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The Pathway of Antigen Uptake and Processing Dictates MHC Class II-Mediated B Cell Survival and Activation1

Toufic O. Nashar and James R. Drake2

The influence of the pathway of Ag uptake and processing on MHC class II (CII)-mediated B cell function is unknown. In this study, we investigate in resting and activated (via the BCR or CD40) B cells the biological properties of CII-peptide complexes (CII-peptide) generated by either the BCR-mediated Ag processing (type I complex) or fluid phase Ag processing (type II complex). Compared with type I complex, ligation of type II complex by either specific Ab or the TCR in Ag-presenting assay results in significant decreases in B cell survival rate (50–100%) and expression levels of CII, CD86, and CD54. Loss of B cells following ligation of type II complex occurs in the presence of a comparatively good level of specific CD4+ T cell division, indicating that B cell loss is a late event following T cell stimulation. Comparative analysis of T and B cell conjugates after Ab ligation of type I or II complex reveals decreased efficiency of the latter in forming conjugates. Neither initial differential levels of CII and other studied surface markers, B cell type inherent differences, BCR signaling, T cell proliferation, nor initial density of CII-peptide complexes could explain the T cell-induced B cell loss. We propose that the context in which CII-peptide complexes are present in the membrane following BCR uptake and processing leads to B cell survival. Thus, appropriate targeting of Ag ensures generation of relevant immune responses. The Journal of Immunology, 2005, 174: 1306–1316.

The classical function of MHC class II (CII)3 molecules as Ag-presenting moieties for CD4+ T cell stimulation has been well documented in a number of studies. CII molecules are important in shuttling antigenic peptides from specialized intracellular compartments for their surface expression on professional APC, including B cells. Although in B cells Ag can access CII compartments by different pathways, the most common route is through binding to the specific (BCR). This ensures that relevant B cells are recruited to recognize and internalize Ag even when present in low amount in vivo. Other nonspecific ways that Ag may be internalized include fluid phase uptake, and this pathway may be particularly important in disease when B cells are challenged with a large amount of self Ags derived from destruction of tissue, such as in advanced stages of autoimmunity or from necrosis following infection.

This classical function of CII gives the impression of molecules with restricted function in Ag presentation. However, this has been challenged more recently in a number of studies. Thus, it is clear in both human and murine B cells, CII are signaling molecules that can either inhibit or activate B cells depending on the context in which these molecules function. The signaling activities of CII are brought about by binding of microbial Ags, and ligation via the TCR or Abs. In resting murine B cells, ligation of CII elicits the production of cAMP and promotes translocation of protein kinase C (1), resulting in cell death (2). However, simultaneous ligation of CII and CD95 protects CD40-activated murine B cells from CD95-induced apoptosis (3). Moreover, activation of mouse B cells by ligation of the BCR in the presence of IL-4 primes CII for induction of Ca2+, inositol lipid hydrolysis, and tyrosine kinase activation, and leads to B cell proliferation (4). CII signaling is also reportedly coupled to the protein tyrosine kinase-dependent signaling pathway in human B cells and cell lines (5, 6). However, ligation of CII on human activated B cells results in cell death in vitro (7, 8), while resting human B cells appear to be protected (9). More recently, ligation of peptide-loaded CII on EBV-transformed B cells has been shown to induce apoptosis following cognate MHC-TCR interaction (10). This complex and dynamic feature of CII signaling suggests that it can be used to modulate outcome of immune responses. Importantly, the influence of Ag uptake and processing on CII-peptide-mediated B cell survival has not been addressed. The route of Ag uptake and processing might regulate the density of CII-peptide complex and the context in which they are present in membrane domains, such as ligation via the TCR or mAbs would lead to different biological outcome.

In this study, we investigate the biological consequences of CII ligation in resting and activated B cells. By using B cells from BCR transgenic mice in which B cells are specific for hen egg lysozyme (HEL), as well as nontransgenic littermates, we were able to distinguish the effects resulting from ligation of CII-peptide complex formed via BCR-mediated and fluid phase Ag processing, using both peptide-Ia-specific mAbs and CD4+ T cells. We find significant differences in B cell recovery in culture, and in the levels of B cell surface markers, including CII, CD86, and CD54 upon mAb or TCR interaction with type I and II peptide-Ia complexes. This correlates with differences in the ability of B and T cells to form conjugates. The results demonstrate the influence of Ag uptake and processing on CII regulation of B cell survival and activation.

Materials and Methods

Mice

BCR transgenic mice (MD4.B10.BR, μ−, H-2d) expressing HEL-specific BCR (11, 12) that have been bred onto the B10 background over 15 generations

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3. Abbreviations used in this paper: CII, MHC class II; DiIC16, 1.1-dihexadecyl-3,3’,3’,3’-tetramethylindocarbocyanine perchlorate; FSC, forward light scatter; HEL, hen egg lysozyme; MFI, mean fluorescence intensity; PI, propidium iodide; IC, isotype control.

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were used from 7 to 12 wk of age. The 3A9.B10.BR/J TCR transgenic (H-2d, TCRVβ8) (11) were originally obtained from The Jackson Laboratory and backcrossed onto B10.BR over seven generations in our animal facility. Mice were housed under specific pathogen-free conditions at the Albany Medical College animal breeding facility.

Reagents
C4H3 mAb (rat IgG2b) was either purified from ascites fluid or used from ammonium sulfate precipitate of cell supernatant. HEL_{pc-61} peptide was a gift from R. German (National Institutes of Health, Bethesda, MD). Biotin anti-mouse I-A^k (11.5.2, mouse IgG2b), biotin anti-mouse CD25 (7D4, rat IgM), FITC anti-mouse CD54 (3E2, hamster IgG1), PE anti-mouse CD86 (GL1, rat IgG2a), FITC anti-mouse anti-CD25 (R12-1, rat IgG2a), purified hamster anti-mouse CD40 (HM40-3), FITC hamster IgG1 isotype control (IC) (A19-3), PE rat IgG2a IC (R35-95), biotin rat IgM IC (R4-22), and FITC StreptAvidin were all from BD Pharmingen. Goat anti-mouse m Ab(F(ab')2) was from The Jackson Laboratory, HEL was from Sigma-Aldrich; mitomycin C from Sigma-Aldrich; and CFSE from Molecular Probes. The 1,1-dihexadecyl-3,3',3''-tetramethylindocarbocyanine perchlorate (DiIC16) and CellTracker Green were from Molecular Probes.

Cell purification
Splenic cell suspensions were recovered and incubated with MACS Ab-conjugated magnetic beads and then fractionated on columns, according to instructions supplied by the manufacturer. B cells were negatively selected with anti-CD43 Ab-conjugated microbeads. CD4^+ T cells were negatively selected with a mixture of MACS Abs, including biotin-conjugated mAbs against CD8a (Ly-2), CD11b (Mac-1), CD45R (B220), DX5, and Ter-119, followed by anti-biotin-conjugated microbeads. The purity of these populations was >90%, as revealed by FACSscan flow cytometer (BD Biosciences), using anti-B220 and anti-CD4 Abs.

Assays for determination of the dose and kinetics of HEL
To determine the dose response of HEL for generation of either type I (BCR uptake and processing) or II (fluid phase uptake and processing) CII-peptide complexes, splenocytes were isolated from BCR transgenic or nontransgenic mice and incubated overnight in 37°C in RPMI 1640 containing 10% FCS and 50 μM 2-ME in the presence of an increasing dose of HEL, as indicated (Fig. 1A). Following incubation, cells were labeled on ice with the HEL_{pc-61}-A^k-specific C4H3 mAb (13) for 30 min, followed by anti-rat IgG FITC (to detect bound C4H3 mAb) incubated for an additional 30 min. C4H3 binding was detected by flow cytometry. The binding is reported as mean fluorescence intensity (MFI) units of C4H3 in B2^20^ cells after subtraction of background staining from all values.

To examine the kinetics of generation of type I and II complexes in resting B cells, splenic cells were isolated from HEL-specific BCR transgenic mice and incubated in the presence of 100 nM HEL (BCR transgenic) or 100 μM HEL (nontransgenic) HEL for the indicated time points. At each time point, the cells were collected and stained with anti-B220 PE Ab and 1 μg/ml C4H3 mAb, followed by anti-rat IgG FITC. Specific C4H3 binding was detected as above.

To measure density of type I and II complexes on surface of anti-CD40-activated B cells, pure CD43- B cells were isolated from BCR transgenic and nontransgenic mice and incubated overnight in RPMI 1640 containing 0.5% fresh autologous mouse serum and 2 μg/ml hamster anti-CD40 mAb. Following incubation, 100 nM or 100 μM HEL was added to wells and incubated for 4 h at 37°C. Wells containing cells incubated in the absence of Ag were also included. Cells were washed and put back in culture for an additional 20 h. Cells were then labeled with C4H3 mAb or IgG2b IC, followed by FITC or Cy5, as described above. The cells were then collected, washed, and incubated with propidium iodide (PI) for 10 min. Flow cytometry analysis was performed after gating on viable (PI-negative) cells.

Assays for ligation of CII-peptide complexes with C4H3 mAb and its effect on B cell recovery
Splenocytes were isolated from BCR transgenic or nontransgenic mice and incubated overnight at 37°C in RPMI 1640 containing 10% FCS and 50 μM 2-ME in the presence of 100 nM or 100 μM HEL with or without 1 μg/ml HEL_{pc-61}-A^k-specific C4H3 mAb, and with or without 10 μg/ml F(ab')2 of goat anti-murine m Ab. Samples designated medium contained 1 μg/ml HEL_{pc-61} added to cells in the absence of HEL. The cultures were then collected, and number of B cells was enumerated by cell counting and determination of the percentage of B2^20^ cells. Dead cells were excluded by PI staining.

For cell cycle analysis of DNA staining, the method described previously (14) was used. Briefly, pure CD43^- B cells were isolated from BCR nontransgenic mice and incubated overnight with 100 μM HEL with and without 1 μg/ml C4H3 mAb. Samples designated medium contained 1 μg/ml HEL_{pc-61} added to cells in the absence of HEL. Cells were then layered over Ficoll-Paque (Amersham Biosciences) to collect whole cells and remove cell debris and nuclei, and then fixed with cold ethanol added drop-wise. Then 50 μg/ml PI (Sigma-Aldrich) and 40 μg/ml RNase A (DNase free) (Sigma-Aldrich) were added and incubated for 1 h at room temperature. The relative intensity of DNA staining with PI was determined.

Ag presentation assays
Pure CD43^- B cells were isolated from BCR transgenic and nontransgenic littermates. Cells were activated by overnight incubation with 2 μg/ml hamster anti-CD40 mAb. Cells were washed and incubated with and without 100 nM HEL (BCR transgenic) or with and without 100 μM HEL (nontransgenic) in the presence or absence of 10 μg/ml goat anti-mAb (F(ab')2) for 4 h at 37°C, or left untreated. Excess Ag was washed off. CD4^+ T cells were isolated from HEL-specific TCR transgenic 3A9 mice and labeled with a predetermined concentration of 5 μM CFSE. CD40-activated B cells were then cultured at 0.5, 1, 2, 4, and 8 × 10^5 with 1 × 10^5 T cells in a total volume of 200 μl in 96-well plate, for 70 h. All incubations were in RPMI 1640 containing 0.5% fresh mouse autologous serum. Following incubation, cells were stained with PI and analyzed by flow cytometry. Only CD4^+ T cells were gated from PI-negative and CFSE^- cells. T cells that underwent at least one cell division were expressed as percentage of total CFSE^-labeled T cells minus the background (in the absence of HEL). The percentage of viable B cells was determined by gating on CFSE^- cells and by calculating the number of those cells that excluded PI. B cells cultured under similar conditions derived from above were CII^- as determined by flow cytometry. Values were calculated as percentage of total B cells treated with HEL minus percentage values from untreated cells. To inhibit DNA replication and protein synthesis, B cells were pretreated with 25 μg/ml mitomycin C for 30 min at 37°C, washed thoroughly, and cultured with CD4^+ T cells, as described above. To examine B cell type-specific effects and its consequences on T cell stimulation, CD4^- B cells were activated with HEL_{pc-61} on CD40 Ab overnight, washed, and incubated with 200 μM HEL_{pc-61} synthetic peptide for 4 h at 37°C. Cells were subsequently washed to remove excess peptide and incubated with mitomycin C, as described above. CD4^- T cells were isolated from 3A9 mice. B and T cells were then cultured and analyzed, as described above.

Analysis of activation/adhesion B cell markers
CD43^- pure B cells were isolated from HEL-specific BCR transgenic mice and nontransgenic littermates. Cells were cultured in RPMI 1640 containing 0.5% fresh autologous serum with 100 nM HEL + 2 μg/ml anti-CD40 (transgenic), 100 μM HEL + 2 μg/ml anti-CD40 (nontransgenic), or anti-CD40 alone for ~2 h to allow for HEL processing. Following incubation, 2 μg/ml C4H3 mAb was added, and the cultures were incubated for an additional 40 h at 37°C. Cells were washed and labeled on ice in HBSS/0.5% BSA/NaNO_3 with biotin anti-I-A^k, PE anti-CD86, FITC anti-CD45, and PE anti-CD11c. B cells were then counted by flow cytometry. For biotinylated Abs, FITC-StreptAvidin was added and incubated on ice. Cells were then washed and analyzed by flow cytometry.

Quantification of B:T cell conjugates by flow cytometry
The formation of B and T cell conjugates was measured using a method previously described (15), with some modifications. Briefly, CD43^- pure B cells were isolated from HEL-specific BCR transgenic mice and nontransgenic littermates. Cells were added to HEL_{pc-61} 3A9 mice. B cells were cultured with 2 μg/ml anti-CD40 mAb and with and without 100 nM HEL (transgenic), or with and without 100 μM HEL (nontransgenic) HEL. A non saturating dose of 1 μg/ml HEL_{pc-61} mAb (saturating dose = 10 μg/ml) was then added, and cultures were incubated for an additional 40 h. A total of 1 × 10^6 B cells from each treatment was then stained with 1 μg/ml lipophilic dye DiIC16 in PBS and on ice for 30 min. A total of 1 × 10^5 pure HEL_{pc-61} T cells was labeled with 50 nM CellTracker Green, in PBS for 30 min at 37°C. B and T cells were washed four times in PBS/5% FCS, combined, pelleted at 400 × g for 5 min, and incubated for 15 min at 37°C. Cells were then gently resuspended in PBS/FCS and analyzed by flow cytometry.

Results
Dose response of Ag and kinetics of CII-peptide processing
Initially, we determined the dose response of HEL processing and presentation and kinetics of generation of HEL peptide complexes
by either the BCR or fluid phase uptake of the Ag, in splenic B cells isolated from BCR transgenic mice and nontransgenic littermates. Cells were incubated overnight with the indicated concentrations of HEL (Fig. 1A). The cells were collected and stained with PE anti-B220 and C4H3 mAb (rat IgG) that specifically recognizes SDS stable HEL$_{46-61}$-I-A$^k$ (13), followed by anti-rat IgG FITC. Binding to CII-peptide complexes was detected by flow cytometry. Fig. 1A reveals two phases of generation of CII-peptide complexes on the surface. The first phase is seen in B cells from transgenic animals and is indicated by the increase in C4H3 staining between 0.1 and 10 nM HEL, with no further increase between 10 nM and 1 nM. This phase is not seen in B cells from nontransgenic mice, and therefore, must be due to uptake and processing by the BCR. The second phase of Ag processing is indicated by increasing staining between 1 nM and 1 mM, is observed in both BCR transgenic and nontransgenic littermates, and must therefore be due to the processing of HEL by fluid phase endocytosis. Importantly, while saturation of the first phase is directly related to saturation of the BCR (data not shown), the second phase of Ag processing is not saturated with Ag concentration as high as 1 mM HEL. It should be noted that BCR signaling fails to alter the efficiency of fluid phase processing (16). Furthermore, in this system, the generation of HEL$_{46-61}$-I-A$^k$ complexes via both fluid phase and BCR-mediated processing occurs exclusively via newly synthesized CII molecules (17, 18) (also our unpublished data).

We then determined the kinetics of CII-peptide complex generation (Fig. 1B). Splenic cells were incubated with either 10 nM (BCR transgenic) or 100 μM (nontransgenic) HEL over 20 h. Cells were sampled at intervals as indicated, and stained with PE anti-B220 and C4H3 mAb, followed by rat anti-IgG FITC. Specific C4H3 binding was determined by flow cytometry. There was no significant difference between the densities of CII-peptide complexes generated by fluid phase and BCR-mediated uptake and processing of HEL during the first 8 h. Subsequently, higher density of CII-peptide complexes was detected after fluid phase uptake. It should be noted that both BCR and fluid phase uptake and processing of HEL could occur in BCR transgenic cells at the highest dose of HEL (100 μM) (Fig. 1A).

**Differential effect of Ab ligation of type I and II complexes on B cell recovery**

To start addressing the biological properties of type I and II CII-peptide complexes, we examined B cell recovery following Ab ligation of these complexes. Splenocytes isolated from BCR transgenic and nontransgenic littermates were incubated overnight at 37°C in medium containing 10 nM HEL (BCR uptake) or 100 μM HEL (fluid phase uptake) with and without the HEL$_{46-61}$-I-A$^k$ specific mAb C4H3 (as indicated in Fig. 2, A and B). The addition of C4H3 mAb alone to medium did not affect B cell recovery. However, significant differences in B cell recovery were observed following ligation of type II complex compared with type I complexes. Thus, ~50% less B cell recovery was detected following ligation of type II complexes (Fig. 2, A and B). As survival of BCR transgenic B cells could be due to signals generated following ligation of the BCR by HEL, we examined whether BCR ligation with F(ab')$_2$ of goat anti-murine μ Ab could rescue the loss of B cells. However, the addition of F(ab')$_2$ of goat anti-murine μ Ab did not have any effects on the rate of B cell recovery (Fig. 2A) (the slightly higher percentage number of treated B cells recovered compared with medium (100%) is due to higher B cell viability in the former; however, this finding was not reproducible). Furthermore, pulsing cells isolated from BCR transgenic mice with 100 μM HEL (to generate type I and II complexes) did not improve B cell recovery (Fig. 2B), suggesting that the depletion resulting from ligation of type II complexes is dominant over the lack of depletion observed following ligation of type I complexes. Thus, not only does BCR signaling fail to alter processing of fluid phase Ag (16), but also our data suggest that the signaling function of the type II complex that leads to B cell loss is similarly not significantly affected.

Cell death following ligation of total CII by Abs has been reported in human (7–9, 19, 20) and mouse (2) B cells. CII-mediated cell death in activated human B cells has been shown to be associated with changes in morphology characteristic of cells undergoing apoptosis. This type of B cell death has been distinguished from classical apoptosis (19). Specifically, CII-induced apoptosis is rapid and does not involve the typical ladder pattern of DNA fragmentation (7), and in some cases is independent of caspases (19, 20). In contrast, ligation of CII on activated mouse B cells protects from apoptosis (3, 4), whereas mouse resting B cells are induced to die (2). We therefore examined whether ligation of CII-peptide leading to the loss of B cells observed above is associated with DNA fragmentation, a hallmark of cells undergoing the

![Figure 1](http://www.jimmunol.org/)
classical type of apoptosis. For this purpose, we used our established method for cell cycle analysis of primary cells to detect DNA fragmentation (14). Purified B cells from the spleen of nontransgenic mice were incubated overnight with 100 μM HEL with and without C4H3 mAb. Cells were then layered over Ficoll (to collect whole cells and to remove cell debris and nuclei), fixed with ethanol, and stained with PI. Cells were then analyzed by flow cytometry. Evidence for DNA fragmentation was noted when an increase in cell counts occurred in the sub-G0/G1 stage of the cell cycle (Fig. 2C). The addition of C4H3 mAb alone to purified B cells resulted in background of 3% of cells being detected in the sub-G0/G1 region. The addition of HEL alone increased the number of cells to 12%. Compared with HEL alone, the addition of HEL and C4H3 resulted in no increase in the percentage of cells (both at 12%). These results demonstrate the absence of DNA fragmentation following ligation of CII-peptide by C4H3. The latter is contrary to what has been reported previously in human and mouse B cells using Abs to CII. The different results in our study from those reported before may be due to the binding properties of the different Abs or the type of CII. Indeed, in the mouse study (2), the rate of cell death was related to the type of the Ab used, and different classes of HLA have been found to signal differently following their ligation with anti-HLA Abs (21). Furthermore, in the former study, staining with ethidium bromide has been taken as evidence for B cells undergoing apoptosis in the absence of data showing specific DNA fragmentation. In our study, ligation of type II complexes on B cells made cells similarly permeable to cell viability dyes such as PI and trypan blue. This suggests that binding of C4H3 mAb to type II complex affects mainly the integrity of the plasma membrane. Finally, our data reflect a more physiological outcome compared with the studies above, as they examined CII effect on cell death in the context of a naturally processed Ag.

Differential effect of ligation of type I and II complexes following MHC-TCR cognate interaction

To start addressing the physiological relevance of the findings above, we used CD40-activated B cells and naive T cells isolated from TCR transgenic 3A9 mice. T cells from 3A9 mice recognize the same epitope as C4H3, i.e., HEL46–61-I-Ak (11, 13). This epitope is buried within HEL, and therefore must be processed in late endosomal compartments (17, 18). This is expected to occur in compartments that are accessed by both fluid phase and BCR uptake of HEL. The use of anti-CD40-activated B cells allowed us to examine the signaling function of CII-peptide in the presence of CD40 signals, thus mimicking (to a certain extent) the engagement of CD40 by activated T cells through CD40L. The use of activated B cells was also necessary to examine B cell-specific effects on T cell division, because resting B cells are poor stimulators of naive T cells. Pure B cells were isolated from the BCR transgenic mice...
and the nontransgenic littermates. Cells were activated by over-night incubation with hamster anti-CD40 mAb. The activation of B cells with anti-CD40 mAb led to similar expression levels of CII, CD86, CD54, and CD25 (IL-2Rα) in both transgenic and nontransgenic cells (see below). Cells were then incubated with 100 μM HEL ± 10 μg/ml goat anti-murine μ F(ab′)2 (BCR nontransgenic) or 100 nM HEL (BCR transgenic) for 4 h at 37°C, or left untreated. Excess Ag was washed off. Subsequently, naive CD4+ T cells were isolated from 3A9 mice and labeled with CFSE. A fixed number of T cells (10^5) was then cultured with an increasing number of pure CD43+ B cells for 70 h, as indicated in Fig. 3.

As shown (Fig. 3A), HEL processing and presentation by both transgenic and nontransgenic B cells resulted in an increase in T cell division with apparently different kinetics. However, this difference in the kinetics of the T cell response is not due to lower efficiency of BCR transgenic B cells in activating T cells compared with the nontransgenic because the total number of T cells maintained in culture was higher in the former (data not shown), resulting in lower calculated percentage levels of T cell division. In addition, to take into account BCR-signaling effects on HEL processing and presentation, nontransgenic B cells were incubated with HEL and goat anti-μ F(ab′)2 before mixing with T cells, as described in Materials and Methods. As shown (Fig. 3A), the addition of goat anti-μ F(ab′)2 did not significantly alter the kinetics of the T cell response. The difference in the dose response of T cells between transgenic vs nontransgenic is not due to the density of CII-peptide or to other B cell type inherent differences (see below). Thus, under the current experimental conditions, both type I and II complexes induce T cell division, but with different kinetics.

The noted effects of C4H3 mAb-specific binding on B cell recovery (Fig. 2, A and B) suggested that ligation of CII-peptide complexes by the TCR might result in similar outcome, particularly because both C4H3 mAb and 3A9 T cells recognize the same epitope, i.e., HEL46–51-I-Ak. We therefore examined B cell recovery in the cultures described above. Maximal B cell recovery of ~50% was detected in BCR transgenic cells, while recovery of nontransgenic B cells did not exceed 12% (Fig. 3B). Comparative forward light scatter (FSC) analysis revealed significant numbers of blasts in samples containing BCR transgenic cells, whereas few of the nontransgenic B cells showed any increase in cell size (data not shown).

**FIGURE 3.** Differential effects of cognate TCR type I and II complex interaction on B cell recovery. A, T cell division: pure CD43+ B cells were isolated from BCR transgenic and nontransgenic littermates. Cells were activated by overnight incubation with anti-CD40 mAb. Cells were washed and incubated with and without 100 nM (transgenic) or with and without 100 μM (nontransgenic) HEL ± 10 μg/ml goat anti-μ F(ab′)2, for 4 h at 37°C, or left untreated. Excess Ag was washed off. CD4+ T cells were isolated from TCR transgenic 3A9 mice and labeled with CFSE. B and T cells were cultured for 70 h, as indicated. Following incubation, cells were stained with PI to exclude dead cells. Data represent number of T cells that have undergone at least one cell division, and were expressed as percentage of total CFSE+ T cells minus the background (in the absence of HEL). The inset shows an example of how CFSE-labeled cells were gated so as to determine the percentage of T cells that underwent cell division, after gating out CFSE+ cells. The percentage of levels of T cell division in nontransgenic culture was higher than those in BCR transgenic cultures due to higher numbers of total T cells in the latter. B, B cell recovery: the percentage of viable B cells (from A) was determined by gating on CFSE+ B cells and by calculating the number of cells that excluded PI. Cells cultured under the conditions described above were CII+, as determined by flow cytometry. Calculated values represent percentage of total B cells treated with HEL minus percentage values from untreated cells. The data are representative of two similar experiments.
not shown). Significantly, at higher doses of nontransgenic B cells (2 x 10^6–8 x 10^7), B cell recovery levels were lower than background (in the absence of HEL). The latter was present despite comparable T cell division levels in both transgenic and nontransgenic cell cultures (Fig. 3A, 4 x 10^7 and 8 x 10^6 B cells). Finally, prestimulation of nontransgenic B cells with goat anti-μ F(ab')2 Ab could not rescue the loss of B cells, thus confirming our earlier results with the C4H3 mAb in resting B cells (Fig. 2, A and B) and our data on the inability of BCR signaling to alter fluid phase uptake and processing of HEL (16).

To examine whether the difference in the dose response of T cells was due to density of CII-peptide or to other B cell type inherent differences, we looked at the level of expression of type I and II complexes on the surface of anti-CD40-activated B cells (Fig. 4A). In addition, we compared the ability of BCR transgenic and nontransgenic B cells to present HEL(66–61) synthetic peptide (Fig. 4B). The incubation of anti-CD40-activated B cells with Ag for 4 h, followed by washing and incubation for further 24 h, resulted in detection of comparable densities of type I and II complexes (Fig. 4A; also see Fig. 1B up to 8 h). Furthermore, the presentation of cognate peptide added to either BCR transgenic or nontransgenic B cells led to essentially identical levels of T cell responses (Fig. 4B). These results demonstrate the absence of any significant differences between BCR transgenic and nontransgenic B cells in their ability to stimulate T cell responses.

Overall, these results demonstrate that ligation of CII-peptide complexes generated via BCR-mediated or fluid phase Ag processing leads to significant differences in B cell recovery in the presence of comparable T cell division. Moreover, the differential rate of B cell loss induced following cognate TCR interaction with type I and II complexes is independent of the density of CII-peptide and of B cell type inherent differences.

**FIGURE 4.** Comparable densities of type I and II complexes and absence of B cell type inherent effects. A, Density of type I and II complexes: pure CD43- B cells were isolated from BCR transgenic and nontransgenic mice, and activated by overnight incubation with anti-CD40 mAb. Following incubation, 100 nM or 100 μM HEL was added to wells and incubated for 4 h at 37°C. Medium contained cells incubated in the absence of HEL. Cells were then washed and put back in culture for additional 20 h. To detect CII-peptide complexes, cells were labeled with C4H3 mAb or IgG2b isotype control (data not shown), followed by incubation with anti-rat IgG2b FITC. Flow cytometry analysis was performed after gating on viable (PI-negative) cells. B, Absence of B cell type-specific effects on T cell stimulation; CD43- B cells were isolated from BCR transgenic and nontransgenic mice and activated by overnight incubation with anti-CD40 mAb. Cells were washed and incubated with 200 μM HEL(66–61) synthetic peptide for 4 h at 37°C. Cells were then incubated with 25 μg/ml mitomycin C for 30 min at 37°C, and thoroughly washed. CD43+ T cells were isolated from 3A9 mice and labeled with CFSE. B and T cells were then cultured, and T cell division was determined, as described in the legend of Fig. 3A. The data are representative of two experiments.

**Differential requirement for DNA replication and protein synthesis following ligation of type I and II complexes**

Previous reports demonstrated protection from death following ligation of the BCR or CD40 (22, 23) that was dependent upon the transcription of new proteins. Moreover, both the BCR and CD40 have been reported to induce B cell proliferation (22). However, in our study, the B cell loss resulting from ligation of type II complexes occurred even in the presence of CD40 and/or BCR signals (Figs. 2 and 3B). This suggested that some CII signals were dominant over other B cell proviability signals, and led us to examine whether there is a differential requirement of type I and II complex-mediated B cell survival and death, respectively, for DNA or protein synthesis. We used mitomycin C, an agent that blocks DNA replication, but that has also been implicated along with other DNA-binding drugs in blocking protein synthesis by affecting synthesis of key regulators in mRNA translation (24). Purified B cells were stimulated overnight with anti-CD40. HEL (100 nM or 100 μM for BCR transgenic and nontransgenic B cells, respectively) was then added, and the cultures were incubated for additional 4 h at 37°C. The cells were then washed and incubated with 25 μg/ml mitomycin C for 30 min at 37°C. Following incubation, cells were thoroughly washed and incubated with purified, CFSE-labeled CD4+ T cells isolated from 3A9 mice (Fig. 5A). After 70 h of incubation, cells were collected, stained with PI, and analyzed, as described above. B cells expressing type I and II complexes stimulated T cell division of kinetics similar to those in mitomycin C-unreated cultures (Fig. 3A). Moreover, the ratio of T cell division in transgenic over that of nontransgenic cultures was similar in both mitomycin C treated (Fig. 5A) and nontreated (Fig. 3A) (the ratio for both was ~0.6 and 0.85 for 2 x 10^4 and 4 x 10^3 B cells, respectively). This indicates that the treatment of B cells with mitomycin C does not affect the overall T cell stimulatory properties of type I and II complexes. B cell recovery after treatment with mitomycin C is shown in Fig. 5B. Maximal B cell recovery was ~58% (BCR transgenic) and 52% (nontransgenic). Maximal B cell recovery levels in BCR transgenic cells were comparable to those detected in the absence of mitomycin C (Figs. 3B and 5B). In contrast, the recovery levels of nontransgenic compared with BCR transgenic B cells declined rapidly with increasing numbers of B cells to a value lower than background (in the absence of HEL). This demonstrates that treatment with mitomycin C alters the threshold of type II complex-induced B cell death. We conclude that the B cell loss observed following ligation of type II complexes is at least partially dependent on DNA replication or protein synthesis.
Differential effect of Ab ligation of type I and II complex on expression of B cell surface markers

Although the direct binding of C4H3 mAb induced ~50% of B cell loss (Fig. 2), the more dramatic outcome on B cell recovery following cognate TCR-MHC interaction (80–100%) suggested the contribution of additional factors in the regulation of B cell fate. We hypothesized that ligation of type I and II complexes differentially alters the expression levels or function of adhesion/activation molecules on B cells, thus contributing to the increase and decrease of B cell survival, respectively. In support for a role of adhesion molecules in the regulation of B cell fate, blocking Abs against the CD54/CD11a system have been found to decrease the rescue effect from cell death by anti-CD40 Ab in anti-μ-stimulated human B cells and cell lines (25), and were crucial for CD4⁺ T cell-mediated B cell apoptosis of anti-CD40-activated mouse cells (26). In the latter report, molecules such as CD86 and CD28 did not play a role in B cell death. We therefore examined the surface expression of CII, CD86, CD54, and CD25 (IL-2Ra, a marker that predicts B cell activation) in anti-CD40-activated transgenic and nontransgenic B cells that were incubated with and without HEL and C4H3 mAb (Fig. 6).

Pure B cells were isolated from BCR transgenic and the nontransgenic littermates and pulsed with HEL + anti-CD40 or HEL + C4H3 mAb + anti-CD40 mAb. The sample designated medium contained cells incubated with anti-CD40 in the absence of HEL. Cells were incubated for 40 h at 37°C. Following incubation, cells were stained with Abs to markers, as indicated (Fig. 6). Analysis was performed after gating on cells that excluded PI and exhibited high FSC profile (an indicator of an increase in cell size). In both BCR transgenic and nontransgenic B cells, the addition of anti-CD40 alone significantly increased expression of CII, CD86, CD54, and CD25 compared with untreated cells (Fig. 6, and data not shown). The addition of HEL to BCR transgenic cells resulted in a slight increase in the expression of CD86 and CD25. The expression level of CII decreased compared with medium, although this finding was not observed in repeat experiments. The incubation with HEL did not alter the expression level of CD54. The incubation of B cells with HEL + C4H3 mAb had little effects on the level of markers examined above. In contrast, the incubation of nontransgenic B cells with HEL + C4H3 led to significant decrease in expression levels of CII, CD86, and CD54 compared with cells treated with HEL alone. CD25 expression was slightly altered, as shown by the appearance of two populations of cells with different expression levels.

To rule out that some of the effects on the markers observed above were indirectly influenced by the selective death of B cells present in culture, we followed in initial experiments the time course of CII-peptide-triggered cell death in nontransgenic B cells (data not shown). We found that cell death (measured by PI staining) occurred rapidly within 4 h after addition of C4H3. At 4 h after addition of C4H3, a large number (~35%) of B cells were PI⁺, which did not dramatically increase as it reached a platform of 50% after 16 h. This pattern of cell death has also been reported following cognate HLA-DR-peptide interaction (10). We then examined the effects of C4H3 on the expression of surface markers at 16 h in the nontransgenic B cells. Data from the latter experiment (our unpublished observation) showed no significant effects of C4H3 on the level of expression of the studied markers after 16 h. These experiments rule out that the observed down-regulation of surface markers shown in Fig. 6 was due to the presence of dead cells in culture.

The findings above (Fig. 6) suggest that the higher rate of B cell loss following cognate MHC-TCR interaction (Fig. 3B), compared with the direct effects resulting from C4H3 binding (Fig. 2), may be partly due to decreased expression of adhesion/activation molecules. The latter would destabilize B/T cell conjugates, leading to B cell loss by lack of sustained essential activation/maintenance signals from T cells.

Differential effect of ligation of type I and II complexes on formation of T:B conjugates

To investigate whether the decreased expression of adhesion/activation markers observed above would lead to reduced cell-cell contact following ligation of type II complexes, we examined formation of cell conjugates between B and T cells. Pure B cells were isolated from BCR transgenic and nontransgenic mice, as described above. Cells were incubated with anti-CD40 mAb with and without HEL for 2 h, followed by addition of a subsaturating dose.
of 1 μg/ml C4H3 mAb (saturating dose = 10 μg/ml). Cells were then cultured for an additional 40 h. The use of a low nonsaturating dose of C4H3 mAb and a long incubation time with B cells minimized any blocking effects that the Ab might have on their subsequent conjugation with T cells. Cells were washed and counted, and 1 × 10⁶ B cells from each treatment were labeled with DiIC16 (red). Pure CD4⁺ T cells were isolated from 3A9 transgenic mice, and 1 × 10⁶ were labeled with CellTracker Green. A total of 1 × 10⁶ B cells and 1 × 10⁶ T cells was combined, pelleted, and incubated for 15 min at 37°C. Cells were gently resuspended, and 2 × 10⁵ cells were analyzed by flow cytometry for conjugate formation. A detailed fluorescence analysis (Fig. 7) reveals a significantly higher proportion of conjugates in the BCR transgenic compared with the other groups. It should be noted that the efficiency of cell labeling with the lipophilic dye DiIC16 was found reduced in the treated nontransgenic B cells compared with the other treatments. This is most likely due to effects resulting from prior ligation of type II complexes with C4H3 mAb (shown to compromise the integrity of the plasma membrane). However, despite this technical difficulty, the percentage of labeled B cells that formed conjugates with T cells was reproducibly and significantly less compared with other treatments (15, 60, and 47% for nontransgenic, BCR transgenic, and no Ag treatments, respectively). Similar decrease in the efficiency of B cells from nontransgenic, compared with BCR transgenic, to form conjugates with cognate CD4⁺ T cells was found when B:T ratio was 1:3 (data not shown). The presence of background conjugates in the no Ag treatment is most likely due to the limitation of the technique used, which relies on gentle pipetting and incubation at 37°C to disperse nonspecific clustering of cells after centrifugation. Furthermore, the lower proportion of conjugates in the nontransgenic cells compared with the no Ag treatment may reflect the significantly lower expression of adhesion markers in the former after ligation with C4H3 mAb (Fig. 6). We conclude that the efficiency of B cells to form conjugates with T cells is significantly reduced following ligation of type II complexes.

**Discussion**

This study addresses the influence of the pathway of CII-peptide generation on the CII regulation of B cell activation and survival. This investigation was possible by the use of B cells transgenic for HEL-specific BCR and nontransgenic littermates, and an Ag dose that engages either BCR or fluid phase uptake. Moreover, the use of an Ab and T cells both specific for the same peptide HEL₄₆₋₆₁-I-A^k complex allowed detailed analysis of the contribution of accessory molecules signaling in B cell survival. We show that compared with type I complexes, ligation of type II complexes results in significantly lower level of B cell survival.
overnight incubation with anti-CD40 mAb with and without 100 nM HEL were isolated from TCR transgenic 3A9 mice. B cells were activated by specific BCR transgenic mice and nontransgenic littermates. CD4 were incubated for an additional 40 h. B cells from each treatment were saturated dose of 1 cell survival.

in the membrane, leading to differential signaling properties and B cell death is independent of the activation status of the cell. Anti-CD40-activated B cells are induced to die, indicating that the formation of HEL4 6–6 1-I-Ak peptide-class II complexes recognized by both C4H3 mAb and TCR transgenic 3A9 T cells requires the invariant chain and the neosynthesis of CII molecules (17). Moreover, HEL processing involves an acidic compartment related to late endosome or the MIIC (18). Because the 46–61 epitope is buried in HEL molecule, the requirement for its processing might be less sensitive to Ab (BCR)-mediated regulation. Therefore, processing of this peptide should be expected to be independent of the route of uptake of HEL. By using BCR transgenic cells and an increasing dose of HEL, we determined the amount of HEL protein to be used for generation of either type I or II complexes. Furthermore, we examined the kinetics of processing of these complexes by either route. Ligation of type II complex in resting or anti-μ-activated B cells by C4H3 mAb induced ~50% B cell loss; contrastingly, ligation of type I peptide-class II complexes failed to induce B cell to die. The recovery rate obtained with type II complexes in this study is comparable to HLA-DR (8, 27) or peptide-loaded HLA-DR-induced B cell death (10). A more pronounced loss of B cells resulted from the HMC-TCR cognate interaction in our study (~88%), and B cell loss was even higher than that observed in control samples at higher numbers of B cells (Fig. 3B). These results demonstrate profound depletion of B cells expressing type II complexes. Interestingly, the decrease of B cell survival could not be altered by signals from the BCR (anti-μ) that have been shown to alter the biology of Ag processing in B cells (28). This suggests that the induction of B cell loss following ligation of type II HEL4 6–6 1-I-Ak is either independent from BCR signals or a dominant effect. Alternatively, anti-CD40 signals might override those signals generated after BCR ligation.

One possibility for explaining the differential rate of B cell recovery resulting from ligation of type I and II peptide-class II complexes is their level of expression on the surface of B cells. Differential expression levels of type I and II complexes might result in differential signaling that could alter the threshold for signals involved in cell death. Our data, however, show that B cells loaded with identical densities of either type I or II complex were still differentially susceptible to cognate TCR-MHC-induced B cell loss when expressing type II complexes (Figs. 3B and 4A). Similarly, B cell death induced by HLA-DR-TCR cognate interaction occurred even at suboptimum dose of specifically loaded synthetic peptide (10). These results argue against the contention that the loss of B cells is caused by higher density of CII-peptide on the surface of B cells.

The precise mechanism responsible for the mouse B cell death remains unclear. We were unable to demonstrate DNA fragmentation (Fig. 2C), a hallmark of cells undergoing apoptosis, contrary to what has been reported previously using Abs to CII (2). In human B cells, rather modest DNA fragmentation is more common, and was only visible using techniques with the possibility of detecting ssDNA cleavage (7, 20). The different results in our study from those reported before (2) may be due to the binding properties and type of the different Abs; the outcome of CII ligation on the death of mouse resting B cells varied depending on the nature of the Ab used (2). Furthermore, the results from the latter study lacked direct evidence for DNA fragmentation. Instead, conclusions have been made based on measurements of cells that were permeable to ethidium bromide. In our study, B cells expressing type II complexes were similarly permeable to cell viability dyes such as PI and trypan blue. This suggests that binding of C4H3 mAb affects mainly the integrity of the plasma membrane. However, our data would argue against B cell death as a result of necrosis. The results in Fig. 4B show that B cell loss was partially restored in mitomycin C-treated B cells, indicating the requirement decreased expression of activation/adhesion markers, and reduced efficiency of forming B and T cell conjugates. Both resting and anti-CD40-activated B cells are induced to die, indicating that the B cell death is independent of the activation status of the cell. Neither differential initial levels of CII and other studied surface markers, cell type-specific effects, BCR signaling, T cell proliferation, nor initial density of CII-peptide complexes could explain the T cell-induced B cell loss. Furthermore, a more pronounced B cell loss was induced following cognate TCR-MHC interaction compared with the specific Ab alone, suggesting that the former was induced mainly following direct ligation of CII-peptide, but also by indirect resulting effects such as the reduced efficiency in forming cell-cell conjugates. We propose that Ag processing influences the context in which CII-peptide complexes are present in the membrane, leading to differential signaling properties and B cell survival.
of B cell loss for DNA replication and protein synthesis. Thus, although similarities exist between mouse and human B cells in the functional outcome of CII ligation (i.e., B cell loss), the precise mechanism of death in mouse B cells is still unclear.

Our experiments on the influence of cognate TCR-MHC interaction on B cell survival reveal profound induction of B cell loss following ligation of type II complexes. B cell loss was noticeable in the presence of an ongoing T cell division. This indicates that B cell loss was induced following HEL processing and stimulation of T cell division. The results also argue against the presence of suboptimal levels of IL-2, a B cell growth-promoting cytokine, being responsible for the B cell loss. Indeed, ligation of CII-peptide complexes by C4H3 alone induced B cell loss in the absence of growth-promoting cytokines (Fig. 2). In addition, B cell loss was induced even when T cell division levels were high in cultures containing BCR transgenic and nontransgenic B cells (Fig. 3). Furthermore, B cell loss still occurred (although at reduced level) after treatment of the B cells with mitomycin C (Fig. 5). The effects on B cell survival of mitomycin C-treated B cells suggest that the bulk of signals responsible for the induced B cell loss was generated following direct ligation of CII-peptide complex on the surface of B cells. Finally, BCR transgenic and nontransgenic B cells loaded with synthetic HEL46–61 peptide stimulated almost identical kinetics and levels of T cell responses (Fig. 4A), and had comparable recovery rate in culture (data not shown), thus highlighting the specificity of the B cell loss reported above (Figs. 3B and 5B). These results further highlight the absence of significant inherent differences between BCR transgenic and nontransgenic B cells, and the unique nature of peptide-CII complexes formed via BCR-mediated Ag processing.

Other contributing signals to the observed B cell death could result from engagement of activation/adhesion molecules on the B cell following cognate MHC-TCR interaction. In support for a role of adhesion molecules, blocking Abs against the CD54/CD11a system were found to decrease the rescue effect from cell death by anti-CD40 Ab in anti-μ-stimulated human B cells and cell lines (25) and were critical for CD4+ T cell-mediated B cell apoptosis of anti-CD40-activated mouse cells (26). Other possible molecules such as the interaction of CD86 and CD28 were found not to play a role in B cell death (26). Our analysis of the effects following ligation of CII-peptide with C4H3 mAb revealed significant differences in the level of activation/adhesion markers that was dependent on the Ag uptake and processing (Fig. 6). Decreased levels of CII, CD86, CD54, and to some extent CD25 occurred following ligation of type II complexes. In contrast, there was no significant alteration in the levels of these markers after ligation of type I complexes. Importantly, this led to reduced ability to form conjugates with T cells (Fig. 7). This latter result is in conformity with those reported by other studies following cognate MHC-TCR interaction, in which APC loaded with no peptide failed to form conjugates (10, 15). Specifically, the decreased expression of CII and CD54 observed in this study may explain the reduced ability of cells to form conjugates. Recently, it has been shown that the immunological synapse formed between an APC and T cell is dynamic and involves constant remodeling (29). Because CD54 has been shown to be important in the formation of the synapse (29), and CII ligation involves an actin-dependent process (30), their reduced expression may contribute to destabilization of the synapse following APC engagement by the T cell.

How might Ag processing regulate CII-peptide signaling? BCR-targeted Ags access the endocytic compartments very efficiently, and this property contributes to their enhanced processing and presentation compared with uptake of fluid phase-added Ag. The processing of HEL involves the generation of HEL46–61-I-Ak complexes in late endosomal compartments (17, 18). Both 3A9 T cells and the C4H3 mAb recognize the peptide-MHC complex (16, 31). Using a B cell hybridoma, four HEL peptides varying in length, but containing the core dominant sequence 52–61, were found complexed to I-Ak, in addition to one peptide, HEL48–60 (32). The 3A9 T cell hybridomas appear to recognize less well epitope 52–61 compared with 48–61, whereas other peptides from the core sequence were not recognized (31). It is not known whether BCR-mediated processing of HEL generates different arrays of peptides from the core segment compared with HEL processing by fluid phase (this pathway was used for HEL processing in the studies above). Nevertheless, the presence of such peptide complexes in the plasma membrane might play an indirect role in the outcome of HEL46–61-I-Ak signaling in B cells, as CII loaded with irrelevant peptide have been shown to play a significant role in enhanced T cell activation to agonist CII-peptide complexes (15, 33). Moreover, the presence of dimeric or multimeric forms of the same CII-HEL peptide on surface of APC has been documented (34), and shown to influence T cell-specific recognition (31, 35). The different cell distribution patterns of the class II chaperone HLA-DM compared with the HLA-DM/H2M regulator HLA-DO/H2O might also influence processing of the Ag within nonterminal late endosomes. Thus, BCR-Ag complexes exhibited prolonged persistence in late endosomal compartments compared with Ag internalized via fluid phase endocytosis, and had higher degree of colocalization with HLA-DM/H2M than with HLA-DO/H2O (36). Another possibility, which may explain the influence of Ag processing, is the appropriate targeting and recruitment of adaptor molecules essential for CII signaling. BCR priming is essential for the recruitment and association of CD79a and CD79b, which function as signal transducers with CII (37). Although both fluid phase and BCR-targeted HEL would access late acidic endosomal compartments, it is not known whether these compartments are identical and whether they similarly contain CD79a and CD79b, or other yet unknown adaptor molecules essential for CII signaling. Ag processing may also influence later events of Ag presentation, such as the expression of activation/adhesion molecules, as shown in this study. Most noticeable is the down-regulation of CII, because of its association with essential signaling elements in membrane microdomains. Thus, relevant microdomains containing peptide-MHC have been shown to interact with CD82 and other members of the tetraspan family of integral membrane proteins (38). CII has also been shown to associate with CD19 and CD20, molecules that may couple CII to the protein tyrosine kinase signaling pathway (21). Other molecules have also been reported to be associated with CII, including CD40 (39). Moreover, central cluster formation in the APC is based on a transport process sensitive to MHC-peptide strength and number, and the transient interaction of TCR and MHC peptide complex is remodeled in the immunological synapse as a result (29). Therefore, any significant reduction in the number of CII in the membrane microdomains would destabilize association of signaling molecules and cytoskeletal elements with CII and would indirectly impact on the context in which CII-peptide signaling is initiated. The nature of those signals is currently being investigated in our laboratory.

The physiological relevance of the findings above in immunization, autoimmunity, and microbial infection should not be underestimated. First, appropriate targeting of rare Ag to the BCR ensures Ag processing, B cell activation, and generation of immune responses. Second, microbial Ags may reach the extracellular environment; their processing and presentation, however, could lead to a loss of the B cell, thus focusing B cell function on dealing with rare microbial protein Ags. Finally, the generation of self peptide-CII complexes (e.g., in advanced stages of autoimmune
reaction or in necrosis) could lead to B cell loss, and would consequently limit expansion of potentially autoreactive T cells present in the immune repertoire.

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References

