NF-κB1 and c-Rel cooperate to promote the survival of TLR4-activated B cells by neutralizing Bim via distinct mechanisms

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The nuclear factor-κB (NF-κB) pathway is crucial for the survival of B cells stimulated through Toll-like receptors (TLRs). Here, we show that the heightened death of TLR4-activated nfkbi−/− B cells is the result of a failure of the Tpl2/MEK/ERK pathway to phosphorylate the proapoptotic BH3-only protein Bim and target it for degradation. ERK inactivation of Bim after TLR4 stimulation is accompanied by an increase in A1/Bim and Bcl-xL/Bim complexes that we propose represents a c-Rel–dependent mechanism for neutralizing Bim. Together these findings establish that optimal survival of TLR4-activated B cells depends on the NF-κB pathway neutralizing Bim through a combination of Bcl-2 prosurvival protein induction and Tpl2/ERK-dependent Bim phosphorylation and degradation. (Blood. 2008;112:5063-5073)

Introduction

Different microbial components activate cells of the innate and the adaptive immune system by binding specific Toll-like receptors (TLRs).1 Bacterial lipopolysaccharide (LPS) is a TLR4 ligand that promotes both B-cell proliferation and differentiation.2 Signaling networks downstream of TLR4 in B cells include the Rel/nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. Rel/NF-κB transcription factors are dimers composed of 5 related subunits (c-Rel, RelA, RelB, p50NF-κB1, and p52NF-κB2). In the absence of stimulatory signals, Rel/NF-κB factors are retained within the cytosol as IκB proteins.3 Signals emanating from cell surface or intracellular receptors promote Rel/NF-κB nuclear translocation by engaging an IκB kinase (IKK) that phosphorylates IκBs, targeting them for degradation.4 In mature B cells,5−7 both c-Rel and NF-κB1 are required for B-cell proliferation and survival.8,9 After B-cell receptor (BCR) or TLR4 stimulation.

Little is known about the role of MAPKs in TLR4-activated B cells. MAPK signaling uses kinase cascades that activate the effector serine/threonine kinases extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. MAP kinase kinase kinases (MAP3Ks) are at the apex of these pathways.10 TLR4 uses distinct MAP3Ks to activate different MAPK effectors; transforming growth factor–activated kinase 1 (TAK1) induces JNK and p38, whereas Tpl2 activates ERK.11,12 IKK is essential for both TLR4 activation of Rel/NF-κB and ERK.13 In unstimulated cells, Tpl2 forms a complex with p105NF-κB1, the p50NF-κB1 precursor.14 TLR4 activation of IKK2 targets p105 for proteasomal degradation, leading to the release of Tpl2, which then engages the mitogen-activated protein kinase kinase (MEK)/ERK pathway.13 nfkbi−/− macrophages lack detectable Tpl2 because of its rapid turnover,12 resulting in a failure to activate ERK after TLR4 stimulation.11,12

Given microbial products can induce apoptosis,15,16 higher organisms have evolved mechanisms to prevent this cell death. In many instances, activated B cells use the intrinsic survival pathway,17 which is regulated through interactions between members of 3 subgroups of Bcl-2 proteins: prosurvival proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1), multi-BH (Bcl-2 homology) domain proapoptotic proteins (Bax, Bak, and Bok), and proapoptotic BH3-only proteins (Bim, Bad, Bid, Bik, Bmf, Hrk, Noxa, and Puma).18 Bax and Bak, which trigger apoptosis by disrupting mitochondrial outer membrane integrity,19 are kept in check by Bcl-2 prosurvival homologs. BH3-only proteins activated via various transcriptional and posttranslational mechanisms20 are thought to initiate apoptosis either by binding Bcl-2 prosurvival homologs associated with Bax and Bak, thereby leading to their liberation and activation,18,21,22 or in the case of BH3-only proteins such as Bim, by directly binding to and activating Bax or Bak.23 Irrespective of which model of Bax/Bak activation is correct, increased or decreased levels of Bcl-2-like prosurvival and BH3-only proteins, respectively, tip the balance in favor of survival, whereas increases in BH3-only protein expression or reductions in Bcl-2-like prosurvival proteins induce apoptosis.

Among BH3-only proteins, Bim plays a prominent role in hematopoietic cell apoptosis.24,25 BimB and BimL are the most abundant isoforms26,27 and bind with high affinity to all Bcl-2-like prosurvival proteins.28 Bim proapoptotic activity is regulated by transcriptional and posttranslational mechanisms.20 In the latter instance, Bim can be regulated by phosphorylation,29 with ERK inactivating Bim by targeting it for ubiquitination and proteasomal degradation,30,32 whereas JNK enhances Bim proapoptotic activity.33

Here we show p105NF-κB1 promotes B-cell survival after LPS stimulation by a novel nontranscriptional mechanism involving Tpl2-dependent ERK activation that leads to Bim phosphorylation and degradation. LPS stimulation also leads to a c-Rel–dependent increase in A1 and Bcl-xL, which bind Bim. Consistent with both NF-κB1 and c-Rel promoting TLR4 mediated B-cell survival, loss of Bim prevented the elevated death of LPS-stimulated nfkbi−/− and c-rel−/− B cells. Furthermore, LPS caused more apoptosis in nfkbi−/− c-rel−/− B cells than either nfkbi−/− or c-rel−/− B cells.

The online version of this article contains a data supplement.


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demonstrating that optimal survival after TLR4 stimulation requires Bim to be neutralized by the c-Rel–dependent induction of A1 and Bcl-xL, plus p105NF-κB1/Tpl2/ERK–mediated Bim phosphorylation and degradation.

Methods

Mice

All experimental mice are on a C57BL/6 background and were 7 to 12 weeks of age. nfkb1+/−, c-rel−/−, nfkb1−/−, and vav-bcl-2 transgenic (bcl-2−/−) mice were maintained as inbred strains. nfkb1−/− Bim−/− and nfkb1−/− Bad−/− mice were generated by intercrossing nfkb1−/− and bim−/− or nfkb1−/− and bad−/− mice, whereas nfkb1−/−, c-rel−/−, bcl-2−/−, and nfkb1−/− c-rel−/− bcl-2−/− mice were generated by intercrossing the mutant and bcl-2−/− mice. All animal experiments were conducted in accordance with guidelines of the National Health and Medical Research Council (Australia).

Reagents and antibodies

Antibody sources were: ERK and c-Raf phosphospecific antibodies (Cell Signaling Technology, Danvers, MA), ERK, Tpl2, and actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti–Bcl-xL, and hamster anti–mouse Bcl-2 antibodies (BD Biosciences, San Jose, CA) and monoclonal rat anti-Bim antibody (clone 3C5), rat anti-A1 (Aldrich (St Louis, MO). Phosphorylation and degradation.

Cell proliferation and survival

Purified B cells (106 cells/population) before and after LPS stimulation. LPS treatment did not alter Mcl-1 expression rescued B cells. LPS-induced death in culture.8 Initially, we determined whether nfkb1−/− B cells do not impair the expression of Bcl-2 B1 independently regulates survival and proliferation of TLR4-stimulated B cells, consistent with previous reports.9,9 Loss of NF-κB1 led to enhanced spontaneous B-cell death in culture over 48 hours (Figure 1A). LPS stimulation further heightened the death of nfkb1−/− and bcl-2−/− B cells. This was assessed using nfkb1−/− mice expressing a hemopoietic lineage-driven bcl-2 transgene (bcl-2−/−). Consistent with previous reports,9,9 loss of NF-κB1 led to enhanced spontaneous B-cell death in culture over 48 hours (Figure 1A). LPS stimulation further heightened the death of nfkb1−/−, but not wt B cells. Bcl-2−/−, expressed at equivalent levels in wt and nfkb1−/− B cells (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article) blocked enhanced spontaneous plus LPS-induced nfkb1−/− B-cell apoptosis (Figure 1B). Although bcl-2−/− expression rescued TLR4-stimulated nfkb1−/− B-cell survival, proliferation remained defective (Figure 1B). These data show that NF-κB1 independently regulates survival and proliferation of TLR4-stimulated B cells, with death that occurs in the absence of NF-κB1 mediated through the cell-intrinsic pathway.

Retrovirus production, infections, and bone marrow engraftment

Viral stocks of pMy-internal ribosomal entry site (IRES)–green fluorescent protein (GFP)38 and pMy-IRES-GFP-Raf/Estrogen Receptor (ER) were generated as described.31 Bone marrow chimera s were made by injecting irradiated (2 × 550 rad) C57BL/6 Ly5.1 mice with approximately 106 virus-infected Ly5.2+ wt or nfkb1−/− hemopoietic progenitors.

B-cell isolation

Splenic B cells were isolated by MACS purification (Miltenyi Biotec, Auburn, CA) or cell sorting.39 B cells prepared using both protocols were routinely of more than 95% purity. Splenic B cells from chimeras were stained with phycoerythrin (PE)-conjugated anti-B220 anti- sIgM, affinity-purified goat antimouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), or both stimuli. PD98059 and 4-HT were used at 20 μM and 50 nM, respectively. Cell proliferation was quantified by 3H-thymidine article) blocked enhanced spontaneous plus LPS-induced nfkb1−/− B-cell apoptosis (Figure 1B). Although bcl-2−/− expression rescued TLR4-stimulated nfkb1−/− B-cell survival, proliferation remained defective (Figure 1B). These data show that NF-κB1 independently regulates survival and proliferation of TLR4-stimulated B cells, with death that occurs in the absence of NF-κB1 mediated through the cell-intrinsic pathway.

Results

nfkb1−/− B cells undergo excessive apoptosis after LPS stimulation

Mature nfkb1−/− B cells exhibit heightened spontaneous and LPS-induced death in culture.8 Initially, we determined whether perturbation of the cell intrinsic survival pathway41 was responsible for the abnormal death of nfkb1−/− B cells. This was assessed using nfkb1−/− mice expressing a hemopoietic lineage-driven bcl-2 transgene (bcl-2−/−). Consistent with previous reports,8,9 loss of NF-κB1 led to enhanced spontaneous B-cell death in culture over 48 hours (Figure 1A). LPS stimulation further heightened the death of nfkb1−/−, but not wt B cells. Bcl-2−/−, expressed at equivalent levels in wt and nfkb1−/− B cells (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article) blocked enhanced spontaneous plus LPS-induced nfkb1−/− B-cell apoptosis (Figure 1B). Although bcl-2−/− expression rescued TLR4-stimulated nfkb1−/− B-cell survival, proliferation remained defective (Figure 1B). These data show that NF-κB1 independently regulates survival and proliferation of TLR4-stimulated B cells, with death that occurs in the absence of NF-κB1 mediated through the cell-intrinsic pathway.

Loss of NF-κB1 does not impair the expression of Bcl-2 prosurvival family members

NF-κB control of transcription for genes encoding Bcl-2 prosurvival family members42,43 prompted a comparison of Bcl-2, Mcl-1, Bcl-xL, and A1 expression in wt and nfkb1−/− B cells (Figure 1C). Bcl-2 and Mcl-1 were expressed at similar levels in unstimulated wt and nfkb1−/− B cells. LPS treatment did not alter Mcl-1 expression rescued B cells.

Cell proliferation and survival

Purified B cells seeded at 3 × 106 cells/mL in Dulbecco modified Eagle medium/10% fetal bovine serum/50 μM 2-mercaptoethanol were unstimulated, treated with LPS (20 μg/mL), affinity-purified goat antimum IgM (Fab2) fragments (25 μg/mL; Jackson ImmunoResearch Laboratories, West Grove, PA), or both stimuli. PD98059 and 4-HT were used at 20 μM and 50 nM, respectively. Cell proliferation was quantified by 3H-thymidine uptake. B-cell survival in culture was assessed by flow cytometric analysis of cells stained with propidium iodide.

Two-dimensional gel electrophoresis and Western blotting

B-cell lysates were fractionated on isoelectric focusing gels (ZOOM IPG strips; Invitrogen, Carlsbad, CA), after which gel strips were resolved in the second dimension on NuPAGE MOPS/SDS ZOOM gels (Invitrogen). proteins were transferred onto nitrocellulose membranes and Western blotting performed as described.31 Immunofluorescence staining

For 3-color FACS analysis of B lymphocytes,34 106 splenocytes were incubated with FITC-conjugated antiCD23, APC-conjugated anti-CD21, and PE-conjugated anti-IgM antibodies and viable cells examined using a FACScalibur (BD Biosciences).

Real-time polymerase chain reaction (PCR)

mRNA was isolated from FACS-purified IgM+ wt and nfkb1−/− splenic B cells (106 cells/population) before and after LPS stimulation. bim mRNA levels were measured by real-time PCR40 using cDNA generated from equivalent amounts of RNA. bim mRNA levels were normalized by performing quantitative PCR for gapdh mRNA. Primer sequences are available on request.

Immunoprecipitation–Western blots

Total cell extract isolated from 5 × 107 magnetic-activated cell separation purified wt and nfkb1−/− splenic B cells for each time point were lysed41 and a sample of each subjected to Western blotting using anti-HSP70 antibodies to quantify protein content in each lysate. The remainder of the samples was incubated with rat anti-Bim monoclonal antibody (3C5), followed by immunoprecipitation (IP) with protein G-Sepharose beads. After washing, samples were eluted, fractionated on 12% sodium dodecyl sulfate-polyacrylamide gels and the proteins transferred onto nitrocellulose membranes. Filters were incubated with either mouse monoclonal anti–BCL-xL, hamster anti-mouse Bcl-2, or monoclonal rat anti-A1 antibodies and bound antibody revealed by enhanced chemiluminescence (GE Healthcare, Little Chalfont, United Kingdom) using horseradish peroxidase-conjugated goat anti-mouse, goat anti-hamster or goat anti-rat antibodies (Southern Biotechnology Associates, Birmingham, AL).
expression, whereas Bcl-2 expression increased marginally (~2-fold) in wt but not nfkb1−/− B cells after 12 hours. Basal levels of Bcl-xL and A1, plus their induction by LPS, which is c-Rel dependent,42,43 was normal in nfkb1−/− B cells. This indicates that the abnormal death of nfkb1−/− B cells was not the result of major differences in the expression of Bcl-2 prosurvival family members.

Loss of Bim prevents the heightened death of LPS-stimulated nfkb1−/− B cells

Our attention next turned to the proapoptotic BH3-only proteins, with a focus on Bim, given its prominence in B-cell death.44 Bim involvement in nfkb1−/− B-cell death was determined using nfkb1−/− bim−/− mice. Like the parental strains, nfkb1−/− bim−/− mice exhibit no gross developmental abnormalities (A.B., unpublished data, October 2006). A comparison of spleen cellularity, plus follicular and marginal zone (MZ) B-cell populations in wt, nfkb1−/−, bcl2T, and nfkb1−/− bcl2T mice (Table S1; Figure S2) revealed that increased follicular B-cell numbers that occur in the absence of Bim24 were not further altered in nfkb1−/− bim−/− mice. However, reduced MZ B-cell numbers in the nfkb1−/− and bim−/− mutants were compounded in nfkb1−/− bim−/− mice. bcl-2T expression had a similar effect on MZ B cells in nfkb1−/− mice as loss of Bim (Figure S2).

With follicular B-cell development intact in nfkb1−/− bim−/− mice, spontaneous and LPS-induced death was examined in these mutant B cells (Figure 2). During the initial 24 hours in culture, the high spontaneous death of nfkb1−/− B cells was abrogated by loss of Bim (Figure 2A, lanes 1-4). However, spontaneous nfkb1−/− bim−/− B-cell

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**Figure 1.** Transgenic Bcl-2 protects nfkb1−/− B cells from spontaneous and LPS-induced apoptosis. Purified B cells from wt, nfkb1−/−, bcl2T, and nfkb1−/− bcl2T mice were cultured with or without LPS for the indicated times. (A) Levels of cell death. The data are mean plus or minus SEM for 3 experiments. (B) B-cell proliferation. Results represent mean plus or minus SEM of 3 experiments, each performed in triplicate. (C) Bcl-2, Mcl-1, Bcl-xL, and A1 expression in B cells after LPS stimulation. This blot is representative of 3 experiments.
apoptosis after 48 hours (~20%, lane 8), although less than for nfkbl
B cells (~50%, lane 6), was higher than that of bim
B cells (~8%, lane 7). Notably, bel-2 expression limited the spontaneous death of nfkbl
B cells to approximately 10% (Figure 1A). This indicated that the elevated spontaneous death of nfkbl
B cells involves Bim-dependent and -independent processes, with Bim-independent apoptosis operating predominantly at later times. In contrast, the higher levels of LPS-induced nfkbl
B-cell death were completely inhibited by loss of Bim, at both early and late time points (Figure 2B).

### The enhanced death of nfkbl
B cells coincides with a failure to block the transient reduction of Bim after LPS stimulation

The finding that Bim was critical for the enhanced spontaneous and LPS-induced death of nfkbl
B cells prompted an examination of the connection between ERK and the transient reduction in Bim activity. First, a comparison of bim mRNA levels in wt and nfkbl
B cells (Figure 3A) established that, despite modest increases in bim mRNA after LPS treatment, bim expression was equivalent in cells of both genotypes. Next, Bim levels were examined in LPS-activated wt and nfkbl
B cells at 6-hour intervals over 18 hours (Figure 3B). Bim expression was equivalent in unstimulated wt and nfkbl
B cells, gradually increasing in response to LPS, such that, by 18 hours, Bim in cells of both genotypes was 2- to 3-fold higher than in prestimulated cells.

In the absence of differences during sustained LPS activation, Bim expression was examined early during LPS stimulation (Figure 3C). Interestingly, Bim levels in LPS-stimulated wt but not nfkbl
B cells was lower after 2 hours compared with B cells cultured without LPS. Given our bim mRNA expression data, this change in Bim levels probably reflected Bim turnover. Because ERK phosphorylation of Bim targets it for degradation,29,30 we investigated the connection between ERK and the transient reduction in Bim after LPS stimulation. Initially, Bim levels were measured in wt B cells stimulated with LPS plus the MEK inhibitor PD98059 (Figure 3D). The transient decrease in Bim after 2 hours of LPS stimulation was blocked by PD98059, indicating that it was ERK-dependent.

p105NF-xB1 serves as a scaffold for the ERK-specific MAP3K, Tpl2,12,14 with an absence of p105 in macrophages and fibroblasts leading to very low levels of Tpl2 that result in defective LPS-induced ERK activation.11,12 Tpl2 was undetectable in nfkbl
B cells (Figure 3E), and this coincided with a defect in LPS-induced ERK activation (Figure 3F). Consistent with LPS-induced ERK activation in B cells being dependent on Tpl2 and not c-Raf, the MAP3K that activates ERK downstream of many receptors, including the BCR,47 S338 phosphorylation of c-Raf is absent in LPS-stimulated B cells (Figure 3F).

### Increased TLR4-induced nfkbl
B-cell death is associated with defective MEK/ERK signaling

The link between impaired ERK activity in LPS-treated nfkbl
B cells and increased apoptosis was reinforced by showing apoptosis in LPS-stimulated wt B cells was enhanced by PD98059 (Figure 4A). To confirm that impaired survival coincided with the ERK defect, B cells were isolated from mice engrafted with hemopoietic progenitors transduced with retroviruses coexpressing GFP and a c-Raf/estrogen receptor (Raf/ER) fusion protein that served as a 4-HT–inducible activator of ERK in B lymphoma cells (Figure S3A). Spontaneous (Figure 4B) and LPS-induced (Figure 4C) apoptosis was examined in these cells over 48 hours. Raf/ER activation diminished the spontaneous death of wt and nfkbl
B cells after 24 hours (~6% and ~18%, respectively) compared with GFP+ control B cells (~17% and 30% for wt and nfkbl
B cells, respectively). Sustained ERK activation had less impact on spontaneous apoptosis after 24 hours, with approximately 22% and approximately 42% death for Raf/ER expressing wt and nfkbl
B cells, respectively (T48 hours) compared with approximately 40% and 51% for GFP+ wt and nfkbl
B cells.

In contrast, activated Raf/ER protected nfkbl
B cells against LPS-induced apoptosis over 48 hours (Figure 4C). The reduced levels of apoptosis were not the result of increased viability resulting from proliferation, as sustained Raf/ER activation blocked wt and nfkbl
B-cell division (Figure S3B), as previously reported for fibroblasts.48 The finding that the Bim-dependent death of nfkbl
B cells was associated with a defect in transient Bim degradation accompanying TLR4 activation, suggested that this death is normally prevented by ERK inhibiting Bim function during a critical period after LPS stimulation. This hypothesis was examined by monitoring apoptosis in LPS-treated nfkbl
B cells expressing Raf/ER, where delayed ERK activation was achieved by adding 4-HT to cultures at specific times after LPS stimulation.
Remarkably, activation of Raf/ER within 4 hours of initiating LPS stimulation was necessary to prevent the enhanced death of \(nfkb1^{-/-}\) B cells; activating Raf thereafter had no significant impact on apoptosis. Collectively, these biochemical and genetic data indicate that the abnormally high death of LPS-stimulated \(nfkb1^{-/-}\) B cells is a consequence of a defect in ERK activation.

LPS stimulation induces ERK-dependent phosphorylation of Bim in B cells

Despite many signals inducing ERK-dependent phosphorylation of Bim, this remained to be established for TLR signaling. Two-dimensional gel electrophoresis combined with Western blotting was used to examine Bim modification in LPS-stimulated B cells. Before stimulation, Bim(p), the major form of Bim expressed in B cells, exhibited an identical pattern of 3 isoforms (Figure 5A, labeled 1-3) in \(wt\) and \(nfkb1^{-/-}\) B cells. After LPS stimulation for 2 hours (Figure 5A), the time when Bim levels normally decrease, 3 additional negatively charged Bim(EL) isoforms (4-6) were detected in \(wt\) cells, whereas only isoforms 1 to 4 were seen in \(nfkb1^{-/-}\) B cells. ERK activation was required for the induction of isoforms 5 and 6 given they were absent in LPS plus PD98059-treated \(wt\) B cells (Figure 5B). The LPS-induced ERK-dependent isoforms were detectable within 60 minutes, peaking in abundance at approximately 2 hours and then gradually decreased until they were undetectable by 6 hours (Figure S4A). This reinforced that the kinetics of LPS-induced Bim phosphorylation reflect the transient activity of ERK. We also confirmed the relationship between Bim phosphorylation and ERK in W231 B lymphoma cells using the 4-HT induction of Raf/ER (Figures S3A, S4B). Together, these results establish that \(nfkb1^{-/-}\) B cells responding to LPS are defective in ERK-dependent Bim phosphorylation.

BCR ligation restores normal Bim phosphorylation and survival in LPS-stimulated \(nfkb1^{-/-}\) B cells

Engaging ERK via an alternate MAP3K should promote the survival of LPS-activated \(nfkb1^{-/-}\) B cells. Because BCR cross-

(Figure 4D). Remarkably, activation of Raf/ER within 4 hours of initiating LPS stimulation was necessary to prevent the enhanced death of \(nfkb1^{-/-}\) B cells; activating Raf thereafter had no significant impact on apoptosis. Collectively, these biochemical and genetic data indicate that the abnormally high death of LPS-stimulated \(nfkb1^{-/-}\) B cells is a consequence of a defect in ERK activation.
Linking activates ERK via c-Raf, we examined whether BCR and TLR4 costimulation overcame the Bim phosphorylation and survival defects seen in LPS-treated nfkb1−/− B cells. We first confirmed that c-Raf activation (S338 phosphorylation) and ERK phosphorylation were normal in nfkb1−/− B cells after BCR cross-linking (Figure 5C). Survival was then determined for wt and nfkb1−/− B cells activated with anti-IgM antibodies, LPS, or both stimuli (Figure 5D). BCR signals prevented the LPS-induced death of nfkb1−/− B cells, with the pattern of Bim isoforms after BCR plus TLR4 costimulation comparable between wt and nfkb1−/− B cells (Figure 5E). This establishes that B-cell survival linked to ERK-dependent Bim phosphorylation can be independently regulated via distinct MAP3Ks activated by different signals.

TLR4 signals lead to increased association between Bim and the prosurvival proteins A1 and Bcl-xL

Despite a transient ERK-dependent drop in Bim levels in wt B cells, Bim expression is restored within 6 hours of initiating LPS activation (Figure 3B). This indicated that mechanisms distinct from ERK-dependent Bim turnover prevented Bim-induced apoptosis during sustained LPS stimulation. Because Bim interaction with Bcl-2-like prosurvival proteins has also been shown to promote cell survival, these associations were examined in LPS-stimulated B cells.

To ensure that relevant times were chosen to assess Bim and pro-survival protein interactions, Bcl-2-like protein expression was re-examined at early times in LPS-activated B cells. In the absence of changes in Bcl-2 and Mcl-1 levels (Figure 1C), attention focused on A1 and Bcl-xL. In wt and nfkb1−/− B cells, A1 levels increased within 2 hours and were sustained for at least 18 hours (Figures 1C, 6A). Bcl-xL induction was slower, with a small increase in expression first seen after 4 hours (Figure 6A). Like A1, LPS-induced Bcl-xL expression was sustained. IP-Western blots were then performed to examine Bim interaction with A1, Bcl-xL, and Bcl-2 in LPS-treated wt and nfkb1−/− B cells (Figure 6B). Interactions between Bim and A1 or Bcl-xL were undetectable in quiescent B cells but increased after LPS stimulation. Although Bim levels were reduced in wt but not nfkb1−/− B cells after 2 hours (Figure 6B, compare lanes 2 and 6), paradoxically, A1 association with Bim in nfkb1−/− B cells was less compared with wt B cells. After 6 hours, the abundance of the A1/Bim complex was equivalent in cells of both genotypes. Bcl-xL/Bim association was detected in wt and nfkb1−/− B cells after 6 hours and increased by 12 hours. Unlike the A1/Bim complex, the pattern of Bcl-xL and Bim association was equivalent in wt and nfkb1−/− B cells over 12 hours. Bcl-2 association with Bim and the abundance of this complex did not alter significantly in either cell type during LPS stimulation.

Because c-Rel is required for the transcriptional induction of A1 and bcl-xL in LPS-stimulated B cells, we determined whether the
prosurvival role of c-Rel during TLR4 signaling involved neutralizing Bim proapoptotic activity. To this end, c-rel1−/− bim−/− mice were generated and B-cell survival in response to LPS examined (Figure 6C). As expected, LPS-induced apoptosis was abnormally elevated in c-rel1−/− B cells but was blocked by the loss of Bim, demonstrating that c-Rel, like NF-κB1, promotes TLR4-activated B-cell survival by neutralizing Bim.

c-Rel and NF-κB1 cooperate to counteract Bim-dependent apoptosis in TLR4-stimulated B cells

The findings that TLR4 signals trigger Bim degradation and increased binding of Bim to A1 and Bcl-xL raised the question as to whether these 2 processes are part of one pathway or represent distinct pathways for neutralizing Bim. This was tested by comparing LPS-induced apoptosis in nfkbi1−/−, c-rel−/−, and nfkbi1−/−c-rel−/− B cells (Figure 6D). Although LPS-induced apoptosis was abnormally elevated in both nfkbi1−/− (lane 6) and c-rel−/− (lane 7) B cells, celllying death was even higher for nfkbi1−/−c-rel−/− B cells (lane 8). bcl-2 expression blocked the death of LPS-stimulated nfkbi1−/−c-rel−/− B cells, confirming it involved the Bcl-2-regulated intrinsic pathway. This shows that NF-κB1 and c-Rel promote survival of LPS-stimulated B cells by nonredundant mechanisms that converge to neutralize Bim.


discussion

Here we show that NF-κB1 enhances the survival of TLR4-stimulated B cells by regulating the MAP3K Tpl2 required for ERK-dependent Bim phosphorylation and degradation. Coupled with the c-Rel-dependent induction of A1 and bcl-xl that result in the formation of A1/Bim and Bcl-xL/Bim complexes after LPS stimulation, these distinct NF-κB1 and c-Rel-regulated survival mechanisms synergize to neutralize Bim proapoptotic activity in TLR4-activated B cells.

Loss of p105NF-κB1 increases spontaneous plus TLR4 activation induced B-cell apoptosis. Bcl-2 transgene expression prevents sustained spontaneous nfkbi1−/− B-cell death; loss of Bim, although blocking the initial apoptosis, affords only partial protection thereafter. Similarly, constitutive ERK activation only inhibits spontaneous wt and nfkbi1−/− B-cell death during the first 24 hours. Therefore, the initial phase of spontaneous nfkbi1−/− B-cell death is Bim- and ERK-dependent, whereas subsequent death involves other BH3-only proteins that are not ERK regulated. In contrast, elevated TLR4-induced nfkbi1−/− B-cell apoptosis is blocked by loss of Bim or sustained ERK signaling. However, death in LPS-stimulated nfkbi1−/−bim−/− B-cell cultures, although equivalent to that of bim−/− and wt cells (Figure 2), remains higher than either wt or nfkbi1−/− B cells expressing Bcl-2 (Figure 4A) and wt cells expressing activated Raf/ER (Figure 4C). Collectively, these findings are best reconciled in a model where multiple BH3-only proteins contribute to TLR4-induced B-cell death, with their apoptotic function neutralized by Bcl-2 or constitutive ERK signaling. Transgenic Bcl-2, unlike endogenous Bcl-2, would presumably be expressed at levels sufficient to neutralize multiple LPS-activated BH3-only proteins. Whereas Bcl-2 transgene expression is equivalent in wt and nfkbi1−/− B cells (Figure S1), a direct comparison of endogenous mouse and transgenic human Bcl-2

Figure 5. LPS stimulation results in ERK phosphorylation of Bim. (A,B) wt or nfkbi1−/− B cells were cultured for 2 hours without or with LPS in the absence or presence of PD98059. Cell lysates were subjected to 2-dimensional gel electrophoresis and Western blotting using Bim-specific antibodies. The data are representative of 5 experiments. (C) Western blots for phospho–c-Raf, phospho-ERK, and ERK in wt and nfkbi1−/− B cells stimulated with anti-IgM antibodies. Data are representative of 3 experiments. (D) Apoptosis in wt and nfkbi1−/− B-cell cultures untreated or stimulated for 48 hours with LPS, anti-IgM antibodies, or both agents. The data represent the mean plus or minus SEM from 4 experiments. (E) Bim Western blots on 2-dimensional gels for wt and nfkbi1−/− B cells left untreated or stimulated (2 hours) with anti-IgM antibodies in the absence or presence of LPS. Data are representative of 3 experiments. The symbols “+” and “−” (panels A, B, and C) refer to the anode and cathode, respectively, and numbers refer to Bim isoforms.
levels in these cells is precluded by an inability of the antibodies used here to recognize both forms of Bcl-2. Why Bim appears to be more important in the LPS-induced death of \( \text{nfkb}^1 \) B cells than \( \text{wt} \) cells is unclear but may indicate that the hierarchical importance of particular BH3-only proteins in cell death varies on different genetic backgrounds.

Here we demonstrate that Tpl2/ERK signaling in B cells initiated through TLR4 leads to transient Bim phosphorylation and degradation, with disruption of this pathway resulting in the enhanced death of LPS-activated \( \text{nfkb}^1 \) B cells. This represents a cell-type-specific mechanism for protecting cells from TLR4-induced apoptosis, given that the ERK defect in LPS-activated \( \text{nfkb}^1 \) macrophages\(^{11,12} \) does not result in increased apoptosis (Figure S5). Transient ERK-dependent Bim degradation in B cells over a 3- to 4-hour period, initiated within approximately 60 minutes of LPS stimulation (Figure S5), points to this being a critical window for preventing Bim-mediated apoptosis. This conclusion is supported by Raf/ERK activation of ERK in LPS-treated \( \text{nfkb}^1 \) B cells needing to occur within this time frame to protect these cells from enhanced death. This points to Bim activation at this early juncture in LPS stimulation triggering subsequent cell death via a “hit-and-run” mechanism (Figure 4D). Although ERK signaling prevents apoptosis in TLR4-activated \( \text{nfkb}^1 \) B cells by counteracting Bim, our data suggest that constitutive ERK signaling inhibits this apoptosis by acting on additional targets. One candidate, Bad, another BH3-only proapoptotic protein controlled by ERK,\(^{50} \) was excluded from having a role in the spontaneous or TLR4 stimulation-

![Figure 6](image_url)
induced death of wt or \( \mathbf{n f k b l} \mathbf{k b 1} ^ {/-} \) B cells (Figure S6A,B). Furthermore, despite compelling biochemical and genetic data presented here for the importance of Tpl2/ERK in preventing B-cell death, we cannot exclude a role for p50NF-κB transcription in protecting cells against TLR4 proapoptotic signals.

The observation that Tpl2/ERK signaling only induces a transient drop in Bim levels during sustained LPS activation indicates that this mechanism alone probably does not fully protect B cells from Bim-induced apoptosis. The finding that exaggerated levels of death for LPS-stimulated \( \mathbf{c - r e l} \mathbf{k b 1} ^ {/-} \) B lymphocytes were blocked by loss of Bim, despite ERK activation and Bim phosphorylation being normal in \( \mathbf{c - r e l} \mathbf{k b 1} ^ {/-} \) B cells (A.B., unpublished data, July 2007), indicates that c-Rel promotes survival by inhibiting Bim via a different mechanism. Consistent with NF-κB1 and c-Rel neutralizing Bim in TLR4-activated B lymphocytes through nonredundant mechanisms, the death of LPS-stimulated \( \mathbf{n f k b l} \mathbf{k b 1} ^ {/-} \mathbf{c - r e l} ^ {/-} \) B cells was greater than that of \( \mathbf{n f k b l} \mathbf{k b 1} ^ {/-} \) or \( \mathbf{c - r e l} ^ {/-} \) B cells. The increased death of LPS-activated \( \mathbf{c - r e l} ^ {/-} \) B cells coincides with a failure to up-regulate A1 and Bcl-xL expression, which in \( \mathbf{w t} \) cells leads to the generation of Bim/A1 and Bim/Bcl-xL complexes. The binding of Bim to A1 precedes its binding to Bcl-xL, consistent with the kinetics of A1 and Bcl-xL induction. That A1 and Bcl-xL bind Bim after LPS stimulation indicates that both proteins probably contribute to the inhibition of Bim. This is consistent with models in which the neutralization of multiple prosurvival Bcl-2 family members by BH3-only proteins is required to trigger apoptosis.\(^{18,22}\) Surprisingly, less A1 bound Bim at the 2-hour time point in LPS-activated \( \mathbf{n f k b l} \mathbf{k b 1} ^ {/-} \) B cells, despite Bim not being targeted for degradation in these cells. This unexpected finding may indicate that Bim phosphorylation enhances binding to A1; this remains to be determined.

Results presented here support an integrated signaling model in which ERK- and c-Rel–dependent pathways coordinated through IKK promote optimal survival of TLR4-activated B cells (Figure 7). IKK2 activation leads to the nuclear translocation of c-Rel dimers required for A1 and bcl-xl transcription, with elevated expression of these Bcl-2 prosurvival proteins leading to the formation of Bim/A1 and Bim/Bcl-xL complexes. IKK2 also induces Tpl2-dependent ERK activation, resulting in Bim phosphorylation and turnover. Why both of these mechanisms are necessary to neutralize Bim is unclear. Furthermore, our results establish how Bim is inactivated in TLR4-stimulated B cells but do not shed light on how Bim activates Bax or Bak.

For example, ERK phosphorylation of Bim can inhibit Bim/Bax interaction,\(^{51}\) a finding consistent with a model whereby excess Bim in LPS-treated \( \mathbf{n f k b l} ^ {/-} \) B cells promotes cell death by directly binding and activating Bax. However, increased binding of A1 and Bcl-xL to Bim in LPS-activated B cells is compatible with models in which Bim only binds to prosurvival proteins. Although we have failed to detect Bim and Bax interactions in B cells (R. Grumont, unpublished data, February 2007), it is unclear whether this result is real or instead reflects technical issues, such as the low abundance of these complexes in primary cells or differences in their stability in particular detergents.\(^{52,53}\) Future work is required to resolve this issue and to ascertain the relationship between Bim phosphorylation and binding to Bcl-2–like proteins and whether multiple Bcl-2–like proteins...
proteins are necessary to protect B cells from TLR4-induced apoptosis.

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Authorship

Contribution: A.B. and S.G. designed research; A.B. and R. Grumont performed most of the experiments; R. Gugasyan did the FACS analysis on splenic B cells; C.W. did the macrophage survival analysis and A.B.A.S., and S.G. wrote the manuscript.

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NF-κB1 and c-Rel cooperate to promote the survival of TLR4-activated B cells by neutralizing Bim via distinct mechanisms

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