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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 dispensable 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 IKK*αβ*, 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 nonapoptotic 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)[3] (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 IκB 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

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[3] The abbreviations used are: DR, death receptor; LPS, lipopolysaccharide; FACS, fluorescent-activated cell sorter; VSV, vesicular stomatitis virus; LTA, lipoteichoic acid; DISC, death-inducing signaling complex; FADD, Fas-associated death domain protein; FLIP, FLICE-like inhibitory protein); RIP-1, receptor-interacting protein-1; LN, lymph node; BM, bone marrow; TLR, toll-like receptor; NK, natural killer cells; PFU, plaque-forming units.

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).

A link between TLR pathways and DISC components has been identified in recent studies of caspase-8, FADD, Flip (FLICE-like inhibitory protein), and RIP-1 (receptor-interacting protein-1) (29–32). Flip is recruited to the DISC and functions as an endogenous dominant-negative molecule by inhibiting FADD-caspase-8 interactions and thereby inhibits DR apoptosis (33). Deletion of FADD or FLIP in mouse embryonic fibroblats (MEFs) enhanced NFκB activation in response to LPS stimulation (29, 30), whereas RIP-1 deficiency resulted in decreased NFκB activation or survival following TLR3 or TLR4 stimulation, respectively (31, 32). The molecular mechanisms linking FADD, Flip, and RIP-1 with TLR pathways remain to be identified.

In this study, we have investigated the roles of caspase-8 in development, apoptosis, and proliferation of B lymphocytes. As expected, caspase-8-deficient B cells were resistant to CD95-mediated killing; however unlike T lymphocytes (20), we found that caspase-8 is not required for development and homeostasis of B lymphocytes. Our study identifies an impaired response of caspase-8-deficient B cells to TLR4 and links this defect to a delayed nuclear translocation of NFκB, and defective transcription of its target genes in the absence of caspase-8. We also report that loss of caspase-8 leads to reduced phosphorylation of p65 at serine 536, which is known to regulate p65 nuclear translocation (34). Finally, in response to TLR4 stimulation, caspase-8 was found to be transiently recruited to a complex that contains IKK$\alpha\beta$ thereby placing this caspase in a signaling complex important for cell survival and immune responses.

EXPERIMENTAL PROCEDURES

Mice—*Casp8*-conditional mutant mice (20) were intercrossed with *CD19Cre* transgenic mice (35) to obtain *bcasp8* mice. All mice studied were in a mixed 129/J × C57BL/6 genetic background and were genotyped by PCR (primer sequences and PCR conditions available upon request). Controls used in the different experiments correspond to either wild-type, *casp8*$^{fl/wt}$ *CD19-Cre*, or *CD19-Cre* mice. The former two genotypes produced similar results compared with the wild-type controls. Mice used for each experiment were littermates. All experiments were performed in compliance within the Ontario Cancer Institute Animal Care Committee Guidelines.

Flow Cytometry Analysis—Single cell suspensions prepared from thymus, spleen, lymph lodes (cervical, inguinal, axillary, brachial, and inguinal lymph nodes were pooled), and bone marrow from mice 8–12 weeks of age were stained with antibody at 4 °C in phosphate-buffered saline, 10% fetal calf serum (Invitrogen). The following antibodies were used to analyze cell populations: anti-CD4, anti-CD8, anti-Thy.1, anti-B220, anti-CD43, anti-IgM, anti-IgD, anti-CD21/35, and anti-CD23. Lymphocytes were analyzed by flow cytometry (FACScalibur; Becton Dickinson) with CellQuest software (Applied Biosystems). Cells were sorted using a FACSVantage flow cytometer (Becton Dickinson).

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 [^{3}H]thymidine (1 μCi/well) (Amersham Biosciences), and incorporation of [^{3}H]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 (10^6) 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 FACS analysis.

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 (2×10^6 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 (10^5) were used for cytospin centrifugation. Cytospin 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 Immunoresearch). Cells were stained with Hoescht for 5 min and then mounted with Fluoromount (Southern Biotec). Images were collected on a Leica TCS-NT/SP confocal microscope (Leica Microsystems) using a ×63 oil immersion objective NA 1.32, zoom X.

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-IKK$\alpha\beta$ (H-470, Santa Cruz Biotechnology) and anti-caspase-8 (epitope SNKDDRRNKGKQMP; 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-caspase-8 (epitope SNKDDRRNKGKQMP; amino acids 453–466 of murine caspase-8), rat monoclonal anti-caspase-8 antibody (kindly provided by Dr. A Strasser), anti-phospho-p38 MAPK, anti-phospho-IKBα, anti-IKBα, anti-PARP, anti-phospho-p65 (S536), and anti-β-actin (Sigma), and anti-NFkB-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′-AACAGGAAGCCCATCACCATCTT-3′ and 5′-GCCCTTCCACAATGCCAAAGTT-3′); TNF-α (5′-CCCTCACACTCAGATCATCTTCT-3′ and 5′-GCTACGACGTGGGCTACAG-3′); IL6 (5′-TAGTCCTTCCTACCCCAATTTCC-3′ and 5′-TTGGTCCTTAGCCACTCCTTC-3′), INF-β (5′-CAGCTCCAAGAAAGGACGAAC-3′and 5′-GGCAGTGTAACTCTTCTGCAT-3′) and IP10 (5′-CCAAGTGCTGCCGTCATTTTC-3′and 5′-GGCTCGCAGGGATGATTTCAA-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$^{fl/fl}$;CD19Cre,* also called *bcasp8$^{-/-}$*) were generated by crossing mice homozygous for the caspase-8-floxed allele (*casp8$^{fl/fl}$*) (20) with mice expressing the Cre recombinase under the control of the B-cell-specific CD19 promoter (*CD19Cre*) (35). Southern blot analysis indicated the highly efficient deletion of caspase-8 gene in purified peripheral B cells from *bcasp8$^{-/-}$* mice (Fig. 1*A*). Western blot analysis of serial dilutions of protein extracts prepared from purified B cells confirmed the loss of caspase-8 protein in *bcasp8$^{-/-}$* cells (Fig. 1*B*).

Analysis by flow cytometry of cell subpopulations in bone marrow (BM), spleen, and lymph nodes (LN) did not show any differences between *bcasp8$^{-/-}$* mice and control littermates (Fig. 1*C* and data not shown). The proportion of T and B cells in LN and spleens and total lymphocyte numbers in thymus, spleen, LN, and BM were similar in control and *bcasp8$^{-/-}$* mice (Fig. 1*D* 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.

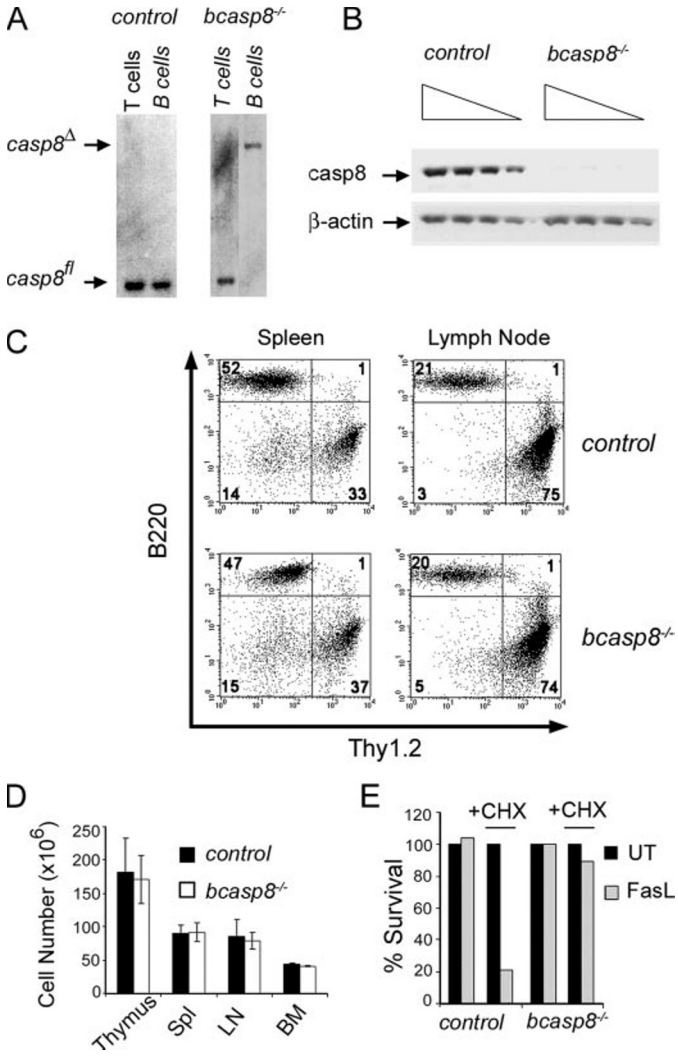

FIGURE 1. **Caspase-8 is dispensable for B-cell homeostasis but required for Fas killing.** *A*, Southern blot analysis of caspase-8 deletion in B cells. Caspase-8 floxed allele (*casp8^{fl}*) and mutant caspase-8 allele lacking exons 3 and 4 (*casp8$^{\Delta}$*) are indicated. *B*, Western blot of caspase-8 and actin (loading control) using 2-fold serial dilutions of cell extracts from purified B cells from control and *casp8$^{fl/fl}$ CD19-Cre* (*bcasp8$^{-/-}$*) mice. *C*, FACS analysis of B lymphocytes populations in spleen (*Spl*) and LN of *bcasp8$^{-/-}$* and control mice. *D*, cell numbers in thymus, Spl, LN, and BM. Data are presented as the mean ± S.D. of 12 mice for each genotype (*p* values are 0.32 for thymus, 0.48 for spleen, 0.7 for LN, and 0.5 for BM). *E*, Fas ligand (*FasL*) induced cell death in control and *bcasp8$^{-/-}$* B lymphocytes. The percent of surviving fraction is shown. *UT*, untreated; *CHX*, cycloheximide.

To confirm the functional deletion of caspase-8, we assessed the sensitivity of *casp8$^{-/-}$* 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, *bcasp8$^{-/-}$* cells were resistant (Fig. 1*E*). 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,

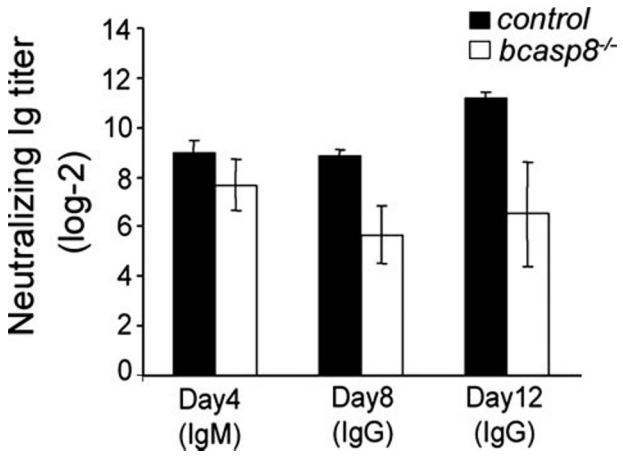

FIGURE 2. **Impaired immunoglobulin production in *bcasp8*$^{-/-}$ mice following VSV infection.** Levels of anti-VSV neutralizing IgM and IgG production are shown at days 4, 8, and 12 post-VSV infection of *bcasp8*$^{-/-}$ and control mice. *p* values are 0.1 for IgM at day 4, 0.01 for IgG at day 8, and 0.02 for IgG at day 12 post-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 2×10^6 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 [^{3}H]thymidine incorporation following treatment with increasing doses of different *Escherichia coli*-derived LPS, ultrapure LPS, CpG, LTA, or poly (I:C) to identify TLR4, TLR9, TLR2, and TLR3 responses, respectively. Whereas the stimulation by CpG consistently produced similar B-cell expansion in the control and *bcasp8*$^{-/-}$ backgrounds, responses of *casp8*$^{-/-}$ B cells to poly(I:C), LPS (Fig. 3*A* and supplemental Fig. S1), and LTA (supplemental Fig. S2) were reduced compared with controls, demonstrating that caspase-8 does indeed play a role in TLR2, TLR3, and TLR4 signaling. Similarly, CFSE labeling indicated defective expansion of *casp8*$^{-/-}$ B cells in response to LPS and poly(I:C) stimulation (Fig. 3*B* and data not shown). These defects could not be attributed to reduce TLR3 and TLR4 protein levels in bcaps8$^{-/-}$ cells as their cell surface expression levels comparable to controls (data not shown). No major defect in proliferation of *bcasp8*$^{-/-}$ B cells was observed in response to anti-IgM, anti-IgM, and IL4, or anti-IgM and anti-CD40 compared with wild-type controls (data not shown).

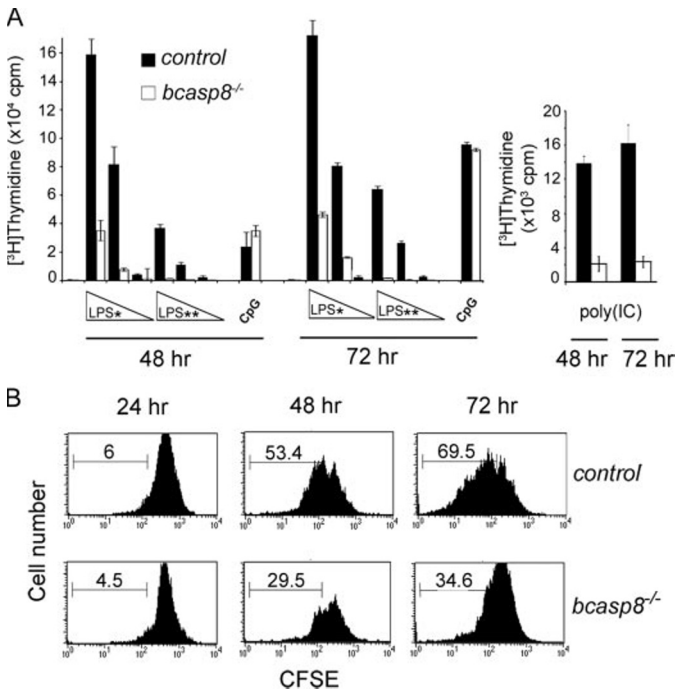

FIGURE 3. **Impaired TRL3- and TLR4-mediated B-cell activation in the absence of caspase-8.** *A*, reduced B-cell expansion of *bcasp8*$^{-/-}$ following LPS (TLR4) poly(I:C) (TLR3), but not CpG (TLR9) stimulation. Cell expansion was determined by measurement of [^{3}H]thymidine incorporation by purified B cells following LPS (*, 0111:B4; **, 055:B5), poly(I:C), and CpG activation. *B*, kinetics of CFSE loss following LPS activation. CFSE levels in *bcasp8*$^{-/-}$ and control B cells were evaluated by FACS at 24, 48, and 72 h following LPS activation. Percents of viable and dividing cells post-LPS stimulation are indicated.

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-G_1 population, the defective expansion of *casp8*$^{-/-}$ B cells in response to TLR4 stimulation was attributed to increased cell death (Fig. 4*A*). 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. 4*B*). 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.

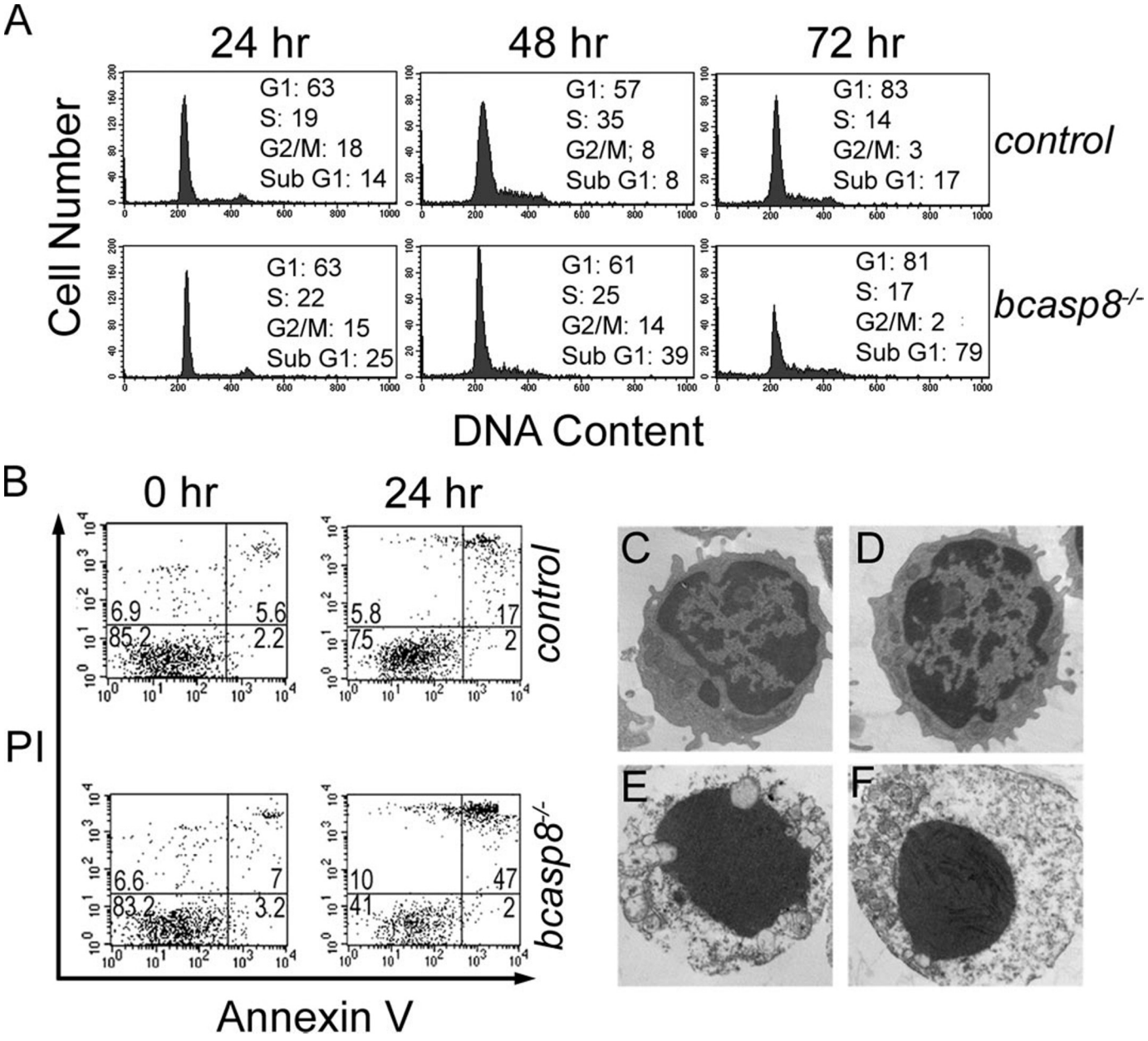

FIGURE 4. **Increased apoptosis of caspase-8-deficient B cells in response to LPS stimulation.** *A*, cell cycle progression and sub-G_1 populations were determined using propidium iodide staining and FACS analysis of LPS-stimulated (10 μg/ml) *casp8*$^{-/-}$ and control B cells. Increased cell death but no defects in cell cycle were observed. *B*, increased apoptosis in *casp8*$^{-/-}$ B-cells in response to LPS stimulation (10 μg/ml). Analysis was performed by FACS using Annexin-V/propidium iodide staining. *C–F*, electron microscopy of *casp8*$^{-/-}$ B cells before (*C* and *D*) or 24-h post-stimulation with LPS (*E* and *F*). Nuclear condensation, a feature of apoptosis is seen in *E* and *F*.

Immunoprecipitation was performed using anti-IKK$\alpha\beta$ and/or anti-caspase-8 antibodies on protein extracts derived from untreated or LPS-stimulated *casp8*$^{-/-}$ or control B cells. These studies indicated that caspase-8 co-immunoprecipitates with IKK$\alpha\beta$ following 30 min of LPS stimulation of control B cells (Fig. 5*A*). The specificity of the IKK$\alpha\beta$ immunoprecipitations was shown by Western blot using anti-IKK$\alpha\beta$ antibodies. The recruitment of caspase-8 to a complex containing IKK$\alpha\beta$ in response to LPS stimulation was confirmed in 3T3-immortalized fibroblasts. As shown in Fig. 5*B*, IKK$\alpha\beta$ 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 IKK$\alpha\beta$, an important regulator of NFκB functions.

NFκB is an important downstream target of IKK$\alpha\beta$ 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-

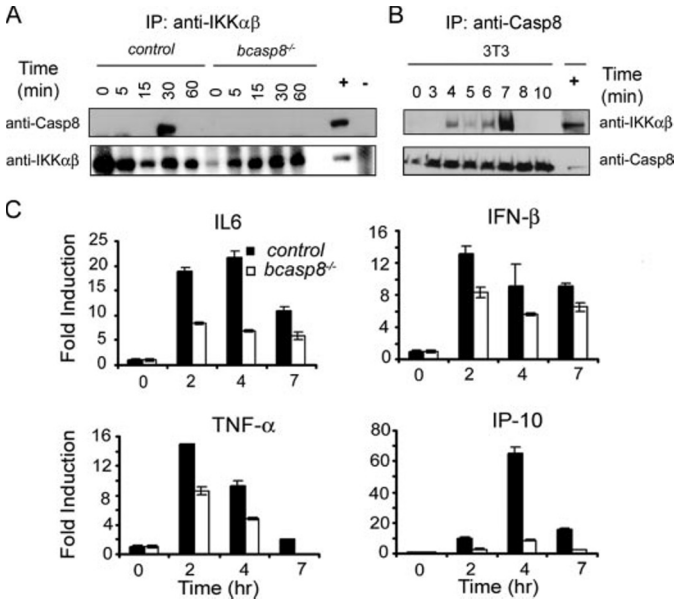

FIGURE 5. **A role for caspase-8 in NFκB signaling.** *A*, caspase-8 is transiently recruited to IKK complex following LPS/TLR4 stimulation. Immunoprecipitation using anti-IKK$\alpha\beta$ antibodies and cell extracts derived from *casp8*$^{-/-}$ and control B cells activated with LPS (10 μg/ml) for 5, 15, 30, and 60 min. Co-immunoprecipitation of caspase-8 and IKK$\alpha\beta$ was revealed by Western blot analysis using anti-caspase-8 (*upper panel*) and anti-IKK$\alpha\beta$ antibodies (*lower panel*). Total cell extract (+) and an irrelevant polyclonal antibody (−) were used as controls. *B*, recruitment of caspase-8 to IKK$\alpha\beta$ complex in LPS-stimulated 3T3 fibroblasts. Association of IKK$\alpha\beta$ 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-IKK$\alpha\beta$ 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.

tion of these genes over control was significantly lower in *casp8*$^{-/-}$ B cells (Fig. 5*C*). Therefore, caspase-8 is important for NFκB transcriptional activation in response to TLR4-stimulated B cells.

To investigate the mechanisms leading to the defective NFκB-mediated responses of TLR4-stimulated *casp8*$^{-/-}$ B cells, we focused on known signaling events downstream of LPS/TLR4 activation, including Akt, MAPK-p38, and IkBα phosphorylation. LPS stimulation of control and *casp8*$^{-/-}$ B cells for 30 min, 1 h, and 2 h showed comparable levels of expression and phosphorylation of Akt and MAPK-p38 (Fig. 6*A* and data not shown). Phosphorylation of IκBα results in its proteasome-mediated degradation followed by nuclear translocation and transcriptional activation of NFκB (44). Expression levels and phosphorylation of IkBα were similar in *casp8*$^{-/-}$ and control B cells at all time points after LPS stimulation (Fig. 6*A*).

To further investigate the mechanisms for the defective TLR4-induced NFκB transcriptional activation in *casp8*$^{-/-}$ B cells, nuclear translocation of the NFκB-p65 subunit was assessed by immunofluorescence using control and *casp8*$^{-/-}$ B cells stimulated with LPS for 0, 30, 45, 60, and 120 min. Similar to *controls*, NFκB-p65 was almost exclusively cytoplasmic in untreated *casp8*$^{-/-}$ B cells (Fig. 6, *B* and *C*). However, LPS-induced NFκB-p65 nuclear translocation was delayed in *casp8*$^{-/-}$ B cells at 30, 45, 60, and 120 min post-LPS treatment relative to controls (Fig. 6, *B* and C). Similarly, Western blot analysis of cytoplasmic and nuclear fractions from *bcasp8*$^{-/-}$ B cells and their controls confirmed a delayed LPS-induced nuclear translocation of NFκB-p65 in the absence of caspase-8 (Fig. 6*D*). Similar delayed NFκB-p65 nuclear translocation was observed in *bcasp8*$^{-/-}$ B cells in response to TLR3 stimulation (supplemental Fig. S3).

Phosphorylation of NFκB-p65 at serine 536 is known to play a major role in its nuclear translocation and transactivation (34), and therefore we assessed the effect of loss of caspase-8 on this NFκB-p65 phosphorylation. Interestingly, the level of phosphorylated NFκB-p65 at serine 536 was reduced in both cytoplasmic and nuclear fractions from LPS-stimulated *bcasp8*$^{-/-}$ B cells compared with controls (Fig. 6*D*). Therefore defective phosphorylation of NFκB-p65 at serine 536 could potentially contribute to the NFκB-impaired shuttling and function in LPS-stimulated *casp8*$^{-/-}$ B cells.

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.

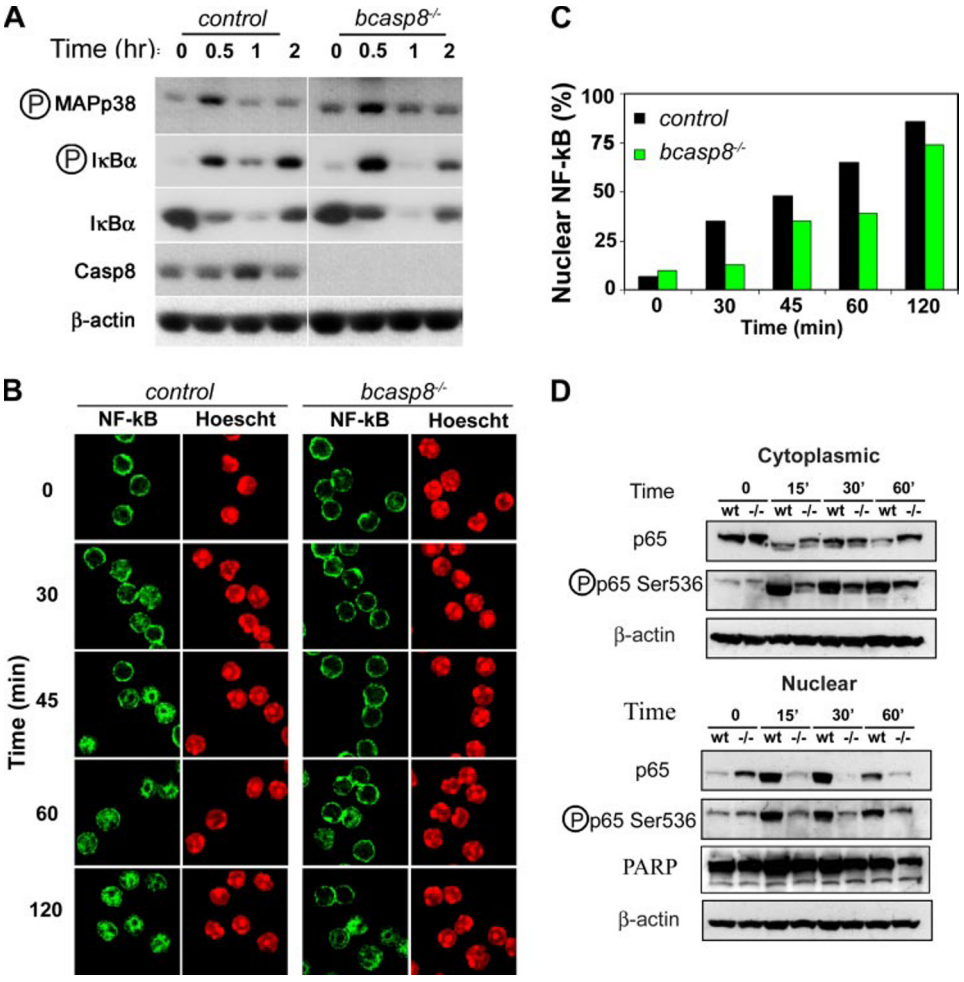

FIGURE 6. **Delayed NFκB nuclear translocation in LPS-stimulated *bcasp8*$^{-/-}$ B cells.** *A*, Western blots showing the levels of expression and phosphorylation of MAPp38 and IkBα following LPS stimulation (10 μg/ml) of purified control and *bcasp8*$^{-/-}$ B cells for 0, 0.5, 1, and 2 h. Levels 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 Ser^{536}, 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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