Role of Complement-Binding CD21/CD19/CD81 in Enhancing Human B Cell Protection from Fas-Mediated Apoptosis

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Defective expression of Fas leads to B cell autoimmunity, indicating the importance of this apoptotic pathway in eliminating autoreactive B cells. However, B cells with anti-self specificities occasionally escape such regulation in individuals with intact Fas, suggesting ways of precluding this apoptosis. Here, we examine whether coligation of the B cell Ag receptor (BCR) with the complement (C3)-binding CD21/CD19/CD81 costimulatory complex can enhance the escape of human B cells from Fas-induced death. This was warranted given that BCR-initiated signals induce resistance to Fas apoptosis, some (albeit not all) BCR-triggered events are amplified by coligation of BCR and the co-stimulatory complex, and several self Ags targeted in autoimmune diseases effectively activate complement. Using a set of affinity-diverse surrogate Ags (receptor-specific mAb:dextran conjugates) with varying capacity to engage CD21, it was established that BCR:CD21 coligation lowers the BCR engagement necessary for inducing protection from Fas apoptosis. Enhanced protection was associated with altered expression of several molecules known to regulate Fas apoptosis, suggesting a unique molecular model for how BCR:CD21 coligation augments protection. BCR:CD21 coligation impairs the generation of active fragments of caspase-8 via dampened expression of membrane Fas and augmented expression of FLIP_L. This, in turn, diminishes the generation of cells that would be directly triggered to apoptosis via caspase-8 cleavage of caspase 3 (type I cells). Any attempt to use the mitochondrial apoptotic protease-activating factor 1 (Apaf-1)-dependent pathway for apoptosis (as type II cells) is further blocked because BCR:CD21 coligation promotes up-regulation of the mitochondrial antiapoptotic molecule, Bcl-2.

The extent to which protective signals are generated controls whether responses to foreign Ag are prolonged and responses to self Ag are aborted (11, 12). Thus, BCR signals ensure the continued viability of activated cells as long as foreign Ag is present. On the other hand, the impaired BCR signaling that characterizes most mature B cell engagements with self Ag probably promotes Fas-mediated elimination of autoreactive B cells (11–13). Anti-self cells that escape deletion while immature or that are generated as a result of later somatic mutation typically engage self Ag weakly and/or are in an anergic state. This is thought to preclude induction of BCR-mediated protective signals and to encourage the demise of potentially pathogenic clones activated upon contact with CD40 ligand (CD40L)-bearing T cells (11–13). Consistent with this interpretation, deficiencies in Fas or FasL expression result in increased numbers of activated autoimmune B cells in both mice and patients with autoimmune lymphoproliferative disease (14–16). Additionally, the germinal centers of Fas-deficient mice harbor an elevated number of highly mutated B cells. These may represent B cells with lowered affinity for the immunogen and increased reactivity for self (17, 18).

The B cell costimulatory complex, comprised of complement (C3)-binding CD21(CR2), CD19, and CD81, significantly lowers the Ag:BCR binding threshold for inducing several (albeit not all) BCR-triggered phenomena (19–23). This raises the important issue of whether protection against Fas apoptosis is more effectively achieved by complement-bound Ags. Viruses, bacteria, and Ag:Ab complexes promote deposition of C3 fragments via either the classic or alternative complement activation pathways (24). An ability of such C3d(g)-bearing BCR ligands to lower the BCR threshold for inducing protection might be important in sustaining B cell viability as concentrations of foreign Ag become limiting. Additionally, the complement-activating potential of certain atypical self Ags (25–29) might promote the escape of autoreactive B cells from Fas-triggered apoptosis despite the low affinity of the latter’s BCR.
The present study directly examines whether coengagement of BCR and the C3d-binding costimulatory complex on CD40-activated human follicular B lymphocytes influences their resistance to Fas-mediated apoptosis. The effects of coligation were investigated with surrogate C3dg-free and C3dg-bearing Ags, i.e., a set of previously described Ab:high molecular weight dextran (dex) conjugates bearing affinity-diverse anti-human IgM mAbs, with and without coconjugated anti-CD21 mAb (20, 30). As discussed previously (20, 21), the moderate multivalency of these ligands simulates the organized presentation of epitopes and C3 fragments on genuine foreign and self antigenic substrates such as bacteria, viruses, apoptotic cells, and Ag:Ab complexes. We here report that under conditions of limiting receptor engagement, a ligand’s ability to coengage both BCR and CD21 dex indeed significantly enhance resistance to Fas-mediated death. A molecular explanation for augmented protection is presented based on the degree to which BCR:CD21 coligation influences the expression of several anti- and proapoptotic proteins. The findings suggest that attempts to promote BCR:CD21 coligation during vaccine administration or, alternatively, to reduce BCR:CD21 coligation during the exacerbating flares of B cell autoimmune disease might help in optimally managing the Fas apoptosis-inducing pathway.

Materials and Methods

Monoclonal Ab:dex conjugates

A previous report has described the synthesis and binding properties of the mAb:dex conjugates employed in this study, i.e., high m.w. dex covalently linked to two distinct mAb, i.e., anti-IgM mAb (or IgGl isotype control mAb) and THB-5 anti-CD21 mAb (or IgG2a isotype control mAb) (20). The BCR binding avidity of these ligands was varied by constructing conjugates with three different affinity-diverse murine mAb specific for that same (or proximal) epitope on the C4d2 domain of human IgM; mAb HB57 (DA4.4), Fab" (Kd = 5 x 10^-7 M^-1), mAb MuS3 (Kd = 2 x 10^-7 M^-1), and mAb P24 (Kd = 2 x 10^-6 M^-1) (31, 32). The soluble conjugates have 10-12 anti-IgM mAb (or control mAb) and 10-12 anti-CD21 mAb (or control mAb) per dex molecule (20). For clarity of discussion we here occasionally refer to anti-IgM:anti-CD21:dex conjugates as C3dg-bound Ab and anti-IgM:isotype control:dex conjugates as C3dg(g)-free Ag. However, it is acknowledged that we have not eliminated the possibility that the later mAb:dex conjugates might not be fully free of C3dg(g) and might have acquired some C3dg as the result of C3 synthesis from a low number of contaminating monocytes/dendritic cells in culture.

BCells and culture conditions

Human tonsil tissue from 3- to 15-year-old donors was obtained from New York University Medical Center, New York, NY, with the cooperation of Dr. S. McCormick and the surgery and pathology staffs. Tissue was used according to the guidelines of the institutional review board of New York University Medical Center. High density, T cell-depleted B cells were obtained by rosetting and Percoll gradient density centrifugation as previously described (32-34). Naive follicular B cells were further enriched by depletion of CD27" B cells (memory, germinal center, and subepithelial marginal zone B cells) (35) with anti-human CD27 magnetic beads (Miltenyi Biotec, Auburn, CA). These were, on the average, 88% IgM", 83% CD23", and ≤1% CD27". Where indicated, some experiments were performed with high density B cells without CD27 depletion (on the average, 65% IgM", 64% IgG", 27% CD27", and 97% CD21). Additionally, human spleen B cells (National Disease Research Interchange and Cooperative Human Tissue Network) were tested. Cells were cultured in microtiter wells at 1-2 x 10^4/200 μl in RPMI 1640, 15% FCS, 5 x 10^-5 M 2-ME, and gentamicin. For lystate preparation, cells were cultured in 24-well plates at a density of 3-5 x10^6 cells/1.5 ml/well. In all but one experiment, B cells were derived from a single donor. After 3 days of culture, cells were washed twice with cold PBS and lysed in 50 μl of lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM HEPES (pH 7.6), and 1% Nonidet P-40) containing protease inhibitor cocktail (Roche, Indianapolis, IN). After 15 min on ice, lysates were cleared of debris by centrifugation and were frozen at −70°C. For each experiment, 40 μg of protein was loaded; 5-20 μg for Bcl-2 and Bcl-x assays, 40-50 μg for FLIP assays, and 5-40 μg for caspase-8, Fas-associated death domain (FADD), and Fas assays resolved on an SDS PAGE gel (12.5%), transferred to nitrocellulose, blocked with 5% nonfat dry milk, and probed with 1) mouse anti-human Bcl-2 (clone 124; 1/500 dilution; DAKO), 2) rabbit anti-Bcl-x (S-18; 1/500 dilution; Santa Cruz Biotechnology), or 3) mouse anti-FLIP (mAb N6F; 1/10 dilution of culture supernatant; gift from Drs. P. Kramer and A. Krueger, DKFS, Heidelberg, Germany) (37). Abs bound to protein bands were detected with HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) or HRP-conjugated goat anti-rabbit IgG (BD Biosciences) and ECL detection reagent (Amersham Pharmacia Biotech, Buckingham, MA) or HRP-conjugated goat anti-rabbit IgG (BD Biosciences, and MBL (Watertown, MA)) or mouse IgM control (TEPC-183) at a final concentration of 0.5 μg/ml. Cells were harvested after 12-18 h and assayed for the level of apoptotic cells by staining with FITC-annexin as previously described (36). Apoptotic cells exhibit a decrease in light scatter (36). Maximum change value for specific anti-Fas-induced apoptosis under each culture condition was calculated by subtracting background apoptosis (i.e., percentage of FITC-annexin positive cells in culture with control IgM) from the value for percentage of apoptosis in a replicate culture containing anti-Fas. Percent protection from anti-Fas-mediated apoptosis was calculated using the following formula: 1 - (specific Fas-induced apoptosis in CD40L-stimulated cultures containing mAb:dex/percent specific Fas-induced apoptosis in CD40L-stimulated cultures containing medium)) x 100.

Flow cytometric assays for B cell expression of cell surface and intracellular molecules

For analysis of surface molecules, cells were stained with FITC-mAb XG4 (31), FITC-anti-CD40L (31), CD8, HLA-DR (BD Pharmingen, San Diego, CA), and FITC- and PE-isotype controls (BD Pharmingen). Staining intensity was quantitatively measured with a FACSscan and associated software (BD Biosciences, Mountain View, CA). For analysis of intracellular molecules, Ficoll-Hypaque-isolated viable cells (1-2 x 10^6) from day 3 of culture (and before culture) were fixed with 2% paraformaldehyde, pH 7.2, in PBS for 10 min at room temperature, washed, and made permeable with 0.1% saponin in 50% heat-inactivated human AB serum diluted in PBS/HEPES (assay buffer). Cells were then incubated with 0.5 μg of Ab: mouse anti-human Bcl-2 (DAKO, Carpinteria, CA), mouse anti-human Bcl-xL (H-5, Santa Cruz Biotechnology, Santa Cruz, CA), or mouse IgG control mAb (MOPC-21) in 50 μl of the above saponin-containing assay buffer for 30 min at room temperature, washed, and then stained with R-PE-conjugated goat F(ab)_2 anti-mouse IgG (H+L) (Southern Biotechnology, Birmingham, AL) for an additional 30 min at 4°C. After washes, cells were resuspended in 1% paraformaldehyde and analyzed with a FACSscan (same settings for day 0 and day 3 cells).

Assay for Fas-mediated apoptosis

After 66-72 h incubation with CD40L and mAb:dex ligands, cell cultures were pulsed with CH11 anti-Fas mAb (Oncor (Gaithersburg, MD) and MBL (Waternow, MA)) or mouse IgM control (TEPC-183) at a final concentration of 0.5 μg/ml. Cells were harvested after 12-18 h and assayed for the level of apoptotic cells by staining with FITC-annexin as previ-
arbitrarily given a maximal value of 100%, and the ratios of the other lanes were expressed as a percentage of this maximal value.

Results

Analysis of BCR binding thresholds for inducing protection from Fas-mediated death with and without recruitment of the CD21/CD19/CD81 costimulatory complex

High density, CD27-depleted tonsil lymphocytes cultured for 3 d with CD40L and high affinity HB57 anti-IgM:dex were significantly protected from Fas-mediated apoptosis compared with B cells cocultured with CD40L and IgG control:dex (Fig. 1). BCR-mediated elicitation of a protective state was indicated by diminished incidence of apoptotic cells upon treatment with a surrogate for FasL, i.e., CH11 anti-Fas Ab (2, 7). The decision to assay for Fas-mediated apoptosis on day 3 of culture was based on the results of several other studies with CD40-activated human B cells (2, 3, 7). Although maximal levels of Fas had been noted by 48 h of culture, B cells became most susceptible to Fas-mediated apoptosis at 72 h.

To investigate whether the capacity of BCR ligand to coengage the CD21/CD19/CD81 complex might facilitate protection, B cells were stimulated with CD40L and various concentrations of a set of affinity-diverse anti-IgM:dex ligands with or without coconjugated anti-CD21 (Fig. 2A). Alternatively, cells were cocultured with mAb:dex conjugates bearing isotype control mAb with or without anti-CD21 mAb (Fig. 2A, left). The results indicate that BCR:CD21 coengagement significantly augments protection against Fas-mediated death, provided that BCR engagement is limiting. Enhancement was quite notable at 0.001–0.01 μg/ml concentrations of ligands with intermediate to high BCR binding sites (Fab’ $K_a = 2 \times 10^5$ to $5 \times 10^6$ M$^{-1}$) and at 0.01–0.1 μg/ml concentrations of the low affinity BCR ligand (Fab’ $K_a \approx 2 \times 10^6$ M$^{-1}$). At high mAb:dex concentrations (1 μg/ml), the ligand that engaged CD21 alone also induced substantial protection (Fig. 2A, left, ●). Whether this is mediated independently of the BCR signaling complex or whether this reflects the low level intrinsic association between CD19 and BCR (38) is unclear. Observations similar to all the above were made with high density B cells from human spleens (data not shown). Taken together, results with these polyclonally activating, surrogate CD3-free and CD3-bearing Ags indicate that BCR:CD21 coligation significantly reduces the BCR binding requirements for eliciting protection from Fas-mediated apoptosis.

Heightened protection from Fas-mediated death at very low concentrations of the affinity-diverse BCR:CD21 coengaging ligands was associated with heightened B cell S phase entry compared with cultures containing CD40L plus the respective monospecific BCR ligands (data not shown). Synergy between low concentrations of multivalent BCR ligands and CD40L in promoting B cell proliferation has been previously described (39, 40), and the latter findings indicate that this synergy becomes even more pronounced with multivalent BCR ligands that recruit the CD21/CD19/CD81 costimulatory complex.

Relative expression of antiapoptotic molecules, Bcl-2, Bcl-xL, and FLIPs, in B cells stimulated with CD40L and limiting BCR ligand with or without CD21 binding sites

Previous studies have established that high density resting tonsil B cells express high levels of Bcl-2, low levels of Bcl-x, and nearly undetectable levels of the two c-FLIP isoforms, FLIPL, and FLIPs, and Fas (7, 41). CD40 engagement transiently up-regulates levels of Bcl-x, FLIPL, and FLIPs (but not Bcl-2) to a maximum between 24–48 h of culture. While Fas expression is also rapidly induced and remains elevated through day 3, all the above antiapoptotic proteins significantly decline by day 3 (7, 41). If BCR ligand is concomitantly present however, increases in Bcl-xL and FLIPs are sustained (7, 9, 41). The latter is thought to contribute to BCR-mediated protection from death following Fas ligation.

To examine whether BCR:CD21 coengagement promotes greater expression of these or other antiapoptotic molecules, B cells were cultured with CD40L plus intermediate affinity Mu53 anti-IgM:dex with or without anti-CD21 (or control mAb:dex with or without anti-CD21) at a concentration manifesting the greatest protective effects of BCR:CD21 coligation (0.005–0.01 μg/ml). Day 3 lysates from five experiments were assessed for the expression of Bcl-2, Bcl-xL, FLIPL, and FLIPs by immunoblotting (Fig. 3). Consistent with other studies (7, 8, 41), cultures with CD40 alone exhibited detectable levels of Bcl-xL, FLIPL, and Bcl-2 despite susceptibility to Fas apoptosis (Fig. 3A). The additional presence of a limiting concentration of BCR:CD21 coengaging ligand significantly up-regulated the expression of Bcl-2 and FLIPL.

![Graph](http://www.jimmunol.org/)

**FIGURE 1.** High affinity anti-IgM:dextran can protect CD40-activated human follicular B cells from Fas-induced apoptosis. High density, CD27-depleted tonsil B cells were cocultured with CD40L and IgG control:dex or moderately multivalent HB57 anti-IgM:dex (1 μg/ml, a dose found optimal for the induction of several BCR-triggered phenomena (20, 21)). On day 3 cells were pulsed with apoptosis-inducing CH11 anti-Fas or IgM control (0.1 μg) and after 10 h were assessed for FITC-annexin staining (A) and light scatter (B) by flow cytometry. The degree of apoptosis is indicated as the percentage of the total population analyzed that exhibited high FITC-annexin binding (A) or low forward scatter (B).
while monospecific BCR ligand generally did not (Fig. 3, A and B). The alternative FLIP isoform, i.e., FLIP$_S$ (42), was weakly detectable in two of the five experiments. In these two cases, FLIP$_S$ was augmented upon exposure to either BCR ligand or BCR:CD21 coengaging ligand, but the levels were not statistically different from that in control cultures. In three subsequent experiments (not shown in Fig. 3) FLIP$_L$ and FLIP$_S$ were assessed by immunoblotting with a more sensitive ECL substrate. The mean ± SEM values for maximal expression of FLIP$_L$ per constant protein loaded were 19 ± 1, 9 ± 9, 80 ± 2, and 100 ± 2% for CD40-activated cultures containing Ig control:dex, anti-CD21:dex, anti-IgM:dex, and anti-IgM:anti-CD21:dex, respectively. The mean ± SEM values for maximal FLIP$_S$ expression were 37 ± 20, 51 ± 17, 76 ± 14, and 97 ± 3%, respectively. BCR:CD21 coligation did not augment the expression of Bcl-x$_L$ above that seen with monospecific anti-IgM:dex (Fig. 3, A–C). Indeed, in two of five experiments, the levels of Bcl-x$_L$ in cultures with CD40L and anti-IgM:anti-CD21:dex were less than those observed in cultures with CD40L alone. This was never observed in cultures receiving signals from CD40L and monospecific BCR ligand.

Interestingly, in three of five experiments (one shown in Fig. 3A) CD21 engagement alone augmented Bcl-2 expression above
that in both control cultures and cultures with anti-IgM:dex. However, in none of the experiments did CD21 engagement alone result in levels of Bcl-xL, FLIP_L, or FLIP_S that exceeded those in control cultures or those in cultures with anti-IgM:dex (Fig. 3; data not shown).

The above analysis does not account for the fact that the amount of protein recovered from 10^6 viable cells in cultures containing the BCR ligands was, on the average, 1.7- to 2.3-fold greater than the amount of protein recovered from an equal number of viable cells in cultures containing CD40L plus Ig control:dex or anti-CD21:dex (viability, as determined by trypan blue exclusion, was almost identical, i.e., the range in the four treatment groups was 80–84%). To obtain what may be a better assessment of the relative level of Bcl-2, Bcl-xL, and FLIP_L per cell, the data are additionally presented as normalized expression per 10^6 cells (Fig. 3C). These normalized values also indicate that at a low ligand concentration of 0.005–0.01 μg/ml, BCR:CD21 coligation can significantly augment Bcl-2 and FLIP_L expression in CD40-activated cells, while BCR engagement alone does not. Although by this analysis the levels of FLIP_S were up-regulated by BCR:CD21 coligation, the significance of this increase is questionable due to the small number of positive samples and the weak density of the protein bands.

Additional intracellular staining analyses (Fig. 4) showed that Bcl-2 levels were most effectively sustained in CD40-activated B cells receiving additional signals from BCR:CD21 coengaging ligand or CD21-engaging ligand (Fig. 4, C and D). When the results of seven such experiments (Fig. 4 and data not shown) were analyzed by Student’s t test, only the level of Bcl-2 in B cells exposed to BCR:CD21 coengaging ligand was significantly different from that in control cultures (p = 0.03). Although the level of Bcl-xL was occasionally elevated by BCR engagement (Fig. 4F), when the results of seven experiments were pooled (Fig. 4 and data not shown), Bcl-x levels were not significantly greater in cells receiving additional signals via BCR with or without CD21 ligand vs those receiving CD40 signals alone. This in part reflected the fact that in four additional experiments in which the sensitivity for detecting Bcl-xL was higher (due to use of a polyclonal rabbit anti-Bcl-xL Ab) cultures receiving additional signals via BCR or BCR:CD21 exhibited a major subpopulation of cells with Bcl-x levels less than those seen in cultures containing IgG control:dex. Whether this truly reflected a decline in Bcl-x expression or, alternatively, a change in the compartmentalization or intermolecular association of Bcl-x with other molecules (with a resulting diminished accessibility to anti-Bcl-x Ab) is not clear. In the above intracellular staining analyses, no significant level of anti-FLIP staining above isotype control background was noted with any of the cells analyzed (data not shown).

Taken together, the above results suggest that at limiting doses of Ag, BCR:CD21 coengagement may protect against Fas-mediated death by augmenting B cell expression of the antiapoptotic molecules, Bcl-2, FLIP_L, and possibly FLIP_S. Since signaling via BCR alone does not readily promote Bcl-2 up-regulation (7, 23) (present results), it appears that the net effect of B cell engagement with C3d-bound Ag is the expression of a wider spectrum of antiapoptotic molecules.
Relative expression of proapoptotic molecules, i.e., caspase-8, FADD, and Fas, in B cells stimulated with CD40L and a limiting concentration of BCR ligand with or without CD21 binding sites

Additional immunoblotting experiments were performed to investigate whether BCR:CD21 coengagement might reduce the expression of molecules needed for death-inducing signal complex (DISC) formation, i.e., Fas, FADD, and caspase-8 (43). These latter studies showed that BCR engagement, particularly with CD21 coligation, up-regulated, rather than down-regulated, the day 3 expression of caspase-8 and FADD (data not shown). The magnitude of the increase was <2-fold. Whether this up-regulation might be related to the recently described additional roles of caspase-8 and FADD in promoting lymphocyte proliferation (44–47) is not clear. Unlike caspase-8 and FADD, the expression of the protein Fas was not enhanced upon BCR:CD21 coligation. Indeed, in three of five experiments, Fas levels within the lysates of B cells stimulated for 3 days with CD40L and BCR:CD21 coengaging ligand were almost 2-fold lower than those in lysates of B cells stimulated with CD40L and BCR ligand or B cells stimulated with CD40L alone (data not shown).

Because the level of membrane Fas is more physiologically relevant than total protein levels, flow cytometry was used to investigate whether recruitment of the costimulatory complex affects B cell expression of membrane Fas. Surface Fas was assessed both before culture (Fig. 5A) and after 3 days of culture with CD40L and intermediate affinity anti-IgM:dex with or without CD21 binding sites, or Ig control:dex with or without CD21 binding sites (0.01 μg/ml; Fig. 5B). While membrane Fas was augmented in all CD40L-containing cultures, cells exposed to BCR:CD21 coengaging ligand exhibited approximately half as much Fas as B cells triggered via CD40 alone. The mean ± SEM values for the maximal Fas expression in a total of five such experiments were 100 ± 0, 78 ± 3, 85 ± 8, and 59 ± 8% for cells exposed to isotype control:dex, anti-CD21:dex, anti-IgM:dex, and anti-IgM:anti-CD21:dex, respectively. The difference in Fas expression between cells cultured with Ig control:dex and anti-IgM:anti-CD21:dex was highly significant (p = 0.006, as assessed by paired Student’s t test). It was observed with concentrations of staining Ab that were clearly saturating, i.e., 4 times that recommended by the supplier (data not shown). Other previous studies had indicated that BCR signaling can significantly reduce Fas expression in germinal center B cells, but not other tonsil B cell populations (6, 48). It is

FIGURE 4. Effect of BCR:CD21 coligation on intracellular expression of antiapoptotic molecules in CD40-activated B cells. High density, CD27-depleted tonsil B cells were cultured with CD40L and the mAb:dex ligands described in Fig. 3 (at 0.01 μg/ml). Prior to culture (day 0) and on day 3 of culture, viable cells were analyzed for intracellular levels of Bcl-2 and Bcl-x. Shown are the flow cytometric profiles from two representative experiments (A, C, and E (experiment I) and B, D, and F (experiment II)). A and B, Background staining with mouse IgG1 control Ab; C and D, staining with anti-Bcl-2 (IgG1); E and F, staining with anti-Bcl-x (IgG1). The slightly higher background staining consistently observed on cells cultured with CD21- and BCR-specific dex conjugates probably represents residual cell-bound mAb:dex detected by the R-PE-conjugated anti-mouse IgG probe. Δ MFI (mean fluorescence intensity values above background) for Bcl-2 and Bcl-x are shown to the left of the respective histograms. In these and five additional experiments, the Δ MFI values for cultured cells were expressed as a percentage of the maximum within each experiment, and a comparison of these latter values (n = 7) for each of the culture conditions was made. Cultures containing IgG control:dex, anti-CD21:dex, anti-IgM:dex, and anti-IgM:anti-CD21:dex exhibited values of 78 ± 3, 83 ± 5, 73 ± 7, and 94 ± 6%, respectively, of maximum values for Bcl-2 expression; for Bcl-x expression they exhibited values of 67 ± 7, 86 ± 7, 69 ± 11, and 53 ± 11%, respectively. In these pooled experiments, while Bcl-2 expression in cells exposed to BCR:CD21 coengaging ligand was significantly different (p < 0.01, by Student’s t test) from that in control cultures (or cultures exposed to monospecific BCR ligand), the values for Bcl-x expression under the diverse culture conditions were not significantly different.
therefore important to note that the high density, CD27-depleted B cells placed in culture have the phenotype of follicular B cells, but not germinal center B cells (35, 49, 50) i.e., they are almost uniformly IgM⁺, CD23⁺, and CD27⁺ (Fig. 5A).

Interestingly, down-regulated Fas expression was also observed in B cells stimulated with CD40L and high concentrations of monospecific anti-IgM:dex (Fig. 6, B and C-c). Under these circumstances as well, the reduction in Fas correlated with an augmented protection from Fas-mediated apoptosis (Fig. 6, A and C-a). Thus, it would appear that Fas down-regulation is not dependent upon an Ag’s capacity to recruit CD21/CD19, but can be induced by C3d(g)-free Ags of moderate multivalency provided the Ag concentration is sufficiently high.

B cells stimulated by CD40 and BCR:CD21 coengaging ligand exhibit a lesser level of caspase-8 fragmentation upon acute exposure to Fas ligand, i.e., CH11 anti-Fas mAb.

A decline in membrane Fas, taken together with a rise in FLIP (which binds FADD with higher affinity than procaspase-8 and can compete with the latter for binding to FADD) (37, 42, 43), might be expected to impair DISC formation and cleavage of procaspase-8 into its active fragments. To investigate whether B cells activated by CD40L and a limiting concentration of BCR:CD21 coengaging ligand do indeed exhibit diminished caspase-8 activation, cultures costimulated with CD40L and anti-IgM:dex with or without anti-CD21, or Ig control:dex with or without anti-CD21 (0.005 µg/ml) were exposed to Fas-cross-linking mAb (or control mAb) on day 3. Lysates prepared after 6 h of anti-Fas exposure

**FIGURE 5.** BCR:CD21 coligation results in a diminished level of membrane Fas on B cells costimulated by CD40L. High density, CD27-depleted tonsil B cells were analyzed by flow cytometry for the expression of various surface molecules prior to culture (A) and after 3 d of culture with CD40L and mAb:dex (B; as in Fig. 3; dose of 0.01 µg/ml). Background staining with FITC-labeled IgG control is indicated by the light histogram to the left of each plot, and the relative levels of Fas are shown as the mean fluorescence intensity (MFI). Percent protection from anti-Fas-induced apoptosis in other replicate cultures (assayed as described in Fig. 3) was as follows: IgG control:dex, 13%; anti-CD21:dex, 30%; anti-IgM:dex, 33%; and anti-IgM:anti-CD21:dex, 73%.

**FIGURE 6.** At high ligand concentrations, BCR-specific ligand devoid of CD21 binding sites can down-regulate Fas expression. High density CD27-depleted tonsil B cells were cultured with CD40L and mAb:dex ligands (as described in Fig. 3). Dex-conjugated mAb were present at a concentration of either 1 or 0.01 µg/ml. After 3 days one replicate set of cultures was analyzed for the expression of Fas by flow cytometry with FITC-anti-Fas (B, C-c, and C-d). CH11 anti-Fas or IgM control was added to other sets of replicate cultures during the last 8–10 h of culture, and the percent protection from Fas-mediated death was determined (A, C-a, and C-b).
were analyzed for the expression of uncleaved and processed forms of caspase-8. As shown in Fig. 7, B cells receiving concomitant activation signals from CD40L and BCR:CD21 coengaging ligand were notably resistant to anti-Fas-induced cleavage of caspase-8 into its intermediate (p43/41) and active p18 fragments. When compared to the levels in anti-Fas-treated cultures stimulated with CD40L alone, cleavage to p41/43 was less affected than cleavage to p18 (65 vs 84% inhibition, respectively). Anti-Fas-induced cleavage of the pro form of FLIPL into its p43 fragment (which results from the proximity of FLIPL and caspase-8 within the DISC) (42, 51) was also reduced in B cells activated by CD40L and BCR:CD21 coengaging ligand (Fig. 7). Generation of the p43 FLIPL fragment, like generation of p43/p41 caspase-8, was suppressed to a lesser degree by BCR:CD21 coligation than generation of p18 caspase-8. These differences might be linked to the stepwise manner in which DISC-associated proteins are cleaved and to the relative degree that FLIPL inhibits generation of the p43/p41 and p18 fragments of caspase-8 (41, 51, 52). Following Fas cross-linking in cells bearing moderate levels of FLIPL, both caspase-8 and FLIPL are recruited to the DISC. Indeed, at such levels FLIPL can promote caspase-8 recruitment and activation. The initially recruited caspase-8 initiates cleavage of FLIPL to its p43 fragment as well as autocatalytically cleaves itself, generating the proteolytically active p43/p41 fragment (42, 51, 52). Generation of the p18 fragments of caspase-8 (important in downstream caspase 3 activation) is thought to occur only under conditions where molecules of procaspase-8 or its p43/p41 fragments lie adjacent to one another, i.e., when levels of competing FLIPL molecules are low, and transproteolytic cleavage is possible (42, 51, 52).

**Discussion**

Using soluble receptor-specific mAb dextrans conjugates as surrogate Ags, the present study has noted that coengagement of BCR and C3 fragment-binding CD21 lowers the Ag concentration threshold for eliciting protection from Fas apoptosis by 2–3 orders of magnitude. This protection was evident over a wide range of BCR:ligand binding affinities. Because the in vitro B cell-activating properties of these moderately multivalent anti-IgM/dextran ligands (34) appear comparable with those of moderately multivalent Ags such as bacteriophage (53), we consider that these findings have some parallels in vivo. Thus, with the caveat that B cell responses to certain Ags:CD3(d) complexes in vivo may be influenced by parameters not present in this model system, the present study strongly suggests that the innate immune system's capacity to tag Ags with fragments of complement (C3) contributes significantly to the escape of CD40-activated B cells from Fas-mediated death. Because the deposition of complement is greatly augmented on the surfaces of microorganisms, compared with most body cells (24), T cell-dependent B cell responses to microorganisms can be selectively amplified over those to most self Ags. Problems may arise, however, with occasional self antigenic substrates that also effectively activate complement, e.g., apoptotic cells and IgG in Ab:Ag complexes. Coligation of BCR and CD21 on autoreactive B cells specific for these Ags may under some circumstances drive autoantibody production.

The possibility that the coligation of BCR and the C3(dg)-binding costimulatory complex might prevent Fas-mediated elimination of B cells during an immune response was indeed previously suggested from the in vivo murine studies by Carroll and colleagues (54). These investigators found that follicular survival of moderate affinity B cells with defective expression of the Cr2 gene products, i.e., CD21 and CD35, was significantly reduced after immunization with a T cell-dependent Ag compared with that of wild-type B cells of the same affinity (54). However, no difference in the follicular survival of Cr2−/− and Cr2+/− B cells was noted if the B cells were deficient in Fas/CD95/lpr (55). In the above-cited study, B cells of high affinity for Ag were not dependent upon Cr2 for follicular survival, but were dependent on Cr2 for germinal center entry and/or survival (54). Whether the diminished recovery of high affinity Cr2−/− cells within this site reflected impaired resistance to Fas-mediated apoptosis and/or an impaired receipt of viability signals from Ags:CD3dg complexes on germinal center dendritic cells was not determined.

Several factors argue that the CD21/CD19 costimulatory complex might be of relevance in protecting high affinity B cells from germinal center Fas-mediated apoptosis. Firstly, Fas apoptosis appears to play a role in B cell selection within this site (50, 56). Secondly, within germinal centers the dose of Ag, i.e., the availability of free antigenic epitopes, is probably quite limiting. Finally, from the present study it is clear that when BCR:ligand affinity is high, low doses of ligand are needed to observe dependency upon the costimulatory complex.

The fact that BCR coligation with CD21 failed to increase the protection elicited by high doses of the higher affinity ligands is consistent with past observations that in vivo Ab responses elicited under conditions of near-optimal BCR engagement (high doses of Ag or high antigenic valency) are relatively independent of C3 and CD21/CD19 (19, 22, 57). However, it was somewhat unexpected.
that recruitment of the costimulatory complex did not enhance the suboptimal protection achieved at high doses of the low affinity BCR ligand. Given that the mAbs conjugated to dex were intact IgG Abs, it is possible that at high concentrations, the inhibitory influences of FcγRIIB engagement (58, 59) over-ride the positive influences of recruiting CD21/CD19/CD81. However, in two experiments in which 100 µg/ml of FcγRIIB-blocking mAb (AT10) (30) was added to cultures 30 min before addition of CD40L and the mAb:dex conjugates, little to no augmentation in protection was noted (P.K.A.M., unpublished observations).

The present study’s examination of how BCR:CD21 coligation affects the expression of various anti- and proapoptotic molecules provides important insights into probable mechanisms for augmented protection. The reduced density of membrane Fas in B cells exposed to BCR:CD21 coengaging ligand is probably a major factor responsible for diminished susceptibility, since 1) formation of the active DISC is dependent upon aggregation of preformed Fas oligomers (43, 60), and 2) 2-fold changes in the density of other receptors can have notable effects on cell behavior (61–63). Additionally, the slight, but statistically significant, augmentation in FLIP L expression upon BCR:CD21 coligation may contribute to the diminished susceptibility to Fas apoptosis. FLIP L bears the FADD binding domain of caspase-8, but lacks the latter’s catalytic activity (42, 43). In a manner quite dependent upon the expression level, FLIP dampens Fas-mediated apoptosis by competing with caspase-8 for binding to FADD (42, 43, 51, 52). Importantly, FLIP’s higher affinity for FADD (37) may promote this interference despite the higher intracellular levels of caspase-8. A major finding, which was consistent with a diminished formation of Fas: FADD: caspase-8 DISC complexes in B cells costimulated with CD40L and BCR:CD21 coengaging ligand, is the observation that such B cells were significantly compromised in the cleavage of caspase-8 into its p43/41 and p18 fragments following cross-linking of Fas.

Diminished cleavage of caspase-8 is expected to affect the two cellular programs available for executing Fas apoptosis, i.e., type I and type II, to differing degrees. As discussed previously (3, 64, 65), Fas apoptosis in type I cells requires extensive caspase-8 activation at the DISC to initiate direct cleavage and activation of downstream caspase-3. It is unaffected by antiapoptotic molecules within the mitochondria, i.e., Bcl-2 and Bcl-x. In contrast, Fas apoptosis in type II cells requires low levels of DISC activation. In such cells, although the level of caspase-8 activation is not sufficient for cleavage and activation of caspase-3, it is sufficient for cleavage of the cytosolic proapoptotic molecule, BID. Truncated BID translocates to the mitochondria, where it induces the release of cytochrome c and the activation of an alternative apoptotic protease-activating factor 1 (Apaf-1)-dependent apoptotic program (43, 64, 65). Thus, although the level of caspase-8 activation following Fas aggregation in cells exposed to BCR:CD21 coengaging ligand might be too low to activate direct type I apoptosis, it might be sufficient to initiate type II apoptosis were it not for the heightened level of the mitochondrial antiapoptotic molecule, Bcl-2. Importantly in this regard, deliberate overexpression of Bcl-2 in a B cell line was shown to reduce Fas-mediated apoptosis (66). Although the increases in Bcl-2 noted here are generally ≤2-fold, they may very well be relevant to cell survival. It has been noted that only a 30% increase in Bcl-2 expression in vivo can effectively rescue B cells from apoptosis (67).

The present study’s correlation between CD21-promoted resistance to Fas apoptosis and Bcl-2 expression is consistent with other findings linking the CD21/CD19 complex to increased B cell viability and Bcl-2 expression (68–70). Of greatest relevance to the present work, Bonnefoy et al. (69) reported that in vitro engagement of CD21 on human germinal center B cells promoted cell viability via a Bcl-2-dependent mechanism (70). Additionally, Roberts and Snow (23) noted that engagement of CD19 on LPS-activated murine B cells promoted both increased Bcl-2 expression and increased proliferation. In another parallel with the present study, the latter investigators noted that CD19 engagement up-regulated Bcl-2, but not Bcl-x, and conversely, BCR engagement up-regulated Bcl-x, but not Bcl-2. Additionally, coligation of BCR and CD19 was found to result in Bcl-2 expression greater than that observed with CD19 engagement alone (23).

Although prior studies found that BCR induced down-regulation of Fas only in germinal center B cells (48), the present report clearly indicates that this phenomenon can occur in CD40-activated follicular cells as well. There are several reasons why this may not have been observed previously (5, 6, 9, 48). Firstly, it appears that without recruitment of the costimulatory complex, down-modulation of Fas requires high concentrations of moderately multivalent BCR ligand. This level of BCR engagement might not have been reached in other studies with nongermineral center B cells. Secondly, B cell heterogeneity, e.g., incidence of cells binding the BCR ligand, may affect the degree to which Fas down-regulation is detected. Thirdly, the time at which BCR signals are delivered relative to CD40 signals may affect the degree to which Fas expression is modulated. Of relevance, the addition of anti-IgM:dex or anti-IgM:anti-CD21:dex (0.01 µg/ml) late after CD40 activation (an approach used in some studies (5, 8)) resulted in no down-modulation of Fas. Although protection was less pronounced than when mAb:dex ligands were added at the beginning of culture, the BCR:CD21 coengaging ligand remained more effective than the ligand that engaged solely BCR (P.K.A.M., unpublished observations). Thus, upon delayed exposure to BCR: CD21 coengaging ligand, CD40-activated B cells are protected by a mechanism(s) independent of a reduction in membrane Fas. Protection might involve modulation of the above antiapoptotic molecules or others not presently explored, e.g., Fas apoptosis inhibitory molecule (10). Alternatively, protein kinase B (Akt) phosphorylation and inactivation of proapoptotic BAD (71, 72) might be important, since the costimulatory complex is known to amplify signaling pathways that lead to augmented protein kinase B (Akt) function (19, 74, 75).

Importantly from a therapeutic perspective, several lines of evidence suggest that the C3-binding costimulatory complex could be a relevant target for blocking the activation of mature autoreactive B cells. Firstly, as shown here, the costimulatory complex plays an important role in protecting CD40-activated B cells from Fas-mediated death when BCR signal transduction is suboptimal, a situation expected when autoreactive escapes from immature B cell deletion confront self Ag in the periphery (76). Secondly, Foote et al. (77) showed that anergic B cells are not incapable of signaling a Fas-resistant state, but require higher levels of BCR cross-linking to do so. BCR engagement with CD21/CD19/CD81 may lower the degree of BCR engagement required to convert anergic B cells into a Fas-resistant state. Thirdly, although extensive deposition of C3 on most body cells is prevented by specific complement regulatory mechanisms and the presence of high levels of sialic acid, two self Ag substrates known to be targeted in autoimmune diseases, i.e., apoptotic cells expressing DNA and nucleoproteins on surface blebs (25–29) and complexes of Ag and IgG Ab, have a proclivity to bind C3 fragments. Interestingly, mice and humans deficient in Fas characteristically exhibit augmented production of autoantibodies specific for these self Ags (14, 78–81). Indeed, mice deficient in Fas accumulate rheumatoid-factor producing cells within the T cell-rich regions of lymphatic tissue (81). Thus, deliberate blocking of C3d:CD21 interactions might be
expected to enhance Fas-mediated elimination of mature autoreactive B cells.

Paradoxically, on certain genetic backgrounds, mice with defective expression of both Cd2 (CD21/CD35) and Fas have greater levels of lupus-like autoantibodies than do mice with either defect alone (82, 83). Part of the explanation may lie in the fact that the murine Cd2 gene encodes both Cr1 (CD35) and CR2 (CD21), two molecules with differing expression patterns and functions (83, 84). Additionally, coengagement of BCR and the CD21/CD19/CD81 costimulatory complex might have differing functional effects depending upon the state of the target autoreactive B cell (83). Thus, such receptor coligation might augment the tolerance of immature B cells that reside in or are recent emigrants from the bone marrow. In mature B cells that have not received strong costimuli such as CD40L, coligation might help promote anergy. In developing interventional therapy for B cell autoimmune diseases, it will be important to consider ways of targeting costimulatory complex-blocking agents to the latter cell population.

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