

## Cutting Edge: Innate Immunity Conferred by B Cells Is Regulated by Caspase-8<sup>1</sup>

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*Caspase-8 is an essential component of death receptor-mediated apoptosis. Along with Fas-associated death domain protein, it is also essential for T cell proliferation in response to antigenic or mitogenic stimuli. To determine whether caspase-8 is also required for B cell proliferation, we generated mice with a B cell-specific Casp8 deficiency. Unlike T cells, caspase-8 was not required for Ag receptor-driven proliferation or Ab formation. Rather, Casp8-deficient B cells failed to proliferate in response to dsRNA and LPS, ligands for TLR3 and TLR4, respectively, but responded normally to the TLR9 agonist CpG DNA. Similarly, Ab production to trinitrophenol-LPS was selectively reduced in B cell-specific Casp8-deficient mice. The activation of NF- $\kappa$ B or IFN regulatory factor 3 was found to be unaffected by the loss of caspase-8, implicating it in a novel pathway important for some forms of innate immunity mediated by B cells. The Journal of Immunology, 2005, 175: 3469–3473.*

The extrinsic pathway of cell death, mediated by death receptor signaling, is poorly characterized with respect to physiological lymphocyte activation and survival. Receptor ligation leads to the recruitment of an adaptor molecule, Fas-associated death domain protein (FADD),<sup>3</sup> along with caspase-8. The protease activity and subsequent self-processing of recruited caspase-8 sets off a proteolytic cascade that ultimately results in apoptosis (1). Enigmatically, FADD and caspase-8 are required for the survival of T cells undergoing activation. Loss of FADD or caspase-8 function results in aborted T cell activation as well as defective immune responses in vivo (2–7). In addition, human beings harboring a mutation in *Casp8* exhibit defects in T and B cell activation (8), and a recent study provides evidence associating a loss of caspase-8 with a deficit in NF- $\kappa$ B activation (9).

To examine the role of caspase-8 in B cell responses, mice with a B cell-conditional deficiency in *Casp8* were examined for Ag receptor and TLR-mediated activation. Results show that caspase-8 is necessary for several aspects of B cell activation re-

lated to innate immunity, and yet mouse B cells with a conditional mutation in *Casp8* showed no detectable loss of NF- $\kappa$ B activation as measured in four different ways.

### Materials and Methods

#### Mice

Exon 3 of *Casp8* was flanked by *loxP* recombination sites by homologous recombination, with details available upon request. *Cd19<sup>tm1(cre)Cgn</sup>* mice (abbreviated *Cd19-Cre*) were provided by Dr. R. Rickert (10). *Irf3*-deficient mice were generously provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan).

#### Cell culture and B cell isolation

Cell culture was performed as described elsewhere (6). Resting splenic B cells were purified magnetically by depletion with CD43, Thy1.2, and CD11b MACS beads (Miltenyi Biotec). The purity of these negatively selected cells for all experiments was determined to be >95% B220<sup>+</sup> by flow cytometry.

#### In vitro proliferation

Purified B cells were plated in triplicate at  $5 \times 10^4$  cells/well in 96-well plates and cultured with increasing concentrations of anti-CD40 (eBioscience), IL-4 (PeproTech), loxoribine (InvivoGen), anti-IgM F(ab')<sub>2</sub> (Jackson ImmunoResearch Laboratories), poly(I:C) (Sigma-Aldrich), certified endotoxin-free poly(I:C) (InvivoGen), CpG ODN1826/2138 (Coley Pharmaceuticals), or LPS (Sigma-Aldrich) for 2 days. Proliferation assessed by thymidine incorporation or CFSE dilution was performed as described previously (6).

#### Flow cytometry

Cells were stained for 20 min at 4°C using FITC-, PE-, PerCP-, allophycocyanin-, and biotin-conjugated Abs against B220, CD4, CD23, CD69, CD80, CD86 (eBioscience), CD5, CD8, CD21, CD43, IgD, IgM, IA<sup>b</sup>, streptavidin (BD Biosciences), CD3, annexin V (Caltag Laboratories), and 7-aminoactinomycin D (7AAD; Molecular Probes). TUNEL staining was performed as described previously (6).

#### Western blotting and EMSA

Western blotting using whole cell and nuclear extracts was conducted according to methods described previously (6, 11). The following Abs were used: I $\kappa$ B $\alpha$ , p65, STAT1, phospho-Akt, phospho-Erk, poly(ADP-ribose)polymerase, Bcl-x<sub>L</sub> (Cell Signaling Technology), receptor-interacting protein (BD Biosciences),  $\beta$ -tubulin (Upstate Biotechnology), and phospholipase C $\gamma$ 1 (Santa Cruz Biotechnology). Anti-caspase8 was a gift from Idun Pharmaceuticals in San Diego, CA. Gel shift assays were performed on nuclear extracts from untreated cells or cells stimulated with 100  $\mu$ g/ml poly(I:C) for the indicated amounts of time as described previously (11).

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<sup>3</sup> Abbreviations used in this paper: FADD, Fas-associated death domain protein; 7AAD, 7-aminoactinomycin D; TNP, trinitrophenol; WT, wild type; KO, knockout.

### Immunizations and Ab assays

Mice were immunized with 25  $\mu$ g trinitrophenol (TNP)-Ficoll, 25  $\mu$ g TNP-LPS, or 100  $\mu$ g TNP-OVA (Biosearch Technologies) in 100  $\mu$ g CFA (Fisher Scientific). TNP-specific Ig was assayed by ELISA as described previously (12). HRP-conjugated rat anti-mouse IgM, IgG1, and IgG2a were purchased from BD Pharmingen. Plates were developed using the Super AquaBlue ELISA substrate system (eBioscience). Each plasma sample was titrated, and the curves were modeled by a two-parameter logit function (13). The plotted values are equal to the plasma dilutions, resulting in a constant ELISA value of 20% of maximum.

## Results and Discussion

### Generation of B cell-specific Casp8-deficient mice

To generate Casp8-deficient B cells, Casp8<sup>fl/+</sup> mice were crossed to Cd19<sup>tm1(cre)Cgn</sup> mice (Cd19-Cre) (10). To assess caspase-8 deletion, B cells were purified from the spleens of Casp8<sup>fl/fl</sup>;Cd19-Cre mice along with Casp8<sup>fl/fl</sup> mice as controls and assessed by immunoblot for caspase-8 (Fig. 1A). As representative of eight experiments, deletion appeared to be almost complete in B cells while remaining at wild-type (WT) levels in T cells.

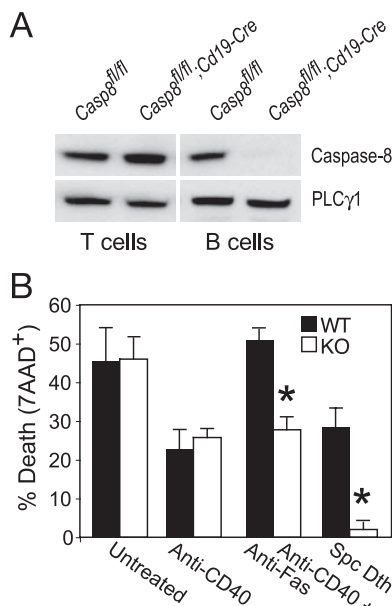
Previous work has shown that caspase-8 is required for Fas-induced apoptosis in many cell types, including T cells (5, 7, 14). To determine whether caspase-8 is also required for Fas-induced apoptosis in B cells, purified splenic B cells were first stimulated with anti-CD40 to induce the expression of Fas, followed by the addition of agonistic anti-Fas Ab. Cell viability was then determined by counting (data not shown) as well as flow cytometry (Fig. 1B). The results showed that Fas-mediated apoptosis was completely abrogated in Casp8-deficient B cells.

To determine whether caspase-8 deficiency had an effect on B cell development, primary and secondary lymphoid organs were collected and B cell compartments were assessed by flow

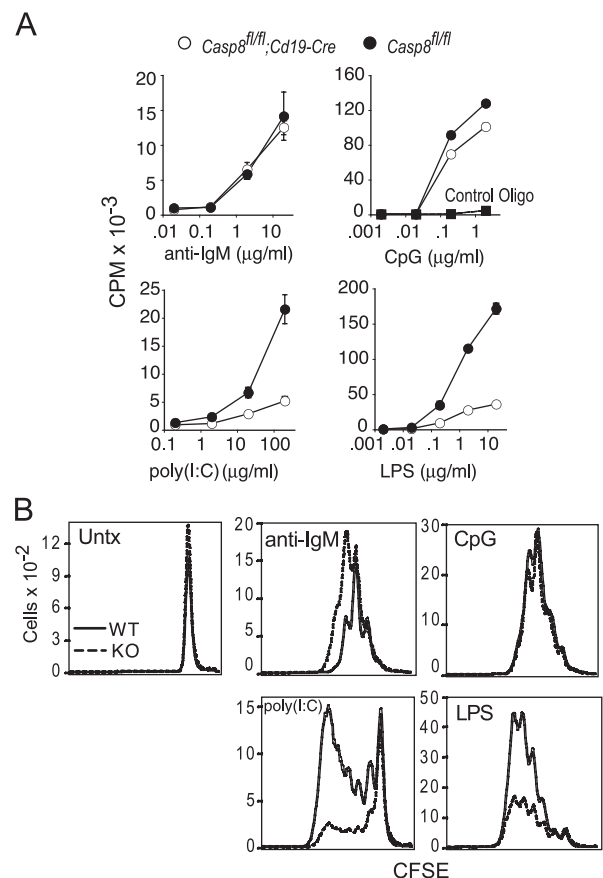
cytometry. There were normal proportions of Pre/Pro (B220<sup>+</sup>, IgM<sup>-</sup>) and immature B cells (B220<sup>+</sup>, IgM<sup>+</sup>) in the bone marrow and modest reduction in B1 cells (B220<sup>low</sup>CD5<sup>low</sup>) in the peritoneal cavity (WT = 19%  $\pm$  2.9% vs knockout (KO) = 9.6%  $\pm$  3.4%). The total number of spleen cells from Casp8-deficient mice was increased an average of 30% (103  $\times$  10<sup>6</sup>,  $n$  = 17 WT vs 134  $\times$  10<sup>6</sup>,  $n$  = 26 KO,  $p$  < 10<sup>-7</sup> Student's  $t$  test), yet the proportion of mature B cells in the spleen (CD23<sup>+</sup>CD21<sup>low</sup>) was unchanged. Consistent with the analysis of B1 cells in the peritoneum, there was a reduction in the percentage of splenic marginal zone B cells (CD23<sup>-</sup>CD21<sup>+</sup>, WT = 5.9%  $\pm$  1.2% vs KO = 3.5%  $\pm$  1.9%). At most, there appears to be a modest disproportion of mature B cells over marginal zone B cells in Casp8-deficient mice.

### Caspase-8 is dispensable for Ag receptor-induced proliferation but required for proliferation induced by specific TLR ligands

Previous studies have shown that caspase-8 is essential for Ag-mediated T cell proliferation (5). To determine whether B cells were similarly affected, purified splenic B cells were cultured with anti-IgM F(ab')<sub>2</sub> and proliferation was measured by [<sup>3</sup>H]thymidine incorporation and the dilution of CFSE. By either criterion, Casp8-deficient B cells were indistinguishable from controls (Fig. 2).



**FIGURE 1.** Generation of B cell-specific caspase-8 KO mice. *A*, Western blot analysis for caspase-8 in T and B cells confirms cell type-specific deletion of Casp8. *B*, B cells were cultured in the presence or absence of anti-CD40 and anti-Fas, and the proportion of 7AAD<sup>+</sup> cells was enumerated by flow cytometry. Specific death (Spc Dth) refers to the percentage of dead cells in anti-CD40-treated cultures subtracted from the percentage in anti-CD40- plus anti-Fas-treated cultures. The data depict the average of three experiments and the error bars represent the SD from the mean. The asterisk indicates statistical significance ( $p$  < 0.0001, Student's  $t$  test).



**FIGURE 2.** Ligand-specific defects in B cell proliferation in the absence of caspase-8. *A*, B cell proliferation was assessed by [<sup>3</sup>H]thymidine incorporation after 48 h of culture with increasing concentrations of the indicated stimuli. The data represent the mean of triplicate cultures and the bars indicate the SD from the mean. The data are representative of eight experiments. *B*, B cell proliferation was also assessed by the dilution of CFSE. The  $y$ -axis is scaled to reflect cell number for each culture condition. Untx, Untreated.

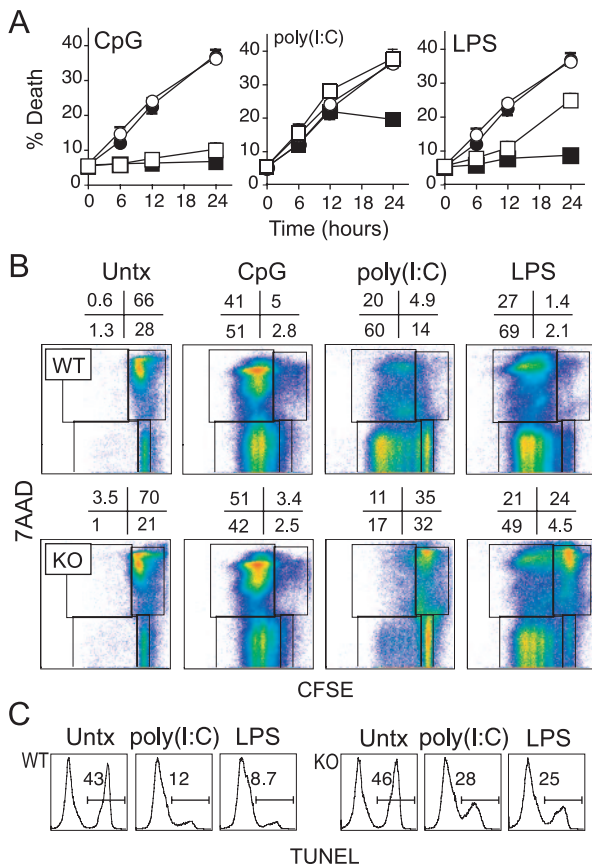
Additionally, B cells can be induced to proliferate by other stimuli including several pathogen-associated molecular patterns as well as through the ligation of CD40. DNA synthesis and cell division of *Casp8*-deficient B cells were unchanged compared with control cells stimulated with CD40, CpG oligonucleotides, and loxoribine (Fig. 2 and data not shown). However, a significant reduction in the proliferative response was observed in *Casp8*-deficient B cells in response to LPS and dsRNA (Fig. 2). These results indicate that caspase-8 is selectively required for activation of B cells by specific pathogen-associated molecular patterns, while being largely dispensable for activation via other stimuli.

#### *Caspase-8 is required for survival signals induced by dsRNA and LPS*

To determine whether caspase-8 is paradoxically responsible for transducing a survival signal, B cells from *Casp8<sup>fl/fl</sup>;Cd19-Cre* or *Casp8<sup>fl/fl</sup>* mice were cultured with or without a mitogenic stimulus and cell death was monitored by 7AAD staining (Fig. 3A). B cells cultured in medium alone exhibited a time-dependent increase in cell death that was not affected by the absence of

caspase-8. This cell death was almost completely overcome by the addition of CpG oligonucleotides, and the rescue was not dependent on caspase-8. In contrast, *Casp8*-deficient B cells stimulated with dsRNA failed to receive a survival signal, while control B cells exhibited a significant decrease in cell death at 24 h. *Casp8*-deficient B cells stimulated with LPS exhibited early survival that waned after 12 h. In either case, the requirement for caspase-8 only became apparent after 12 h of activation.

To understand the progression of cell death with respect to cell division, we combined CFSE dilution with 7AAD staining and analyzed the results as a bivariate flow cytometric plot (Fig. 3B). Similar to the results shown above, the majority of B cells cultured in medium alone died during the 72-h culture period, and, again, this was independent of caspase-8 (Fig. 3B). B cells from *Casp8<sup>fl/fl</sup>;Cd19-Cre* mice stimulated through TLR9 or the Ag receptor underwent proliferation and cell death at a rate similar to B cells from control mice (Fig. 3B and data not shown). However, *Casp8*-deficient B cells stimulated with dsRNA or LPS exhibited a substantial increase in cell death, and this cell death appeared to have occurred before cell division based on CFSE dilution. To determine whether the cell death occurring in *Casp8*-deficient B cells was consistent with apoptosis, DNA fragmentation was analyzed by TUNEL staining. An increase in DNA fragmentation was observed in *Casp8*-deficient B cells stimulated with LPS or dsRNA compared with controls, while stimulation through the B cell receptor resulted in similar levels of DNA fragmentation (Fig. 3C). These data demonstrate that caspase-8 plays an essential role in the survival of B cells stimulated with dsRNA and LPS, while being dispensable for survival signals transmitted by other stimuli.



**FIGURE 3.** Decreased survival of dsRNA and LPS-activated B cells in the absence of caspase-8. *A*, Cell death was measured by 7AAD after stimulation with CpG oligonucleotides (1  $\mu$ g/ml), LPS (2  $\mu$ g/ml), or poly(I:C) (100  $\mu$ g/ml) at the indicated time points represented by unstimulated cells (● and ○) and cells cultured with various stimuli (■ and □). ○ and □, *Casp8*-deficient B cells; ● and ■, control B cells. Error bars indicate the SD from the mean, and the data are representative of three experiments. *B*, B cell proliferation and survival were simultaneously determined by combining CFSE and 7AAD. Purified B cells were cultured with medium alone, CpG (2  $\mu$ g/ml), poly(I:C) (100  $\mu$ g/ml), or LPS (10  $\mu$ g/ml) for 72 h. *C*, DNA fragmentation was assessed by TUNEL assay on cells stimulated with poly(I:C), CpG oligonucleotides, or LPS for 24 h. Untx, Untreated.

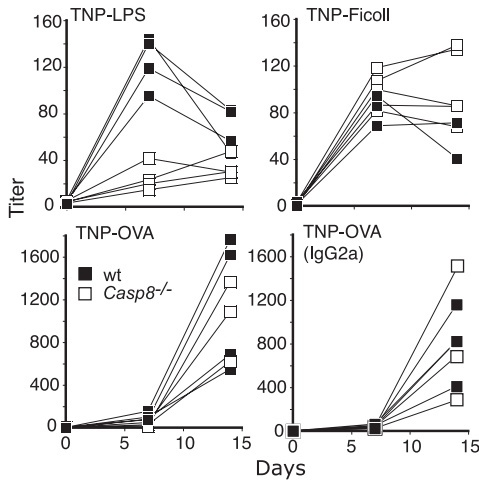
#### *Caspase-8 is required for T-independent type I Ab response*

The primary *in vivo* function of B cells is to produce Abs to promote both innate and adaptive immunity. To determine whether caspase-8 is also required for *in vivo* Ab responses, cohorts of *Casp8<sup>fl/fl</sup>;Cd19-Cre* mice along with *Casp8<sup>fl/fl</sup>* controls were immunized with LPS, Ficoll, or OVA haptenated with TNP. Plasma titers were determined at 7 and 14 days postimmunization. Consistent with the results presented above, *Casp8<sup>fl/fl</sup>;Cd19-Cre* mice mounted an inferior IgM response to the T-independent Ag TNP-LPS (Fig. 4, upper left). By contrast, the B cell-specific caspase-8 deficiency did not affect the TNP-specific Ab responses to the T cell-independent Ag TNP-Ficoll (Fig. 4, upper right) or the T cell-dependent Ag TNP-OVA (Fig. 4, lower right). In addition, there was no difference detected in the amount of IgG2a produced by *Casp8<sup>fl/fl</sup>;Cd19-Cre* mice, demonstrating that caspase-8-deficient B cells can carry out T cell-dependent and Ag-specific class switching (Fig. 4, lower right).

#### *TLR signaling in the absence of caspase-8*

A number of signaling cascades have been characterized for the response to dsRNA and LPS through TLR3 and TLR4, respectively. The most prominent pathways shared by these two receptor-ligand pairs are the activation and nuclear localization of NF- $\kappa$ B and IFN regulatory factor 3. In this study, NF- $\kappa$ B activation was measured in four separate ways. The loss of I $\kappa$ B was analyzed by Western blot (15), and the results reveal that I $\kappa$ B degradation occurred similarly in control and *Casp8*-deficient B





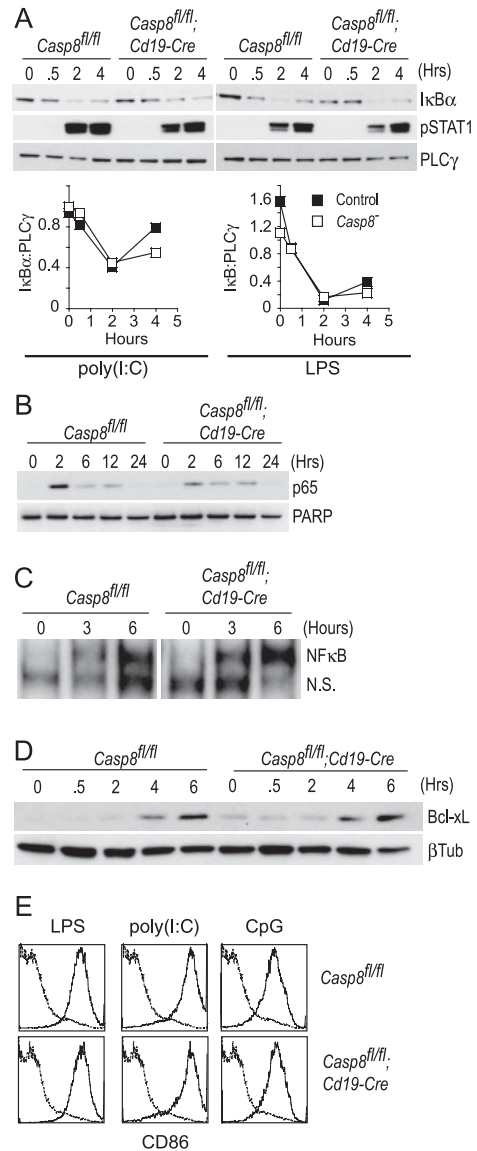
**FIGURE 4.** TNP-specific Ab responses in the absence of caspase-8. Cohorts of *Casp8<sup>fl/fl</sup>/Cd19-Cre* mice along with controls were immunized with TNP-LPS (A), TNP-Ficoll (B), and TNP-OVA (C) in CFA. TNP-specific Ab levels were determined by ELISA.

cells stimulated with LPS or dsRNA (Fig. 5A). Upon degradation of  $\text{I}\kappa\text{B}$ , NF- $\kappa\text{B}$  dimers localize to the nucleus, and RelA/p65 is abundantly present in these complexes. In five experiments stimulating with either LPS or poly(I:C), we found a large nuclear localization of p65 with no consistent defect found for *Casp8*-deficient B cells (Fig. 5B).

NF- $\kappa\text{B}$  DNA-binding activity can also be measured directly by EMSA, and, again, NF- $\kappa\text{B}$  DNA binding induced by dsRNA and LPS was unaffected by the deletion of *Casp8* (Fig. 5C and data not shown). As a fourth measure, NF- $\kappa\text{B}$  activity can be assessed by the induction of NF- $\kappa\text{B}$  target genes (16, 17), and both Bcl- $x_L$  (Fig. 5D) and  $\text{I}\kappa\text{B}\alpha$  (data not shown) were induced to a similar extent in WT and *Casp8*-deficient B cells. In addition, B cells uniformly induced CD86, CD80, and MHC class II in response to dsRNA, LPS, or CpG oligonucleotides, and this induction was not affected by a *Casp8* deficiency (Fig. 5E and data not shown).

One of the major consequences of IFN regulatory factor 3 translocation to the nucleus is the production of IFN- $\beta$ . IFN- $\beta$  in turn binds to its receptor, which leads to the phosphorylation and activation of STAT1. As depicted in Fig. 5A, STAT1 was phosphorylated after treatment with LPS or dsRNA in both control and *Casp8*-deficient cells. Consistent with these data, *Irf3*-deficient B cells showed no defect in their response to either LPS or poly I:C (data not shown). Phosphorylation of Akt and Erk1,2 was likewise unaffected by *Casp8* deletion (data not shown).

Our results differ in almost every respect from those recently published by Su et al. (9). Although their studies were mainly focused on T cells, experiments showed that human B cells, deficient in caspase-8 expression, did not exhibit p65 nuclear localization stimulated by either anti-IgM or LPS. In these experiments, the localization of p65 was assayed by fluorescence microscopy. The experiments described in this report show that B cells, with a *Casp8* deficiency, respond normally to stimuli that involve Ag receptor-mediated activation. Neither proliferation in culture nor Ab production in vivo was affected by a loss of caspase-8. In contrast, there was a marked reduction in cellular responses associated with LPS and dsRNA activation, both in culture and in TNP-LPS-specific Ab responses; yet, we de-



**FIGURE 5.** Signal transduction in the absence of caspase-8. A,  $\text{I}\kappa\text{B}$  and STAT1 tyrosine phosphorylation were determined by Western blot after stimulation with 100  $\mu\text{g/ml}$  poly(I:C) or 10  $\mu\text{g/ml}$  LPS for the indicated amount of time. A separate experiment was analyzed by densitometry and plotted as the ratio of  $\text{I}\kappa\text{B}:\text{PLC}\gamma$  vs time in culture. B, Nuclear translocation of p65 in response to 100  $\mu\text{g/ml}$  poly(I:C) was tracked by subcellular fractionation and Western blot. C, Gel shift assays were performed on nuclear extracts derived from 100  $\mu\text{g/ml}$  poly(I:C)-stimulated cells using a probe specific for NF- $\kappa\text{B}$ . D, Bcl- $x_L$  expression was monitored by Western blot after stimulation with 100  $\mu\text{g/ml}$  poly(I:C) for the indicated amount of time. E, Costimulatory molecule up-regulation was assessed by stimulation for 24 h with LPS (2  $\mu\text{g/ml}$ ), poly(I:C) (100  $\mu\text{g/ml}$ ), CpG oligonucleotides (1  $\mu\text{g/ml}$ ), or medium alone. Expression was determined by gating on B220<sup>+</sup> cells and background is expression by cells cultured in medium alone.

tested no consistent deficiency in NF- $\kappa\text{B}$  activation to any of the stimuli tested. Consistent with these results, preliminary data show that macrophages from *Casp8;LysM-Cre* mice produce a normal TNF response to LPS. Other than a possible difference between human and mouse B cells, we cannot resolve these differences at present.

Notwithstanding the wide-ranging and detailed analyses on the role of caspases in cell death (18–20), a role for caspase-8 as

a survival factor in lymphocyte activation is not easily understood. Although caspase-8 may constitute part of the signaling cascade that promotes NF- $\kappa$ B nuclear localization through a potential interaction with c-FLIP (21) or I $\kappa$ B $\alpha$  (22), we find that it is dispensable for the hallmarks of NF- $\kappa$ B activation described above. It has also recently been shown to affect hemopoietic and nonhematopoietic differentiation (7) and the proliferation of hemopoietic progenitor cells (23). One possibility is that caspase-8 is required for initial entry into the cell cycle, and cells that fail this “checkpoint” die. Whether caspase-8 is necessary for survival or cell cycle regulation, it is clearly an essential and pleiotropic signaling molecule that affects cell physiology in ways that have yet to be fully appreciated.

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## Disclosures

The authors have no financial conflict of interest.

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