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Essential Role for Caspase-8 in Toll-like Receptors and NFκB Signaling*

Bénédicte Lemmers, Leonardo Salmena, Nicolas Bidère, Helen Su, Elzbieta Matysiak-Zablocki, Kiichi Murakami, Pamela S. Ohashi, Andrea Jurisicova, Michael Lenardo, Razqallah Hakem, and Anne Hakem

From the 1Ontario Cancer Institute, 2The Advanced Medical Discovery Institute, and the 3Department of Medical Biophysics, University of Toronto, Toronto, Ontario MSG 2C1, Canada, the 4Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892, and the 5Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario MSG 2X1, Canada

In addition to its pro-apoptotic function in the death receptor pathway, roles for caspase-8 in mediating T-cell proliferation, maintaining lymphocyte homeostasis, and suppressing immunodeficiency have become evident. Humans with a germline point mutation of caspase-8 have multiple defects in T cells, B cells, and NK cells, most notably attenuated activation and immunodeficiency. By generating mice with B-cell-specific inactivation of caspase-8 (bcasp8−/−), we show that caspase-8 is indispensable for B-cell development, but its loss in B cells results in attenuated antibody production upon in vivo viral infection. We also report an important role for caspase-8 in maintaining B-cell survival following stimulation of the Toll-like receptor (TLR)2, -3, and -4. In response to TLR4 stimulation, caspase-8 is recruited to a complex containing IKKB and its loss resulted in delayed NFκB nuclear translocation and impaired NFκB transcriptional activity. Our study supports dual roles for caspase-8 in apoptotic and non-apoptotic functions and demonstrates its requirement for TLR signaling and in the regulation of NFκB function.

Caspase-8 has been best characterized as a cysteine protease that cleaves specific substrates to transmit apoptotic signals downstream of death receptors (DR)1 (1–4). During DR signaling, procaspase-8 is recruited to the death-inducing signaling complex (DISC), where oligomerization drives its own activation by self-cleavage to form an activated caspase-8 tetramer complex in a process termed “proximity-induced activation” (5). Once activated, caspase-8 mediates apoptosis through a cell-specific type I or type II DR pathway. In type I cells, active caspase-8 at the DISC can directly process and activate caspase-3, leading to apoptosis. In type II cells, caspase-8 processes the BH3-only molecule Bid to form a truncated Bid molecule that translocates to the mitochondrial membrane and triggers cell death through the mitochondria (6).

In addition to their apoptotic roles, a new paradigm for caspases in non-apoptotic roles has become clear. In various studies, activation of caspases was detected in non-apoptotic-activated lymphocytes (7–10). Additionally, lymphocyte activation and proliferation was defective in the presence of selective caspases inhibitors (11–14). T-cell-specific deletion or overexpression of a dominant negative form of Fas-associated death domain protein (FADD), another DISC component, demonstrated its requirement for proper T-cell activation and IL2-dependent proliferation (15–18). Together these findings implicate caspases and other DISC components in non-apoptotic functions.

Until recently, embryonic lethality prevented the generation and phenotypic characterization of adult mice missing caspase-8, necessitating tissue-specific inactivation (19–21). We recently reported the conditional inactivation of caspase-8 in the T-cell lineage (tcasp8−/−) and identified important novel non-apoptotic roles for caspase-8 in maintaining peripheral T-cell homeostasis, in mediating expansion of T-cells upon in vitro activation, and in inducing an effective in vivo T-cell immune response against viral infections (20). Studies of human patients with a caspase-8 point mutation also support a non-apoptotic role for caspase-8. These patients are immunodeficient, show reduced activation of B, T, and NK cells in vitro, exhibit decreased levels of circulating antibodies and impaired immunoglobulin production in response to antigenic stimulation (22). Impaired activation of T, B, and NK cells from these patients was associated with defective NFκB nuclear translocation (23). In response to TCR stimulation of T cells, caspase-8 contributes to the formation of the activation receptor-induced signalosome (ARIS) that contains CARMA1-BCL10-MALT1 and the IkB kinase (IKK) complexes. Loss of caspase-8 impairs ARIS formation leading to defective NFκB signaling in response to TCR stimulation. Toll-like receptors are important components of innate immunity and provide a first-line of
defense against microbial infections. Binding of specific TLRs by their appropriate antigens activates B cells, induces their antigen-presenting ability, and stimulates the production of neutralizing antibodies (24, 25).

At least 10 members of the Toll-like receptor (TLR1–10) family have been identified in human, and 13 in mice (26). Examples of such antigens activating specific TLRs are: bacterial lipoprotein for TLR2, double-stranded viral RNA (or analog, poly(I:C)) for TLR3, bacterial lipopolysaccharide (LPS) for TLR4, flagellin for TLR5, single-stranded DNA for TLR7, and unmethylated CpG DNA for TLR9 (13, 27, 28).

The MHC class II molecules present the processed antigens on the cell surface for recognition by CD4+ T cells. TLRs recognize pattern recognition receptors (PRR) for TLR4, TLR3, TLR7, TLR8, and TLR9 that recognize bacterial and viral components. The TLR family have been identified in human, and 13 in mice (26).

B-cell Purification and Thymidine Incorporation—For all the experiments, B cells were purified by negative selection or selected by activated cell sorting (FACS). For negative selection, splenocytes were subjected to red blood cell lysis, depleted of macrophages using anti-rat IgG magnetic beads (Dynabeads, Dynal) following anti-CD11b and anti-Gr-1 (eBioscience) labeling. T cells were eliminated using anti-Thy 1.2-bound magnetic beads (Dynabeads, Dynal). All B-cell experiments were performed with purified B cells with a purity ≥90%, LPS (10, 1 and 0.1 μg/ml) (serotype 0111:B4; 055:B5; Sigma), CPG (1 μg/ml) (Coley Pharmaceutical ODN 1826/2138), and poly(IC) (100 μg/ml) (invivoGen). Cells were harvested at 24 or 48 h after an 18-h pulse with [3H]thymidine (1 μCi/well) (Amer sham Biosciences), and incorporation of [3H]thymidine was measured with a Matrix 96 direct β-counter system (Canberra Packard). Lipoteichoic acid (LTA) and ultrapure LPS (invivoGen) were used at 10 μg/ml and 5 μg/ml, respectively.

Cell Cycle and CFSE Analysis—For cell cycle analysis, purified B cells were fixed in 70% ethanol and stained with 5 μg/ml of propidium iodide as previously described (20). Purified splenic B cells (10⁶) were collected in serum-free medium and incubated with CFSE (Molecular Probes-5 μM) at 37 °C for 10 min. Following three washes in Iscove’s medium, 10% fetal calf serum, the CFSE-labeled cells were stimulated for 24, 48, and 72 h with LPS (10 μg/ml). For each time point, cells were stained for survival using 7-amino-actinomycin D (7-ADD), and the levels of CFSE incorporation determined by FACScalibur (Becton Dickinson).

Apoptosis—Purified B cells were stimulated with anti-IgM + IL4 (20 μg/ml, 5 ng/ml) for 3 days and then with CD95L (recombinant hCD8-mCD95L fusion protein 1 μg/ml) with or without cycloheximide (0.1 μg/ml) for 24 h. Apoptosis and cell death were measured after treatment using Annexin-V-FITC/PI Apoptosis Detection kit (R&D Systems) or 7-AAD (Sigma).

Vesicular Stomatitis Virus Infections—Mice were immunized with VSV-Indiana (2 × 10⁶ PFU, intravenously). After 4, 8, and 12 days post-infection, sera were collected, and neutralizing IgM and IgG immunoglobulin titers determined as described (36).

Immunofluorescence—Cells (10⁶) were used for cytospin centrifugation. Cytosin specimens of B cells were fixed with 4% PFA for 30 min at room temperature. Cells were incubated with 1:50 dilution of anti-p65 Ab (C-20, Santa Cruz Biotechnology) for 45 min at room temperature. Labeling was revealed using anti-rabbit-conjugated to fluorescein isothiocyanate (Jackson Immuno research). Cells were stained with Hoescht for 5 min and then mounted with Fluoromount (Southern Biotech). Images were collected on a Leica TCS-NT/SP confocal microscope (Leica Microsystems) using a ×63 oil immersion objective NA 1.32, zoom X.
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Immunoprecipitation and Western Blot Analysis—Total protein extracts from purified B cells were prepared as previously described (20). Immunoprecipitation was performed using protein A-Sepharose following incubation of cell extracts with anti-IKKαβ (H-470, Santa Cruz Biotechnology) and anti-caspase-8 (epitope SNKDDRNNGKQMP; amino acids 453–466 of murine caspase-8) antibodies. Optimal Ab concentrations and conditions for immunoprecipitation were determined in pilot studies. The separation of cytosol and nucleus was performed as previously described (23). Proteins were separated on 4–20% Tris-glycine gels (Novex).

The following antibodies were used for Western blotting in 5% powdered milk (Carnation) in PBS-T: rabbit polyclonal anti-IKKαβ, anti-phospho-IκBα, anti-phospho-p38 MAPK, anti-phospho-IκBα, anti-IκBα, anti-PARP, anti-phospho-p65 (S536), and anti-β-actin (Sigma), and anti-NFκB-p65 (Santa Cruz Biotechnology, C-20).

Real Time PCR—Cell-sorted B cells were stimulated with LPS (10 μg/ml) for 0, 2, 4, and 7 h. Cells were harvested and total RNA was made using the TRIzol reagent (Invitrogen). cDNA was made using the Invitrogen kit. The SYBR green kit from Applied Biosystems was used for real-time PCR. The following oligonucleotides were used for real-time PCR: GADPH (5′-AACAGGAAGCCCATCACCCT-3′ and 5′-GCCCTTCCACAATGCCAAAGTT-3′); TNF-α (5′-CCCTCAACACAGATCATTCTTTT-3′ and 5′-GCTACGACGTGGGCTACTT-3′); IL6 (5′-TAGTCCTTCTACCACCCAATTCC-3′ and 5′-TTGTGCCTTAGCCACTCTTCT-3′); INF-β (5′-CAGCTCCAAGAAAGGACGGAC-3′ and 5′-GGCGTGTAACTCTCTTCTTG-3′) and IP10 (5′-CAGATCATCTTCT-3′ and 5′-GGCTGCAGGGATGATTTCAA-3′). All PCR was performed on the Applied Biosystems 7900HT Fast Real Time PCR System.

RESULTS

To study the function of caspase-8 in B lymphocytes, mice carrying the caspase-8 mutation targeted to the B-cell lineage (casp8<sup>δδ</sup>/CD19<sup>Cre</sup>, also called bcas8<sup>−/−</sup>) were generated by crossing mice homozygous for the caspase-8-floxed allele (casp8<sup>fl/fl</sup>) (20) with mice expressing the Cre recombinase under the control of the B-cell-specific CD19 promoter (CD19<sup>Cre</sup>) (35). Southern blot analysis indicated the highly efficient deletion of caspase-8 gene in purified peripheral B cells from bcas8<sup>−/−</sup> mice (Fig. 1A). Western blot analysis of serial dilutions of protein extracts prepared from purified B cells confirmed the loss of caspase-8 protein in bcas8<sup>−/−</sup> cells (Fig. 1B).

Analysis by flow cytometry of cell subpopulations in bone marrow (BM), spleen, and lymph nodes (LN) did not show any differences between bcas8<sup>−/−</sup> mice and control littermates (Fig. 1C and data not shown). The proportion of T and B cells in LN and spleen and total lymphocyte numbers in thymus, spleen, LN, and BM were similar in control and bcas8<sup>−/−</sup> mice (Fig. 1D and data not shown). Therefore, unlike its function in the T-cell lineage, caspase-8 is not essential for the maintenance of B-lymphocyte homeostasis.

To confirm the functional deletion of caspase-8, we assessed the sensitivity of casp8<sup>−/−</sup> B cells to CD95-induced apoptosis. Purified peripheral B cells were activated in vitro for 3 days with anti-IgM/IL4, and then treated with Fas ligand, in the presence or absence of cycloheximide, an inhibitor of protein biosynthesis known to potentiate Fas killing (37). Annexin-V/PI staining demonstrated that while B cells from control mice were sensitive to CD95-induced killing, bcas8<sup>−/−</sup> cells were resistant (Fig. 1E). These results confirm the essential role for caspase-8 in DR-mediated cell death.

Vesicular stomatitis virus (VSV) infection in mice induces a primary infection phase, which consists of a T-cell-independent response characterized by the production of neutralizing IgM antibodies by B cells. The second phase of the infection,
which is T-cell-dependent, leads to the production of IgG immunoglobulin (36, 38). We investigated the role of caspase-8 in the in vivo B-cell response to VSV infection. Control and bcasp8−/− mice were subjected to intravenous immunization with 2 × 10⁶ PFU of VSV, and the production of VSV-neutralizing IgM and IgG antibodies was measured 4, 8, and 12 days later (39). While the levels of IgM antibodies directed against VSV were not affected in bcasp8−/− mice, the production of VSV-neutralizing IgG was reduced in bcasp8−/− mice (Fig. 2). This finding is consistent with the increased susceptibility of human patients deficient for caspase-8 to viral infections (22). These findings suggest that caspase-8 deficiency in B cells leads to impaired in vivo responses to VSV infection.

B cells are essential components of the innate immune response against microbial infection (24). TLR signaling is important for the activation of B cells and other components of the immune response by microbes (40). Given the connection between DISC components and TLR signaling (29–32), we investigated the effect of caspase-8 deficiency on TLR signaling. This was accomplished by treating purified B-cell populations with antigens specific for different TLRs. B-cell expansion was measured by [3H]thymidine incorporation following treatment with antigens specific for different TLRs. B-cell expansion was thus investigated by treating purified B-cell populations with antigens specific for different TLRs. B-cell expansion was measured by [3H]thymidine incorporation following treatment with antigens specific for different TLRs.

Cell cycle and cell death were analyzed in response to TLR4 stimulation to characterize the lack of expansion of LPS-stimulated casp8−/− B cells. Based on the PI-stained cell cycle profiles and the quantification of the sub-G₁ population, the defective expansion of casp8−/− B cells in response to TLR4 stimulation was attributed to increased cell death (Fig. 4A). Annexin-V/PI staining experiments were also performed and indicated that the increased cell death of TLR4 stimulated casp8−/− B cells was attributed to apoptosis (Fig. 4B). Apoptotic cell death was further confirmed by electron microscopy (Fig. 4, C–F) and increased caspase-3 processing in LPS-stimulated casp8−/− B cells compared with controls (data not shown). Together, these data suggest that caspase-8 is required for the activation of B cells and maintaining their survival in response to TLR3 and TLR4 stimulation.

Upon ligand binding, TLRs initiate signaling cascades that culminate in nuclear translocation of NFκB, and transcriptional activation of NFκB target genes (41–43). NFκB protein members include a family of Rel domain-containing proteins; e.g. p65 (Rel A), Rel B, c-Rel, p50 (NF-B1), and p52 (NF-B2) (44). Activation and nuclear translocation of NFκB result in increased transcription of its target genes such as TNF-α, IL6, IL1-α, IL12p40, and INF-β (45).

The IKK complex, composed of IKKα, β, and γ or NEMO plays an important role in mediating NFκB nuclear translocation in response to TLR (46). We therefore tested whether caspase-8 associates to this complex upon TLR4 activation.
Immunoprecipitation was performed using anti-IKK and/or anti-caspase-8 antibodies on protein extracts derived from untreated or LPS-stimulated casp8−/− B cells. These studies indicated that caspase-8 co-immunoprecipitates with IKK following 30 min of LPS stimulation of control B cells (Fig. 5A). The specificity of the IKK immunoprecipitations was shown by Western blot using anti-IKK antibodies. The recruitment of caspase-8 to a complex containing IKK in response to LPS stimulation was confirmed in 3T3-immortalized fibroblasts. As shown in Fig. 5B, IKK association to caspase-8 in 3T3 was detected as early as 4 min following TLR4 activation and appeared to be optimal at 7-min post-LPS stimulation. Thus, our data identify that TLR4 stimulation drives caspase-8 transient recruitment to IKKαβ, an important regulator of NFκB functions.

NFκB is an important downstream target of IKKαβ and its activation, in response to TLR4 stimulation, leads to transcriptional activation of a subset of target genes. Thus, the effect of caspase-8 mutation on TLR4-induced NFκB transcriptional activation was assessed using real time PCR to quantify the transcription levels of the NFκB target genes including IL6, TNF-α, IFN-β, and IP-10. cDNA, representative of total cellular mRNA, was prepared from cell-sorted control and casp8−/− B cells activated with LPS for 0, 2, 4, and 7 h. As expected, increased expression of these genes was observed in B cells after treatment with LPS; however, the level of transcriptional induc-
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FIGURE 5. A role for caspase-8 in NFκB signaling. A, caspase-8 is transiently recruited to IKK complex following LPS/TLR4 stimulation. Immunoprecipitation using anti-IKKαβ antibodies and cell extracts derived from casp8−/− and control B cells activated with LPS (10 μg/ml) for 5, 15, 30, and 60 min. Co-immunoprecipitation of caspase-8 and IKKαβ was revealed by Western blot analysis using anti-caspase-8 (upper panel) and anti-IKKαβ antibodies (lower panel). Total cell extract (+) and an irrelevant polyclonal antibody (−) were used as controls. B, recruitment of caspase-8 to IKKαβ complex in LPS-stimulated 3T3 fibroblasts. Association of IKKαβ with caspase-8 in 3T3 cell extracts prepared after 0, 3, 4, 5, 7, 8, and 10 min of LPS treatment was revealed by anti-IKKαβ Western blot on anti-caspase-8 immunoprecipitates (upper panel). The level of caspase-8 protein was assessed by Western blot analysis using anti-caspase-8 antibodies (lower panel). Total cell extract (+) was used as a control. C, defective transcriptional activation of NFκB target genes in bcasp8−/− B cells in response to LPS stimulation. Real time PCR was performed on cDNA derived from purified control and bcasp8−/− B cells activated by LPS for the indicated times. The expression of IL6, IFN-β, TNF-α, and IP-10 were assessed.

DISCUSSION

CASPASE-8 deficiency has been recently associated with human diseases. These diseases include immunodeficiency, cancer, and metastasis (22). Characterization of CASPASE-8 function in immunodeficient patients has demonstrated that in addition to its apoptotic functions, caspase-8 also carries important non-apoptotic functions.

In this study, we report important roles for caspase-8 in the regulation of NFκB function in response to TLR4 stimulation. We have previously reported that caspase-8 is dispensable for thymocyte development but required for peripheral T-cell homeostasis and T-cell survival in response to activation stimuli (20). Deletion of caspase-8 in early lymphoid progenitors was also reported to cause early differentiation arrest indicating its important role in early hematopoietic development (21). Similarly, deletion of caspase-8 in all hematopoietic cells using Vav-iCre transgenic mice that express Cre in early hematopoietic progenitors including T- and B-cell progenitors, results in embryonic lethality.4

Our present study demonstrates that CD19-Cre-mediated caspase-8 deletion in B lymphocytes does not affect B-cell development or homeostasis: B-lymphocyte subpopulations were similar in bcasp8−/− mice compared with controls. We also identified defective in vivo production of neutralizing antibodies, one of the main functions of B cells, in the absence of caspase-8 in B cells. Because helper T cells in bcasp8−/− mice express caspase-8 and are fully functional, the deficient immunoglobulin production following VSV infection of bcasp8−/− mice is likely a result of defects in (i) T/B-cell interactions, (ii) immunoglobulin switch, or (iii) B-cell expansion. Our present study supports a key role for caspase-8 in the immune response of B cells and for the proper production of pathogen-neutralizing antibodies.

Toll-like receptors are important components of innate immunity and provide a line of defense against microbial infections. Stimulation of specific TLRs induces a distinct pattern of

4 B. Lemmers, data not shown.
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![Diagram](image_url)

**FIGURE 6. Delayed NFκB nuclear translocation in LPS-stimulated bcasp8−/− B cells.** A. Western blots showing the levels of expression and phosphorylation of MAPp38 and IkBα following LPS stimulation (10 μg/ml) of purified control and bcasp8−/− B cells for 0, 0.5, 1, and 2 h. Levels of expression of casp8 and β-actin (loading control) are shown. B, confocal microscope representative merged views of the p65 subunit of NFκB (green) and Hoescht (red) from control and bcasp8−/− B cells stimulated with LPS for 0, 30, 45, 60, and 120 min. Data shown are representative of five independent experiments. C, percent of nuclear NFκB as measured by anti-p65 nuclear staining as in B. D, Western blot analysis of the levels of NFκB-p65 in cytoplasmic and nuclear fractions from LPS-stimulated purified B cells from bcasp8−/− and control mice. B cells were stimulated with LPS as in A, and cell fractions prepared and used for Western blots with anti-p65, anti-p65 Ser536, anti-PARP, and anti-actin.

gene expression, which leads to antigen-specific acquired immunity and activation of the innate immune response pathway. Caspase-8-interacting proteins such as FADD and c-FLIP, or their downstream responsive molecules such as RIP, have been implicated in the regulation of the signaling by some TLR such as TLR3 and TLR4 (29–32). TRAIL-R, known to induce apoptosis and to form a death signaling complex with FADD, and caspase-8 was reported recently to negatively regulate TLR2, TLR3, and TLR4 signaling in macrophages and BM-derived dendritic cells (47). Our study demonstrates that caspase-8 plays an essential role in B-cell activation and expansion in response to TLR2, TLR3, TLR4, but not TLR9.

In contrast to a recent study by Beisner et al. (48), our data support a role for defective NFκB signaling in the impaired response to TLR3 and TLR4 stimulation of B cells deficient for caspase-8. A possible explanation for this discrepancy is that we have analyzed the effect of caspase-8 loss on NFκB-p65 nuclear translocation at earlier time points post-TLR3 and -TLR4 stimulation. However, Beisner et al. have analyzed this effect at much later time points when the delay of NFκB-p65 nuclear translocation is not obvious anymore. Thus, similar to human B cells from caspase-8 mutant patients (23), loss of caspase-8 in mouse B cells impairs nuclear translocation of NFκB-p65.

TLR4 stimulations activate two major signaling pathways, both dependent on NFκB-mediated transactivation of genes encoding pro-inflammatory cytokines and survival molecules, among other immune mediators. One pathway is a MyD88-dependent and involves the sequential activation of IRAK-4, binding of TRAF6 to phospho-IRAK1, activation of AP-1 through the MAP kinase pathway, and NFκB nuclear translocation (49). This pathway induces the early phase of NFκB-mediated transactivation of genes including IL6 and TNF-α. The second LPS-activated pathway is MyD88-independent, and activates the late phase of NFκB-mediated gene expression, driving the production of IFN-β and the IFN-inducible gene IP-10 (50).

Our real-time PCR analysis identified that in response to TLR4 stimulation, casp8−/− B cells exhibit defective expression of early and late NFκB-dependent pro-inflammatory cytokines and other immune mediators. We have also identified a temporal delay in the nuclear translocation of NFκB-p65 in TLR4-stimulated casp8−/− B cells. Delayed NFκB nuclear translocation has been shown to compromise NFκB-mediated gene expression, and cell function upon activation (51). These observations highlight the importance of the delayed nuclear translocation of NFκB in casp8−/− B cells, in causing the defective production of inflammatory cytokines upon LPS treatment. Thus, we propose a model whereby caspase-8 is involved in the timely activation of NFκB downstream signals in response to TLR4 activation. In the absence of caspase-8, NFκB signaling in response to TLR4 activation is not blocked, but delayed. Nevertheless, this delay is sufficient to impair the transduction of NFκB survival signals in TLR4-stimulated caspase-8-deficient B cells.

Interestingly, we have also identified that in response to TLR4 stimulation, caspase-8 transiently associates with the IKKαβ, proteins important for NFκB signaling (52). The IKK complex is responsible for the phosphorylation of various substrates including NFκB-p65 in response to various stimuli.
including pro-inflammatory signals (52). Interestingly, we have identified that in response to LPS/TLR4 stimulation, caspase-8 transiently associates with the IKK complex. We also demonstrate that caspase-8 deficiency in TLR4-stimulated caspase-8-deficient B cells leads to decreased phosphorylation of NFκB-p65 at serine 536. This phosphorylation is mediated by the IKK complex, modulates NFκB nuclear translocation, and therefore, its impairment could contribute to the defective NFκB nuclear translocation and functions observed in TLR4-stimulated bcasp8-/- cells. Our findings support a role for defective NFκB signaling in the impaired responses to TLR4 activation in the absence of caspase-8 and provide a molecular mechanism for the immunodeficiency of caspase-8 mutant patients.

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