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Cutting Edge: B Cell Receptor (BCR) Cross-Talk: The IL-4-Induced Alternate Pathway for BCR Signaling Operates in Parallel with the Classical Pathway, Is Sensitive to Rottlerin, and Depends on Lyn

Benchang Guo,* Derek Blair,† Thomas C. Chiles,† Clifford A. Lowell,‡ and Thomas L. Rothstein2*

B cell exposure to IL-4 alters subsequent BCR signaling such that ERK phosphorylation becomes signalosome-independent; however, the nature of this new, alternate signaling pathway and its relationship to the classical, signalosome-dependent signaling pathway are not known. In this study, we report that the alternate and classical pathways for BCR signaling are differentially affected by rottlerin, and by Go6976 or LY294002, respectively. Furthermore, in B cells lacking protein kinase C (PKC)β, the classical pathway for BCR signaling is blocked, whereas the alternate pathway is little affected. Conversely, in B cells lacking Lyn, the alternate pathway for BCR signaling is blocked, whereas the classical pathway is little affected. The rottlerin-sensitive element is not PKCβ, inasmuch as the alternate pathway is not blocked in PKCβ-deficient B cells. These results indicate that the rottlerin-sensitive, Lyn-dependent alternate pathway, and the classical pathway, for BCR signaling operate in parallel when BCR engagement follows IL-4 exposure.


Surface Ig constitutes the B cell Ag-binding moiety, engagement of which initiates downstream signaling through associated proteins in the BCR complex. This triggers an initial signaling cascade involving several early signaling molecules that have been collected in a conceptual framework termed the signalosome (1). The prototype for this group is Bruton’s tyrosine kinase, mutation or deletion of which results in diminished numbers of mature B cells and complete failure of BCR signaling. Equivalent phenotypes are found in mice constructed with genetic deficiencies of the p85α subunit of PI3K, the p110δ subunit of PI3K, B cell linker, phospholipase C (PLCγ)-γ2, and protein kinase C (PKC)β (1, 2). Thus, each of these molecules is individually required for successful BCR signal transduction, which eventuates, among other outcomes, in phosphorylation of ERK and other MAP kinases that mediate activation of AP-1 components. Importantly, although multiple inputs appear capable of influencing ERK activation following BCR engagement, the vast majority of ERK phosphorylation is dependent on signalosome signaling and is blocked by inhibitors of PI3K (3).

IL-4 is a pleiotropic cytokine produced by multiple cell types that plays a key role in B cell physiology by enhancing proliferation, maintaining viability, fostering isotype switching, and altering surface Ag expression (reviewed in Ref. 4). In exploring the interactions between IL-4R engagement and BCR signaling, we unexpectedly found that triggering the former produced an alternate pathway for the latter, in which BCR signaling for ERK phosphorylation became independent of, and thus bypassed the need for, PI3K and PLC signalosome mediators (5). This reprogramming of BCR signaling was not produced by multiple other cytokines examined (5).

To clarify the relationship between BCR signaling via the alternate and classical pathways and the molecular mechanisms involved, we examined the role of several potential mediators, specifically including members of the PKC family and Lyn. The results of these studies demonstrated that after IL-4 treatment the signalosome-independent alternate pathway for BCR signaling coexists with, but does not replace, the classical pathway.

Materials and Methods

Animals

Male BALB/cByJ mice at 8–14 wk of age were obtained from The Jackson Laboratory. PKCβ-deficient mice (Prkcb1tm1Tara on a mixed C57BL/6 and 129×1/SvJ background) generated by Dr. M. Leitges (Max-Planck-Institute of Experimental Endocrinology, Hannover, Germany) (6) were obtained from Dr. G. King (Joslin Diabetes Center, Boston, MA) and bred at Boston College.

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3 Abbreviations used in this paper: PLC, phospholipase C; PKC, protein kinase C; pERK, phosphorylated ERK; MZ, marginal zone.

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PKCδ-deficient mice (Prkdc<sup>−/−</sup> on a mixed C57BL/6 and 129×1/SvJ background) were provided by Dr. R. Messing (University of California, San Francisco, CA) and bred at Boston University Medical Center. Lyn-deficient mice (B6.129S4-Lyn<sup>−/−</sup>) have been described previously (7). Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines, and studies with these mice were approved by institutional review committees.

B cell isolation

B cells were prepared from spleen cell suspensions by negative selection and cultured at 2 × 10^7/ml in RPMI 1640 medium as described previously (8).

B cell stimulation

B cells were stimulated by F(ab′)2, goat anti-mouse IgM (anti-Ig) after incubation in medium for 3 h, or after treatment with IL-4 for 24 h followed by incubation in medium for 3 h. Inhibitors were added 1 h before stimulation with anti-Ig.

Western immunoblot analysis

Proteins were extracted from B cell pellets with radioimmunoprecipitation assay lysis buffer. In each experiment, equal amounts of protein for each condition (15–30 µg) were subjected to SDS-PAGE followed by immunoblotting as described previously (9). Immunoreactive proteins were detected by ECL (Amersham Biosciences). Immunoblots were stripped and reprobed with control Ab to verify that equal amounts of protein were loaded in each lane.

Reagents

Affinity-purified F(ab′)2, polyclonal goat anti-mouse IgM (anti-Ig) was obtained from Jackson ImmunoResearch Laboratories. Anti-phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), anti-ERK1/2, and secondary Abs for immunoblotting were obtained from Cell Signaling Technology. Anti-phospho-ERK1/2 was obtained from Sigma-Aldrich. LY294002, Go6983, Go6976, PP2, PP3, and rottlerin were obtained from Cell Signaling Technology. Anti-actin Ab was obtained from Pharmingen.

Results

To explore the requirements for BCR signaling via the alternate pathway, we tested several PKC inhibitors with differing specificities. We stimulated B cells with anti-Ig in the presence or absence of one or another PKC inhibitor, with or without prior IL-4 treatment, and then examined ERK phosphorylation (Fig. 1). In naive B cells, anti-Ig-stimulated ERK phosphorylation was eliminated by the broad-spectrum PKC inhibitor, Go6983 (10), which inhibits PKCαβγδ, as well as by the more limited PKC inhibitor, Go6976 (11), which primarily inhibits the conventional PKC isoforms PKCαβ (consistent with the role of PKCβ as a signalosome element integral to BCR signaling via the classical pathway), but not by rottlerin (12), which is relatively specific for PKCδ (notably, optimal inhibitor doses were determined by separate titrations in data not shown and were similar to those used in other reports (13, 14)). The level of phosphorlated ERK (pERK) (as a percentage of uninhibited control values) present at 15 min after anti-Ig-stimulation of inhibitor-treated naive B cells, in three independent experiments was as follows: Go6983, 5.30 ± 3.48% (mean ± SEM); and Go6976, 3.62 ± 3.58%. The situation was quite different, however, for IL-4-treated B cells. After IL-4 treatment, anti-Ig-stimulated pERK was eliminated by Go6983 (4.07 ± 3.52% pERK remaining; n = 3), as with naive B cells (see above), but was substantially resistant to Go6976 (52.3 ± 9.36% pERK remaining; n = 3) and rottlerin (58.9 ± 8.07% pERK remaining; n = 3). These results seemed counterintuitive in that alternate pathway pERK was inhibited by a PKC inhibitor that blocks PKCαβγδ but not to the same extent by more restricted inhibitors that primarily block either PKCαβ or PKCδ. This suggested that two PKC isoforms may be involved, operating in parallel, both sensitive to Go6983. To address this possibility, we treated B cells with the two inhibitors, Go6976 and rottlerin, individually and together. Importantly, in IL-4-treated B cells, anti-Ig-stimulated pERK was completely eliminated by the combination of Go6976 plus rottlerin (4.03 ± 2.23% pERK remaining; n = 3).

To more fully address the possibility that IL-4 treatment adds an alternate BCR signaling pathway to a preexisting classical one, we treated B cells with LY294002 and rottlerin, individually and together, inasmuch as the ability to circumvent the need for PI3K defines the alternate pathway (5). Importantly, in IL-4-treated B cells, anti-Ig-stimulated pERK was substantially resistant to LY294002 alone, indicating the presence of alternate pathway signaling (59.2 ± 4.89% pERK remaining; n = 3), and to rottlerin alone (see above), but was completely eliminated by the combination of LY294002 plus rottlerin (2.70 ± 1.46% pERK remaining; n = 3). Thus, the alternate, PI3K- and PKCβ-independent pathway for BCR signaling induced by IL-4 requires a rottlerin-sensitive mediator, which the classical pathway for BCR signaling does not, and ERK phosphorylation stimulated by anti-Ig in IL-4-treated B cells is only blocked when inhibitors of both the alternate and the classical pathways are used together, suggesting that these two pathways operate in parallel.
If the alternate and classical pathways are truly separate and distinct, then B cells from PKCβ-deficient mice that lack classical, signalosome-dependent BCR signaling, should, after IL-4 treatment, still express the alternate BCR signaling pathway, much like Go6976-inhibited wild-type B cells. To test this prediction, we examined B cells obtained from PKCβ-deficient mice (Fig. 2). In naive PKCβ-deficient B cells, anti-Ig produced very little ERK phosphorylation that was much less than that produced by anti-Ig in naive control B cells, as expected due to crippled signalosome signaling. However, after IL-4 treatment of PKCβ-deficient B cells, anti-Ig produced a marked increase in ERK phosphorylation that was blocked by rottlerin, much like the LY294002-resistant fraction of pERK stimulated by anti-Ig in littermate control B cells. Thus, although PKCβ-deficiency severely disrupts the classical pathway for BCR signaling, it has little or no effect on the IL-4-induced alternate pathway, demonstrating that the latter is independent of the former at the level of PKC.

We then addressed whether PKCθ is in fact the rottlerin-sensitive mediator of the alternate pathway. To test this, we examined B cells obtained from PKCθ-deficient mice (7). In naive PKCθ-deficient B cells, anti-Ig-stimulated ERK phosphorylation was inhibited by LY294002. Unexpectedly, after IL-4 treatment of PKCθ-deficient B cells, anti-Ig-stimulated ERK phosphorylation was substantially resistant to LY294002, mimicking the situation in IL-4-treated littermate control B cells (Fig. 3). In other words, BCR signaling in IL-4-treated PKCθ-deficient B cells recapitulated BCR signaling in IL-4-treated control B cells in this respect, indicating that loss of PKCθ did not hamper BCR-triggered signal propagation via the alternate pathway. Furthermore, rottlerin eliminated BCR signaling for LY294002-resistant pERK in B cells lacking PKCθ just as it did in littermate control B cells (data not shown). These results were reproduced with B cells obtained from a second line of PKCθ-deficient mice (provided by Dr. A. Tarakhovsky, Rockefeller University, NY, NY). Thus, although the alternate pathway for BCR signaling is clearly rottlerin-sensitive, PKCθ cannot be considered the rottlerin-inhibitable central mediator of the alternate pathway.

To further explore alternate pathway signaling requirements, we examined B cells exposed to PP2, a src kinase inhibitor, or PP3, its relatively inactive congener. In IL-4-treated B cells, anti-Ig-stimulated LY294002-resistant ERK phosphorylation was blocked by PP2, but not by PP3 (Fig. 4 A). These results demonstrate that BCR signaling via the alternate pathway, like BCR signaling via the classical pathway, depends on src kinase activity.

To identify more precisely the point at which BCR signaling via the alternate and classical pathways diverge, we examined the role of Lyn, the predominant src kinase in B cells, by testing B cells obtained from Lyn-deficient mice. In naive Lyn-deficient B cells, anti-Ig stimulated substantial ERK phosphorylation, and this anti-Ig-triggered pERK was fully inhibited by LY294002. Thus, BCR signaling in naive Lyn-deficient B cells recapitulated BCR signaling in naive littermate control B cells.

**FIGURE 2.** The IL-4-induced alternate pathway for BCR signaling operates independently of PKCβ. B cells were obtained from PKCβ-deficient mice (PKCβ KO) and from wild-type littermate controls (WT). B cells were cultured in medium alone (MED) or with IL-4 at 10 ng/ml for 24 h (IL-4), after which viable cells were stimulated with anti-Ig at 15 μg/ml (alg) for 15 min, as indicated. B cells were exposed to LY294002 at 20 μM (LY) and rottlerin at 10 μM (Rottl), as indicated, starting 60 min before addition of anti-Ig. Whole cell extracts were prepared and Western blotted as described in Fig. 1.

**FIGURE 3.** The IL-4-induced alternate pathway for BCR signaling does not require PKCθ. B cells were obtained from PKCθ-deficient mice (PKCθ KO) and from wild-type littermate controls (WT). B cells were handled as described in the legend to Fig. 2, as indicated. Whole cell extracts were prepared and Western blotted as described in Fig. 1.

**FIGURE 4.** The IL-4-induced alternate pathway for BCR signaling depends on src kinase activity and Lyn. A, B cells were cultured in medium alone or with IL-4 as described above, after which B cells were stimulated with anti-Ig at 15 μg/ml (alg) for 15 min. B cells were exposed to LY294002 at 20 μM (LY), the src kinase inhibitor, PP2 at 20 μM, or the inactive congener, PP3 at 20 μM, starting 60 min before addition of anti-Ig, as indicated. Whole cell extracts were prepared and Western blotted as described in Fig. 1. One of three comparable experiments is shown. B, B cells were obtained from Lyn-deficient mice (Lyn KO) and from wild-type littermate controls (WT) and handled as described in the legend to Fig. 3. Whole cell extracts were prepared and Western blotted as described in Fig. 1. One of four comparable experiments is shown.
Notably, previous reports have shown that BCR signaling is little perturbed and, in some respects, enhanced, by the loss of Lyn (15–18), in light of which our results with naive Lyn-deficient B cells are not unexpected. The surprising result came from IL-4-treated Lyn-deficient B cells. Anti-Ig stimulated substantial pERK, which far outweighs the proportion of MZ B cells, because Lyn-deficient mice lack MZ B cells (21) (although against this is the relative level of alternate pathway vs classical pathway pERK, which far outweighs the proportion of MZ B cells), but this possibility was ruled out by sorting experiments in which follicular B cells were isolated, treated with IL-4, stimulated with anti-Ig, and shown to express the alternate pathway identically to total splenic B cells (data not shown).

Discussion

Previous work showed that B cell exposure to IL-4 alters the nature of subsequent BCR signaling so that ERK phosphorylation proceeds through an alternate pathway without the need for signalsosome elements (5). It has remained unclear as to whether BCR signaling via the alternate pathway induced by IL-4 supplants, or supplements, classical pathway BCR signaling. Taken together, the results reported herein now indicate that the IL-4-induced alternate pathway for BCR signaling operates in parallel with, but does not replace or supersedes, the classical signaling pathway. This conclusion is based on specific inhibition of classical pathway signaling by LY294002, Go6976, and PKCβ-deficiency, with minimal effect on alternate pathway signaling, and specific inhibition of alternate pathway signaling by rottlerin and Lyn-deficiency, with minimal effect on classical pathway signaling. A diagram depicting the proposed two pathway model for BCR signaling in IL-4-treated B cells is shown as Fig. 5. This work further emphasizes that previous paradigms for BCR signaling requirements based on studies of naive B cells may not be fully applicable to B cells in the midst of an immune response in which cytokine exposure is more likely than not.

Elevated levels of IL-4 are associated with severe infectious, allergic, and other disorders (22–24) (notably, in our in vitro experiments, IL-4 at 1 ng/ml was as effective as 10 ng/ml at inducing the alternate signaling pathway; data not shown). Thus, at various times throughout life, B cells can be expected to be exposed to IL-4 in an Ag-nonspecific fashion. This may facilitate productive immune responses; however, inasmuch as IL-4-overexpressing transgenic animals break tolerance and manifest serological autoactivity plus immune complex-mediated autoimmune dyscrasias (25–27), this may predispose to, or facilitate the development of, autoimmunity. These outcomes may relate to the well-described enhancing effect of IL-4 on B cell proliferation in response to BCR engagement (28).

We now suggest that the alternate, Lyn-dependent BCR signaling pathway induced by IL-4 exposure plays a role in mediating both the positive immune and negative autoimmune effects of IL-4 on B cell responses; thus, the alternate pathway in general, and Lyn in particular, may represent potential targets for therapeutic intervention in IL-4-associated autoimmunity, and, furthermore, the IL-4-induced alternate pathway or Lyn itself may prove useful in enhancing the efficiency of intentional immunization.

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Disclosures

The authors have no financial conflict of interest.

References