implication of β2 integrin ligation in BCG engulfment–induced apoptosis. (A and B) Raw 264.7 cells were incubated with β2 integrin agonist C3bi (10 μg/ml) for 3 d. Then, viable cell number was measured by WST assay (A), and the percentage of apoptotic cells was measured by TUNEL assay (B). (C) Raw 264.7 cells were incubated with BCG in the presence or absence of anti–β2 integrin Abs (10 μg/ml) for 3 d. The number of viable Raw 264.7 cells in each experimental condition was measured by WST assay. *p < 0.05. ctrl, control; un, untreated.
FIGURE 6. TLR2 and β2 integrin are receptors responsible for inducing BCG-engulfment–induced apoptosis in Raw 264.7 cells. (A) Raw264.7 cells were incubated with BCG in the presence of Abs (10 μg/ml) against both TLR2 and β2 integrin for 3 d. The number of viable Raw 264.7 cells was measured by WST assay. (B) Raw 264.7 cells were incubated with noncoated beads or Pam3CSK4 (Pam) and C3bi-coated beads at 1:10 for 3 d. Viable cell number of Raw 264.7 cells was measured by WST assay. *p < 0.05. ctrl, control; un, untreated.

Supplemental Fig. 2B). The administration of a function-blocking Ab against TLR2 combined with an anti–β2 integrin Ab completely rescued Raw 264.7 cells from BCG-induced apoptosis (Fig. 6A). Furthermore, the viability of Raw 264.7 cells after the engulfment of latex beads that were coated with agonists for TLR2 and β2 integrin decreased to a similar extent as that induced by infection with BCG (Fig. 6B). Thus, our data suggest that the contribution of TLR4 to BCG infection–induced macrophage apoptosis is much lower than that of TLR2 and β2 integrin. Indeed, in our study, the BCG-induced apoptosis of macrophages was independent of TNF-α secretion (data not shown), although Means et al. (19) reported that proapoptotic TNF-α production is induced by Mycobacterium tuberculosis ligation through TLR4. We have no clear answer to explain this discrepancy regarding the participation of TLR4, but speculate that our observations merely indicate the simplest response of macrophages against mycobacteria. The other signaling systems, including TLR4–derived signaling, might act synergistically with the responses observed in our study, depending on the virulence of the pathogen. In other words, the signaling pathways observed in our study might work broadly as a basic host response during infection with Gram-positive pathogens.

As an executive factor for BCG-induced apoptosis, we focused on the Bcl-2 family because the pivotal role of these proteins in the regulation of apoptotic cell death is well established. Among the Bcl-2 family, the BH3-only protein Bim is reportedly a major accelerator of apoptosis induction in immune cells (29, 30). The activity of proapoptotic Bim is reportedly regulated by its subcellular localization (31–34). In particular, the release of Bim proteins from the mitochondria is thought to be an important process in Bim-related apoptosis (31). In our experiments, infection with BCG induced the translocation of Bim from the microtubules to the mitochondria (Fig. 4A–C), and complex formation of the released Bim with Bcl-2 was observed subsequently (Fig. 4D). Because the siRNA-based downregulation of Bim led to a significant rescue of macrophages from apoptosis (Fig. 3F), BCG-induced macrophage apoptosis might be regulated through Bim. It is well known that three major isoforms of Bim are generated by alternative splicing; however, we could detect only BimEL in macrophages, even when they were infected with BCG (Supplemental Fig. 2E). Thus, BimEL might play a major role in the induction of apoptosis in macrophages in response to BCG infection.

Notably, the presence of phosphorylated BimEL was detected in the nontubulin fraction of BCG-infected macrophages (Fig. 4A). We did not detect a band indicating the presence of nonphosphorylated Bim in this fraction. These results suggest that the translocation of BimEL was triggered by its phosphorylation. In the case of apoptosis induction, accumulating evidence indicates the importance of JNK in Bim phosphorylation (32–34), whereas other MAPKs, such as ERK and p38, are also activated in macrophages following Mycobacterium tuberculosis infection or TLR2 ligation (35–37). However, in our study, a JNK inhibitor failed to increase the number of viable macrophages (Supplemental Fig. 1). Conversely, an MEK/ERK inhibitor rescued BCG-infected macrophages from apoptosis (Fig. 1A–C), which was accompanied by the complete suppression of BimEL phosphorylation (Fig. 3A, 3D). Thus, MEK/ERK could be a candidate kinase responsible for BimEL phosphorylation leading to apoptosis.

In fact, a number of studies have indicated the participation of MEK/ERK in Bim phosphorylation; however, the majority of these reports summarized the role of MEK/ERK activation as an antiapoptotic signal. MEK/ERK-mediated BimEL phosphorylation reportedly leads to proteasomal degradation, resulting in the

FIGURE 7. Induction of MEK/ERK-dependent apoptosis in Raw 264.7 cells infected with NTM or in primary macrophages infected with BCG. (A) Raw 264.7 cells were incubated with Mycobacterium sp. No. 2 (MOI 10) for 3 d. The number of viable Raw 264.7 cells was measured by WST assay. (B) TGC-macrophages (TGC Mφ) were incubated with BCG (MOI 10) in the presence or absence of U0126 (10 μM) for 3 d. The percentage of apoptotic cells was measured by TUNEL assay. *p < 0.05. un, untreated.
suppression of proapoptotic effects (38–42). In the current study, we observed that the 23-kDa form of BimEL was degraded consistently in an MEK/ERK-dependent manner (Fig. 3A, 3E, Supplemental Fig. 2E). However, when proteasomal degradation was suppressed by MG132, no enhancement in the intensity of the phosphorylated 26-kDa BimEL band was detected in BCG-infected Raw 264.7 cells, whereas the nonphosphorylated 23-kDa BimEL band accumulated (Fig. 3E, Supplemental Fig. 2E). These observations indicate that MEK/ERK-mediated Bim phosphorylation might rescue proapoptotic BimEL from constant degradation. In support of our hypothesis, it has been mentioned that ERK activation could lead to the phosphorylation of Bim at sites associated with Bim activation and increase proapoptotic activity (43, 44). Taken together, BCG-induced apoptosis might mainly be carried out by MEK/ERK-mediated Bim phosphorylation, which is triggered by TLR2 ligation through the engulfment of BCG.

In the current study, we could not determine the amino acid residue of Bim phosphorylation responsible for the induction of BCG infection–induced apoptosis, although it might be the serine residue and not the threonine residue (Fig. 3D). However, Stang et al. (44) showed that ERK could directly phosphorylate the Ser69 residue of Bim, which is responsible for the activation of its proapoptotic function, in 1,2-diacetylgluceral–induced B cell apoptosis. Therefore, Ser69 might possibly be the residue relevant for the induction of apoptosis as a result of MEK/ERK activation. Further examination is required to define the residue of Bim relevant for MEK/ERK-mediated apoptosis induction.

In this study, we highlighted a unique mechanism underlying BCG infection–induced apoptosis of macrophages. In particular, MEK/ERK worked as an activator of proapoptotic Bim in the TLR2–BimEL–apoptosis pathway. Moreover, we found that this proapoptotic pathway via TLR2/MEK/ERK/BimEL might work in the case of tuberculosis mycobacteria infection. We also ascertained that MEK/ERK/BimEL-dependent apoptosis is induced in primary macrophages with BCG infection. The results obtained in this study show that this signaling pathway might serve as a major functional route in the induction of apoptosis following Mycobacterium infection. In sharp contrast, it has been well accepted that MEK/ERK signaling is largely implicated in the promotion of cell-beneficial events, such as cell survival and proliferation. As mentioned above, the induction of apoptosis following infection by microorganisms is considered to be a host defense mechanism. From this point of view, MEK/ERK-dependent apoptosis seems to be beneficial to the host following infection with BCG. Meanwhile, it has been well discussed that infection with virulent M. tuberculosis induces necrosis rather than apoptosis (4–7). Zhang et al. (6) reported that a virulent M. tuberculosis strain has the ability to upregulate Bcl-2 expression, which could attenuate the proapoptotic function of Bim. To establish a new clinical anti–M. tuberculosis treatment based on our findings, further examinations are needed that clarify the role of the proapoptotic response observed in this study during virulent M. tuberculosis infection. This will be one of our next research targets.

Disclosures

The authors have no financial conflicts of interest.

References

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