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B Cell Receptor (BCR) Cross-Talk: CD40 Engagement Enhances BCR-Induced ERK Activation

Takuya Mizuno* and Thomas L. Rothstein2*†

Bystander B cells may be initially stimulated through CD40, which enhances susceptibility to Fas-mediated apoptosis, before encountering Ag, which produces Fas resistance. A key issue in this process is to what extent CD40 cross-talk might affect subsequent BCR signaling. It has previously been shown that CD40 engagement bypasses or mitigates the need for Bruton’s tyrosine kinase in subsequent BCR signaling for NF-κB activation. However, the full extent of the effects of CD40 on BCR signaling has not been delineated. In the present study we evaluated the possibility that CD40-mediated cross-talk also affects another principal outcome of BCR signaling: MAPK activation. We found that prior stimulation of primary murine B cells with CD40L markedly enhanced the level of ERK and JNK (but not p38 MAPK) phosphorylation produced by subsequently added anti-Ig Ab, and much, but not all, of this enhancement was independent of PI3K and phospholipase C. CD40L treatment similarly enhanced BCR-induced MAPK kinase (MEK) phosphorylation, and MEK was required for enhancement of ERK. Although BCR-induced c-Raf phosphorylation was also enhanced by prior CD40L treatment, c-Raf was not required for MEK/ERK phosphorylation. These results identify a novel system of receptor cross-talk between CD40 and BCR and indicate that the effects of CD40 engagement on subsequent BCR stimulation spread beyond NF-κB to involve the MAPK pathway. The Journal of Immunology, 2005, 174: 3369–3376.

B cell receptor engagement is vital for the development, survival, and immune responsiveness of B lymphocytes (1–3). These diverse and complex outcomes are supported by the multiple signaling pathways activated by BCR triggering (4). Typically, BCR-generated signals do not operate in isolation; rather, in mature B cells presentation of antigenic peptides leads to T-dependent effects that include stimulation of CD40, which promotes germinal center formation and B cell differentiation (5). In this paradigm a restricted set of B cells is initially activated by Ag binding and then further stimulated through CD40 by peptide-specific T cells, ensuring the specificity of resultant serological immune responses.

However, B cells are not always stimulated in this sequence. In some cases naive B cells are first stimulated through CD40 (6). The long period of time during which activated B cells experience CD40 signaling would seem to provide ample opportunity for accidental stimulation of non-BCR-engaged, bystander B cells (7–10); in fact, bystander B cell activation has been demonstrated in a number of ways, most convincingly through responsiveness to a number of ways, most convincingly through responsiveness to a nonlinked, normally immunogenic protein as a result of immunogenic, T-dependent Ag administration (11). Recently, Rajewsky and colleagues (12) demonstrated non-Ag-linked germinal center formation in the absence of BCR expression and in three different strains of transgenic mice expressing nonautoreactive BCRs. Thus, naive B cells may be stimulated, aberrantly or in special locales, through CD40 first. Such stimulation poses a risk of Ag-nonspecific B cell responses, including production of autoreactive Ab (13), and so must be tightly controlled. As we and others have shown, B cell stimulation through CD40 is accompanied by up-regulated expression of the Fas death receptor and greatly enhanced sensitivity to Fas-mediated apoptosis (14–17). Thus, mice lacking Fas in the B cell compartment succumb to autoimmune disease accompanied by marked hypergammaglobulinemia and autoantibody production (18, 19). However, concurrent, or in keeping with the report by Casola et al. (12), sequential, BCR stimulation promotes Fas resistance and in so doing protects B cells from Fas-induced cytotoxicity, thereby promoting Ag specificity in B cell responses (14, 16, 17, 20). A key issue for this process is the nature of BCR signaling that occurs after CD40 engagement, that is, the extent to which CD40 cross-talk might influence subsequent BCR signaling and lower, or alter, the bar for BCR-triggered downstream events.

Recently, we reported that CD40 cross-talk has a profound effect on subsequent BCR signaling (21). We monitored induction of NF-κB, because this is indispensable for induction of Fas resistance (22). We found that after primary B cell treatment with CD40L, BCR-induced IκBα degradation and NF-κB activation, like BCR-induced Fas resistance, proceeded in the absence of functioning Bruton’s tyrosine kinase (Btk) (21, 23). This stands in marked contrast to the situation in naive B cells, in which BCR signaling for NF-κB requires Btk (24, 25). More recently, we have shown that after CD40L treatment, BCR signaling for NF-κB activation occurs in the absence of other signalosome elements, including PI3K and phospholipase C-γ2 (PLC-γ2). These results

1 Abbreviations used in this paper: Btk, Bruton’s tyrosine kinase; GA, geldanamycin; MEK, MAPK kinase; PLC, phospholipase C; RBD, Ras-binding domain.

2 Address correspondence and reprint requests to Dr. Thomas L. Rothstein, Immunobiology Unit, Evans Memorial Department of Clinical Research, Boston University Medical Center, 650 Albany Street, Boston, MA 02118. E-mail address: tr@bumc.bu.edu

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suggest that CD40 engagement establishes an alternate or enhanced pathway for BCR signaling that bypasses or mitigates the need for several otherwise required intracellular mediators.

Among the mediators stimulated by BCR engagement are the MAPKs, including ERK, JNK, and p38 MAPK (26). Although there is evidence that MAPK activation and NF-κB induction represent downstream outcomes of distinct BCR-triggered pathways (27–30), interconnections between these pathways likely exist. Thus, ERK activation has been shown to be at least partially dependent on PI3K and/or protein kinase C, mediators associated with BCR-induced NF-κB activation (31–36), that are, at least in the case of PI3K, bypassed upon BCR engagement that follows CD40 signaling. Further, the observation that CD40L and anti-Ig, added concurrently or sequentially to Btk mutant xid B cells, act in synergy to produce cell cycle progression (21, 37) raises the possibility that CD40 cross-talk involves more downstream events than NF-κB activation alone. This idea is supported by the role that the MEK/ERK axis plays in up-regulating cyclin D2 expression and enhancing proliferation of B cells (38, 39). Thus, we questioned whether BCR signaling for MAPK activation in general, and for ERK activation in particular, might be influenced by CD40 engagement. We undertook a series of experiments to identify cross-talk between these receptors and now report that CD40 engagement greatly enhances subsequent BCR induction of ERK and JNK activation and does so to a great extent independently of PI3K and PLC-γ2.

Materials and Methods

Animals

Male BALB/cByJ mice at 6–8 wk of age were obtained from The Jackson Laboratory. Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

B cell isolation

B cells were prepared from spleen cell suspensions by negative selection as previously described (14). Briefly, splenocytes were depleted of T cells by treatment with anti-Thy 1.2 Ab, followed by complement lysis; the resultant cells were then subjected to density separation using Lymphocyte M (Cedarslane Laboratories) to remove dead cells and RBC. B cells were cultured at 2–4 × 10^6/ml in RPMI 1640 medium (BioWhittaker) supplemented with 5% heat-inactivated FBS (Sigma-Aldrich), 10 mM HEPES (pH 7.25), 50 μM 2-ME, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

B cell stimulation

B cells were stimulated by F(ab’2) goat anti-mouse IgM (anti-Ig) with or without prior CD40L treatment. B cells stimulated without CD40L treatment (CD40L(−)) were cultured in medium for 3 h before addition of anti-Ig. B cells stimulated with CD40L treatment (CD40L(+)) were cultured with soluble recombinant CD40L (see below) for 48 h (except as noted), washed with medium three times, then cultured in medium for 1 h before addition of anti-Ig. Inhibitors were added 30 min prior to stimulation with anti-Ig.

Western immunoblot analysis

Cytoplasmic proteins were extracted from cell pellets with 1% Nonidet P-40 lysis buffer containing protease inhibitors, and equal amounts (10–30 μg) were subjected to SDS-PAGE, followed by immunoblotting as previously described (21). Anti-phospho-ERK1/2 (Thr183/Tyr185), anti-ERK1/2, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-JNK1/2 (Thr183/Tyr185), anti-JNK, anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-MEK1/2 (Ser217/221), anti-MEK, and anti-phospho-c-Raf (Ser338) Abs were obtained from Cell Signaling Technology. Anti-p38 MAPK and anti-c-Raf Abs were obtained from Santa Cruz Biotechnology. Anti-Ras Ab was obtained from BD Pharmingen. Rabbit or mouse secondary Abs were obtained from Jackson ImmunoResearch Laboratories. Immunoblotting were detected by ECL (Amersham Biosciences). Immunoblots were stripped and reprobed with anti-actin Ab (Sigma-Aldrich) to verify that equal amounts of protein were loaded in each lane.

GST pull-down assay

Ras activation was evaluated after GST pull-down as described by Taylor et al. (40). Briefly, cytoplasmic extract from stimulated B cells (200 μg) was mixed with glutathione-Sepharose 4B beads (Amersham Biosciences) conjugated with GST-Ras-binding domain (GST-RBD) protein (containing the Raf RBD that binds only activated, GTP-bound Ras; kindly provided by Dr. Z. Luo, Boston University, Boston, MA) for 1 h and washed with lysis buffer. Precipitated Ras-GTP was subjected to SDS-PAGE, followed by Western blotting.

Cell surface staining

CD69 expression of purified B cells was determined by immunofluorescent staining in the presence of 2,4G2 anti-FeR Ab, followed by flow cytometric analysis, as previously described (41).

Reagents

Affinity-purified F(ab’2) of polyclonal goat anti-mouse IgM Ab (anti-Ig) were obtained from Jackson Immunoresearch Laboratories and used at 15 μg/ml. Preparation of soluble CD40L/CD8α (42) and cross-linking anti-CD8α Ab have been described previously (21, 43); these reagents were used at 1/10 and 1/40 dilutions of diazylated supernatants as previously described (43). LY294002 and U73122 were obtained from Calbiochem. U0126 was obtained from Promega. FITC-labeled anti-CD69 Ab was obtained from BD Pharmingen.

Results

CD40 engagement enhances BCR-induced MAPK phosphorylation regardless of the presence of a PI3K inhibitor

To evaluate the possibility that CD40 engagement influences subsequent BCR signaling for MAPK activation, we stimulated primary murine splenic B cells with anti-Ig with or without prior CD40L treatment and then examined extracts by Western blotting using MAPK phosphospecific Abs. We used the same period of CD40L treatment (48 h) that was recently demonstrated to produce a new signaling pathway for BCR induction of NF-κB activation (21). Results are shown in Fig. 1.

Stimulation of naive B cells with anti-Ig produced substantial phosphorylation of ERK1 and ERK2 within 5 min, in keeping with previous work (36). Inhibition of PI3K with LY294002 interfered with BCR-induced ERK activation to a greater extent (ERK2) or lesser extent (ERK1) 1) protein, and, although CD40L itself induced transient ERK1/2 phosphorylation (Fig. 2A) (44), phospho-ERK was at or near baseline after 48 h (Fig. 1A). However, addition of anti-Ig at this time produced much higher levels of ERK1/2 phosphorylation than observed with anti-Ig-stimulated naive B cells. Further, LY294002 only partially inhibited these elevated levels of ERK phosphorylation, so that the amount of anti-Ig-stimulated phospho-ERK1 and phospho-ERK2 in CD40L-treated, LY294002-inhibited B cells was still much greater than the amount of phospho-ERK1/2 present after anti-Ig stimulation of naive (CD40L-untreated) B cells. Thus, CD40L treatment enhances BCR signaling for ERK1/2 phosphorylation through a pathway that is, at least in part, PI3K independent.

Anti-Ig stimulation of naive B cells produced substantial phosphorylation of JNK2 within 5 min, in keeping with previous work (32). Again, PI3K inhibition with LY294002 substantially interfered with BCR-induced JNK2 activation. With JNK2, as with ERK1/2, the situation for CD40L-treated B cells was quite different. CD40L treatment produced little change in the level of JNK2 protein, but greatly enhanced the level of phosphorylated JNK2 triggered by subsequently added anti-Ig compared with anti-Ig-treated naive B cells. Moreover, LY294002 produced only partial inhibition of the elevated level of BCR-induced phospho-JNK2, so that the amount of anti-Ig-stimulated phospho-JNK2 in CD40L-treated, LY294002-inhibited B cells was greater than the amount

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3370
of phospho-JNK2 present after anti-Ig stimulation of naive (CD40L-untreated) B cells. Thus, CD40L treatment enhances BCR signaling for JNK2 phosphorylation, in addition to ERK phosphorylation, through a pathway that is, at least in part, PI3K independent.

Anti-Ig stimulation of naive B cells produced substantial phosphorylation of a third MAPK, p38 MAPK, within 5 min, as previously reported for human tonsillar B cells (35). However, BCR-triggered p38 MAPK behaved differently as compared to ERK1/2 and JNK2. Inhibition of PI3K with LY294002 had little effect on phosphorylation of a third MAPK, p38 MAPK, and BCR signaling for p38 MAPK activation was not enhanced after CD40L treatment.

The possibility that CD40L-induced B cell activation altered the penetration of, metabolism of, or sensitivity to LY294002 was addressed by evaluating PI3K-mediated Akt activation (Fig. 1D). LY294002 at doses of 1 and 10 μM, and wortmannin at doses of 0.01 and 0.1 μM, inhibited phosphorylation of Akt to the same extent in naive B cells and CD40L-treated B cells, with complete inhibition apparent at the higher doses in both cases. These Akt results discount the possibility that differential sensitivity to PI3K inhibitors explains the increased LY294002 resistance of BCR-induced ERK phosphorylation in CD40L-treated B cells.

The acknowledged presence of ERK in the nucleus as well as the cytosol of B cells raised the possibility that incomplete MAPK phosphorylation, through a pathway that is, at least in part, PI3K independent.

Anti-Ig stimulation of naive B cells produced substantial phosphorylation of a third MAPK, p38 MAPK, within 5 min, as previously reported for human tonsillar B cells (35). However, BCR-triggered p38 MAPK behaved differently as compared to ERK1/2 and JNK2. Inhibition of PI3K with LY294002 had little effect on

FIGURE 1. CD40 engagement enhances BCR-induced phosphorylation of ERK and JNK regardless of the presence of a PI3K inhibitor. B cells were cultured in medium alone for 3 h (CD40L−) or with CD40L/CD8α plus cross-linking anti-CD8 Ab for 48 h (CD40L+), then stimulated with F(ab′)2 of goat anti-mouse IgM Ab (αlg) for the indicated times. B cells were exposed to the PI3K inhibitor, LY294002, at 10 μM (LY) or to diluent control DMSO (DM) starting 30 min before addition of anti-Ig.

Whole cell extracts were prepared and Western blotted as described in Materials and Methods with anti-phospho-ERK (A), anti-phospho-JNK (B), and anti-phospho-p38 MAPK (C) Abs. Blots were stripped and reprobed with anti-actin Ab to verify equal loading of extracted protein. One of three comparable experiments is shown. D, B cells were cultured in medium alone or with CD40L as described above, then stimulated with αlg for 5 min. B cells were exposed to LY294002 and wortmannin at the indicated doses starting 30 min before addition of anti-Ig. Whole cell extracts were prepared and Western blotted with anti-actin Ab. Blots were stripped and reprobed with anti-Akt Ab and anti-actin Abs, respectively, to verify equal loading of extracted protein. One of two (A and B) or two (C) comparable experiments is shown. E, B cells were cultured in medium alone or with CD40L, then stimulated with αlg in the presence or absence of LY294002 as described above. Cell pellets were directly dissolved in SDS loading buffer before denaturing electrophoresis and Western blotting with anti-phospho-ERK Ab. Blots were stripped and reprobed with anti-ERK and anti-actin Abs to verify equal loading of extracted protein. One of two comparable experiments is shown.

FIGURE 2. CD40L-mediated enhancement of BCR-induced ERK phosphorylation requires time to develop. A, Anti-Ig and CD40L do not act in synergy to induce ERK phosphorylation. Naive B cells were stimulated with anti-Ig, CD40L, or the combination of anti-Ig plus CD40L for the indicated times in the presence of LY294002 (LY) or control diluent, DMSO (DM), starting 30 min before addition of anti-Ig. Whole cell extracts were prepared and Western blotted with anti-phospho-ERK Ab. Blots were stripped and reprobed with anti-actin Ab to verify equal loading of extracted protein. One of three comparable experiments is shown. B, CD40L treatment enhances BCR-induced ERK phosphorylation in a time-dependent fashion. B cells were cultured in medium alone for 3 h (0 h) or with CD40L for 12, 24, or 48 h, as indicated, after which anti-Ig was added for the indicated times in the presence of LY294002 (LY) or control diluent DMSO (DM), as described above. Whole cell extracts were prepared and Western blotted with anti-phospho-ERK Ab. Blots were stripped and reprobed with anti-actin Ab to verify equal loading of extracted protein. One of three comparable experiments is shown.
that is, CD40L treatment might be expected to produce less, not more, anti-Ig-induced ERK phosphorylation (45).

Taken together, these findings suggest that BCR-induced MAPK activation pathways are substantially enhanced by CD40 engagement in a way that is largely independent of PI3K, and that this enhancement is specific for pathways leading to ERK and JNK phosphorylation and does not involve pathways leading to p38 MAPK.

**CD40L-mediated enhancement of BCR-induced ERK phosphorylation requires time to develop**

To examine the possibility that enhancement of ERK phosphorylation simply reflected direct synergy between CD40 and BCR signaling pathways, we stimulated naive B cells with either anti-Ig, CD40L, or the combination of the two. In this and subsequent experiments, we focused on ERK activation. Results are shown in Fig. 2. As noted above (Fig. 1), anti-Ig stimulation induced phosphorylation of ERK1 and ERK2 within 5 min which was partially blocked by inhibition of PI3K with LY294002. CD40L stimulation also induced phosphorylation of ERK1/2, although this did not occur at 5 min, but instead began at 15 min; as expected, CD40L-induced phospho-ERK was not altered by LY294002. Combined stimulation with anti-Ig and CD40L induced ERK phosphorylation early, within 5 min, but the level did not exceed that observed for CD40L alone at any subsequent time point. Thus, CD40L-mediated enhancement of BCR-induced ERK phosphorylation does not result from simple synergy between CD40 and BCR intracellular signaling pathways in real time.

These results suggested that enhancement of ERK phosphorylation may be a time-dependent process with respect to CD40L treatment. To evaluate this possibility, we treated B cells with CD40L for 0, 12, 24, or 48 h prior to stimulation with anti-Ig (in the presence or absence of LY294002). Although synergy was not apparent when CD40L and anti-Ig were added together (Fig. 2A), sequential treatment produced marked enhancement of BCR-induced ERK phosphorylation that was evident after 12, 24, and 48 h of CD40L exposure (Fig. 2B), much like that observed in Fig. 1. Despite the early induction of ERK phosphorylation by CD40L (Fig. 2A), after 12–48 h phospho-ERK levels were at or near baseline before anti-Ig was added. At all periods of CD40L treatment, LY294002 partially inhibited subsequent anti-Ig-induced ERK phosphorylation, but, in all cases, LY294002-resistant, BCR-triggered phospho-ERK exceeded the level of phospho-ERK induced in naive B cells by anti-Ig alone. Moreover, the degree of enhancement increased as a function of the duration of CD40L treatment. However, CD40L treatment did not increase the total level of ERK (data not shown), indicating that the increase in phospho-ERK did not simply reflect an increase in the level of ERK. Thus, CD40L-mediated enhancement of BCR signaling for ERK phosphorylation is a time-dependent process with respect to CD40L exposure.

**BCR-induced ERK phosphorylation enhanced by CD40L treatment depends on MEK activation**

MEK is an upstream MAPK kinase that directly phosphorylates ERK (46). To elucidate the mechanism responsible for enhanced ERK phosphorylation, we stimulated B cells with anti-Ig, with or without prior CD40L treatment, and then evaluated MEK phosphorylation. Results are shown in Fig. 3A. Anti-Ig stimulation of naive B cells produced phosphorylation of MEK within 5 min, in keeping with previous work (39). PI3K inhibition by LY294002 substantially abrogated detectable MEK phosphorylation. However, CD40L treatment markedly altered the MEK response to BCR engagement. After CD40L treatment, anti-Ig induced much more phospho-MEK, without altering the baseline level of MEK protein. Although LY294002 partially inhibited the enhanced level of MEK phosphorylation, the level of phospho-MEK induced by anti-Ig in CD40L-treated, LY294002-inhibited B cells was much higher than the level triggered by anti-Ig in naive B cells, similar to the pattern observed with ERK1/2 phosphorylation.

To determine whether MEK regulates CD40L-enhanced BCR-induced ERK phosphorylation, we used the MEK-specific inhibitor, U0126, to block MEK activation (47). Results are shown in Fig. 3. U0126 blocked induction of ERK phosphorylation following anti-Ig stimulation of naive B cells in a dose-dependent manner between 1 and 10 μM, with complete elimination of phospho-ERK at doses of U0126 above 5 μM (Fig. 3B). When U0126 at 10 μM was added to CD40L-treated B cells before anti-Ig, BCR-induced ERK phosphorylation was virtually completely eliminated (Fig. 3C). Thus, inhibition of MEK blocks BCR-induced ERK phosphorylation with or without prior CD40L treatment, strongly suggesting that enhancement of MEK activation is directly connected to enhancement of ERK phosphorylation.

**CD40L-enhanced, BCR-induced ERK phosphorylation is associated with c-Raf phosphorylation, but does not depend on c-Raf**

In the classical BCR-induced ERK activation pathway, Raf is primarily responsible for MEK phosphorylation (48). Among the family of Raf proteins, consisting of c-Raf (Raf-1), B-Raf, and A-Raf, c-Raf appears to be the most important for B cells because it is expressed in B cells, and an activated form of c-Raf rescues...
defective B cell development produced by dominant negative Ras (49).

To determine the role of c-Raf in the pathway leading to enhanced ERK phosphorylation, we stimulated B cells with anti-Ig with and without prior CD40L treatment and then evaluated c-Raf phosphorylation. The results are shown in Fig. 4. Anti-Ig stimulation of naive B cells induced c-Raf phosphorylation within 5 min, in keeping with previous work (27), and PI3K inhibition with LY294002 inhibited anti-Ig-induced c-Raf phosphorylation. However, CD40L treatment markedly altered the c-Raf response to BCR engagement. After CD40L treatment, anti-Ig induced much more phospho-c-Raf than observed in naive B cells, even in the presence of LY294002.

Raf may be activated by Ras, among other mediators (50). To extend our analysis of signaling molecules affected by CD40L-mediated enhancement of BCR triggering, we stimulated B cells with anti-Ig with and without prior CD40L treatment and then evaluated Ras activation by pull-down assay using the RBD of Raf that only binds activated, GTP-bound Ras, followed by Western blotting with Ras-specific Ab. Results are shown in Fig. 4B. Anti-Ig stimulation of naive B cells induced Ras activation within 5 min, in keeping with previous work, and BCR-induced Ras activation was inhibited somewhat by LY294002 (36). CD40L treatment modestly enhanced Ras activation in response to BCR engagement, but, unlike ERK, MEK, and c-Raf, this enhancement was minimally sensitive to LY294002. Overall, the degree of enhanced BCR-induced Ras activation was much less than the degree of enhancement produced by CD40L in BCR-triggered ERK, JNK, MEK, and c-Raf phosphorylation.

We also evaluated the role of c-Raf with geldanamycin (GA), as previously described (39, 51). B cells were depleted of Raf by treatment with GA during the last 12 h of 48-h cultures with CD40L. Raf depletion of naive B cells (during overnight culture with GA) was confirmed in two ways. After GA treatment, Western blotting failed to reveal c-Raf protein, and anti-Ig failed to induce MEK phosphorylation (Fig. 4C). This approach was then applied to CD40L-treated B cells (Fig. 4D). As before, GA depleted immunoreactive c-Raf. However, after CD40L treatment, BCR-induced MEK phosphorylation was reduced little, if at all, by Raf depletion, and this was true regardless of the presence of LY294002, which partially blocked MEK phosphorylation. Thus, the presence or absence of GA had little or no effect on the phosphorylation of MEK or the degree of inhibition produced by LY294002, strongly suggesting that the enhancement of BCR-induced MEK phosphorylation produced by CD40L treatment does not require c-Raf, or other GA-sensitive Raf isoforms, despite CD40L-mediated enhancement of BCR-triggered Raf phosphorylation.

**CD40 engagement enhances BCR-induced MAPK phosphorylation independently of PLC-γ2**

PLC-γ2 is located downstream of PI3K and generates second messenger molecules that activate protein kinase C. To determine whether CD40L-mediated enhancement of BCR signaling for MAPK activation occurs independently of additional signalosome elements, we stimulated B cells with anti-Ig in the presence or absence of the PLC inhibitor, U73122, with or without prior CD40L treatment, and then examined ERK phosphorylation. We and others have shown that U73122 at 1 μM blocks BCR-induced increased Ca2+ (36) and NF-κB activation (data not shown) in primary B cells. Results are shown in Fig. 5.

Anti-Ig stimulation of naive B cells produced substantial phosphorylation of ERK within 5 min, as noted above, which was strikingly reduced by inhibition of PLC-γ2 with U73122. CD40L treatment greatly enhanced subsequent BCR-induced ERK phosphorylation, and this was only partially inhibited by U73122, so that the amount of anti-Ig-stimulated phospho-ERK in CD40L-treated, U73122-inhibited B cells was much greater than the amount of phospho-ERK present after anti-Ig stimulation of naive (CD40L-untreated) B cells. Thus, CD40L treatment enhances

**FIGURE 4.** CD40L-enhanced, BCR-induced ERK phosphorylation is associated with c-Raf phosphorylation, but does not depend on c-Raf. A, CD40 engagement enhances BCR-induced phosphorylation of c-Raf regardless of the presence of a PI3K inhibitor. B cells were cultured in medium (CD40L (−)) or with CD40L (CD40L (+)), and then stimulated with anti-Ig (αIg) for the indicated times in the presence of LY294002 (LY) or control diluent DMSO (DM), as described in Fig. 1. Whole cell extracts were Western blotted with anti-phospho-c-Raf and anti-c-Raf Abs. One of three comparable experiments is shown. B, CD40 engagement enhances BCR-induced Ras activation independently of PI3K. B cells were treated as described in A. Ras-GTP was precipitated from whole cell extracts by incubation with GST-RBD protein and detected by Western blotting with anti-Ras Ab, as described in Materials and Methods. One of three comparable experiments is shown. C, GA depletes B cell c-Raf and blocks BCR-induced MEK phosphorylation. B cells were cultured with GA at 2 μM for 12 h, washed, and then stimulated with anti-Ig for the time indicated in minutes. Whole cell extracts were Western blotted with anti-c-Raf and anti-phospho-MEK Abs. Blots were stripped and reprobed with anti-MEK Ab to verify equal loading of extracted protein (not shown). D, BCR-induced MEK phosphorylation does not depend on c-Raf activity. B cells were cultured with CD40L for 48 h, with (+) or without (−) GA added during the last 12 h. B cells were washed, and then stimulated with anti-Ig for the indicated times in the presence of LY294002 (LY) or control diluent DMSO (DM), as described in Fig. 1. Whole cell extracts were Western blotted with anti-c-Raf, anti-phospho-MEK, and anti-MEK Abs, as described above.

**FIGURE 5.** CD40 engagement enhances BCR-induced MAPK phosphorylation independently of PLC-γ2. B cells were cultured in medium (CD40L (−)) or with CD40L (CD40L (+)), and then stimulated with anti-Ig (αIg) for the indicated times, as described in Fig. 1. B cells were exposed to the PLC-γ2 inhibitor, U73122 at 1 μM, or diluent control DMSO (DM) starting 30 min before addition of anti-Ig. Whole cell extracts were prepared and Western blotted with anti-phospho-ERK and anti-ERK Abs as described in Fig. 1. One of two comparable experiments is shown.
BCR signaling for ERK phosphorylation through a pathway(s), that operates at least in part independently not only of PI3K, but also of the signalosome element, PLC-γ2, indicating that the findings in this study are not limited to a single mediator or a single inhibitor.

**BCR-induced ERK activation after CD40-mediated receptor cross-talk promotes downstream events**

To determine whether CD40L-mediated enhancement of BCR signaling for ERK activation enhances ERK-dependent downstream events, we examined CD69 expression in the presence or absence of LY294002 (38). Results are shown in Fig. 6. Anti-Ig stimulation of naive B cells produced an increased level of CD69 expression, which was completely eliminated by LY294002. However, after CD40L treatment, anti-Ig triggered up-regulated CD69 expression, which was then resistant to PI3K inhibition. These results indicate that the enhanced and partially PI3K-independent MAPK activation produced by anti-Ig stimulation following CD40L treatment promotes at least one typical ERK-dependent downstream activation event, suggesting that the alternate pathway has physiological consequences.

**Discussion**

The present study demonstrates that BCR induction of MAPK activation is markedly enhanced by prior B cell treatment with CD40L and in so doing identifies a novel system of receptor cross-talk between CD40 and BCR. In this case, engagement of CD40 up-regulated MAPK activation produced by anti-Ig added after, rather than concurrently with, CD40L, and the effect was greater with increased duration of CD40L exposure. Both ERK and JNK were affected, whereas p38 MAPK was not, which fits well with previous work indicating that p38 MAPK activation is distinct from ERK/JNK activation (27, 52). CD40-mediated enhancement of BCR-triggered ERK and JNK occurred even when PI3K or PLC-γ2 was blocked, suggesting that it operates, at least in part, through a nonsignalosome-dependent pathway. At the same time, the level of enhancement was reduced by inhibition of PI3K and PLC-γ2, just as the level of BCR-induced ERK activation in naive cells is reduced by PI3K inhibition (34–36), suggesting that signalosome elements are involved, at least in part, at some level.

Enhancement of phosphorylation and partial dependence on PI3K extended to the upstream ERK activators MEK and c-Raf, although c-Raf did not appear to be part of the pathway for enhanced BCR-induced ERK activation. The functional relevance of increased and PI3K-independent BCR-triggered MAPK activation resulting from CD40 receptor cross-talk is supported by the demonstration of anti-Ig-induced up-regulated CD69 expression and enhanced thymidine incorporation in the presence of LY294002. These results are summarized diagrammatically in Fig. 7.

The use of PI3K inhibitors in these experiments presented distinct advantages over the use of B cells obtained from PI3K-deficient animals, because PI3K-deficient B cells do not proliferate normally in response to CD40 engagement (53, 54), indicating that CD40 signaling is deranged. Thus, failure to establish an alternate pathway because of abnormal CD40 signaling would be indistinguishable from failure to bypass PI3K. Moreover, “knockdown” of PI3K with siRNA was impractical because of the potentially confounding effects of IFN production (55) and because the requirement of primary B cell stimulation for retroviral transduction would make it impossible to compare the effects of PI3K reduction on naive and CD40L-stimulated B cells.

Although the precise nature of the BCR-triggered pathway leading to ERK/JNK phosphorylation that is enhanced by CD40L treatment remains uncertain, the results presented in this study suggest the roles of some mediators. Like ERK, BCR-induced MEK phosphorylation was enhanced by prior CD40L treatment and was partially blocked by PI3K inhibition; further, inhibition of MEK activity interrupted BCR-induced ERK phosphorylation. Thus, MEK seems to be intimately involved in enhancing ERK activation. Like ERK and MEK, BCR-induced c-Raf phosphorylation was enhanced by prior CD40L treatment and was partially blocked by PI3K inhibition; however, unlike MEK, inhibition of c-Raf had little or no effect on BCR-induced MEK phosphorylation, although complementation by B-Raf, whose sensitivity to GA has not been defined, remains a possibility (56). Thus, c-Raf may not be involved in the enhancement pathway and input to MEK

![FIGURE 6](image-url)  
**FIGURE 6.** BCR-induced ERK activation following CD40-mediated receptor cross-talk promotes downstream events. B cells were cultured in medium (CD40L(−)) or with CD40L (CD40L(+)) for 24 h, then stimulated with anti-Ig (αIg) for 24 h. B cells were exposed to LY294002 starting 30 min prior to addition of anti-Ig, as indicated. B cells were unstained (thin line) or were stained with anti-CD69 Ab (thick line) and analyzed for fluorescence by flow cytometry. One of three comparable experiments is shown.

![FIGURE 7](image-url)  
**FIGURE 7.** Model depicting reprogramming of BCR signaling for ERK phosphorylation as described in this report. The dotted line indicates the CD40L-induced, alternate pathway for enhanced BCR-mediated MAPK activation that is largely, but not completely, PI3K independent. Note that only a selected set of the mediators that propagate signals from BCR to Ras/Raf1 are depicted.
probably comes from another source. This also implies that Ras is not involved in producing enhanced BCR-induced MEK/ERK phosphorylation, unless MEK derives input from Ras (directly or indirectly, without c-Raf, plus signalosome elements), although it may be involved in c-Raf enhancement.

A direct connection between CD40 cross-talk for enhanced BCR-induced MEK/ERK phosphorylation and CD40 cross-talk for signalosome-independent NF-κB activation seems unlikely. B cells deficient in Bam32 failed to properly activate ERK and JNK in response to BCR triggering, whereas activation of NF-κB (and p38 MAPK) proceeded normally (27). In contrast, B cells deficient in Carma1 failed to activate NF-κB in response to BCR triggering, whereas activation of ERK proceeded normally (28–30). It has been suggested, then, that Bam32 and Carma1 channel BCR engagement into two distinct pathways, one operating through MEK and ERK, and the other propagating signalosome-derived signals to IKK phosphorylation and NF-κB induction (57). However, as noted above, interconnections between these pathways probably exist, although some of these may be cell type-specific. Whereas elements of the Ras/Raf/Mek/ERK pathway have been reported to be involved in NF-κB induction triggered by various ligands in different cell types (58–62), in B cells interruption of PI3K completely blocked BCR-induced NF-κB, suggesting that the ERK pathway does not play a major role in this outcome (63, 64). Thus, there is little evidence at this time to suggest whether the effects of CD40L on BCR signaling for ERK and NF-κB operate through a common, central element. However, we recently found that IL-4 induces an alternate, PI3K-independent pathway for ERK phosphorylation, but not for NF-κB activation, suggesting that these outcomes are separately reprogrammed as a result of receptor cross-talk.5

The enhanced phosphorylation of MEK/ERK/JNK described in this study might be thought to be similar to enhanced ERK activation produced by engagement of CD19 and BCR (65). However, enhancement produced by CD19 and BCR occurred when these receptors were triggered concurrently, which was not the case for CD40 and BCR, and enhancement produced by CD19 and BCR involved both ERK and p38 MAPK, whereas p38 MAPK was not affected by sequential engagement of CD40 and BCR.

The enhanced BCR signaling produced by CD40 engagement identified here for MAPK activation and elsewhere for NF-κB induction (21) strongly suggests that coincident with CD40 signaling the biological outcome of CD40 signaling is dependent on the duration of CD40 and Fas (CD95) ligands modulated by the B cell antigen receptor. Cell 87:319.


Han, A., K. Sajo, I. Mecklenbrauker, A. Takaroхovsky, and M. C. Nussenzev. 2003. Bam32 links the B cell receptor to ERK and JNK and mediates B cell proliferation but not survival. Immunity 19:621. 


1 B. Guo and T. L. Rothstein. B cell receptor cross-talk: IL-4 creates an alternate pathway for BCR-induced ERK activation that is PI3K-independent. Submitted for publication.

References


