c-Rel Is Required for the Protection of B Cells from Antigen Receptor-Mediated, But Not Fas-Mediated, Apoptosis

Alexander M. Owyang, Joseph R. Tumang, Brian R. Schram, Constance Y. Hsia, Timothy W. Behrens, Thomas L. Rothstein and Hsiou-Chi Liou

*J Immunol* 2001; 167:4948-4956; doi: 10.4049/jimmunol.167.9.4948

http://www.jimmunol.org/content/167/9/4948

---

**References**

This article cites 48 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/167/9/4948.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
c-Rel Is Required for the Protection of B Cells from Antigen Receptor-Mediated, But Not Fas-Mediated, Apoptosis

Alexander M. Owyang,* Joseph R. Tumang,‡ Brian R. Schram,† Constance Y. Hsia,* Timothy W. Behrens,§ Thomas L. Rothstein,‡‡ and Hsiou-Chi Liou*‡

The NF-κB/Rel transcription factor family has been shown to protect many cell types from apoptotic signals. However, it is not known whether NF-κB is required for all survival pathways and whether each NF-κB member plays a unique or a redundant role. Here we describe the results of studies on the role of c-Rel in survival. Mature B cells from c-Rel−/− mice exhibit defects in survival, including sensitivity to Ag receptor-mediated apoptosis as well as increased sensitivity to ionizing radiation and glucocorticoids. Transgene expression of Bcl-xL, a c-Rel target gene, rescues c-Rel−/− B cells from their survival defects. Thus, c-Rel-dependent survival pathways are crucial for protection from apoptotic signals that target the mitochondrial pathway. Despite a lack of Bcl-xL, c-Rel−/− B cells can still be rescued from Fas-mediated apoptosis via B cell receptor signaling. The Fas apoptosis inhibitor molecule and FLICE inhibitory protein (c-FLIP) proteins are up-regulated normally in c-Rel−/− B cells, and these two molecules may play a more physiological role in the Fas pathway. Furthermore, unlike the TNF sensitivity of RelA−/− fibroblasts, c-Rel-deficient fibroblasts are refractory to TNF-mediated cell death. Thus, c-Rel is dispensable for protection against death receptor-mediated apoptosis. Taken together, our data suggest that distinct NF-κB/Rel members are required for protecting cells from different types of apoptotic signals. The Journal of Immunology, 2001, 167: 4948–4956.

Apoptosis is an important cellular process that is required for tissue remodeling as well as for maintaining homeostasis (1). In the immune system apoptosis also provides an important mechanism for eliminating potentially autoreactive lymphocytes (2). Proapoptotic signals can be generated by ligation of death receptors such as TNF and Fas or by death receptor-independent cytotoxic agents, such as glucocorticoids (dexamethasone), ionizing irradiation, and chemotherapeutic drugs. The death signals can be integrated directly or indirectly via mitochondria, leading to the eventual activation of caspase proteolytic pathways and disruption of cell integrity. Survival proteins such as the anti-apoptotic Bcl-2 family members, inhibitor of apoptosis proteins, FLICE inhibitory protein (c-FLIP) (3), and Fas apoptosis inhibitor molecule (FAIM) (4) are used by cells to counteract the death pathways.

B lymphocytes are subject to apoptotic regulation by many death signals, including the B cell Ag receptor, Fas, gamma irradiation, and glucocorticoids (5). For example, the B cell lymphoma lines WEHI231 and CH31 have been used extensively in the studies of anti-IgM-induced cell death of immature B cells (6–8). Alternatively, activation of mature B cells by CD40 up-regulates Fas expression and can sensitize them to Fas-mediated apoptosis. Such CD40-sensitized Fas-mediated death was found to be protected by Ag receptor signaling (reviewed in Ref. 9). Subsequently, several survival proteins, including Bcl-xL, FAIM, and c-FLIP, were shown to be responsible for anti-IgM-mediated protection mechanisms (4, 10, 11).

NF-κB/Rel, a family of dimeric transcription factors, has been shown to protect many cells from apoptotic signals (reviewed in Ref. 12). For example, the RelA knockout mouse fails to develop beyond embryonic day 15 and is characterized by massive apoptosis in the liver (13). This death was shown to be mediated mostly by TNF-α, as RelA/TNF-α double knockout mice are rescued from embryonic lethality and have normal livers (14). There are also examples where NF-κB/Rel was found to be pro-apoptotic. For example, NF-κB/Rel was implicated in the apoptosis of CD4+CD8+ double positive thymocytes, as double positive thymocytes expressing the IκBα super-repressor were protected from anti-CD3-mediated apoptosis (15). In embryonic fibroblasts, RelA was shown to play a role in Fas-induced death (16), and in NIH-3T3 cells it was shown to be important for expression of Fas as induced by TNF-α (17). Thus, the contribution of NF-κB/Rel in either protecting or inducing apoptosis has to be considered in the context of stimuli, cell types, and differentiation stages of the cells.

Much effort has been focused on characterizing the pro-survival genes that are up-regulated by NF-κB/Rel. Some of the genes identified include TNFR-associated factor 1, TNFR-associated factor 2, the Bcl-2 family members Bcl-xL (18, 19) and Bcl-2 (20, 21), FAS-L, and the inhibitor of apoptosis proteins (22). However, not all these molecules are definitive candidates for anti-apoptotic NF-κB targets. While several studies have established a link among NF-κB, Bcl-xL, and survival (18, 19), others have not (21–23). These findings are complicated by the fact that the results were obtained in transformed cell lines of different tissue types, using various means of activating or inhibiting NF-κB. Therefore, additional studies are necessary to sort out the specific regulatory

*Division of Immunology, Department of Medicine, Weill Graduate School of Medical Sciences, Cornell University, New York, NY 10021; Departments of ‡Microbiology and §Medicine, Boston University School of Medicine, Boston, MA 02118; and †Center for Immunology, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455

Received for publication May 8, 2001. Accepted for publication August 27, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grants CA68155 and T32AI07621.

2 Address correspondence and reprint requests to Dr. Hsiou-Chi Liou, Weill Medical College, Cornell University, 515 East 71st Street, Room 5-210, New York, NY 10021. E-mail address: hcliou@med.cornell.edu

3 Abbreviations used in this paper: c-FLIP, FLICE inhibitory protein; EF, embryonic fibroblast; FAIM, Fas apoptosis inhibitor molecule; PI, propidium iodide; RDA, representational difference analysis; Tg, transgenic.

Copyright © 2001 by The American Association of Immunologists 0022-1767/01/$02.00

Downloaded from http://www.jimmunol.org/ by guest on April 16, 2017
relationships among activating signals, NF-kB/Rel, and survival proteins in the context of primary B lymphocytes.

c-Rel is a lymphoid-specific member of the NF-κB/Rel family; thus, c-Rel knockout (c-Rel−/−) mice provide a unique system in which to study the requirements for NF-κB/Rel in immune cells. Previous studies have shown that c-Rel-deficient B lymphocytes fail to receive anti-IgM-mediated activation, proliferation, and survival signals (24, 25). Furthermore, Myc-transformed c-Rel-deficient cell lines are still sensitive to apoptosis when cross-linked with anti-IgM (21). In this case, however, it is unclear whether the death sensitivity is due to the transformation by Myc.

In the present study we address the following questions. 1) Is c-Rel required for protection from all, or just selective, types of apoptosis in B lymphocytes? 2) What are the survival genes regulated by c-Rel in primary B cells that are responsible for this protection? Since transformed B cell lines may not faithfully reflect the survival requirements of their untransformed counterparts, we decided to examine the role of c-Rel in the modulation of survival/apoptosis using primary splenic B cells derived from c-Rel knockout mice. Here we show that c-Rel−/− B cells, when compared with wild-type cells, are more sensitive to anti-IgM-, γ irradiation-, and dexamethasone-induced death in the presence of survival signals such as LPS or anti-CD40. We also confirm Bcl-xL, and A1/Bfl1 as bona fide c-Rel target genes in mature splenic B cells. By introducing a Bcl-xL transgene into the c-Rel−/− background, we were able to rescue the survival defect in B cells. Interestingly, we find that c-Rel−/− B cells have no defect in their ability to be rescued from CD40-sensitized Fas-mediated apoptosis. These results indicate that while c-Rel and its target genes are required for B cell protection against certain forms of programmed cell death, c-Rel and Bcl-xL may not play as large a protective role in the Fas pathway. The implications of these findings in the context of the regulation of apoptosis are discussed.

Materials and Methods

Mice

c-Rel−/− mice were generated in the C57BL/6 background as described previously. They were subsequently crossed with the B cell-restricted Bcl-xL transgenic mice (26) (from T. W. Behrens), c-Rel−/−;Bcl-xL transgenic (Tg) F1 mice were intercrossed to generate the c-Rel−/−;Bcl-xL Tg F2 progeny.

Cell culture and proliferation assays

B cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, and 50 μM 2-ME. B cells were purified by complement-mediated lysis in the presence of J11 (anti-Thy1.2; a gift from Dr. J. Nikolic-Zugic, Sloan-Kettering Institute, New York, NY), GK1.5 (anti-L3T4), and 3.115 (anti-Ly2) to remove T cells. Enriched B cell populations were >95% B220+ (22). For proliferation assays, B cells were plated at 104 cells in 96-well U-bottom plates in triplicate. Where indicated, cells were stimulated with 5 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), 10 μg/ml goat anti-mouse IgM (Fab’2) (anti-IgM, Jackson ImmunoResearch Laboratories, West Grove, PA), or 10 μg/ml anti-CD40 (mAb 1C10 provided by Drs. M. Howard (Corixa, Seattle, WA) and A. Heath (University of Sheffield Medical School, Sheffield, U.K.). Before the indicated time points, cultures were incubated with 0.5 μCi [3H]thymidine (Amersham, Arlington Heights, IL). Cells were then harvested, and incorporation into DNA was quantified by scintillation.

T cells

The T cell clone 2C2 was provided by Dr. A. Houghton. T cells were cultured in RPMI 1640 medium containing 10% FBS, 1% penicillin-streptomycin, 1 mM nonessential amino acids, and 50 μM 2-ME. They were maintained by stimulating with irradiated splenocytes pulsed with pigeon cytochrome c.

Representational difference analysis (RDA)

RDA was performed essentially as described by Hubank and Schatz (27). Splenocytes from wild-type and c-Rel−/− mice were stimulated with anti-

CD40 for 4 h in the presence of 5 μg/ml cycloheximide. Total RNA was isolated using STAT-60 (Tel-Test, Friendswood, TX), and poly(A+) RNA (isolated with Oligotex, Qiagen, Chatsworth, CA) from these populations was then used to generate cDNA. Three rounds of RDA were performed using wild-type representations as the tester population. Difference product 3 was cloned into pBSKII+. Dot blots yielded 18 independent clones, which were sequenced and compared with the nucleotide and dbEST databases. Inducibility of message was verified by RNase protection.

RNase protection assay

Bcl-xL and A1/Bfl1 message induction were initially assayed with the mApo-2 RNase protection assay kit (PharMingen, San Diego, CA), according to the manufacturer’s protocol. RNA used was isolated from purified B cells stimulated for 4 h with the indicated reagent in the presence of 10 μg/ml cycloheximide. Five micrograms of total RNA from each condition were used as samples in the assay. Relative induction of Bcl-xL and A1/Bfl1 was determined by phosphorimager analysis, normalized to L32 and GAPDH.

Western blot

Whole cell lysates were generated by lysing cells in 1× RIPA buffer and sonicitating four times with five pulses of a Branson 250 microtip sonicator (VWR International, West Chester, PA) at 20% duty cycle. Supernatants were separated from pellets and stored at −80°C. Protein concentrations were determined by Bradford assay. Twenty to 30 μg whole cell lysate were loaded onto SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a semidy method. Blots were probed with the following Abs diluted into 1% nonfat milk and Tris-buffered saline (TBS) containing 0.05% Tween 20: rabbit

FIGURE 1. Tissue-specific differences in the sensitivity of c-Rel−/− cells to apoptosis. a, c-Rel−/− embryonic fibroblasts are resistant to TNF-induced apoptosis. EFs from wild-type and c-Rel knockout mice were plated at a density of 3.1 × 104/ml in the presence of the indicated concentration of TNF-α. L929 cells were plated at a density of 7.6 × 104/ml. Cells were counted in trypan blue 3 days later. b, c-Rel−/− B lymphocytes are sensitized to cell death by B cell Ag receptor. B cells from wild-type (solid lines and filled symbols) and knockout (dotted lines and open symbols) mice were cultured in 96-well plates in the presence of medium alone (squares) or with 10 μg/ml anti-IgM (algM; triangles). Samples were collected at 0, 24, 48, and 72 h and were stained with PI. Cell viability was assessed by flow cytometry as described. Cells with DNA content <2 N were considered apoptotic, and cells with DNA content equal to or greater than 2 N were taken to be nonapoptotic, or viable. Error bars indicate the SD for triplicate samples. Lack of error bars indicates that the deviation is smaller than the graph symbol.
anti-human/mouse Bcl-xL (S-18, sc-634, Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-human/mouse c-FLIP (D-2; Alexis Biochemicals, San Diego, CA), rabbit polyclonal raised against full-length FAIM, or anti-mouse actin (Sigma). HRP-conjugated anti-rat or anti-rabbit secondary Ab was purchased from Amersham. The ECL Plus chemiluminescence detection system was used to visualize Western blots (RPN 2132, Amersham). In all experiments equal protein loading was controlled for by stripping blots in 63 mM Tris-HCl (pH 6.8), 2% SDS (w/v), and 100 mM sodium chloride on the immunoblot for Sigma or was verified using nonspecific signals on the immunoblot for comparison.

Cell cycle and apoptosis analysis
B cells were cultured at 5 × 10^5 cells/well in 96-well flat-bottom plates for 2–3 days. Where indicated, cells were treated with 5 μg/ml LPS, 10 μg/ml anti-IgM, or 10 μg/ml anti-CD40. For the irradiation experiments, cells were preirradiated for 18 h before treatment. ^1Ci (Gammacell 1000; MDS Nordion, Kanata, Ontario, Canada) was used as the source of gamma irradiation. Dexamethasone stocks were dissolved in ethanol. The ethanol concentration in culture was 0.004%. At the indicated time points, cells were collected and stained with a solution containing 50 μg/ml propidium iodide (PI), 20 μg/ml RNase A, 0.1% Triton X-100, and 0.1% sodium citrate. Apoptosis was assessed by flow cytometry to quantify the percentage of cells with <2 N DNA content.

Chromium release assays
B cells were stimulated with anti-CD40 for 2 days, with or without overnight stimulation with IgM on the last day. Cells were loaded with 100 μCi sodium chromate (^51Cr) for 1 h at 37°C, washed three times, and counted. B cells were distributed in 96-well plates at 5000 cells/well, and 2C2 T cells were added at E:T ratios of 20:1, 10:1, 5:1, and 2.5:1. Con A (Sigma-Aldrich) was added at 2.5 μg/ml. Plates were incubated for 4 h in a 37°C incubator, then spun for 5 min at 1000 rpm. Supernatants were collected and then assayed with a gamma counter. The percent specific lysis was calculated as: 

\[
\text{Percent specific lysis} = \frac{(\text{Cr release} \times \text{minimum release})}{(\text{maximum release} - \text{minimum release})} 
\]

Results
c-Rel-deficient embryonic fibroblasts (EFs) are resistant to TNF-α-induced cell death
The importance of NF-κB in mediating survival signals was highlighted by studies in the RelA knockout (28). RelA-deficient EFs are exquisitely sensitive to TNF-induced cell death. In contrast, c-Rel-deficient EFs are highly resistant to TNF-induction of cell death. L929 cells were sensitive at the lowest dose used (Fig. 1a). Thus, c-Rel is not specifically required to protect fibroblasts from the effects of TNF-α. One possible explanation is that RelA, which is absolutely required for TNF resistance, could compensate for the lack of c-Rel in EF cells. These data thus suggest that each NF-κB/Rel member plays a distinct death protection role depending upon its expression levels in certain cell types and its response to specific stimuli.

c-Rel-deficient B cells are sensitized to Ag receptor-mediated apoptosis
The predominant expression of c-Rel in the lymphoid and myeloid lineages would predict that it may play a significant role in lymphocytes. We therefore chose to focus our apoptosis studies on B cells. Previous studies indicated that c-Rel^-/-^ B cells fail to be rescued from spontaneous apoptosis by B cell receptor (anti-IgM) signals (25, 29). Our results confirm this finding, and show that, compared with wild-type cells, primary B cells from c-Rel^-/-^ mice are extremely sensitive to anti-IgM-induced death (Fig. 1b). In fact, they exhibit more apoptosis than cells cultured in medium alone, indicating a key role for c-Rel in maintaining the survival of anti-IgM-stimulated cells.

c-Rel-deficient B cells are more sensitive to γ irradiation-induced apoptosis
Resting B cells are exquisitely sensitive to low doses of γ irradiation, and they are protected only after several hours of activation by stimuli such as LPS (30). To further explore the role of c-Rel in survival signaling, B cells from wild-type and c-Rel^-/-^ mice were compared for triplicate samples.

![FIGURE 2. c-Rel^-/-^ B cells are more sensitive to γ irradiation-induced death. B cells from wild-type (solid lines and symbols) and knockout (dotted lines and open symbols) mice were cultured in 96-well plates. After 18 h of prestimulation with medium (a), LPS (b), anti-IgM (c), or anti-CD40 (d), cells were exposed to 0 rad (triangles), 250 rad (squares), or 500 rad (data not shown). Samples were collected at the indicated times, stained with PI, and assessed by flow cytometry. Error bars indicate the SD for triplicate samples.](http://www.jimmunol.org/Downloadedfrom)
were cultured with medium alone, LPS, anti-IgM, or anti-CD40 overnight and then exposed to 0, 250, or 500 rad. Unstimulated wild-type and c-Rel\(^{-/-}\) B cells are equally sensitive to gamma irradiation. Two days after exposure to 250 rad, the viability of the cultures is \(<20\%\) (Fig. 2a). Overnight stimulation with LPS, anti-IgM, or anti-CD40 is able to significantly rescue wild-type B cells from this induced apoptosis (Fig. 2, b–d; compare with Fig. 2a). The ability of these stimuli to rescue is radiation dose-dependent (data not shown). c-Rel\(^{-/-}\) B cells, however, are greatly impaired in their response to these survival signals even at 250 rad, the lowest dose tested. Importantly, c-Rel is required for anti-CD40- or LPS-mediated protection from gamma irradiation (Fig. 2, b and d). As expected, anti-IgM treatment cannot rescue these c-Rel\(^{-/-}\) B cells, and irradiation promotes even greater levels of apoptosis (Fig. 2c). We hypothesize that LPS, anti-IgM, and anti-CD40 exert their protective effects by inducing c-Rel to activate the transcription of anti-apoptotic genes, as all three stimuli have been shown to induce c-Rel nuclear translocation and DNA binding (31, 32).

c-Rel is required for protection against dexamethasone-induced death

We next studied the effect of the glucocorticoid dexamethasone on the survival of c-Rel\(^{-/-}\) B cells. Glucocorticoids are widely used as immunosuppressive agents, and their mechanism of action has been proposed to be via modulation of transcription and/or promotion of apoptosis in lymphocytes (33). Wild-type and c-Rel\(^{-/-}\) B cells were cultured with medium alone, LPS, or anti-CD40, and dexamethasone (100, 200, or 400 nM) was added to the cultures immediately after addition of the indicated stimuli. Although dexamethasone does not further increase spontaneous apoptosis (Fig. 3c), it decreases the survival response of wild-type B cells to LPS and anti-CD40 (Fig. 3, a and b). Significantly, c-Rel\(^{-/-}\) B cells were more sensitive to the suppressive effect of dexamethasone on survival. Thus, c-Rel is also critical for maintaining viability in the presence of dexamethasone.

**Bcl-x\(_L\) is a c-Rel target gene in primary B lymphocytes**

To elucidate the molecular mechanisms responsible for c-Rel’s role in survival, we decided to identify c-Rel-regulated target genes. RDA (27) of cDNA from wild-type and c-Rel\(^{-/-}\) cells was employed. Three rounds of subtractive hybridization and amplification yielded several independent clones. One of the clones was identical to mouse Bcl-x, and this particular representation corresponded to the 3’-untranslated region (34, 35). From this experiment it was not possible to determine whether Bcl-x\(_L\) or Bcl-x\(_S\) message was the source of this representation. To clarify this point and to test expression levels, we performed RNase protection assays, Western blots, and flow cytometry.

In wild-type cells, Bcl-x\(_L\) message is highly induced within 4 h by anti-IgM and anti-CD40 (Fig. 4a). Anti-IgM induces Bcl-x\(_L\) message 2- to 3-fold over the medium control, while anti-CD40 induces the message 9- to 10-fold. c-Rel-deficient B cells have a lower basal level of message, and anti-IgM and anti-CD40 fail to induce Bcl-x\(_L\) to normal levels. As determined by migration distance, the form induced is clearly the anti-apoptotic long form, or Bcl-x\(_L\). Other experiments confirmed that Bcl-xL message is also induced by LPS, but not in c-Rel\(^{-/-}\) B cells (data not shown).

Al/BII1, another member of the Bcl-2 family of proteins, is induced in both wild-type and c-Rel\(^{-/-}\) cells, but levels in c-Rel\(^{-/-}\) cells are significantly lower (Fig. 4a). This is in agreement with a previous report (21). Bax expression remains relatively constant and is not affected by loss of c-Rel. Bcl-2 message is below background in this assay.

We then assessed the level of Bcl-x\(_L\) protein that is induced in B cells by LPS, anti-IgM, or anti-CD40 (Fig. 4b). Unstimulated B cells show low amounts of Bcl-xL protein. In agreement with the RNase protection data, Bcl-x\(_L\) is strongly induced in wild-type B cells by all three stimuli (Fig. 4b, lanes 3, 5, and 7). c-Rel\(^{-/-}\) B cells, on the other hand, are severely deficient in induction of Bcl-x\(_L\) (Fig. 4b, lanes 4, 6, and 8). The pro-apoptotic short form of Bcl-x, Bcl-x\(_S\), was not detected under any conditions (data not shown). These data thus identify Bcl-x\(_L\) as a bona fide c-Rel target gene in primary B lymphocytes.

**Transgene expression of Bcl-x\(_L\) can rescue the c-Rel\(^{-/-}\) survival defects**

To assess the role of Bcl-x\(_L\) in B cell survival, we crossed Bcl-x\(_L\) transgenic mice into the c-Rel\(^{-/-}\) background. These mice (\(-/-\) Tg) appeared normal, although spleen cellularity for both \(+/-\) Tg and \(-/-\) Tg mice was approximately double that of wild-type mice (data not shown). The Bcl-x\(_L\) transgene was able to rescue...
the c-Rel-deficient B cells from anti-IgM-induced death (Fig. 5a). However, it did not correct the proliferation defects described previously (data not shown).

Importantly, Bcl-x\textsubscript{L} was also able to protect c-Rel\textsuperscript{-/-} B cells from gamma irradiation-induced death (Fig. 5b) or dexamethasone-induced death (Fig. 5c), in collaboration with LPS or anti-CD40. For example, when irradiated at 250 rad, anti-CD40-stimulated wild-type B cells are still 73% viable, while c-Rel\textsuperscript{-/-} cells are only 50% viable (Fig. 5b, middle panel). \textminus/- Tg B cells are 68% viable, similar to wild-type cells, and +/- Tg cells are 78% viable, slightly more than wild-type cells.

At the same time, wild-type B cells stimulated with LPS and treated with 200 nM dexamethasone are 65% viable, and c-Rel\textsuperscript{-/-} cells are only 37% viable (Fig. 5c, top panel). However, \textminus/- Tg B cells are 67% viable. In the presence of the transgene, the viability of c-Rel\textsuperscript{-/-} B cells approaches that of wild-type cells. Thus, replacement of the c-Rel target gene Bcl-x\textsubscript{L} can compensate for the loss of c-Rel.

c-Rel and Bcl-x\textsubscript{L} are not required for protection against Fas-mediated apoptosis

The Fas molecule is a key cell surface receptor involved in lymphocyte apoptosis (36). Previous studies have shown that Ag receptor engagement can protect B cells from cell death induced by anti-Fas Ab or by Fas ligand-bearing Th1 T cells (37, 38). This protection is thought to be mediated partly by Bcl-x\textsubscript{L}, because the protein is strongly up-regulated by anti-IgM stimulation with kinetics similar to those of Fas resistance, and because overexpression of Bcl-x\textsubscript{L} can also confer resistance. Thus, in our c-Rel-deficient system, we sought to address the role of c-Rel and Bcl-x\textsubscript{L} in resistance to Fas-mediated cell death.

For this study we sought to reproduce earlier work (37) performed with BALB/c cells (H-2\textsuperscript{b}) and to verify it in the C57BL/6 background (H-2\textsuperscript{b}). In this system stimulation of B cells with anti-CD40 causes up-regulation of surface Fas, which then sensitizes them to Fas-mediated apoptosis via Fas ligand-bearing Th1 T cells. We used the 2C2 T cell clone, which has previously been used to specifically kill Fas-bearing targets of H-2\textsuperscript{b} background (39, 40).

CD40 stimulation up-regulated Fas surface expression equivalently in wild-type and c-Rel\textsuperscript{-/-} B cells (data not shown) and sensitized them to Fas-mediated killing in a dose-dependent manner (Fig. 6a). Surprisingly, anti-IgM signaling was able to rescue both types of B cells from this apoptosis despite the fact that Bcl-x\textsubscript{L} was still deficient in c-Rel\textsuperscript{-/-} B cells (Fig. 6b, lanes 7 and 8). Thus, other molecules that are independent of c-Rel regulation must be mediating the protection.

Two other molecules recently shown to play a role in protection of B cells from Fas-induced death are c-FLIP (11) and FAIM (4). Thus, we sought to investigate the roles of these two proteins in our system. In agreement with previous reports c-FLIP is up-regulated by costimulation with anti-CD40 and anti-IgM (Fig. 6d, lane 4). Interestingly, c-Rel\textsuperscript{-/-} B cells also up-regulated c-FLIP as well as the wild-type cells (Fig. 6d, lane 8). There also appears to be some induction with CD40 alone in the c-Rel\textsuperscript{-/-} cells (Fig. 6d, lane 7).

FAIM was also up-regulated at comparable levels in both wild-type and c-Rel\textsuperscript{-/-} B cells (Fig. 6c). The induction of both c-FLIP and FAAM correlates kinetically with protection from Fas-mediated cell death. These results suggest that the presence of these two molecules in c-Rel\textsuperscript{-/-} B cells could account for this protection.

Discussion

In this study we have examined the role of c-Rel in apoptosis protection in primary B lymphocytes (Fig. 7). We show that c-Rel is indeed crucial for protecting B cells from Ag receptor-mediated death signals. It is also an important component of CD40- and
LPS-mediated survival pathways for preventing death induced by ionizing irradiation and glucocorticoids. Furthermore, we show that c-Rel-mediated protection is partly achieved through the induction of Bcl-xL and A1/Bl1, as supported by the rescuing effect of a Bcl-xL transgene. Interestingly, however, c-Rel-deficient B lymphocytes, which lack Bcl-xL expression, are still able to receive anti-IgM/anti-CD40 costimulatory survival signals in the protection from CD40-sensitized Fas-mediated apoptosis. Taken together, our studies reveal the significance of c-Rel in protecting from some, but not all, types of apoptosis. This emphasizes that the role of a given NF-κB/Rel member in death protection needs to be tested and verified in the context of cell types and specific inducing signals.

One intriguing phenomenon observed in c-Rel−/− B lymphocytes is their death response to anti-IgM signals. While anti-IgM signals are known to trigger proliferation or survival of wild-type lymphocytes, c-Rel−/− B lymphocytes show a death response to anti-IgM signals. This indicates that c-Rel is not essential for the survival signals induced by anti-IgM, but its absence renders the cells susceptible to death, suggesting a role in regulating the balance between survival and death signals.
B cells, the same signals instead induce profound apoptosis of B cells that lack c-Rel. This appears to reflect an active cell death process, since the extent of cell death is higher than that in the medium control (Fig. 1b). This finding implies that anti-IgM ligation can simultaneously trigger both survival and apoptotic signals in mature B lymphocytes. In wild-type B cells the anti-IgM survival signals are mediated in part by c-Rel and its downstream survival proteins, thus protecting them from apoptotic signals. In contrast, in the absence of c-Rel and its survival proteins, the anti-IgM-mediated pro-apoptotic pathway becomes predominant. Therefore, c-Rel is one of the critical components in mature B cells to ensure the proper response to antigenic signals.

In addition to the importance of c-Rel in anti-IgM-mediated responses, our experiments with γ irradiation and dexamethasone-induced death have also uncovered a further requirement for c-Rel in survival responses induced by LPS or anti-CD40. Thus, c-Rel activation can counteract environmental as well as regulatory death signals. We have identified Bcl-xL, as a c-Rel target gene and demonstrated its ability to rescue c-Rel−/− B cells from programmed cell death mediated by γ irradiation, dexamethasone, and anti-IgM (Fig. 5). The protective effect of Bcl-xL correlates well with data showing that these forms of apoptosis are integrated at the level of mitochondria, which controls the release of cytochrome c and the ultimate activation of pro-caspase 9. In support of this view, caspase-9-deficient thymocytes were shown to be resistant to dexamethasone-, γ irradiation-, and etoposide-induced cell death (41).

It is interesting that c-Rel and Bcl-xL are both dispensable for protection of B cells against Fas-mediated cell death. Previously, we and others have found that Bcl-xL, when overexpressed, can protect B cells from Fas-mediated apoptosis (10, 38). Because anti-IgM can induce high levels of Bcl-xL, this led to the hypothesis that anti-IgM can confer protection, because it induces a more prolonged and higher level of Bcl-xL expression than anti-CD40 alone. Our current results suggest that in primary B lymphocytes, Bcl-xL is not necessary for anti-IgM-mediated protection of CD40-activated cells, at least in the time period tested. This raises the possibility that other molecules, such as FAIM and c-FLIP, may play a more significant role in vivo. Indeed, we find that both proteins are up-regulated normally in c-Rel−/− cells, and the timing of induction correlates well with protection. These results are in agreement with earlier work on FAIM and c-FLIP indicating that these molecules, when expressed in B cell lines, can prevent Fas-mediated apoptosis (4, 11). Other candidate protective molecules, such as A1/Bfl1, Bcl-2, and the inhibitor of apoptosis proteins, do not have expression kinetics that match the onset of Fas resistance (B. R. Schram and T. L. Rothstein, unpublished observations; reviewed in Ref. 9).
The differential requirements for c-Rel in protecting from different types of programmed cell death (e.g., irradiation vs Fas) can be reconciled by the fact that there are two major apoptotic pathways in lymphocytes (reviewed in Ref. 42). One is initiated by death receptors and is mediated by caspase-8, and the other is initiated by signals that directly affect the mitochondria, resulting in the activation of caspase-9. Both pathways eventually converge downstream at caspase-3. Work in various caspase knockout mice as well as Apafl knockout mice has revealed that the Fas apoptotic pathway can still function in the absence of caspase-9 and Apaf1, suggesting the involvement of a nonmitochondrial pathway in Fas-mediated cell death (41, 43). On the other hand, casepase-9-deficient thymocytes are resistant to etoposide, dexamethasone, and γ-irradiation; these data correlate well with the requirement for Bcl-xL induction in our B cell model. In addition, the fact that Bcl-xL can rescue c-Rel+/−/− B cells from IgM-mediated apoptosis is in agreement with recent work illustrating that this death pathway involves the mitochondria, and subsequently caspase-9 activation (44, 45).

Work by Scaffidi et al. (46) has identified two types of Fas-expressing cells that differ in their sensitivity to Fas ligation. Type I cells activate caspase-8 rapidly, whereas type II cells have a delayed response. Fas-mediated apoptosis was blocked by Bcl-2 and Bcl-xL in type II cells only. The density of Fas and the amount of caspase-9 determined whether the apoptosis would proceed independently of mitochondrial factors. The authors were able to convert MCF7, a type II breast carcinoma cell previously found to express cells that differ in their sensitivity to Fas ligation. Type I cells activate caspase-8 rapidly, whereas type II cells have a delayed response. Fas-mediated apoptosis was blocked by Bcl-2 and Bcl-xL in type II cells only. The density of Fas and the amount of caspase-9 determined whether the apoptosis would proceed independently of mitochondrial factors. The authors were able to convert MCF7, a type II breast carcinoma cell previously found to be resistant to Bcl-xL, into a type I cell by overexpressing caspase-3. Thus, mouse primary B lymphocytes may be type I cells, which have high levels of Fas and endogenous caspase-8. Indeed, it has been found in both murine and human B cells that caspase-8 is recruited to the death-inducing signaling complex, but only after signaling through Fas (11, 48). Stimulation of murine B cells with anti-IgM inhibits this recruitment, presumably due to c-FLIP up-regulation (11).

Physiologically, it is reasonable to assume that inhibition of the Fas pathway would occur more effectively at the level of the receptor. Thus, c-FLIP and FAIM may play a more important role in vivo. However, our results do not preclude the possibility that Bcl-xL plays an important role in other contexts of Fas-mediated apoptosis, such as higher E:T cell ratios. Previously, we have shown that overexpression of Bcl-xL in transgenic mice diminished the Fas sensitivity of CD40 ligand-stimulated primary B cells (10). Thus, all three molecules may play a role physiologically, but in our in vitro system, FAIM and c-FLIP can fully compensate for the loss of Bcl-xL. Another possibility is that the residual amount of Bcl-xL expression in the c-Rel−/− mouse is sufficient to cooperate with FAIM and c-FLIP. Alternatively, there may be other molecules (perhaps pro-apoptotic) that are affected by the loss of c-Rel. Overall, however, our current results are consistent with the idea that the absence of c-Rel and Bcl-xL does not have a major impact on the protection of B cells from Fas-mediated death.

Acknowledgments
We thank D. Schatz for the RDA protocol and valuable advice on optimization, N. E. Blachere and A. N. Houghton for the 2C2 T cell clone, I. Messaoudi and J. Nikolic-Zugic for help with the 51Cr release assays, and S. Andjelic for help with flow cytometric analysis of apoptotic cells.

References


