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

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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 adaptively 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-C3d, 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. The Journal of Immunology, 2005, 175: 2859–2867.

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 FcyRIIB 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)4 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 within 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 paratrabacular 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).

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4 Abbreviations used in this paper used: HEL, hen egg lysozyme; MD4, HEL immunoglobulin transgene; WT, wild type; TEL, turkey egg white; DEL, duck egg lysozyme; c-FLIP, cellular FLIP; FADD, CD95-associated death domain; DISC, death-inducing-signaling-complex; MNC, mononuclear cell; PNA, peanut lectin (agglutinin); 7-AAD, 7-aminoactinomycin D; AP, alkaline phosphatase streptavidin.

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To address whether CD95 regulation is important in the response of naive B cells to moderate affinity T-dependent Ags and to determine whether coreceptor signaling is involved in protection from the death pathway, both in vivo and in vitro approaches were taken using the well-characterized MD4 transgenic model (4). An advantage of this model is that the MD4 BCR (developed from the HyHEL-10 hybridoma) has different affinities for various forms of avian lysozyme. For example, the transgene encoded receptor binds HEL at a very high affinity (K<sub>I</sub> = 4.5 × 10<sup>10</sup> M<sup>-1</sup>) but DEL with only moderate affinity, e.g., ~1 × 10<sup>7</sup> M<sup>-1</sup> (33). Because of this reduced affinity, it was predicted that MD4 B cells would be more dependent on CD21/CD19 coreceptor signaling for activation when encountering DEL. Cr2<sup>−/−</sup> MD4 mice were bred with Crd5 mutant (Ipr<sup>−/−</sup>) mice (34), and splenic B cells from these mice (or control mice) were adoptively transferred into recipients previously immunized with DEL Ags. We found that CD95 deficiency was protective against elimination of Cr2<sup>−/−</sup> MD4 B cells. To test the role of coreceptor signaling in protection against CD95-killing, Cr2<sup>−/+</sup> MD4 B cells were cultured in vitro in the presence of CD154 and either high or moderate affinity lysozyme coupled to C3d (or noncoupled) and subjected to apoptosis by CD95 cross-linking. B cells exposed to lysozyme alone were more sensitive to CD95-dependent apoptosis than B cells exposed to lysozyme-C3d. Therefore, the CD21/CD19 coreceptor acts to enhance the BCR signal to provide protection from CD95-mediated elimination. In addition, the CD95-Cr2<sup>−/−</sup> pathway is important not only in regulation of self-reactive B cells in the periphery but provides an important checkpoint to eliminate B cells receiving subthreshold signals.

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<sup>−/−</sup>; Ref. 36) intact Cr2 locus. MD4 strains were also crossed to Ipr<sup>−/−</sup> 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.

Adoptive 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. Splenocytes were analyzed 1, 3, and 5 days after transfer by four-color flow cytometry and by immunohistochemistry.

Flow cytometric analysis

Splenic mononuclear cells (MNCs) were isolated by density gradient centrifugation using Lympholyte 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<sup>+</sup> (BD Pharmingen). The allostotype of MD4 BCR is IgM<sup>+</sup>, whereas all endogenous B cells from C57BL/6 mice are IgM<sup>−</sup>. An additional allogenic 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 IgM<sup>+</sup>-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 IgM<sup>+</sup> or, alternatively, IgM<sup>−</sup>. To confirm that transferred cells could be identified as HEL IgM<sup>+</sup> or IgM<sup>−</sup> alone, mice were immunized in the absence of adoptive transfer, and no HEL IgM<sup>+</sup> or IgM<sup>−</sup> 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 × 10<sup>5</sup> sorted Cr2<sup>−/−</sup> and Cr2<sup>−/+</sup> MD4 B cells (pooled from 3–5 mice/group each experiment) were separated by 10% SDSPAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% dry milk (Carnation/PFBS). Fifty-five-kilodalton c-FLIP and 43-kDa-processed c-FLIP<sub>D</sub> were detected by anti-FLIP Ab (Dave-2; Elyte Biochemicals). Anti-Bcl-2 Ab (Ab-2; Oncogene Research Products) detected 26- and 19-kDa molecules. For in vitro experiments, lysates from 1 × 10<sup>6</sup> 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 β<sub>2</sub>-microglobulin expression. c-FLIP<sub>L</sub> and bcl-X<sub>L</sub> expression was determined by real-time PCR (Stratagene) using the following primers (forward/reverse): β<sub>2</sub>-microglobulin (5′-GCCGCCCTCTAATTGAAATCC/GCGTATGTATCAGTCTCAGTGG-3′); c-FLIP<sub>L</sub> (5′-ACGTGCCCTTCCTTCTAGAGG/TTTGCGAGTTGTCCTGGTTGC-3′); bcl-2 (5′-AAGGGATGGTCAGTTGCTAG/TCCTAATGTAAATACAAT-3′). Alternatively, c-FLIP<sub>L</sub> expression was determined by real-time PCR (Stratagene) using the following primers (forward/reverse): c-FLIP<sub>L</sub> (5′-ACGTGCCCTTCCTTCTAGAGG/TTTGCGAGTTGTCCTGGTTGC-3′); bcl-2 (5′-AAGGGATGGTCAGTTGCTAG/TCCTAATGTAAATACAAT-3′).

Lysozyme and lysozyme-C3d<sub>L</sub> production and purification

HEL was purchased from Sigma-Aldrich. DEL was isolated from duck egg whites as described previously (37). Recombinant HEL-C3d<sub>L</sub> 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 rHEL-C3d<sub>L</sub>-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 HEL-C3d<sub>L</sub> 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 rHEL-C3d<sub>L</sub> were identified by 10% SDS-PAGE using GelCode (Pierce) and by Western blot analysis with polyclonal rabbit anti-HEL Ab (BioSource International). Alternatively, HEL-C3d<sub>L</sub> 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 DEL-C3d<sub>L</sub> 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-C3d<sub>L</sub> baculovirus vector DNA. Recombinant DEL-C3d<sub>L</sub>-encoding baculovirus was engineered, and clones producing recombinant protein were identified as described previously (BD Pharmingen).

Ca<sup>2+</sup> 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 10<sup>6</sup> cells. For loading, cells were incubated 30 min at 37°C, then washed with RPMI 1640 containing 2% FCS. Cells were subsequently stained with 2B20FITC (BD Pharmingen), washed, resuspended at 2 × 10<sup>6</sup> cells/ml, and Ca<sup>2+</sup> mobilization measured using a FACS Vantage cytometer with UV laser (BD Biosciences).

Apoptosis assay

Splenic B cells were purified from MD4 mice using MACS (Miltenyi Bio)techn. 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

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(F(ab')2, soluble DEL/HEL and rDEL-C3d/rHEL-C3d, at 1–10,000 pM/1 × 10⁶ cells. After 48 h of culture (exposure to CD154 for 48 h, exposure to BCR ligand to 24 h), dead cells were eliminated by sedimentation on Lympholyte M, and viable cells were labeled by incubating with 0.2 mCi of sodium chromate (Amersham Biosciences) at 37°C. Labeled cells were washed and plated on 96-well round-bottom plates at 10⁵ cells/ml Anti-CD95 Ab (Jo2; BD Pharmingen) was added at 0.9 μg/ml and incubated for 5 h. CD95-specific apoptosis was determined as reported previously (7). Briefly, the following calculation was used (experimental release – spontaneous release)/total release × 100, in which total release was determined by lysing cells with Triton X-100. Cell culture 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⁻/− MD4 B cells are eliminated by CD95-mediated apoptosis in vivo, Cr2⁻/− MD4 B cells lacking functional CD95 were generated by breeding of Cr2⁻/− 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⁺/⁺ or Cr2⁻/− 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⁺/⁺ MD4 B cells are abundant within splenic follicles, whereas negligible numbers of Cr2⁻/− MD4 B cells were observed within the spleen (Figs. 1, a and b, 2, a and b). In contrast, Cr2⁻/− 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⁻/− MD4 lpr B cells within the follicles approached that of Cr2⁺/⁺ MD4 B cells (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.

**FIGURE 1.** Effect of CD95 mutation and constitutive c-FLIP expression on the fate of Cr2⁻/− MD4 B cells in splenic follicles of DEL-immunized recipient mice. Immunohistochemical analysis of splenic follicles of DEL-immunized recipients after adoptive transfer of either Cr2⁺/⁺ (a and c) or Cr2⁻/− (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.** Effect of CD95 mutation on the fate of Cr2⁻/− 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⁺/⁺ (a and c) or Cr2⁻/− (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. In the presence of Cr2⁺/⁺ MD4 B cells, the frequency of donor-derived B cells is similar across all three experiments. Mean values ± SD are shown. Asterisks indicate significant differences compared with frequency of Cr2⁻/− MD4 B cells after transfer (**, p ≤ 0.04; ***, p < 0.07, t test).

**Increased apoptosis and decreased c-FLIPL 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⁻/− and Cr2⁺/⁺ MD4 B cells were examined 1 day after transfer. Day 1 posttransfer was the chosen time point because Cr2⁻/− MD4 B cells exist in similar numbers as Cr2⁺/⁺ MD4 B cells; after day 1, progressive loss of Cr2⁻/− B cells occurs. The transferred MD4 B cells are identified as IgM⁺/HEL-binding double-positive cells (Fig. 3a). Similar levels of CD95 were observed on the surface of transferred Cr2⁻/− and Cr2⁺/⁺ 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⁻/− MD4 = 24, Cr2⁺/⁺ MD4 = 21, p > 0.1; Fig. 3b).

To directly test which B cells lacking CD21/CD35 were undergoing apoptosis, levels of the early apoptotic markers annexin
V (43) and 7-AAD (44) were assessed by flow cytometry. Cr2<sup>−/−</sup> MD4 B cells have ~2-fold higher percentage of 7-AAD<sup>+</sup> cells when compared with Cr2<sup>+/+</sup> MD4 B cells 1 day following transfer into DEL-immunized recipient mice (25–30% vs 10–12% from three separate experiments; Fig. 3c, i–iii). A higher percentage of transferred Cr2<sup>−/−</sup> B cells also stained positive for annexin V staining compared with transferred Cr2<sup>+/+</sup> B cells, confirming the increased apoptosis in Cr2<sup>−/−</sup> B cells suggested by 7-AAD staining (Fig. 3c and data not shown). By day 3 posttransfer, similar frequencies of 7-AAD<sup>+</sup> Cr2<sup>−/−</sup> and Cr2<sup>+/+</sup> 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<sup>−/−</sup> MD4 B cells.

To determine the mechanism responsible for elimination of Cr2<sup>−/−</sup> B cells, the level of c-FLIP, a downstream regulator of CD95, was assessed. 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), and therefore B cells lacking CD21/CD35 may have sub-threshold levels of c-FLIP. To investigate c-FLIP expression upon encounter of moderate affinity Ags, FACS-sorted MD4 B cells from DEL-immunized recipients at day 1 posttransfer were analyzed for levels of c-FLIP by Western blot analysis. Using anti-c-FLIP Ab, two major forms were detected, 55 and 43 kDa, respectively. The 55-kDa form encodes for the proform of c-FLIP and is thought to be inactive until it is proteolytically processed into the 43-kDa active form called c-FLIP<sub>L</sub> (21, 29). A 2- to 3-fold reduction of the active 43-kDa form of FLIP<sub>L</sub> was observed in Cr2<sup>−/−</sup> MD4 B cells compared with Cr2<sup>+/+</sup> MD4 B cells (Fig. 4a; all three experiments summarized in Fig. 4c). Cr2<sup>−/−</sup> MD4 B cells also had reduced levels of the proform of c-FLIP as compared with Cr2<sup>+/+</sup> MD4 B cells, although these differences were not statistically significant. These data suggest that CD21/CD35 coreceptor signaling increases c-FLIP<sub>L</sub> levels and that this may occur in part by regulating processing of the proform.

The Western blots were reprobed to examine levels of BCL-2, a molecule also known to regulate apoptosis (47–49). BCL-2 levels reportedly increase as a consequence of CD21/CD35 signaling (46, 50). In contrast to c-FLIP levels, no significant differences were observed in BCL-2 levels between Cr2<sup>−/−</sup> and Cr2<sup>+/+</sup> MD4 B cells in three independent experiments (Fig. 4, a and d). Therefore, these results suggest that elimination of Cr2<sup>−/−</sup> MD4 B cells during the ongoing immune response to DEL is not due to differential levels of BCL-2.

To further test whether CD21/CD35 signaling regulates c-FLIP<sub>L</sub> levels, Cr2<sup>−/−</sup> and Cr2<sup>+/+</sup> MD4 B cells were isolated by FACS 1 and 3 days after adoptive transfer and analyzed by real-time PCR for c-Flip<sub>L</sub>, bcl-2, and bcl-x<sub>L</sub> mRNA levels (Fig. 4, d–f). No significant differences in bcl-2 and bcl-x<sub>L</sub> mRNA were observed between Cr2<sup>−/−</sup> and Cr2<sup>+/+</sup> 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 Flip<sub>L</sub> mRNA was observed in

<table>
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<th>Donor B Cell Source</th>
<th>Total No. of Follicles&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of Follicles with HEL-Binding Cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of Follicles with HEL-Binding Cells</th>
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<tr>
<td>Cr2&lt;sup&gt;−/−&lt;/sup&gt; MD4 (n = 5)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.3 ± 3.1</td>
<td>16.7 ± 3.4</td>
<td>82.3</td>
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<td>Cr2&lt;sup&gt;−/−&lt;/sup&gt; MD4 (n = 3)</td>
<td>28 ± 1.4</td>
<td>1.5 ± 0.5</td>
<td>5.3&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Cr2&lt;sup&gt;−/−&lt;/sup&gt; MD4 lpr (n = 5)</td>
<td>33 ± 4</td>
<td>29.3 ± 5.1</td>
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<tr>
<td>Cr2&lt;sup&gt;−/−&lt;/sup&gt; MD4 lpr (n = 3)</td>
<td>37 ± 2</td>
<td>24 ± 3.6</td>
<td>64.9&lt;sup&gt;e&lt;/sup&gt;</td>
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* Mean + (SD) number of follicles per section counted.
* Mean + (SD) number of follicles per section counted with ≈10 MD4 B cells.
* n is the total number of mice analyzed.
* Comparison of lpr vs non-lpr Cr2<sup>−/−</sup> MD4 B cells in follicles: p ≤ 0.001 (t test); Cr2<sup>−/−</sup> MD4 lpr vs Cr2<sup>+</sup> MD4 lpr B cells in follicles p > 0.1.

FIGURE 3. Increased apoptosis in Cr2<sup>−/−</sup> 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<sup>−/−</sup> (left panel) and Cr2<sup>+</sup> (right panel) MD4 B cells are identified as surface marker IgM<sup>+</sup> and binding HEL; b, analyzing CD95 expression on Cr2<sup>−/−</sup> or Cr2<sup>+/+</sup> 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<sup>−/−</sup> or Cr2<sup>+</sup> 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<sup>−/−</sup> or Cr2<sup>+/+</sup> MD4 B cells gated as shown in a. Comparison of Cr2<sup>−/−</sup> or Cr2<sup>+/+</sup> MD4 B cells: p < 0.05. Scatterplot summarizes results from three separate experiments.
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-C3d3 and DEL-C3d2,3

The in vivo adoptive transfer model strongly supports a role for CD21/CD35 coreceptor signaling in modulating c-FLIP 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-C3d3 or rDEL-C3d3).

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-C3d3. 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 ~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-C3d3 conferred nearly full protection against CD95 killing. The protection provided by rHEL-C3d3 was also dose dependent, as 1 pM did not protect but 10 pM provided ~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-FLIP levels, 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-FLIP. The levels of c-FLIP continued to increase with addition of higher levels of HEL. However, the levels of c-FLIP were increased even more significantly in HEL-C3d3-stimulated MD4 B cells. Densitometric scans of blots from four independent experiments consistently demonstrated that levels of c-FLIP were approximately equivalent in MD4 B cells stimulated with 100 pM HEL and 1 pM rHEL-C3d3, suggesting that HEL-C3d3 is ~100-fold more potent than HEL alone in up-regulating c-FLIP (Fig. 5c). The relative levels of c-FLIP continued to increase with increasing amounts of rHEL-C3d3, 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-C3d3, indicating that increases in c-FLIP 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-C3d3 (Fig. 5b 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-C3d3 fusion protein enhances survival via c-FLIP and inhibition of CD95 killing. To test whether coupling of C3d3 to DEL also enhances protection, a rDEL-C3d3 fusion plasmid was constructed and protein expressed in sf9 cells (Table II) (33). To test whether the rDEL-C3d3 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 C3d 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 effect 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\(^{-/-}\) MD4 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 lysosome 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<sup>a</sup>

<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>AA3</th>
<th>AA15</th>
<th>AA73</th>
<th>AA75</th>
<th>AA93</th>
<th>AA97</th>
<th>AA103</th>
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<td>HEL</td>
<td>F</td>
<td>H</td>
<td>R</td>
<td>L</td>
<td>K</td>
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<td>K</td>
<td>A</td>
<td>K</td>
<td>A</td>
<td>R</td>
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<sup>a</sup> 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 Rajgal (51) demonstrated that residue 97 is a major immunodeterminant for H2/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<sup>d</sup>-restricted T cells (52).
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 c-FLIPL, an inhibitor of CD95 cell death. Cr2−/− MD4 B cells had reduced levels of active c-FLIPL 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-C3d (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 during activation.

The current study confirms and extends previous examination of the importance of signal strength in regulating mature B cell activation and cell death. Cognate interaction between B and T cells via CD40-CD154 interaction, a recognized early step in B cell activation 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 deletion 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 coengagement 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 transfer experiments using Cr2−/− MD4 B cells (5). The current results suggest that the absence of donor Cr2−/− MD4 B cells from recipient follicles is due to their active elimination by CD95-mediated apoptosis (Table I; Figs. 1–3). Supporting evidence derives from four additional observations: 1) Cr2+/− MD4 B cells are not eliminated when transferred into nonimmunized recipient mice (data not shown; Ref. 5); 2) although small percentages of transferred cells are observed in gut-associated lymphoid tissue of recipient mice, Cr2−/− MD4 B cells do not accumulate in gut-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 (Kd ~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 c-FlipL 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 hypothesis 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-FlipL (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-FlipL (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 consistent with this regulation occurring by increased levels of c-FlipL, 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/C3dg 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 coreceptor signaling on murine and human B cells differ. Alternatively, differences in the nature of ligands used (anti-Ig/C3dg vs lysozyme-C3d) may result in different signals generated, specifically through the BCR where anti-Ig treatment is likely more efficient at cross-linking BCR than lysozyme. CD95 levels are similar following stimulation using several conditions and likely 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 observed in CD95, BCL-2, and BCL-xL levels (Figs. 3b, 4, b, c, e, MD4 B cells).
and h, and 5, b–d), c-FLIP 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-FLIP, 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-FLIP, suggesting that 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-FLIPΔp (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-CDS95 or soluble CD95L treatment (21, 29, 45). Preprocessed c-FLIP (55 kDa) is recruited 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 may represent an important mechanism by which by- stander B cells are censored in the absence of coreceptor activation, thereby preventing unwanted immune responses.

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Disclosures
The authors have no financial conflict of interest.

References
