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*J Immunol* 2005; 175:2859-2867; doi: 10.4049/jimmunol.175.5.2859

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CD21/CD19 Coreceptor Signaling Promotes B Cell Survival during Primary Immune Responses

Robert A. Barrington,* Ming Zhang,‡ Xuemei Zhong,† Helena Jonsson,* Nichol Holodick,‡ Anu Cherukuri,† Susan K. Pierce,‡ Thomas L. Rothstein,‡ and Michael C. Carroll3*

The adaptive immune response is tightly regulated to limit responding cells in an Ag-specific manner. On B cells, coreceptors CD21/CD19 modulate the strength of BCR signals, potentially influencing cell fate. The importance of the CD95 pathway was examined in response of B cells to moderate affinity Ag using an adoptive transfer model of lysozyme-specific Ig transgenic (HEL immunoglobulin transgene (MD4) strain) B cells. Although adoptively transferred Cr2+/− MD4 B cells are activated and persist within splenic follicles of duck egg lysozyme–immunized mice, Cr2+/− MD4 B cells do not. In contrast, Cr2+/− MD4 lpr B cells persist after transfer, suggesting that lack of CD21/CD35 signaling results in CD95-mediated elimination. Cr2 deficiency did not affect CD95 levels, but cellular FLIP (c-FLIP) protein and mRNA levels were reduced 2-fold compared with levels in Cr2+/− MD4 B cells. In vitro culture with Cr2+/− MD4 B cells demonstrated that equimolar amounts of rHEL-C3d1 were more effective than hen egg lysozyme alone in up-regulating c-FLIP levels and for protection against CD95-mediated apoptosis. Collectively, this study implies a mechanism for regulating B cell survival in vivo whereby the strength of BCR signaling (including coreceptor) determines c-FLIP levels and protection from CD95-induced death.


B cells undergo multiple checkpoints as they mature from early development, to activation, and finally formation of memory or effector cells. Each checkpoint is regulated in large part by the strength of signal transduced through the BCR. The overall BCR signal is influenced not only by Ag affinity but also by coreceptors that either enhance or inhibit Ag signaling. For example, the CD21/CD19/CD81 coreceptor complex is a positive regulator of BCR signaling (1), whereas FcγRIIB is a negative regulator (2). Coligation of CD19 and BCR reduces the amount of anti-IgM required for optimal signaling by 10~100-fold (3). Based on this last observation, it was proposed that cross-linking the CD21/CD19 coreceptor serves to lower the threshold for B cell activation (1).

The importance of CD21 B cell coreceptor in vivo was examined directly in the hen egg lysozyme (HEL)3 Ig transgenic model (MD4 strain) (4). Fischer et al. (5) used an adoptive transfer approach in which the survival of Cr2+/− vs Cr2+/− MD4 B cells with splenic follicles wild-type (WT) mice immunized with either high-affinity (turkey egg white (TEL)) or moderate affinity (duck egg lysozyme (DEL)) lysozyme. Both Cr2+/− and Cr2+/− MD4 cells responded and remained in the follicles of TEL-immunized mice. However, in mice immunized with moderate affinity lysozyme, CD21/CD35 coreceptor-deficient MD4 cells failed to survive in contrast to the Cr2+/− MD4 B cells (5). The loss was Ag-specific as normal numbers of CD21/CD35-deficient B cells were identified within splenic follicles of nonimmune WT mice after transfer. These findings support the threshold hypothesis but also raised the question whether CD21/CD35-deficient B cells were eliminated or migrate away from the follicles.

Current understanding of the regulation of peripheral B cells derives in part from transgenic models in which B cells have self-reactive receptors. Anergic B cells, for example, are eliminated by CD95-dependent apoptosis upon interaction with CD4+ T cells (6). Furthermore, acute Ag receptor engagement protects naive B cells from CD95-dependent apoptosis (7), and in the absence of threshold signaling, both naive and anergic B cells are eliminated (8, 9), possibly in the T cell–rich inner splenic periarteriolar lymphoid sheath region (10). CD95 levels, initially low on resting B cells, are strongly induced after CD154–CD40 interaction (7, 8, 11). CD95 is activated by trimerization upon binding to CD95L (12), and its trimerized cytoplasmic region interacts with an adaptor molecule, CD95–associated death domain (FADD) (13–15). The N-terminal region (death effector domain) of FADD binds to caspase-8 (FLICE) and activates caspase-8 by self-cleavage (16–18). CD95 oligomerization induced by the binding of CD95 ligand thereby results in the formation of the death-inducing-signaling-complex (DISC), including CD95, FADD, and FLICE, and transduces signals to apoptosis executioners, the caspases (19, 20). DISC regulation of CD95-mediated apoptosis is achieved by FLIP (18, 21–28). FLIP competes with caspase-8 for FADD binding, thereby inhibiting CD95-mediated apoptosis (21, 29). Importantly, constitutive expression of FLIP inhibits activation-induced cell death in T cells (30) and blocks CD95L–triggered apoptosis in B cell lines (31) in vitro and leads to autoimmunity in vivo (32).
Materials and Methods

Mice

Mice were housed at Harvard Medical School in a specific pathogen-free facility. C57BL/6 mice (The Jackson Laboratory) were used as recipients. MD4 HEL Ig transgenic mice (4, 35) were maintained on a C57BL/6 background, either with or without (Cr2+/−; Ref. 36) intact Cr2 locus. MD4 strains were also crossed to pqr/pqr mice (C57BL/6 background). Animal protocols used were reviewed and approved by the Animal Care and Use Committees at the CBR Institute for Biomedical Research and at Harvard Medical School.

Adaptive transfer protocol

Preparation of recipient mice and transfer of MD4 B cells was performed as described previously (5). Recipient mice were normal and not irradiated. Splenectomies were analyzed 1, 3, and 5 days after transfer by four-color flow cytometry and by immunohistochemistry.

Flow cytometric analysis

Splenocyte mononuclear cells (MNCs) were isolated by density gradient centrifugation using Lymphocyte M (Cedarlane Laboratories). To detect MD4 B cells, MNCs were stained with HEL-Cy5 and counterstained with FITC-conjugated peanut lectin (agglutinin) (PNA) (EY Laboratories), PerCP-conjugated anti-B220 (BD Pharmingen), and PE-conjugated anti-IgM (BD Pharmingen). The allotype of MD4 BCR is IgMh, whereas all endogenous B cells from C57BL/6 mice are IgMh. An additional allotypic marker, CD45.1, was also used to differentiate donor cells from endogenous cells. To determine CD95 levels and the percentage of MD4 B cells undergoing apoptosis, MNCs were stained with IgMh-biotin, followed by streptavidin-FITC, and counterstained with 7-aminoactinomycin D (7-AAD; Molecular Probes) and PE-conjugated CD95. Alternatively, annexin V (BD Pharmingen) was used to identify apoptotic cells in some experiments. Transferred cells were identified as being HEL IgMh or, alternatively, IgMh, alone. To confirm that transferred cells could be identified as HEL IgMh or IgMh alone, mice were immunized in the absence of adoptive transfer, and no HEL IgMh or IgMh cells were detectable.

Immunohistochemical analysis of splenic sections

Spleens were harvested from recipient mice and snap-frozen in OCT compound using a 2-methylbutane/liquid N2 bath. Cryosections (5 µm) were analyzed by immunohistological staining using HEL-biotin and alkaline phosphatase streptavidin (AP, Sigma-Aldrich) and counterstained with FITC-conjugated PNA (EY Laboratories) and HRP-conjugated anti-FITC (Boehringer Mannheim).

Western blot analysis

Lysates from 2.5 × 107 sorted Cr2+/− and Cr2−/− MD4 B cells (pooled from 3–5 mice/group each experiment) were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% dry milk (Carnation/PBS). Fifty-five-kilodalton c-FLIP and 13-kDa processed c-FLIP, were detected by anti-FLIP Ab (Dave-2, Alexis Biochemicals). Anti-Bcl-2 Ab (Ab-2; Oncogene Research Products) detected 26- and 19-kDa molecules. For in vitro experiments, lysates from 1 × 106 purified B cells were used. Blots were developed with substrate 5-bromo-4-chloro-3-indolyl-phosphate/NBT (Roche) after incubation with AP-conjugated, mouse-adsorbed goat anti-rat IgG (Southern Biotechnology Associates) for c-FLIP and AP-conjugated mouse and human-adsorbed goat anti-rabbit IgG (Southern Biotechnology Associates) for Bcl-2. Relative band densities were quantitated by NIH image software package.

Gene expression

RNA was prepared from FACS-sorted B cells using TRIzol (Invitrogen Life Technologies). cDNA was prepared using iSCRIPT (Bio-Rad) and normalized by PCR for β2-microglobulin expression. c-FLIP, bcl-2, and bcl-xL expression was determined by real-time PCR (Stratagene) using the following primers (forward/reverse): β2-microglobulin (5′-CCCCGCTCTAATTGAAATCC/GCGTATGTATCAGTCTCAGTGG-3′); c-FLIP (5′-ACGTGCCCTTCTCCTCAGAGG/TTTGGCAGGTCGTTGTTGGTGC-3′); bcl-2 (5′-CTCTCTTGCCTACCCGC/CTGCCCTGCCTGCGT/CGCGACACCTAAGAAGGCCC-3′); and bcl-xL (5′-GAGAGGCGGAGATGATGTGG/ATCGATGGACCCCGTATTAC-3′).

Lysozyme and lysozyme-C3d production and purification

HEL was purchased from Sigma-Aldrich. DEL was isolated from duck egg white as described previously (37). Recombinant HELC3d4, encoding aa 1–129 of HEL fused to three copies of the human C3d region of complement component C3, was produced using infection of sf9 insect cells with rHELCL3d4-encoding baculovirus (kindly provided by Dr. D. Fearon, Cambridge University, Cambridge, U.K.) (38, 39). Infected sf9 insect cells were cultured using Grace’s insect medium (Invitrogen Life Technologies) supplemented with antibiotics. Recombinant HELC3d4 from baculovirus supernatants was purified by fast protein liquid chromatography (AKT FPLC; Amersham Biosciences) using an anion-exchange column (Pharmacia). Running buffer consisted of 20 mM Tris and 5 mM EDTA. Multiple column fractions were collected after elution with Tris-EDTA buffer containing 1 M NaCl. Fractions containing rHELCL3d4 were identified by SDS-PAGE (using GeCode (Pierce) and by Western blot analysis with polyclonal rabbit anti-Hel Ab (BioSource International)). Alternatively, HELCL3d4 was purified by affinity chromatography as described previously (39). To rule out potential contributions of “carry-over” molecules to B cell activation, DEL and HEL were diluted in mock-infected sf9 cell supernatants, and no differences in Fas-protection were found.

Recombinant DELC3d4 was created using megaprimer PCR-mutagenesis using the nucleotide sequence of HEL in which residues conferring affinity changes for HyHEL-10 binding (the MD4 transgene) were changed (33). Following sequence confirmation of mutations, rDEL was then cloned to replace HEL within the rHEL-C3d4 baculovirus vector DNA. Recombinant DEL-C3d4-encoding baculovirus was engineered, and clones producing recombinant protein were identified as described previously (BD Pharmingen).

Ca2+ flux

Splenocytes were loaded with a 1 mM stock solution of indo-1 AM (Molecular Probes) in anhydrous DMSO at a ratio of 2–5 µL/106 cells. For loading, cells were incubated 30 min at 37°C, then washed with RPMI 1640 containing 2% FCS. Cells were subsequently stained with B220-FITC (BD Pharmingen), washed, resuspended at 2 × 106 cells/ml, and Ca2+ mobilization measured using a FACSVantage cytometer with UV laser (BD Biosciences).

Apoptosis assay

Splenenic B cells were purified from MD4 mice using MACS (Miltenyi Biotech). Cells were incubated with soluble rCD154-CD8 fusion protein at 37°C, followed by anti-CD8 Ab cross-linking as described previously (40). After overnight culture, cells were treated with goat anti-mouse IgM
supernatant was stained with 7-AAD and analyzed by FACS. Alternatively, cell culture supernatant was harvested using a Harvesting Press (Skatron), and chromium release was measured in an automatic gamma counter (PerkinElmer). Negligible B cell proliferation is observed under these culture conditions (data not shown).

Results

CD21/CD35-deficient B cells are eliminated from lymphoid follicles by CD95

To test whether Cr2<sup>−/−</sup> B cells are eliminated by CD95-mediated apoptosis in vivo, Cr2<sup>−/−</sup> B cells lacking functional CD95 were generated by breeding of Cr2<sup>−/−</sup>-HEL Ig transgenic (MD4) mice with CD95-mutant lpr mice (on C57BL/6J genetic background) (12, 41). B cells from these mice were used as donors in adoptive transfer experiments to focus directly on the role of CD21/CD35 in B cell coreceptor signaling independently of follicular dendritic cell expression of CD21/CD35. Lpr MD4 B cells were transferred into DEL-immunized WT recipients (immunized 7 days before B cell transfer). As controls, Cr2<sup>+/+</sup> or Cr2<sup>−/−</sup> MD4 B cells were adoptively transferred into the WT mice previously immunized with DEL. Follicular survival of adoptively transferred MD4 B cells was assessed by immunohistochemistry 5 days after transfer. As reported previously (5), Cr2<sup>+/+</sup> MD4 B cells are abundant within splenic follicles, whereas negligible numbers of Cr2<sup>−/−</sup> MD4 B cells were observed within the spleen (Figs. 1, a and b, 2, a and b). In contrast, Cr2<sup>−/−</sup> MD4 B cells lacking functional CD95 were present 5 days after transfer into DEL-immunized B6 mice (Figs. 1d and 2d). The presence of Cr2<sup>−/−</sup> MD4 lpr B cells within the follicles approached that of Cr2<sup>+/+</sup> MD4 B cells (MD4 B cell-positive follicles are 65 vs 82%, respectively, from three separate experiments) (Table I). Similar results were observed using total splenic cells from the indicated donor strains (42). These data support the hypothesis that CD21/CD35 expression is required for resistance to CD95-mediated elimination.

Increased apoptosis and decreased c-FLIP<sub>L</sub> in CD21/CD35-deficient B cells upon engagement of moderate affinity Ag

One explanation for the difference in survival is that CD95 levels may simply be down-regulated as a result of CD21/CD35 signaling. To test this possibility, Cr2<sup>−/−</sup> and Cr2<sup>+/+</sup> MD4 B cells were examined 1 day after transfer. Day 1 posttransfer was the chosen time point because Cr2<sup>−/−</sup> MD4 B cells exist in similar numbers as Cr2<sup>+/+</sup> MD4 B cells; after day 1, progressive loss of Cr2<sup>−/−</sup> B cells occurs. The transferred MD4 B cells are identified as IgM<sup>+</sup> HEL-binding double-positive cells (Fig. 3a). Similar levels of CD95 were observed on the surface of transferred Cr2<sup>+/+</sup> and Cr2<sup>−/−</sup> MD4 B cells, indicating that differential CD95 levels were not responsible for increased apoptosis of B cells lacking intact CD21/CD35 coreceptor (CD95 mean fluorescence intensity for Cr2<sup>+/+</sup> MD4 = 24, Cr2<sup>−/−</sup> MD4 = 21, p > 0.1; Fig. 3b).

To directly test whether B cells lacking CD21/CD35 were undergoing apoptosis, levels of the early apoptotic markers annexin

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

**FIGURE 1.** Effect of CD95 mutation on the fate of Cr2<sup>−/−</sup> MD4 B cells in splenic follicles of DEL-immunized recipient mice. Immunohistochemical analysis of splenic follicles of DEL-immunized recipient mice. Immunohistochemical analysis of splenic follicles of DEL-immunized recipients after adoptive transfer of either Cr2<sup>+/+</sup> (a and c) or Cr2<sup>−/−</sup> (b and d) MD4 B cells from non-lpr (a and b) or lpr donor mice (c and d). Results shown are from one of three independent experiments. Splenic cryosections were stained by two-color immunohistochemistry demonstrating HEL-binding cells (blue) and PNA-positive germinal centers and outlining follicles (crimson).

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Effect of CD95 mutation on the fate of Cr2<sup>−/−</sup> MD4 B cells in splenic follicles of DEL-immunized recipient mice. Splenocytes from DEL-immunized recipient mice were analyzed by FACS for the presence of Cr2<sup>+/+</sup> (a and c) or Cr2<sup>−/−</sup> (b and d) MD4 B cells from non-lpr (a and b) or lpr donor mice (c and d). Results shown are from one of three independent experiments. e, Summary of the percentage of donor-derived B cells from all three experiments. Mean values ± SD are shown. Asterisks indicate significant differences compared with frequency of Cr2<sup>−/−</sup> MD4 B cells after transfer (*, p ≤ 0.04; **, p < 0.07, t test).
V (43) and 7-AAD (44) were assessed by flow cytometry. Cr2\(^{-/-}\) MD4 B cells have ~2-fold higher percentage of 7-AAD+ cells when compared with Cr2\(^{+/+}\) MD4 B cells 1 day following transfer into DEL-immunized recipient mice (25–30% vs 10–12% from three separate experiments; Fig. 3, i–iii). A higher percentage of transferred Cr2\(^{-/-}\) B cells also stained positive for annexin V staining compared with transferred Cr2\(^{+/+}\) B cells, confirming the increased apoptosis in Cr2\(^{-/-}\) B cells suggested by 7-AAD staining (Fig. 3c and data not shown). By day 3 posttransfer, similar frequencies of 7-AAD+ Cr2\(^{-/-}\) and Cr2\(^{+/+}\) B cells were observed (29 vs 26%; data not shown). These results, together with the lpr experiments described above, suggest a role for the CD95 pathway in the early loss of transferred Cr2\(^{-/-}\) MD4 B cells.

To determine the mechanism responsible for elimination of Cr2\(^{-/-}\) B cells, the level of c-FLIPI, a downstream regulator of apoptosis, was assayed. Although naïve B cells express marginal levels of c-FLIP, cross-linking BCR and/or ligation of CD40 strongly up-regulate c-FLIP expression (45). Similar to CD40, signaling via CD21/CD35 may enhance BCR mediated up-regulation of c-FLIP (46, 47). In contrast to c-FLIP levels, no significant differences were observed between Cr2\(^{-/-}\) and Cr2\(^{+/+}\) MD4 B cells at either 1 or 3 days posttransfer into DEL-immunized recipients (Fig. 4, e and f). However, consistent with protein analysis presented earlier (Fig. 4, a and c), a 2- to 4-fold reduction in FlipL mRNA was observed in Cr2\(^{-/-}\) MD4 B cells.

### Table 1. Morphological analysis of splenic sections 5 days posttransfer of B lymphocytes with and without functional CD95 lpr

<table>
<thead>
<tr>
<th>Donor B Cell Source</th>
<th>Total No. of Follicles(^a)</th>
<th>No. of Follicles with HEL-Binding Cells(^b)</th>
<th>% of Follicles with HEL-Binding Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr2(^{-/-}) MD4 (n = 5)(^c)</td>
<td>20.3 ± 3.1</td>
<td>16.7 ± 3.4</td>
<td>82.3</td>
</tr>
<tr>
<td>Cr2(^{-/-}) MD4 (n = 3)</td>
<td>28 ± 1.4</td>
<td>1.5 ± 0.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Cr2(^{-/-}) MD4 lpr (n = 5)</td>
<td>33 ± 4</td>
<td>29.3 ± 5.1</td>
<td>88.8</td>
</tr>
<tr>
<td>Cr2(^{-/-}) MD4 lpr (n = 5)</td>
<td>37 ± 2</td>
<td>24 ± 3.6</td>
<td>64.9</td>
</tr>
</tbody>
</table>

\(^a\) Mean + (SD) number of follicles per section counted.

\(^b\) Mean + (SD) number of follicles per section counted with ≥10 MD4 B cells.

\(^c\) n is the total number of mice analyzed.

\(^d\) Comparison of lpr vs non-lpr Cr2\(^{-/-}\) MD4 B cells in follicles: p ≤ 0.001 (t test); Cr2\(^{-/-}\) MD4 lpr vs Cr2\(^{+/+}\) MD4 lpr B cells in follicles p > 0.1.

![FIGURE 3. Increased apoptosis in Cr2\(^{-/-}\) MD4 B cells after adoptive transfer. a, Representative dot plot (of three experiments) showing transferred MD4 B cells from splenocytes 1 day after transfer into DEL-immunized recipients. Cr2\(^{+/+}\) (left panel) and Cr2\(^{-/-}\) (right panel) MD4 B cells are identified as surface marker IgM\(^+\) and binding HEL; b, analyzing CD95 expression on Cr2\(^{+/+}\) or Cr2\(^{-/-}\) MD4 B cells. Representative histograms from one of four independent experiments of CD95 staining and negative control (light line) are shown. MFI represents mean fluorescence intensity. Comparison of Cr2\(^{+/+}\) or Cr2\(^{-/-}\) MD4 B cells in the cumulative scatterplot; p > 0.1 (Student’s t test). Horizontal bars in scatter plot represent mean values; c, histogram comparing 7-AAD levels on Cr2\(^{+/+}\) or Cr2\(^{-/-}\) MD4 B cells gated as shown in a. Comparison of Cr2\(^{+/+}\) or Cr2\(^{-/-}\) MD4 B cells; p < 0.05. Scatterplot summarizes results from three separate experiments.](http://www.jimmunol.org/Downloadedfrom)
Reduced levels of c-FLIPL and similar Bcl-2 levels in transferred Cr2\(^{-/-}\) MD4 B cells compared with Cr2\(^{+/+}\) MD4 B cells day 1 posttransfer (Fig. 4d). Furthermore, at day 3 posttransfer, although c-FlipL mRNA increased in both Cr2\(^{-/-}\) and Cr2\(^{+/+}\) MD4 B cells, c-FLIPL mRNA remained reduced in Cr2\(^{-/-}\) MD4 B cells compared with that observed in Cr2\(^{+/+}\) MD4 B cells. Therefore, in combination with the Western blot analysis, these data indicate that increased sensitivity of Cr2\(^{-/-}\) MD4 B cells to apoptosis correlates with a reduction in c-FLIPL, and occurs independent of changes in bcl-2 and bcl-xL mRNA.

**In vitro stimulation with rHEL-C3d\(_3\) and DEL-C3d\(_2,3\)**

The in vivo adoptive transfer model strongly supports a role for CD21/CD35 coreceptor signaling in modulating c-FLIPL levels. Sensitivity to CD95 apoptosis was tested further in vitro with Cr2\(^{+/+}\) MD4 B cells exposed to CD154 with or without lysozyme (HEL or DEL) or recombinant lysozyme fused to three copies of complement fragment C3d (rHEL-C3d\(_3\) or rDEL-C3d\(_3\)).

To test whether signaling through CD21/CD35 is important in protecting B cells against apoptosis, MD4 B cells with an intact coreceptor were stimulated with CD154 and either lysozyme alone or lysozyme-C3d\(_3\). Activated B cells were subsequently subjected to CD95 cross-linking to induce apoptosis, and apoptosis was assessed by staining with 7-AAD. CD95-specific apoptosis was determined as reported previously (7). MD4 B cells cultured in the presence of CD154 alone were more sensitive to CD95-specific apoptosis than in medium alone (Fig. 5a). Although 100 pM HEL were required for \(\sim 50\%\) protection against CD95-apoptosis, the addition of increasing quantities of HEL (up to 10 nM; data not shown) did not significantly change the sensitivity to apoptosis. Comparatively, 100 pM rHEL-C3d\(_3\) conferred nearly full protection against CD95 killing. The protection provided by rHEL-C3d\(_3\) was also dose dependent, as 1 pM did not protect but 10 pM provided \(\sim 50\%\) protection. Therefore, the presence of C3d provided additional protection against CD95-mediated apoptosis.

To determine whether the protection against CD95-mediated apoptosis correlated with increased c-FLIPL, cultured MD4 B cell lysates were analyzed by Western blot analysis and by real-time PCR before CD95 cross-linking (Fig. 5, b–d). Consistent with previous reports, CD154 signal alone resulted in detectable levels of c-FLIPL. The levels of c-FLIPL increased with addition of higher levels of HEL. However, the levels of c-FLIPL were increased even more significantly in rHEL-C3d\(_3\)-stimulated MD4 B cells. Densitometric scans of blots from four independent experiments consistently demonstrated that levels of c-FLIPL were approximately equivalent in MD4 B cells stimulated with 100 pM HEL and 1 pM rHEL-C3d\(_3\), suggesting that HEL-C3d\(_3\) is \(\sim 100\)-fold more potent than HEL alone in up-regulating c-FLIPL (Fig. 5c). The relative levels of c-FLIPL continued to increase with increasing amounts of rHEL-C3d\(_3\), indicating a dose-dependent response similar to that observed for protection against CD95-mediated apoptosis. These results were confirmed by real-time PCR (Fig. 5d). Importantly, no differences in viable cell numbers were found following culture with CD154 alone or in combination with HEL or rHEL-C3d\(_3\), indicating that increases in c-FLIPL levels were not simply due to differences in numbers of MD4 B cells after culture.

The blots were re-examined for BCL-2 levels to assess whether CD21/CD35 cocrross-linking up-regulated BCL-2 as previously reported for anti-IgM/anti-CD19 stimulation (50). Despite an increase in BCL-2 mRNA as measured by real-time PCR, no consistent up-regulation was observed in BCL-2 levels following stimulation with HEL or rHEL-C3d\(_3\) (Fig. 5, b and d). Therefore, increased protection of apoptosis does not correlate with increased levels of BCL-2.

The results from the in vitro assay suggest that coligation of the CD21/CD35 coreceptor and BCR by rHEL-C3d\(_3\) fusion protein enhances survival via c-FLIP and inhibition of CD95 killing. To test whether coupling of C3d\(_3\) to DEL also enhances protection, a rDEL-C3d\(_3\) fusion plasmid was constructed and protein expressed in sf9 cells (Table II) (33). To test whether the rDEL-C3d\(_3\) was a
more potent activator of B cells compared with DEL alone, Ca\(^{2+}\) mobilization assays were performed (Fig. 6, a–d). Approximately 100 nM DEL were required to detect above background levels of intracellular Ca\(^{2+}\) in MD4 B cells (Fig. 6, a and c). In contrast, significant Ca\(^{2+}\) mobilization was detected in MD4 B cells with 1 nM rDEL-C3d3 (Fig. 6b). Therefore, as predicted from earlier studies by Dempsey et al. (38), coupling of Cd3 to DEL greatly enhanced early activation of B cells compared with responses with DEL alone.

To determine whether signaling by rDEL-C3d3 was initiated through CD21/CD35 coreceptor, a blocking assay was performed using 7G6, an Ab specific for the C3d binding site on CD21/CD35 (Fig. 6c). Treatment of Cr2\(^{-/-}\) MD4 B cells with 7G6 alone did not induce Ca\(^{2+}\) flux as expected (data not shown; Ref. 3). After preincubation with 7G6, MD4 B cells were treated with 1 nM rDEL-C3d3 or 100 nM DEL. Ca\(^{2+}\) mobilization with 100 nM DEL was unaffected by preincubation of MD4 B cells with 7G6, indicating that 7G6 did not interfere with signaling through the BCR.

In contrast, although 1 nM rDEL-C3d3 initiated a quick and sustained Ca\(^{2+}\) flux, preincubation with 7G6 completely abrogated this signal. Therefore, blocking of CD21/CD35 with 7G6 abrogated the enhancing affect of C3d. Furthermore, 7G6-mediated blocking was alleviated by stimulating the MD4 B cells with 100 nM rDEL-C3d3, suggesting that sufficient BCR stimulation caused the release of intracellular Ca\(^{2+}\) stores.

To determine whether Cr2\(^{-/-}\) MD4 B cells were nonresponsive to complement-tagged Ag as expected, cells were treated with rDEL-C3d3, and Ca\(^{2+}\) mobilization was assessed. Cr2\(^{-/-}\) MD4 and Cr2\(^{-/-}\) MD4 B cells responded similarly when treated with 100 nM DEL (Fig. 6d), suggesting that BCR signaling was equivalent. In contrast, while treatment with 1 nM rDEL-C3d3 enhanced Ca\(^{2+}\) flux in Cr2\(^{-/-}\) MD4 B cells, the response of Cr2\(^{-/-}\) MD4 B cells was significantly impaired. Therefore, although Cr2\(^{-/-}\) MB4 B cells signal normally through BCR, they have impaired signaling through CD21/CD35.

To determine whether C3d3 enhances protection of MD4 cells stimulated with DEL, CD95-dependent apoptosis was measured using \(^{51}\)Cr release (7). It should be noted that the \(^{51}\)Cr release assay is more sensitive than 7-AAD staining because it measures end-stage apoptosis. Consistent with earlier results using HEL and rHEL-C3d3, coupling C3d3 to DEL conferred better protection against CD95 killing (Fig. 7). The protection against CD95-dependent apoptosis was dose dependent, such that increasing the amount of DEL from 1 to 100 pM increased protection. By comparison, although 50% protection using \(^{51}\)Cr release assay (compare 30% protection with DEL vs 60% with CD154 stimulation alone), 1 pM rDEL-C3d3 conferred \~70% protection against apoptosis. Additional protection by increasing the amount of rDEL-C3d3 to 100 pM was not observed. Therefore, the presence of C3d3 conjugated to moderate affinity lysozyme increases the resistance of Cr2\(^{-/-}\) MD4 B cells to CD95-dependent apoptosis.

### Discussion

In this report, we demonstrate that B cells require complement receptor CD21/CD35 to survive in splenic follicles when encountering moderate affinity protein Ags. CD95 apoptosis eliminates

| Table II. Comparison of amino acid sequences of HEL, DEL, and rDEL* |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lysozyme         | AA3             | AA15            | AA73            | AA75            | AA93            | AA97            | AA103           |
| HEL              | F               | H               | R               | L               | N               | K               | N               |
| DEL              | Y               | L               | K               | A               | K               | R               | D               |
| rDEL             | Y               | L               | K               | A               | K               | R               | D               |

* Only nonconserved amino acid residues affecting binding affinity by MD4 BCR were targeted for mutation in rDEL, with the position indicated. Amino acid sequences for rDEL were predicted from DNA sequence analysis. Epitope mapping studies from Rajpal (51) demonstrated that residue 97 is a major immunodeterminant for H\(_2\)HEL-10 binding; residue 73 is a minor immunodeterminant; residues 15, 73, 93, and 103 are relatively unimportant. Phenylalanine at residue 3 prevents HEL from priming I-A\(^{a}\)-restricted T cells (52).
Ig stimulation alone (3) and in vivo as illustrated by adoptive trans-

transplantation of CD21/CD35 with subthreshold surface Ig cross-linking
documented in vitro. For example, the combination of coengage-

ments unless the BCR is engaged by Ab cross-linking or with Ags
activation in vivo (11), sensitizes B cells to CD95-mediated apo-

tosis and cell death. Cognate interaction between B and T cells
the importance of signal strength in regulating mature B cell ac-

tivation. B cells protects these cells from CD95-mediated elimination dur-

Therefore, we conclude that CD21/CD35 coreceptor signaling on

those B cells lacking CD21/CD35, as revealed by adoptive transfer
experiments where CD95 mutation rescued Cr2−/− MD4 B cells in spleen (Table I; Figs. 1 and 2). The molecular mechanism for
CD21/CD35-regulated follicular survival is controlled in part by

CTLA-4, an inhibitor of CD95 cell death. Cr2−/− MD4 B cells had
reduced levels of active CTLA-4 for 1 and 3 days following adoptive
transfer (Fig. 4). Increased sensitivity to apoptosis occurred in

Cr2−/− MD4 B cells as well as following in vitro activation with
both high (HEL) and moderate (DEL) affinity lysozyme alone
compared with lysozyme-C3d4 (Figs. 5 and 7), demonstrating that
differential sensitivity to apoptosis is not unique to Cr2−/− B cells.
Therefore, we conclude that CD21/CD35 coreceptor signaling on
B cells protects these cells from CD95-mediated elimination dur-

ing activation.

The current study confirms and extends previous examination of
the importance of signal strength in regulating mature B cell ac-
tivation and cell death. Cognate interaction between B and T cells
via CD40-CD154 interaction, a recognized early step in B cell activa-
tion in vivo (11), sensitizes B cells to CD95-mediated apoptosis
unless the BCR is engaged by Ab cross-linking or with Ags (7, 8).
Subthreshold BCR signals subject activated B cells to de-
letion in the outer periarteriolar lymphoid sheath (53, 54). The role
of CD21/CD35 signaling in potentiating B cell activation is well
documented in vitro. For example, the combination of coengage-
ment of CD21/CD35 with subthreshold surface Ig cross-linking
induces B cell differentiation into cell cycle more readily than anti-
Ig stimulation alone (3) and in vivo as illustrated by adoptive trans-
fer experiments using Cr2−/− MD4 B cells (5). The current results
suggest that the absence of donor Cr2−/− MD4 B cells from re-
cipient follicles is due to their active elimination by CD95-mediat-
ad apoptosis (Table I; Figs. 1–3). Supporting evidence derives from
different observations: 1) Cr2−/− MD4 B cells are not
eliminated when transferred into immununized recipient mice
(data not shown; Ref. 5); 2) although small percentages of trans-
ferred cells are observed in gut-associated lymphoid tissue of re-
cipient mice, Cr2−/− MD4 B cells do not accumulate in gut-as-

associated lymphoid tissue with time after transfer (data not shown);
3) transfer of Cr2−/− MD4 B cells into recipient mice immunized with
turkey egg lysozyme, TEL (Km ~ 1 × 10^{-10} M vs 1 × 10^{-7} M
for DEL), results in their accumulation within germinal centers (5); and
4) Cr2−/− MD4 B cells isolated one day posttransfer into TEL-immunized recipients have an approximate 3-fold increase in

%CTLA-4 mRNA compared with Cr2−/− MD4 B cells isolated 1 day
after transfer into DEL-immunized recipients (data not shown).
The results with higher affinity Ags are consistent with the hy-
thesis that sufficient signaling through the BCR bypasses the
requirement for additional CD21/CD35 coreceptor signals at the
follicular checkpoint.

A number of molecules that influence CD95-mediated apoptosis
are described, such as CD95 (46), c-FLIP (31, 46), FAIM (55),
Bcl-2 (50, 56), Bcl-xL (49, 56–60), and caspase-3 (61). Bcl-2
(50, 56), CD95 (46), and c-FLIP (46) were linked previously to
CD21/CD35 signaling and were therefore examined in the current
study. CD21/CD35 signaling may block CD95-mediated apoptosis
either by down-regulation of CD95 or CD95 pathway mediators or
by up-regulation of CD95 pathway inhibitors. Our data are con-
sistent with this regulation occurring by increased levels of
c-FLIPLs, a downstream inhibitor of CD95. Analysis of donor MD4
B cells sorted one day after transfer identified similar levels of
surface CD95 on Cr2−/− and Cr2+/+ B cells (Fig. 3b). These data
contrast with those in a recent study with human follicular B cells
stimulated with anti-Ig/C3d dextran complexes. In that study, the
investigators reported significant reductions in cell surface CD95
levels after stimulation with C3d-containing dextran complexes
(46). Several factors may explain the differences with our findings.
One possibility is that the downstream effects of CD21/CD35 co-
receptor signaling on murine and human B cells differ. Alterna-
tively, differences in the nature of ligands used (anti-Ig/C3d vs
lysozyme-C3d) may result in different signals generated, specifi-
cally through the BCR where anti-Ig treatment is likely more ef-

cient at cross-linking BCR than lysozyme. CD95 levels are sim-
ilar following stimulation using several conditions and likely
represent reflect equivalent early CD40 signaling.

The mechanism of CD95-resistance in our in vivo and in vitro
models is similar in that, along with no significant differences ob-
served in CD95, BCL-2, and BCL-xL levels (Figs. 3b, 4, b, c, e,
and h, and 5, b–d), c-FLIPs levels are differentially up-regulated in B cells receiving signals through CD21/CD35 (Figs. 4, a, c, and d, 5, b, c, and d). The importance of CD21/CD35 signaling in regulating c-FLIPs, is evident at both the protein and mRNA levels. Both the sensitivity of Cr2+/− MD4 B cells stimulated with lysozyme alone in vitro and elimination of Cr2+/− MD4 B cells in vivo correlates with lower levels of c-FLIPs, suggesting the in vivo observations are not simply explained by intrinsic defects of Cr2+/− B cells. Interestingly, while no significant differences in the level of the 55-kDa form of c-FLIP were observed in Cr2+/− and Cr2+/MD4 B cells, c-FLIPs (43 kDa) is reduced consistently in Cr2+/− B cells (Fig. 4a), suggesting that CD21/CD35-mediated signals may influence proteolytic processing of c-FLIP. In vitro studies on CD154-activated primary B cells or c-FLIP transfected B cell lines suggest 55-kDa c-FLIP is processed to 43 kDa after anti-CD95 or soluble CD95L treatment (21, 29, 45). Preprocessed c-FLIP (55 kDa) is retrieved and cleaved at the level of DISC upon CD95 ligation (29, 45). FLIP can be up-regulated by cross-linking BCR using an in vitro culture system of primary B cells or cell lines, providing evidence that strong BCR signaling cross-linking BCR using an in vitro culture system of primary B cells upon interaction with CD4+ T cells. 1. Fearon, D. T., and M. C. Carroll. 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu. Rev. Immunol. 18: 393–422.

The signaling pathway from CD21/CD35 to c-FLIP remains unknown. Because the regulation of c-FLIP level may depend on cell cycle (62), CD21/CD35 may regulate c-FLIP by inducing B cell proliferation (3, 50, 63). However, preliminary results using CFSE-labeled transferred MD4 B cells showed no obvious proliferation within splenic follicles (data not shown). Therefore, although C3d or Ab cross-linking with CD21/CD35 may regulate cell cycle, it does not appear to be a major mechanism during follicular checkpoints. Instead, it may be important later during germinal center responses, during which B cells differentially express c-FLIP (64, 65) and whose survival also depends, at least in part, on CD21/CD35 coreceptor signaling (5, 66). Despite the high-affinity interaction of HEL with the BCR transgene alone (Kd=0.45 × 1010 M−1), HEL-C3d4 also provides increased CD95 resistance to MD4 B cells (Fig. 4e). Because this correlates with increased c-FLIPs levels (Fig. 4b), it is possible that germinal center selection may be dependent on coreceptor cross-linking. However, this possibility was not addressed herein and requires more rigorous testing to determine its validity.

Covalent association of Ags with C3d, a ligand for complement receptors CD21/CD35, leads to augmented humoral responses and led to the proposal that C3d (and C3dg) is a molecular adjuvant (38, 67–70). Because of the well-documented role of CD21/CD35 as a B cell coreceptor as described above, it is speculated that Ag-C3d(g) adds potentiate stronger immune responses in part by eliciting stronger signaling in Ag-specific B cells. A recent report by Haas et al. (71) in which Ag-C3d immunization provoked Ab responses in CD21/CD35-deficient mice suggests there may be other CD21/CD35-independent mechanisms responsible for regulating responses. Both the in vivo and in vitro approaches used in the current study isolate CD21/CD35 defects to the MD4 B cells. We specifically tested whether signaling by lysozyme-C3d conjugates was mediated through CD21/CD35 in vitro by Ca2+ mobilization assay (Fig. 6). C3d-augmented signaling was specifically blocked by anti-CD21/CD3 Ab, and this block was ameliorated by addition of an equivalent concentration of lysozyme to cause mobilization by Ag alone (Fig. 6c). Stimulation of Cr2+/− MD4 B cells with 1 nM rDEL-C3d, failed to stimulate a significant increase in intracellular Ca2+ levels relative to Cr2+/− MD4 B cells, consistent with impaired coreceptor activity (Fig. 6d).

In summary, we found that the CD21/CD35 coreceptor is important in maintaining B cell survival following engagement of the CD95 receptor. The finding that c-FLIP levels are up-regulated following cross-linking of CD21/CD35 supports a direct role for coreceptor signaling in blocking CD95-dependent apoptosis. This regulation may represent an important mechanism by which bystander B cells are censored in the absence of coreceptor activation, thereby preventing unwanted immune responses.

Acknowledgments

We thank T. J. Schneider for critical review of the manuscript, K. Ketman and Dr. N. Barteneva for their aid with the Ca2+ flux assay, and Dr. S. C. Harrison and M. Babynoshev for their guidance with the baculovirus system.

Disclosures

The authors have no financial conflict of interest.

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2867The Journal of Immunology

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