Cutting Edge: Recruitment of the CD19/CD21 Coreceptor to B Cell Antigen Receptor Is Required for Antigen-Mediated Expression of Bcl-2 by Resting and Cycling Hen Egg Lysozyme Transgenic B Cells

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Recruitment of the CD19/CD21 coreceptor is thought to lower the threshold for effective signaling through the B cell Ag receptor. We provide evidence supporting a second role for coreceptor recruitment, and that is to enhance the survival/proliferative potential of the responding B cells. We show that B cell Ag receptor signaling in the absence of coreceptor recruitment induces cellular accumulation of the anti-apoptotic protein Bcl-xL whereas CD19-mediated signals are required for Bcl-2 accumulation. The expression of both anti-apoptotic proteins correlates with the enhanced responsiveness of both resting and cycling B cells to growth-promoting signals delivered through CD40. These results provide further evidence for the necessity of coreceptor recruitment during Ag-dependent B cell activation and indicate that Ags derived from inflammatory sites function as better thymus-dependent Ags than their counterparts not coated with complement fragments. The Journal of Immunology, 1999, 162: 4377–4380.

Bcl-2 is the prototype for the Bcl-2 family of survival/death proteins and represents the first oncoprotein demonstrated to promote cell survival rather than cellular expansion. Members of the Bcl-2 family share homology at functional domains responsible for the formation of homo- and heterodimeric complexes between family members (1). Since Bcl-2 can heterodimerize and thus interfere with the activity of proapoptotic Bcl-2 family members such as Bax, the relative levels of Bcl-2 in relationship to the other family members is important in determining cell fate (2). Bcl-2 interferes with Bax-mediated cell death, at least in part, by blocking Bax-induced release of mitochondrial cytochrome c (3, 4). Once in the cytosol, cytochrome c serves as a required cofactor for the initiation of a caspase cascade. In addition, Bcl-2 acts downstream of cytochrome c release (5), most probably by sequestering the cytochrome c receptor, Apaf-1 (6).

Liu et al. (7) originally showed that cross-linking B cell Ag receptor (BCR)2 expressed by centroblasts/centrocytes recovered from human germinal centers by anti-BCR immobilized onto sheep RBC induces elevated expression of Bcl-2. We extended this observation by showing that restimulation of cycling B cells with soluble F(ab')2 anti-BCR induces accumulation of Bcl-xL while restimulation with immobilized anti-BCR induces both Bcl-xL and Bcl-2 (8). In this study, hen egg lysozyme (HEL)-specific transgenic B cells (9) are used to define the parameters necessary for specific Ag to regulate Bcl-2 expression in both resting and cycling B cell populations. Our results show that although occupancy of BCR by either soluble or immobilized Ag elicits cellular accumulation of Bcl-xL, the CD19/CD21 coreceptor must be recruited to induce Bcl-2 expression. The accumulation of both survival proteins correlates with enhanced responsiveness of resting or cycling B cells to Th cell-induced proliferation. This suggests, therefore, that stimulation of either resting or cycling B cells with Ags derived from inflammatory loci provides a survival and functional advantage to the responding B cells.

Materials and Methods

Mice

A colony of HEL transgenic mice (the MD4 line, a generous gift of Dr. Noelle, Dartmouth Medical School and originally described by Goodnow and colleagues (9)) was maintained by breeding transgenic mice to normal C57BL/6j females. Transgenic male and female mice were used at 6–12 wk of age. The handling and care of all mice were approved by the University of Kentucky Animal Care Committee.

Preparation of resting and cycling B cells

Resting B cells were isolated from spleens of HEL transgenic B cells as described (10). Cycling B cells were prepared from resting B cell preparations as described (8, 10). Briefly, 2.5 × 10^6 resting B cells/ml in complete media (RPMI 1640 supplemented with 10% FCS (Sigma, St. Louis, MO), penicillin, streptomycin, gentamicin, glutamine, and 50 μM 2-ME) were stimulated with 25 μg/ml LPS (purified from Salmonella enteritidis, Sigma) in 100-ml bulk cultures per 162-cm² flasks (Costar, Cambridge, MA) for 48 h. The recovered cells were separated on three-step Percoll gradients (Amersham Pharmacia Biotech, Piscataway, NJ), and the cells

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2 Abbreviations used in this paper: BCR, B cell Ag receptor; HEL, hen egg lysozyme.
bunding to the 50%–0% interface were collected and used as cycling B cells.

**Analysis of Ag-induced resting or cycling HEL transgenic B cell proliferation**

Both resting and cycling HEL transgenic B cells were cultured at $1 \times 10^5$ cells/well in 200 µl of complete media in 96-well flat-bottom plates (Costar), and the cells were stimulated as described in the figure legends. HEL (Sigma) and anti-CD19 (clone 1D, a rat IgG anti-mouse CD19, was a generous gift of Dr. Bondada, University of Kentucky Medical Center) were attached to cyanogen bromide-activated Sepharose 4B as suggested by the vendor, Sigma. CD40 ligand-baculovirus-infected Sf9 cells were a generous gift from Dr. Berton, University of Texas Health Science Center at San Antonio, San Antonio, TX, and were fixed with paraformaldehyde before use. Each well was pulsed with 1 µCi of $[^3]$Hthymidine (ICN, Irvine CA, 67 Ci/mmol) for the final 6 h of culture.

**Western analysis**

Resting or cycling HEL transgenic B cells were cultured at $1 \times 10^7$ cells in 1.0 ml of complete media in 12-well Costar plates and stimulated as described in the figure legends. The Western analyses were done as described (11). The following Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rat anti-mouse Bcl-2 (mAb 4C11), rabbit anti-human/mouse Bcl-xL/S (S-18), goat anti-human actin (C-11), anti-goat IgG conjugated with horseradish peroxidase (sc-2020), and goat anti-rat IgG conjugated with horseradish peroxidase. A goat anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Sigma.

**Results and Discussion**

Using normal B cells, we previously demonstrated that the restimulation of cycling B cells with either soluble F(ab')$_2$ or immobilized anti-µ elicited the transient continuation of B cell proliferation (8). Although both forms of anti-BCR stimulation induced the accumulation of the anti-apoptotic protein, Bcl-xL, only restimulation with immobilized anti-µ induced Bcl-xL. Two possibilities are responsible for regulating Bcl-xL expression, while CD19–coreceptor to sites of cross-linked BCR. The present study addressed the second possibility by stimulating cycling or resting HEL transgenic B cells with HEL coimmobilized with a monoclonal anti-CD19 Ab onto Sepharose beads (H:19:B).

Cycling HEL transgenic B cells recultured in the absence of stimuli displayed a rapid reduction in their proliferation (media in Fig. 1). As we have previously reported (10), restimulation of the cycling cells with HELs, HEL-B, or H:19:B elicited transient increases in radionucleotide incorporation that was always less than seen in cultures restimulated with CD40L alone (Fig. 1A). The coaddition of either HELs or HEL-B with CD40L did not appreciably enhance proliferation beyond levels detected in cultures receiving only CD40L (Fig. 1B). In contrast, restimulation with H:19:B both enhanced and extended CD40L-induced cycling B cell proliferation (Fig. 1B). This result is reminiscent of our previous finding that immobilized anti-µ extended and enhanced cycling B cell responsiveness to CD40-derived proliferative signals (8).

**FIGURE 1.** Impact of CD19-derived signals on BCR- and CD40-induced maintenance of cycling B cell proliferation. The cycling B cells were restimulated with: media, no stimulant; CD40L, a 1:50 ratio of fixed CD40L/S9 cells to input B cells; HELs, 10 µg/ml soluble HEL; HEL:B, 10 µg/ml immobilized HEL; H:19:B, 10 µg/ml HEL coimmobilized with anti-CD19; CD19s, 10 µg/ml soluble anti-CD19; or CD19:B, 10 µg/ml immobilized anti-CD19. All cultures were pulsed with $[^3]$Hthymidine for the final 6 h of culture. This experiment is representative of at least 10 independent experiments.

Since the ability of immobilized anti-µ to potentiate CD40-mediated cycling B cell proliferation correlated with a concomitant increase in both Bcl-2 and Bcl-xL, cycling HEL transgenic B cells restimulated as described in Fig. 1 were examined by Western analysis for expression of the two survival proteins (Fig. 2). As seen when cycling normal B cells were challenged with soluble F(ab')$_2$ anti-µ (9), restimulation of cycling HEL transgenic B cells with either soluble (results not shown) or immobilized (Fig. 2, lane D) HEL induced expression of only Bcl-xL. In contrast, restimulation with HEL coimmobilized with anti-CD19 induced expression of both Bcl-2 and Bcl-xL (Fig. 2, lane E). Restimulation with immobilized anti-CD19 induced accumulation of Bcl-2 but not Bcl-xL (Fig. 2, lane C). This indicates that BCR-derived signals were responsible for regulating Bcl-xL expression, while CD19-mediated signals regulated Bcl-2 expression. Interestingly, restimulation with soluble anti-CD19 did not induce accumulation of
Bcl-2 (Fig. 2, lane F). Thus, simply cross-linking the CD19/CD21 coreceptor was not sufficient to elicit Bcl-2 expression, suggesting that the CD19/CD21 coreceptors must aggregate to some minimal density in the plane of the membrane to impact upon cellular accumulation of Bcl-2. Restimulation of the cycling cells with LPS mimicked restimulation with CD40L (8) by inducing only the expression of Bcl-xL (Fig. 2, lane A). However, when HEL was coimmobilized with anti-CD19, there was a dramatic increase in CD40L-induced B cell proliferation (Fig. 2, lane E). Although immobilized anti-CD19 modestly increased CD40-mediated resting B cell proliferation, soluble anti-CD19 always decreased CD40-mediated B cell proliferation (Fig. 3C). The reason for this is unclear. The same pattern of Bcl-2 and Bcl-xL accumulation seen after restimulation of cycling HEL transgenic B cells was seen when resting HEL transgenic B cells were stimulated with the various forms of Ag (Fig. 4). Both soluble (results not shown) and immobilized HEL induced Bcl-xL accumulation in the absence of Bcl-2 expression. Immobilized, but not soluble, anti-CD19, induced Bcl-2 expression with or without being coimmobilized with HEL. Immobilized anti-CD19 in the absence of HEL, however, failed to elicit Bcl-xL accumulation. Therefore, BCR-derived signals regulated Bcl-xL expression while signals through CD19 regulated Bcl-2 expression.

Next, we determined the ability of BCR-derived signals, with or without colocalization of the CD19/CD21 coreceptor, to regulate Bcl-2 and Bcl-xL expression by resting HEL transgenic B cells. As expected (12), HEL in soluble, immobilized, or coimmobilized with anti-CD19 delivered signals insufficient to elicit resting B cell cycle progression (Fig. 3A). Unexpectedly, however, neither immobilized nor soluble HEL was found to substantially augment the level of resting B cell radionucleotide incorporation induced by CD40L (Fig. 3B). However, when HEL was coimmobilized with anti-CD19, there was a dramatic increase in CD40L-induced B cell proliferation (Fig. 3B). Although immobilized anti-CD19 modestly increased CD40-mediated resting B cell proliferation, soluble anti-CD19 always decreased CD40-mediated B cell proliferation (Fig. 3C). The reason for this is unclear. The same pattern of Bcl-2 and Bcl-xL accumulation seen after restimulation of cycling HEL transgenic B cells was seen when resting HEL transgenic B cells were stimulated with the various forms of Ag (Fig. 4). Both soluble (results not shown) and immobilized HEL induced Bcl-xL accumulation in the absence of Bcl-2 expression. Immobilized, but not soluble, anti-CD19, induced Bcl-2 expression with or without

Bcl-2 (Fig. 2, lane F). Thus, simply cross-linking the CD19/CD21 coreceptor was not sufficient to elicit Bcl-2 expression, suggesting that the CD19/CD21 coreceptors must aggregate to some minimal density in the plane of the membrane to impact upon cellular accumulation of Bcl-2. Restimulation of the cycling cells with LPS mimicked restimulation with CD40L (8) by inducing only the expression of Bcl-xL (Fig. 2, lane A). Unexpectedly, however, neither immobilized nor soluble HEL was found to substantially augment the level of resting B cell radionucleotide incorporation induced by CD40L (Fig. 3B). However, when HEL was coimmobilized with anti-CD19, there was a dramatic increase in CD40L-induced B cell proliferation (Fig. 3B). Although immobilized anti-CD19 modestly increased CD40-mediated resting B cell proliferation, soluble anti-CD19 always decreased CD40-mediated B cell proliferation (Fig. 3C). The reason for this is unclear. The same pattern of Bcl-2 and Bcl-xL accumulation seen after restimulation of cycling HEL transgenic B cells was seen when resting HEL transgenic B cells were stimulated with the various forms of Ag (Fig. 4). Both soluble (results not shown) and immobilized HEL induced Bcl-xL accumulation in the absence of Bcl-2 expression. Immobilized, but not soluble, anti-CD19, induced Bcl-2 expression with or without being coimmobilized with HEL. Immobilized anti-CD19 in the absence of HEL, however, failed to elicit Bcl-xL accumulation. Therefore, BCR-derived signals regulated Bcl-xL expression while signals through CD19 regulated Bcl-2 expression.

CD19 is recruited to BCR aggregated by complement-coated Ags because it is noncovalently associated within the plane of the membrane with CD21, the C3d receptor (13). The importance of this recruitment is highlighted by studies showing that both CD21 (14, 15) and CD19 (16, 17) knockout mice are impaired in their ability to respond to thymus-dependent Ags. One explanation for this dramatic codependence is that CD19/CD21 recruitment effectively lowers the threshold of Ag required to induce B cell cycle progression because CD19 amplifies BCR-mediated activation signals (18). Our results support a previously proposed second alternative (19), that CD19 recruitment to sites of active BCR signaling provides B cells with a survival advantage. Furthermore, the data suggest that this is accomplished by a CD19-mediated induction of Bcl-2 accumulation to complement Bcl-xL induced by virtue of BCR signaling.
Both resting and cycling B cells require BCR-derived signals to either be recruited into or remain part of an ongoing humoral immune response. Our results suggest that whether or not the Ag is derived from inflammatory foci impacts upon the ultimate survival/proliferative potential of the responding B cells. Soluble or immobilized Ags not derived from inflammatory foci only induce the accumulation of Bcl-xL, and elicit only minimal enhancement of CD40-mediated B cell proliferation. Immobilized CD19 induces accumulation of Bcl-2, but impacts only marginally upon CD40-mediated B cell proliferation. The recruitment of the CD19/CD21 coreceptor to sites of active BCR signals results in the accumulation of two survival proteins to complement BCR-derived biochemical signals that interface with the CD40-mediated growth signal. Thus, Ags derived from inflammatory sites are inherently better stimulators for either initiating resting B cell activation or maintaining the continued expansion of cycling B cells.

The results of this study support our previous suggestion (8) that BCR-derived signals determine the window of opportunity that B cells have to locate appropriate Th cell activity. The present results extend the original hypothesis by showing that Ags derived from inflammatory sites are the most effective in extending the time that resting and cycling B cells have to find the right Th cell. Although Th cell-mediated signals through CD40 can induce Bcl-xL expression in either resting or cycling B cells (8, 20), this may not be the most important contribution provided by T cell help since the BCR signal also induces Bcl-xL accumulation. Rather, the more important Th cell contribution may be the ability of CD40-induced signals to regulate B cell expression of key cell cycle-related proteins, such as p21 (21), that are required for the B cell to pass the G1-S boundary and initiate DNA synthesis.

The one caveat to the above hypothesis is raised by our demonstration that immobilized, but not soluble, anti-BCR, which does not recruit the CD19/CD21 coreceptors, induces Bcl-2 accumulation (8). It is possible that multimeric thymus-independent Ags that are capable of inducing extensive and long lasting BCR aggregation can elicit the same pattern of survival protein expression as seen when thymus-dependent Ags are derived from inflammatory sites and coaggregate BCR and CD19/CD21 coreceptors. This possibility will require further attention.

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References