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B cell susceptibility to Fas-mediated apoptosis is regulated in a receptor-specific fashion. CD40 engagement produces marked sensitivity to Fas killing, whereas surface Ig (sIg) engagement blocks Fas signaling for cell death in otherwise sensitive, CD40-stimulated B cell targets, and thus, induces a state of Fas resistance. The signaling mediator, Bruton’s tyrosine kinase (Btk), is required for certain sIg-triggered responses, and Btk is reported to directly bind Fas and block Fas-mediated apoptosis. For these reasons, the role of Btk as a mediator of sIg-induced Fas resistance was examined. Dysfunction of Btk through mutation, and absence of Btk through deletion did not interfere with induction of Fas resistance by anti-Ig. This may be due, at least in part, to induction of Btk-dependent Bcl-2 family members by anti-Ig after CD40 ligand treatment. However, the susceptibility to Fas-mediated apoptosis of B cell targets stimulated by CD40 ligand alone was increased in the absence of Btk. These results indicate that Fas resistance produced by sIg triggering does not require Btk, but suggests that in certain situations Btk modulates B cell susceptibility to Fas killing. The Journal of Immunology, 2002, 168: 2712–2719.
slg-triggered phosphoinositide hydrolysis and Ca$^{2+}$ responses are markedly diminished in xid B cells (37–39). The central role that Btk plays in directing downstream effects produced by slg triggering is demonstrated by the complete failure of proliferation following anti-lg treatment of B cells obtained from xid and Btk$^{-/-}$ mice (40, 41). Thus, it might be thought that slg signaling for Fas resistance, like other outcomes of slg engagement, depends on Btk.

Recently, it was reported that Btk modulates the sensitivity of B cells to Fas-mediated apoptosis (42). In chicken DT40 B cells, Btk bound Fas directly, interfering with signaling for cell death, such that Btk-deficient DT40 cells were much more sensitive to Fas killing than wild-type (WT) control cells. Combined with the observation that Btk protein expression was up-regulated following slg engagement (43), these results raise the possibility that Btk may be responsible for slg-induced Fas resistance, either as a signaling intermediary, or as a terminal inhibitor of the Fas death pathway. The present study was conducted to examine the role of Btk in promoting slg-induced Fas resistance, and the capacity of Btk to modulate the sensitivity of B cells to Fas killing.

### Materials and Methods

#### Animals

Male (CBA/N × A.Bry)F$_1$, xid mice were bred and maintained at the University of Massachusetts Medical School (Worcester, MA). Male Btk-deficient and control C57BL/6, and additional xid and control CBA mice, were obtained at 8–10 wk of age from The Jackson Laboratory (Bar Harbor, ME).

#### B cell purification

B cells were prepared from spleen cell suspensions by negative selection, as previously described (9). Purified B cells were cultured at 37°C with 5% CO$_2$, RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 5% heat-inactivated FBS (Sigma Aldrich, St. Louis, MO), 10 mM HEPES (pH 7.2), 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

#### Cell-mediated cytotoxicity

Following stimulation, B cells were tested as targets for Fas-dependent cytotoxicity in standard lectin-dependent 51 Cr release assays using Vβ2 or of AE7 CD4$^+$ Th1 effector cells, as previously described (9). 3Cr release assays were also conducted using soluble FasL (sFasL) to induce cytotoxicity over a 4- to 8-h period.

#### Flow cytometric analysis

B cells were stained with Jo-2 anti-Fas Ab (BD PharMingen, San Diego, CA) and analyzed by flow cytometry on a FACSscan instrument (BD Biosciences, Mountain View, CA) as previously described (12).

#### RNase protection assay

RNA was obtained from stimulated and unstimulated B cells using UltraSpec reagent (Biotecx Laboratories, Houston, TX) and DNase treated. The expression of Bcl-2 family members was assessed with 2.5 μg total RNA, using the mAPO-2 multitemplate probe set (BD PharMingen), as described by the manufacturer.

#### Reagents

Soluble recombinant CD40 ligand (CD40L) was obtained from transfected 35SL cells that secrete a chimeric CD40L/CD8α fusion protein (44), as previously described (45). A similarly dialeyzed supernatant containing anti-CD8 Ab from the 53-6-72 hybridoma was used to cross-link the fusion protein, as described (45). Affinity-purified Fab(α')$_2$ of polyclonal goat anti-mouse IgM (anti-lg) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). PMA and ionomycin were obtained from Sigma Aldrich. sFasL was obtained from ALEXIS Biochemicals (San Diego, CA).

### Results

The role of Btk in mediating slg-induced Fas resistance was evaluated by testing the capacity of anti-lg treatment to block Fas-mediated apoptosis in CD40L-stimulated B cells containing either mutant (xid) or deleted (Btk knockout (KO)) forms of Btk. The combination of a protein kinase C-activating phorbol ester, PMA, and a calcium ionophore, ionomycin, which are together mitogenic for mutant Btk and Btk-deficient B cells (41, 46), was used to produce Fas resistance by triggering signaling intermediates downstream of Btk. As part of this study, the role of Btk in modulating B cell sensitivity to Fas killing after stimulation by CD40L alone was defined.

#### Fas-mediated apoptosis in xid B cells

B cells from xid and littermate control mice were stimulated with CD40L/CD8α cross-linked with anti-CD8 Ab (CD40L) for a total of 48 h, with or without anti-lg or the combination of PMA plus ionomycin (P/I) added during the last 24 h of culture. B cells were then tested for Fas sensitivity by lectin-dependent chromium release assays in which cytotoxicity was produced by FasL-bearing CD4$^+$ Th1 effector cells that kill in a Fas-dependent fashion (9). The results of two such experiments are displayed in Fig. 1. Stimulation with CD40L alone produced marked sensitivity to Th1 cell-mediated cytotoxicity in both WT control and in xid B cells. This is consistent with previous results indicating that some aspects of CD40 signaling remain intact in Btk mutant B cells (47, 48). As expected, the addition of anti-lg to CD40L-stimulated control B cells led to a marked diminution of subsequent Fas-dependent cytotoxicity; that is, anti-lg produced Fas resistance. Surprisingly, the same was true of xid B cells. Anti-lg produced similar levels of Fas resistance in xid and in control B cells. The combination of P/I also produced Fas resistance in both control and xid

![FIGURE 1.](http://www.jimmunol.org/Downloadedfrom/fig1.jpg)

**FIGURE 1.** slg signaling induces Fas resistance in xid B cells. Primary splenic B cells obtained from xid (Xid) or WT control (WT) mice were cultured with CD40L/CD8α fusion protein cross-linked with anti-CD8 Ab for 48 h (CD40L), or were cultured with CD40L for 48 h plus either P/αβ$_2$ fragments of goat anti-mouse IgM at 10 μg/ml (CD40L/αβ$_2$), or the combination of PMA at 100 ng/ml and ionomycin at 600 ng/ml (CD40L/P + I), added during the last 24 h of culture, as indicated. B cells were then radiolabeled and tested as targets for Fas-dependent cytotoxicity mediated by Vβ2 CD4$^+$ Th1 effector cells in standard lectin-dependent 3Cr release assays. Results obtained at E:T cell ratios of 3:1 and 1:1 are shown for two separate experiments. For each condition, the mean percentage of specific cell lysis of triplicate assays is shown, along with a line indicating the SEM.
B cells, and there was no consistent difference in the relative effectiveness of anti-Ig and P/I in producing Fas resistance in \textit{xid} as compared with control B cells.

To confirm that resistance to Th1 cell-mediated cytotoxicity induced by anti-Ig in \textit{xid} B cells resulted from blocking Fas signaling for cell death, additional chromium release assays were conducted in which apoptosis was produced by triggering Fas with sFasL. The mean results of three such experiments, shown in Fig. 2, essentially recapitulate the results described above. Stimulation with CD40L alone produced marked sensitivity to FasL-induced cytotoxicity, and anti-Ig treatment produced protection against FasL-induced cytotoxicity, in both control and \textit{xid} B cells. The level of Fas resistance produced by anti-Ig (that is, the magnitude of the reduction in Fas-mediated apoptosis derived by contrasting B cells treated with CD40L plus anti-Ig and B cells treated with CD40L alone) was not different for \textit{xid} as compared with control B cells. One experiment from this set is shown in more detail in Fig. 3, which includes the results of FasL dose titration. At each dose of FasL, anti-Ig treatment reduced the level of Fas killing both for control and for \textit{xid} B cells. As previously reported (9), B cell Fas expression was up-regulated by treatment with CD40L, but little changed by the addition of anti-Ig or by the addition of P/I after CD40L stimulation (Fig. 3B); this was true both of normal control and \textit{xid} B cells, and thus, induction of Fas resistance in the presence of mutant Btk cannot be explained by shifts in Fas expression.

\textbf{Fas-mediated apoptosis in Btk-deficient B cells}

B cells from Btk-deficient and control mice were stimulated with CD40L for 48 h, with or without anti-Ig or the combination of P/I added during the last 24 h of culture, and then tested for Fas sensitivity with CD4+ Th1 effector cells, as in the experiments described above. The mean results of four experiments are displayed in Fig. 4. Stimulation with CD40L alone produced sensitivity to Fas-dependent cytotoxicity similarly in both control and in Btk-deficient B cells. However, particularly at higher E:T cell ratios, Btk-deficient B cells appeared to be somewhat more susceptible to Fas-mediated apoptosis than control B cells, after stimulation with CD40L alone. Regardless, anti-Ig addition produced equivalent levels of resistance to Fas killing in Btk-deficient and control B cells. This is especially apparent in comparing Btk-deficient B cells tested at an E:T cell ratio of 1:1.

\textbf{FIGURE 2.} sIg signaling induces resistance to sFasL in \textit{xid} B cells. Primary splenic B cells obtained from \textit{xid} (Xid) or WT control (WT) mice were cultured with CD40L/CD8\alpha fusion protein cross-linked with anti-CD8 Ab for 48 h (CD40L), or were cultured with CD40L for 48 h plus either F(ab’)\textsubscript{2} fragments of goat anti-mouse IgM at 10 \textmu g/ml (CD40L/\textit{xid}), or the combination of PMA at 100 ng/ml and ionomycin at 600 ng/ml (CD40L/P\textsubscript{\textit{xid}}), added during the last 24 h of culture, as indicated. B cells were then radiolabeled and tested as targets for Fas-dependent cytotoxicity mediated by sFasL (50 ng/ml) in standard \textsuperscript{51}Cr release assays. The means of specific lysis for three independent experiments are shown, along with lines indicating the SEM.

\textbf{FIGURE 3.} sIg signaling induces resistance in \textit{xid} B cells at various doses of sFasL. Primary splenic B cells obtained from \textit{xid} (Xid) or WT control (WT) mice were treated as described in the legend to Fig. 2. Separate aliquots of B cells were radiolabeled and tested as targets for susceptibility to Fas-dependent cytotoxicity at the doses of sFasL indicated, and were stained with FITC-labeled Jo-2 monoclonal anti-Fas Ab and analyzed for Fas expression by flow cytometry. A, Mean percentage of specific cell lysis of triplicate assays for each condition, along with a line indicating the SEM. B, Relative cell number as a function of fluorescence intensity for each condition. The results of a single representative experiment are shown.
Either F(ab')2 linked with anti-CD8 Ab for 48 h (CD40L), or with CD40L for 48 h plus either F(ab')2 fragments of goat anti-mouse IgM at 10 μg/ml (CD40L/Ig), or the combination of PMA at 100 ng/ml and ionomycin at 600 ng/ml (CD40L/P + I), added during the last 24 h of culture, as indicated. B cells were then radiolabeled and tested as targets for Fas-dependent cytotoxicity mediated by CD4+ Th1 effector cells in standard lectin-dependent 51Cr release assays. Results obtained at E:T cell ratios of 3:1 and 1:1 are shown. For each condition, the means of specific lysis for four independent experiments are shown, along with lines indicating the SEM.

FIGURE 4. sIg signaling induces Fas resistance in Btk-deficient B cells. Primary splenic B cells obtained from Btk-deficient (Btk KO) or WT control (WT) mice were cultured with CD40L/CD8α fusion protein cross-linked with anti-CD8 Ab for 48 h (CD40L), or with CD40L for 48 h plus either F(ab')2 fragments of goat anti-mouse IgM at 10 μg/ml (CD40L/Ig), or the combination of PMA at 100 ng/ml and ionomycin at 600 ng/ml (CD40L/P + I), added during the last 24 h of culture, as indicated. B cells were then radiolabeled and tested as targets for Fas-dependent cytotoxicity mediated by CD4+ Th1 effector cells in standard lectin-dependent 51Cr release assays. Results obtained at E:T cell ratios of 3:1 and 1:1 are shown. For each condition, the means of specific lysis for four independent experiments are shown, along with lines indicating the SEM.

with control B cells tested at an E:T cell ratio of 3:1 (Fig. 4). The combination of P/I also produced Fas resistance in both normal and Btk-deficient B cells. These results indicate that, as with xid B cells, the induction of Fas resistance by sIg engagement is not abrogated by the loss of Btk function.

To confirm that resistance to Th1 cell-mediated cytotoxicity induced by anti-Ig in Btk-deficient B cells resulted from blocking Fas signaling for cell death, additional chromosome release assays were conducted in which apoptosis was produced by triggering Fas with sFasL. The mean results of three such experiments, shown in Fig. 5, again demonstrate induction of resistance to Fas-mediated apoptosis by anti-Ig in the absence of Btk. Although Btk-deficient B cells stimulated by CD40L alone were more sensitive to Fas-mediated apoptosis than were CD40L-stimulated control B cells (at a dose of 50 ng/ml sFasL), the reduction in Fas sensitivity produced by anti-Ig treatment was similar in comparing Btk-deficient with control B cells. One experiment from this set is shown in more detail in Fig. 6, which includes the results of FasL dose titration. As observed with the pooled results, Btk-deficient B cells were more sensitive than control B cells to Fas-induced cytotoxicity at each dose of FasL tested. Still, at each dose, anti-Ig treatment reduced the level of Fas killing for Btk-deficient as well as for control B cells. As noted above, B cell Fas expression was markedly up-regulated in xid and in control (B) cells by treatment with CD40L, but little changed by the addition of anti-Ig or by the addition of P/I after CD40L stimulation (Fig. 6B). This was also true for Btk-deficient B cells, and thus, induction of Fas resistance in the absence of Btk cannot be explained by shifts in Fas expression. Notably in this experiment, CD40L-stimulated Btk-deficient B cells stained less intensely for Fas (mean fluorescence intensity = 137) than CD40L-stimulated control B cells (mean fluorescence intensity = 191), and yet were more sensitive to Fas-mediated apoptosis, demonstrating that although Fas expression is necessary for Fas killing, there is not always a strict correlation between Fas levels and Fas sensitivity.

To clarify the influence of Btk on Fas sensitivity, the susceptibility to FasL-mediated cytotoxicity of CD40L-stimulated xid, Btk-deficient, and control B cells were compared. To relate independent experiments and avoid saturating levels of FasL, that might induce maximal chromium release in all B cells (thereby obscuring intrinsic differences), cytotoxicity was evaluated for control B cells at the dose of sFasL that produced specific cell lysis nearest to 50% in xid and in Btk-deficient B cells. Results are shown in Fig. 7. The susceptibility of xid and control B cells to Fas killing after stimulation by CD40L did not differ (n = 4). In contrast, the Fas sensitivity of CD40L-stimulated Btk-deficient B cells exceeded that of CD40L-stimulated control B cells by a significant margin (Fig. 5 and data not shown; n = 4, p < 0.03 by Student’s t test), as suggested by the data displayed in Figs. 5 and 6. The Fas sensitivity of stimulated Btk-deficient and control B cells also differed when cytotoxicity was induced by FasL-bearing Th1 effector cells. In this case, specific cell lysis was 51 ± 2.2 vs 38 ± 2.3, for Btk-deficient and control B cells, respectively (Fig. 4 and data not shown; n = 6, p < 0.03). Pooled data yielded values of 51 ± 1.5 and 36 ± 2.5, respectively (n = 10, p < 0.001). Therefore, regardless of the Fas trigger, Btk-deficient B cells appear to undergo apoptosis more readily and/or more extensively at a given level of Fas engagement.

Expression of Bcl-2 family members in Btk-deficient B cells

Bcl-xL has been implicated as a terminal mediator of sIg-induced Fas sensitivity, as noted earlier. However, it has been reported that sIg engagement fails to induce Bcl-xL in xid B cells (49–51). This raises the question of whether the same would be found to be true when anti-Ig is added to CD40L-stimulated B cells. To address this issue, xid and control B cells were stimulated with anti-Ig alone, or were stimulated for 48 h with CD40L in combination with anti-Ig added for the last few hours of culture, after which RNA was obtained and evaluated for expression of Bcl-2 family members by RT-PCR. Results are shown in Fig. 8. Anti-Ig-induced up-regulation of Bcl-xL and of Bcl-1 was blocked by Btk mutation. However, after treatment with CD40L, anti-Ig produced similar levels of Bcl-1 and Bcl-xL expression in xid and control B cells; this was particularly evident for Bcl-1. Therefore, prior CD40L stimulation appears to overcome or circumvent the interruption in sIg signaling imposed by mutation of Btk, at least insofar as the outcomes of Bcl-1 and Bcl-xL expression are concerned. In these experiments, Bcl-2 and Bak were not induced by any of the treatments, as previously observed in our unpublished observations of primary B cell RNA by RT-PCR. Bad, Bax, and Bcl-w were not detected.

FIGURE 5. sIg signaling induces resistance to sFasL in Btk-deficient B cells. Primary splenic B cells obtained from Btk-deficient (Btk KO) or WT control (WT) mice were cultured with CD40L/CD8α fusion protein cross-linked with anti-CD8 Ab for 48 h (CD40L), or with CD40L for 48 h plus either F(ab')2 fragments of goat anti-mouse IgM at 10 μg/ml (CD40L/Ig), or the combination of PMA at 100 ng/ml and ionomycin at 600 ng/ml (CD40L/P + I), added during the last 24 h of culture, as indicated. B cells were then radiolabeled and tested as targets for Fas-dependent cytotoxicity mediated by sFasL (50 ng/ml) in standard 51Cr release assays. The means of specific lysis for three independent experiments are shown, along with lines indicating the SEM.
Discussion

The work presented in this study demonstrates that Ag receptor engagement induces resistance to Fas-mediated apoptosis in primary B cells in the face of loss or mutation of Btk. sIg signaling for S phase entry and for Bcl-xL expression fails in the absence of functioning Btk, and in the present experiments xid and Btk-deficient B cells did not incorporate thymidine (data not shown), nor up-regulate Bcl-xL gene (Fig. 8) or protein (data not shown) expression after stimulation with anti-Ig. Thus, the intracellular signaling pathway responsible for sIg-induced Fas resistance must differ in important ways from the pathway(s) responsible for cell cycle progression and Bcl-xL (and Bfl-1) expression. Moreover, these results indicate that Btk is not required for induction of Fas resistance produced by sIg signaling in primary murine B cells, notwithstanding reports that Btk binds Fas and interferes with apoptosis in DT40 cells (42).

CD40 engagement alone produced up-regulation of Fas expression and acquisition of sensitivity to Fas-mediated apoptosis, despite reports that CD40-triggered proliferative responses are blocked or reduced in xid and Btk KO B cells (41, 52, 53). These apoptotic-related outcomes represent new additions to signaling events known to be preserved under conditions of Btk mutation and/or deficiency, such as up-regulation of CD23 (54). Notably, the complete loss of Btk was associated with significantly increased Fas sensitivity in B cells stimulated by CD40L alone. However, the relative enhancement of susceptibility to Fas signaling for cell death in murine B cells (Fig. 7) is much reduced in comparison to that previously reported for Btk-deficient DT40 cells (42); regardless, these findings support the notion that Btk plays a role in establishing the level of sensitivity to Fas killing of activated mammalian B cells, even if this role is of less consequence than that reported for chicken B cells. In contrast, the xid mutation was not associated with any alteration in the susceptibility of CD40L-stimulated B cells to Fas engagement by sFasL, or by Th1 cells, suggesting that a region other than the pleckstrin homology domain is responsible for the influence of Btk on Fas-mediated apoptosis (28, 29, 55).

Fas resistance was evident in B cells treated with CD40L plus anti-Ig even at higher doses of sFasL that produced substantial levels of cytotoxicity (Figs. 5 and 6). Although the amount of
specific cell lysis that was reversed was similar at higher and lower doses of sFasL, the proportion of apoptosing B cells that were protected declined. This may suggest that higher doses of sFasL recruit B cells into the apoptotic pathway that are incapable of being rescued by sIg engagement. However, Fas resistance is relative, not absolute, and cannot be detected in the face of very strong Fas signaling such as that produced by Jo-2 monoclonal anti-Fas Ab (13). Thus, the loss of proportionate Fas resistance at higher doses of sFasL may reflect the balance between the intensity of pro- and anti-apoptotic signals initiated by Fas and Ag receptor engagement.

The capacity of Bcl-2 family members to oppose Fas-mediated apoptosis in lymphocytes remains controversial (19, 56–59). Some of the discrepancy in previous results may relate to the use of cell lines which rely to different extents on mitochondrial changes and cytochrome c release as intermediates in Fas signaling for cell death (57). In previous work from this laboratory with transgenic mice, Bcl-xL was implicated as a terminal effector of sIg-induced Fas resistance (19). However, in recent work by Liou and colleagues (60) with c-Rel-deficient B cells, Fas resistance was observed in the absence of substantial Bcl-xL induction. Although it had been reported that sIg signaling in naive xid B cells failed to induce Bcl-xL (Refs. 49–51; Fig. 8), the present study specifically probed the expression of Bcl-xL and other Bcl-2 family members in B cells following stimulation by an optimal Fas resistance-inducing regimen, encompassing pretreatment with CD40L and sequential treatment with anti-Ig. Surprisingly, both Bfl-1 and Bcl-xL were up-regulated to the same extent in xid and control B cells when anti-Ig stimulation followed CD40L treatment, even though neither were induced in naive xid B cells by anti-Ig treatment alone. This suggests the possibility that Bfl-1, Bcl-xL, or both may participate in sIg-induced Fas resistance, although neither is induced by anti-Ig in untreated B cells. More importantly, these results suggest that sIg signaling is altered by prior B cell stimulation with CD40L such that an outcome precluded by Btk mutation (e.g., Bfl-1 expression) is now permitted. Elucidation of the sIg signaling pathway that is enabled by prior treatment with CD40L is likely to provide insight into the mechanism by which sIg mediates Fas resistance. The observation that Bfl-1 expression depends on NF-κB (61–64) suggests a potential mediator that may be involved.

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