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Cross-Linking CD21/CD35 or CD19 Increases Both B7-1 and B7-2 Expression on Murine Splenic B Cells

Yuko Kozono,*† Ryo Abe,* Haruo Kozono,* Robert G. Kelly,† Takachika Azuma,* and V. Michael Holers2†

Activation of the complement cascade and ligation of complement C3 receptors on B cells represent an important bridge between innate and Ag-specific acquired immunity. We show here that cross-linking of mouse CD21 (complement receptor type 2, CR2, C3d receptor) and CD35 (complement receptor type 1, CR1, C3b/C4b receptor) or co-cross-linking of CD21/CD35 and surface IgM rapidly up-regulates both B7-1 and B7-2 expression on murine resting splenic B cells. CD21/CD35-mediated up-regulation of both B7-1 and B7-2 expression is observed within 14 h, while other stimuli up-regulate only B7-2 but not B7-1 at this early time point. Consistent with the increase in B7 levels, BALB/c B cells on which surface IgM and CD21/CD35 have been co-cross-linked stimulate C57BL/6 T cells more effectively than controls. This CD21/CD35-enhanced allogeneic mixed lymphocyte reaction is blocked nearly completely by anti-B7-2 mAbs and partially by anti-B7-1 mAbs. In addition, cross-linking of CD19, which is physically associated with CD21/CD35, leads to increased B7-1 and B7-2 expression. These data suggest that CD21/CD35 ligation results in enhanced B cell Ag presentation using costimulatory mechanisms shared with other activators and thus works cooperatively in this process. Rapid up-regulation of B7-1 expression, a unique response to CD21/CD35 and CD19 cross-linking, may be a particularly important effect of C3-containing ligands. We propose that CD21/CD35- and CD19-mediated B7-1 and B7-2 up-regulation is an important mechanism by which complement activation links innate and acquired immunity. The Journal of Immunology, 1998, 160: 1565–1572.

Complement activation is necessary to develop a normal immune response. For example, animals with naturally occurring genetic deficiencies of complement C3 or early classical pathway components manifest an Ag-specific defect in IgM and/or IgG responses following immunization with low doses of T cell-dependent or T cell-independent Ags (1). The same type of defect is observed when an mAb recognizing the B cell complement receptors CD21 and CD35, alternately spliced products of the murine Cr2 gene (2), or a CD21-Ig chimera, both of which block ligand-receptor interactions, are preinjected into mice before low dose Ag immunization (3–5). CD21 and CD35 in mice are physically and functionally associated with CD19 (6, 7), an important B cell immune response regulator that is activated following surface IgM (sIgM)3 cross-linking (8, 9). Mice in which either CD19 or CD21/CD35 expression has been eliminated using homologous recombination demonstrate a profound defect in Ag-specific, T-dependent B cell responses (10–13). These mice, in addition to C3- or C4-deficient (14, 15) mice, also manifest impaired germinal center formation. Finally, C3d itself can enhance an immune response when artificially coupled to a protein Ag such as hen egg lysozyme (HEL) (16). In this particular setting, C3d artificially linked to Ag can bypass the normal requirements for coadministration of adjuvant with the Ag to effectively induce an immune response. For this reason, it has been proposed that C3d normally plays such an adjuvant-like role in vivo (16, 17).

B7-1 (CD80) and B7-2 (CD86) are costimulators that interact with both CD28 and CTLA-4 (CD152) (18, 19). B7-2 is normally expressed at very low levels on splenic B cells and is rapidly up-regulated in vitro within a few hours following sIgM ligation or treatment with activators such as IL-4 or LPS (20, 21). B7-1 is absent from resting B cells, and its expression is also increased following LPS or IL-4 treatment. However, this occurs with much slower kinetics, usually requiring 2 or 3 days for maximal up-regulation, and B7-1 expression is not effectively induced by sIgM ligation alone (21, 22). From these and other studies, it has been suggested that B7-2 would associate with CD28 early after B cell activation to send costimulatory signals to T cells (18), whereas B7-1 may associate with CTLA-4 to send a negative signal that terminates immune responses at a later time point (23, 24). However, unique roles for B7-1 and B7-2 in T cell activation are not definitely established. Other experiments have suggested a possible role for relative B7-1 and B7-2 expression in the determination of Th-1 vs Th-2 responses (25), or of CD8 vs CD4 responses (26).

Both B7-1 and B7-2 have also been shown to play important roles in the formation of germinal centers. For example, treatment with anti-B7-2 mAb results in the lack of germinal center formation following immunization (27). In addition, although B7-1-deficient mice have essentially normal B cell responses, recent studies have shown that in the absence of B7-2 expression, B7-1 is required for the adjuvant-dependent increase in B cell isotype switching and germinal center formation (28). Because of this, a

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3 Abbreviations used in this paper: sIgM, surface IgM; HEL, hen egg lysozyme; PE, phycoerythrin; CD40L, CD40 ligand; mCD40L, mouse CD40 ligand; MAP, mitogen-activated protein; MCF, mean channel fluorescence.
major role for B7-1 in mediating adjuvant-induced costimulation has been proposed (28).

We have previously reported that co-cross-linking of sIgM with CD21/CD35 partially rescues resting splenic B cells and WEHI-231.7 cells from apoptosis induced by sIgM ligation alone (29). The CD21/CD35-derived rescue signal was additive with T cell signals provided by CD40 ligation or IL-4 (29). Herein, we report that co-cross-linking sIgM with CD21/CD35 or cross-linking CD21/CD35 alone also rapidly up-regulates both B7-1 and B7-2 expression on resting B cells. The kinetics of the increased expression is similar for both B7-1 and B7-2. In addition, co-cross-linking CD19 leads to increased B7-1 and B7-2 expression. A rapid increase in B7-1 is a unique response to CD21/CD35/CD19 complex ligation and distinguishes the results of ligation of these molecules from those of other B cell activators previously reported.

Materials and Methods
Antibodies
Cell lines producing rat anti-mouse CD21/CD35 mAb (8D9, IgG2a; 4E3, IgG2a; 7G6, IgG2b; and 7E9, IgG2a) and CD35-specific mAb (8C12, IgG2e) were provided by Dr. Taro Kinoshita, Osaka University (Osaka, Japan) (30, 31). The cell lines producing rat anti-mouse IgM (b-7-6), rat anti-mouse Thy-1 (T24/40), and mouse anti-mouse Thy-1.2 (HO13) were provided by Dr. John Cambier at the National Jewish Center for Immunology (Denver, CO). The control rat anti-mouse Crry/p65 mAb (1F2) was prepared as previously described (33). Briefly, after depleting T cells using anti-mouse Thy-1 mAbs (HO13 and T24/40) and rabbit complement (Life Technologies, Grand Island, NY), resting B cells (1.079) were isolated by density gradient centrifugation (Difco Laboratories, Detroit, MI). Antisera against mouse B7-1 (GI10, 16-10A1) and B7-2 (GL1) mAbs in addition to PE-conjugated anti-B220 mAb (RA3-6B2) were purchased from Pharmingen (San Diego, CA). Anti-CD19 mAb ID3 and rat IgG2a isotype control mAb R35-95 were obtained from PharMingen. Rat anti-mouse B7-2 (GL80), anti-mouse B7-2 (GL1), and anti-mouse class II (M5/114) mAbs in addition to those described above were purified from supernatants using protein G-Sepharose 4 Fast Flow (Pharmacia Biotech, Piscataway, NJ). Purified mAbs were biotinylated by standard methods using N-hydroxysuccinimido-biotin (Sigma Chemical Co., St. Louis, MO) or conjugated with FITC for use in flow cytometric analysis.

Preparation and in vitro treatment of resting splenic B cells
Splenic resting B cells from 5- to 7-wk-old BALB/c (H-2b) mice were prepared as previously described (33). Briefly, after depleting T cells using anti-Thy-1 mAbs (HO13 and T24/40) and rabbit complement (Life Technologies, Grand Island, NY), resting B cells (1.079) were isolated by density gradient centrifugation (Difco Laboratories, Detroit, MI). The purity of cells was 80 to 85%. Resting B cells (1 × 10⁶/ml) were incubated in 10% FCS-Isco’s modified Dulbecco’s medium containing various combinations, described in the text. To cross-link CD21/CD35 or CD19 molecules, 1 µg/ml of rat anti-CD21/CD35, anti-CD35, anti-CD19, or control mAb in addition to 5 to 20 µg/ml of goat anti-rat IgG, Fc fragment-specific (Pierce) Ab (designated secondary Ab herein) were used. To cross-link sIgM, 1 µg/ml of rat anti-mouse IgM (b-7-6) and the same secondary Ab were incubated together. Co-cross-linking experiments included 1 µg/ml of each of the indicated primary Abs in addition to the same secondary Ab. Other conditions included 300 U/ml of mouse IL-4 (Genzyme, Cambridge, MA), 0.1 vol CD40L-containing supernatant from mCD40L-mCD80 transfected J558L cells (34), or 50 µg/ml of LPS, Escherichia coli serotype 055:B5 (Difco Laboratories, Detroit, MI).

All reagents used were used for endotoxin (Limulus amebocyte lysate HS-F, Wako, Osaka, Japan) and, if necessary, absorbed over Polymyxin B-Sepharose (Sigma Chemical Co.).

Flow cytometric analysis
After washing with 1% BSA/PBS/0.1% NaN₃ twice, 1 × 10⁶ cells/condition were stained using either FITC- or PE-conjugated mAbs or biotinylated mAb followed by washing and then streptavidin-FITC (Southern Bio-technology Associates, Birmingham, AL). To minimize nonspecific staining, 10 µg of anti-FcγRII-specific mAb 2.4G2 was added to each staining step. Cells were then analyzed by FACSort (Becton Dickinson, Palo Alto, CA) or Coulter EPICS XL (Irving, TX). Ten thousand cells were examined per sample and were analyzed by CellQuest (Becton Dickinson Immunocytometry Systems, Mansfield, MA).

FIGURE 1. Cross-linking CD21/CD35 up-regulates both B7-1 and B7-2 expression. Resting B cells were incubated with the indicated mAbs and secondary Ab (designated 2° in figures throughout) for 15 h. The cells were then washed and stained with either biotinylated anti-B7-1 (A–C) or anti-B7-2 (D–F) mAbs, followed by streptavidin-FITC and PE-B220. B220-positive cells were then analyzed. Shown in each panel are cells cultured in either medium alone (thin line) or with the indicated reagents (thick line). The MCF of B7-1 expression on cells in which CD21/CD35 had been cross-linked alone is 92.2 (B), while the MCF of cells following cross-linking sIgM alone (A) is 16.5 and co-cross-linking of sIgM with CD21/CD35 (C) is 36.3. The MCF of B7-2 staining on cells in which CD21/CD35 had been cross-linked alone is 153 (E), while the MCF of cells following co-cross-linking sIgM (D) alone is 63.7, and that after co-cross-linking CD21/CD35 with sIgM (F) is 107.9. Results representative of eight different experiments are shown.

Allogeneic MLR
Responder T cells were prepared from inguinal, brachial, axial, cervical, and mesenteric lymph nodes of C57BL/6 (H-2b) mice (6–24 wk old) by negative panning using affinity-purified goat anti-mouse IgG (heavy and light chains) Abs (Rockland, Gilbertsville, PA). The purity of T cells was 90 to 95%. For allogeneic MLR, BALB/c B cells that had been treated for 18 h under the different conditions described above were washed and then either gamma irradiated (500–1000 rad) or used without further manipulation. B cells (3.6–6 × 10⁶) were incubated for 2 to 6 days in triplicate alone or with 1.8 to 6 × 10⁵ freshly isolated T cells in 200 µl using 96-well plates. Individual wells were pulsed with 0.25 µCi/ml [3H]TdR for the last 16 h of culture, harvested through printed Filtermat A (Wallac, Turku, Finland), and counted using a 1205 Betaplate TM counter (Pharmacia/ LKB, Piscataway, NJ) or a 1450 MicroBeta Trilux (Wallac, Turku, Finland).

Results
Cross-linking CD21/CD35 up-regulates B7-1 and B7-2 expression
We have previously shown that co-cross-linking sIgM with CD21/CD35 using mAbs rescues resting splenic B cells from apoptosis induced by sIgM ligation alone (29). To further extend this study, we examined whether cross-linking CD21/CD35 would alter other important B cell phenotypes such as costimulatory molecule expression, and also compared the CD21/CD35-induced changes to those mediated by other activators. As shown in Figure 1A, similar to previous reports (22), B7-1 expression was not significantly increased when sIgM was ligated for only 15 h. In contrast, significant B7-1 expression was observed following CD21/CD35 cross-linking alone at this time point (Fig. 1B). B7-2 expression was also significantly increased following CD21/CD35 cross-linking alone (Fig. 1E).

Many previous studies have shown that CD21/CD35 and CD19 can act to modulate activation signals through sIgM, and that there
is significant cross-talk between the two receptor signaling pathways (35, 36). Therefore, we also examined whether co-cross-linking of sIgM with CD21/CD35 would modify the signal transmitted. In these studies we found that following co-cross-linking of sIgM with CD21/CD35, B cells still maintained an increased B7-1 expression of 110 and 68.2, respectively (Fig. 1C) and B7-2 (Fig. 1F) phenotype compared with that of cells cross-linked with sIgM alone (Fig. 1, A and D). Although in this experiment there was a decrease in B7-1 and B7-2 expression following co-cross-linking of both molecules compared with cross-linking CD21/CD35 alone, we believe that this is due to minor experimental differences or the relative amounts of mAbs used in the co-cross-linking experiments. In other experiments (see Fig. 3 below) levels were not decreased by co-cross-linking, and when we increased the amount of Abs used in the co-cross-linking by twofold, the levels returned to those induced by CD21/CD35 cross-linking alone (data not shown).

A number of specificity controls were performed in these studies. In particular, experiments were performed to determine whether the anti-B7 mAbs used to quantitate expression by flow cytometry were being captured by the cross-linking reagent, thus resulting in an artificial increase in staining intensity. First, as shown in Figure 2, staining with another mAb, anti-Thy-1.2, did not demonstrate significantly enhanced reactivity following CD21/CD35 cross-linking alone or co-cross-linking with sIgM. Anti-CD3 staining was also not significantly altered (data not shown). In addition, expression of other cell surface Ags such as CD2, B220, and Crry/p65, a widely distributed complement regulatory protein, did not change, as assessed by flow cytometry following CD21/CD35 cross-linking (data not shown). From these results, we conclude that expression of B7-1 and B7-2 is specifically altered by CD21/CD35 cross-linking.

**FIGURE 2.** Cross-linking CD21/CD35 on resting B cells markedly increases staining with mAbs recognizing B7-1 and B7-2, but not other molecules. Resting B cells were incubated with indicated Abs for 15 h. The cells were washed and stained with biotinylated mAbs recognizing Thy-1.2, B7-1, or B7-2, followed by streptavidin-FITC and PE-B220. B220-positive cells specifically stained with the anti-B7-1 mAb following cross-linking CD21/CD35 with 7E9 and 4E3, respectively (data not shown). Both anti-CD21/CD35 + anti-IgM + 2* and anti-IgM + 2* resulted in a marked increase in staining of B7-1 and B7-2 relative to the negative control mAb anti-Thy-1.2 and medium autofluorescence. In contrast, staining with mAbs that do not recognize CD21/CD35, such as the anti-CD3 staining, remained unchanged following CD21/CD35 cross-linking compared with other B cell activators known to regulate levels of these proteins. Consistent with previous reports (22), as shown in Figure 3, following treatment of resting B cells with LPS, B7-1 expression increased slowly over 2 or 3 days, while B7-2 expression increased very rapidly. Following sIgM cross-linking, B7-1 levels did not markedly change in this population; however, B7-2 expression rapidly increased beginning at early time points within the first 24 h. In contrast to these other activators, cross-linking CD21/CD35 alone and co-cross-linking sIgM with CD21/CD35 resulted in markedly increased B7-1 expression that peaked between 14 and 24 h. Similar kinetics were found when analyzing B7-2 expression following CD21/CD35 cross-linking with or without co-cross-linking of sIgM.

**FIGURE 3.** Kinetics of B7-1 and B7-2 expression. Resting B cells were incubated with the indicated reagents for 5, 14, 24, 48, or 72 h. The cells were then washed and stained with either biotinylated anti-B7-1 or anti-B7-2, followed by streptavidin-FITC and PE-B220. B220-positive cells were analyzed. Results are shown as the percentage of positive cells compared with the negative control mAb anti-Thy-1.2. Results representative of three different experiments are shown.

**B7-1 and B7-2 expression following CD21/CD35 ligation is primarily enhanced at early time points, and the increase in B7-1 is a unique response compared with those to other activators**

We have also measured the kinetics of B7-1 and B7-2 expression following CD21/CD35 cross-linking compared with other B cell activators known to regulate levels of these proteins. Consistent with previous reports (22), as shown in Figure 3, following treatment of resting B cells with LPS, B7-1 expression increased slowly over 2 or 3 days, while B7-2 expression increased very rapidly. Following sIgM cross-linking, B7-1 levels did not markedly change in this population; however, B7-2 expression rapidly increased beginning at early time points within the first 24 h. In contrast to these other activators, cross-linking CD21/CD35 alone and co-cross-linking sIgM with CD21/CD35 resulted in markedly increased B7-1 expression that peaked between 14 and 24 h. Similar kinetics were found when analyzing B7-2 expression following CD21/CD35 cross-linking with or without co-cross-linking of sIgM.

**Effects of other mAbs recognizing CD21/CD35 or CD35 alone on B7-1 and B7-2 expression**

In mice, CD21 is expressed as an alternative transcript along with CD35 from the C3 gene, and most, if not all, splenic B cells appear to express both proteins (37, 38). Because the carboxyl-terminal two-thirds of the mouse CD35 protein is identical with CD21, including the transmembrane and intracytoplasmic sequences, it has been assumed that the signal transduction events mediated by ligation of each receptor are identical. In the experiments described above, we used the rat anti-CD21/CD35 mAb 7E9 to cross-link CD21/CD35 complexes. We have also analyzed B7 expression following treatment with other mAbs (8D9, 4E3, and 7G6) that, like 7E9, recognize both CD21 and CD35 or, in the case of mAb 8C12, recognize only the longer CD35 molecule. Each of the mAbs we have tested recognizes a unique epitope (39).

As shown in Figure 4, two of the five mAbs have clearly demonstrable effects on B7-1 and B7-2 expression. Treatment with the anti-CD21/CD35 mAb 4E3 is also capable of markedly enhancing expression of both B7-1 and B7-2. In this experiment, cross-linking with mAb 7E9 or 4E3 alone resulted in a mean channel fluorescence (MCF) for B7-1 expression of 110 and 68.2, respectively (Fig. 4, lower left). Sixty-one and sixty percent of the cells specifically stained with the anti-B7-1 mAb following cross-linking with 7E9 and 4E3, respectively (data not shown). Both
mAbs also mediated increased B7-1 expression when co-cross-linked with sIgM (Fig. 4, upper left).

Interestingly, 7E9 and 4E3 were also the most effective when we studied the ability of anti-CD21 mAbs to rescue both resting B cells and WEHI-231 cells from apoptosis induced by sIgM ligation alone (29). We interpret these results as suggesting that these two mAbs recognize epitopes that can mimic the effects of ligand for transducing signals involved in both the rescue from apoptosis and the increased expression of B7-1 and B7-2.

There is no clear relationship between the ability of a mAb to block C3 binding to mouse CD21 and/or CD35 and the up-regulation of B7-1 and B7-2. For example, mAb 7E9, which does not block C3 binding, mediates increased B7 expression, whereas mAb 7G6, which blocks C3d binding, does not. On the other hand, mAb 4E3, which also blocks C3d binding but recognizes a different epitope than 7G6 within the C3d-binding domain (39), does mediate increased B7 expression to a comparable level as 7E9. Although the reason for the lack of correlation is not known, this result is similar to that found when studying human CD21. In this setting, anti-human CD21 mAb HB5, which does not recognize the C3d binding domain and does not block ligand binding, has been extensively used in B cell signaling experiments to demonstrate CD21-mediated enhancement (6, 7).

**Effect of CD21 cross-linking on the allogeneic MLR**

We next wanted to determine whether this early increase in B7-1 and B7-2 expression following CD21/CD35 cross-linking with or without sIgM could be detected in a functional manner by responding T cells. To study the effect of up-regulation of both B7-1 and B7-2 expression at the same time on B cells, we used the allogeneic MLR as a model. This allowed us to minimize the potential effects of CD21/CD35 ligation on Ag uptake and processing. In addition, we studied both the two-way and one-way MLR to be able to measure effects on both B and T cell proliferation.

Figure 5 demonstrates the specific CD21/CD35-mediated effects on a two-way allogeneic MLR analyzed on day 2. As shown, co-cross-linking of sIgM with the control mAb (recognizing Crry/p65) on B cells slightly increased proliferation at the higher T cell dose. This increase with control mAb is probably due to sIgM-mediated effects, as cross-linking sIgM alone in other experiments resulted in a similar increase (data not shown). In comparison, however, co-cross-linking of sIgM with CD21/CD35 resulted in the highest levels of proliferation, demonstrating that CD21/CD35 specifically enhances B and/or T cell activation in this system.

To more clearly distinguish the effects of CD21/CD35 ligation on either B or T cell proliferation, we also studied the one-way allogeneic MLR after irradiation of either cell population. As shown in Figure 6, the major effect of CD21/CD35 is an enhanced T cell proliferative response to irradiated B cells upon which CD21/CD35 had been co-cross-linked with sIgM. Although seen on day 2, the CD21/CD35-related effects are most pronounced after 4 days of culture. These results using one- and two-way MLRs demonstrate an increase in proliferation, but, interestingly, only following co-cross-linking of sIgM with CD21/CD35. We believe that these results are not entirely surprising, however. We have also found that class II expression is not up-regulated by CD21/CD35 ligation (data not shown), whereas sIgM cross-linking, as expected, substantially increases class II expression (40) (data not shown). This result clearly implies that an Ag-specific signal delivered through sIgM is still required to activate T cells even in the context of CD21/CD35-mediated up-regulation of B7 molecules. In a teleologic sense, because Ag-C3d complexes can bind to CD21/CD35 on B cells with any Ag specificity, it would not be advantageous if bystander T cells could be activated by these B cells. Thus, the dual requirement for sIgM and CD21/CD35 coligation appears to be an elegant way to allow CD21 to act as a second signal that enhances activation, but only in a setting in which Ag-specific sIgM molecules have also been ligated.

**Effect of anti-B7-1 and anti-B7-2 mAbs on the CD21/CD35-enhanced allogeneic MLR**

To determine whether the enhanced effect of CD21/CD35 co-cross-linking with sIgM in the MLR was dependent on the up-regulation of B7-2 and/or B7-1 or potentially used another co-stimulatory mechanism, we cultured γ-irradiated B cells and fresh T cells in the presence of blocking anti-B7 mAbs. As controls, the allogeneic MLR, with or without CD21/CD35 enhancement, was suppressed by anti-CD4 and anti-class II mAbs (data not shown). In addition, as shown in Figure 7, anti-B7-2 treatment blocked the proliferation of T cells almost completely for every condition, including co-cross-linking of CD21 with sIgM, whereas anti-B7-1
treatment partially blocked proliferation. This pattern demonstrating a dominant costimulatory effect of B7-2 and a lesser, but clearly detectable, B7-1 component is similar to that recently reported when studying T cell activation requirements in mice in which the B7-1 and/or B7-2 genes have been inactivated (41). It appears, therefore, that CD21/CD35 coligation with sIgM costimulates T cells using the same mechanism as other B cell activators, and that both B7-1 and B7-2 can act in concert to activate or regulate responder T cells following CD21/CD35 ligation.

Similar results were found in the two-way MLR and when the amount of blocking mAb was varied (data not shown). Also, each of the blocking anti-B7-1 mAbs (RM80, 16-10A1, and 1G10) demonstrated comparable inhibition. In addition, similar inhibition of the one-way MLR was found when CD4\(^+\) T cells were used as responders instead of unseparated T cells (data not shown).

CD19 cross-linking also leads to increased B7-1 and B7-2 expression

Because mouse CD19 has been shown to associate with CD21/CD35, and knockouts of both genes demonstrate similar phenotypes, we also determined whether CD19 cross-linking would lead to increased B7 expression. As shown in Figure 8, CD19 cross-linking does lead to increased B7-1 and B7-2 expression to a similar level as CD21/CD35. Cross-linking with isotype control IgG2a mAb had no effect on B7 expression. Therefore, ligation of two members of the CD21/CD35/CD19 complex can lead to the same increase in B7 molecules. We also found that co-cross-linking of CD19 with slgM lead to a similar increase in both B7-1 and B7-2 expression compared with cross-linking CD19 alone (data not shown).

Discussion

We have shown herein a new CD21/CD35- and CD19-mediated phenotype, i.e., rapid B7-1 and B7-2 up-regulation following either cross-linking alone or co-cross-linking with slgM on resting splenic B cells. Consistent with the up-regulation of costimulators, B cells upon which CD21/CD35 had been co-cross-linked with

FIGURE 6. Co-cross-linking sIgM with CD21/CD35 enhances a one-way allogeneic MLR. BALB/c resting B cells were first treated as indicated in the figure for 18 h. After washing, 4.4 \(\times 10^5\) treated B cells, either gamma-irradiated (500 rad) or not, were incubated with 5.5 \(\times 10^5\) freshly isolated C57BL/6 T cells for 2 days (A) or 4 days (B). \[^{3}H\]Tdr was added for the final 16 h. The mean ± SD of triplicate wells are shown. T cells cultured alone had 263 and 713 cpm on days 2 and 4, while irradiated T cells had <40 cpm at each point (data not shown). The p values calculated by Student’s t test for co-cross-linking sIgM with CD21/CD35 compared with co-cross-linking with control mAb in irradiated B cells plus T cells are 0.00043 and 0.00374 for days 2 and 4, respectively. Results representative of three different experiments are shown.

FIGURE 7. Effects of anti-B7-1 and anti-B7-2 mAbs on the CD21/CD35-enhanced allogeneic MLR. BALB/c resting B cells were incubated under the indicated conditions for 18 h. After washing, 5 \(\times 10^5\) treated B cells that had been gamma-irradiated (1000 rad) were incubated with 5 \(\times 10^5\) C57BL/6 T cells in the presence of 10 \(\mu\)g/ml anti-B7-1 or anti-B7-2 for 3 days. \[^{3}H\]Tdr was added for the final 16 h. The mean ± SD of triplicate wells are shown. Results representative of three different experiments are shown.

FIGURE 8. CD19 cross-linking leads to increased expression of B7-1 (C) and B7-2 (F) comparable to that following cross-linking with anti-CD21/CD35 (B and E). No change is seen following cross-linking with control mAb (A and D). Shown in each panel are cells cultured in medium alone (thin line) or with the indicated reagents (thick line). MCF values for cells under each condition following cross-linking are: A, 18; B, 99; C, 197; D, 24; E, 164; F, 314. Results representative of two different experiments are shown.
CD21/CD35 AND CD19 REGULATE B CELL EXPRESSION OF B7-1 AND B7-2

SlgM acted as more potent activators of an allogeneic MLR in a B7-dependent manner. We hypothesize that our activation system mimics the effects of C3d-bound Ag that cross-links CD21/CD35 with slgM. Our observation may thus be able to help explain why CD21/CD35- and CD19-deficient mice have an impaired response to T-dependent Ags (10–13). In this situation the binding of Ag alone to B cells in the absence of a CD21/CD35/CD19-initiated signal could result in a number of defects. One could be due to the impaired enhancement of slgM-mediated signaling resulting in a relative decrease in proliferation and differentiation as previously proposed (42). The second, perhaps mediated by similar signaling pathways as the first, is the lack of up-regulation of B7-1 and B7-2 molecules that is a direct consequence of CD21/CD35/CD19 ligation. This relative lack of B7-1 and B7-2 could also lead to ineffective T cell costimulation and a secondary B cell defect.

The results may also help to explain why recombinant HEL-C3d polymers induced an immune response in vivo at a dose at least 1000-fold lower than HEL alone (16), as the “adjuvant” effect of C3d in the HEL polymer may be due in part to the ability to up-regulate B7-1 and/or B7-2. Our proposal that B7 up-regulation by CD21/CD35 cross-linking is an important effect is also consistent with results demonstrating that CD21/CD35-deficient mice manifest defects in the immune response that can be overcome by the use of adjuvant (12). The defect manifest by CD21/CD35 or C3 deficiency also bears features similar to those found when using anti-B7-2 mAb to block germinal center formation in vivo or when studying B cell responses in mice in which the genes encoding B7-1 and/or B7-2 have been inactivated.

It is not clear yet how B7-1 and B7-2 are up-regulated by CD21/CD35 or CD19 ligation, or whether increased expression following CD21/CD35 cross-linking alone is due to CD19-mediated signaling, a novel CD21/CD35-activated pathway, or a cooperative signaling mechanism requiring all three molecules. It is known that CD19 physically associates with CD21 in the cell membrane, and that CD19 is activated following slgM cross-linking. CD19 can recruit phosphatidylinositol 3-kinase (43–45) or Vav (46), which regulates Ras or Rac. Co-cross-linking of mouse CD19 and slgM increases MAP kinase (ERK2, JNK, and p38) activity to a greater extent than cross-linking slgM alone (47). Thus, a CD19-mediated signaling mechanism that is engaged by CD21/CD35 cross-linking may be important to up-regulate B7 molecules.

In addition, although the most effective enhancement of signaling events mediated by CD19 were shown to follow co-cross-linking with slgM, increases in p38 activity were found with CD19 ligation alone (47). Of interest, CD40 ligation also activates MAP kinases, especially JNK and p38, in addition to leading to increases in B7-2 expression (48–51), while slgM ligation preferentially activates ERK2 (52, 53). By analogy, MAP kinase activation is likely to be involved in the up-regulation of B7-1 and B7-2 expression by the CD21/CD35/CD19 complex.

However, it is still possible that the increase in B7 levels is dependent on CD21/CD35 function alone. First, CD21/CD35 ligation alone efficiently up-regulates both B7-1 and B7-2 in this system, even if slgM and CD19 are not directly ligated. Second, Luxembourg et al. have shown that human CD21 ligation alone will engage a homotypic adhesion pathway on primary B cells and B cell lines (54). And third, CD21 is expressed on cell types that do not express CD19, and ligation of CD21 on CD19 negative human T cells results in the formation of an apparently novel cell surface protein complex (55). Further analysis using CD21/CD35- and CD19-deficient mice is necessary to determine the specific mechanisms used to regulate B7-1 and B7-2.

It is also unclear how the phenotype we have described in mice is related to previous studies of the role of CD21 in human T cell activation. Human CD21 has previously been shown to mediate increased Ag presentation by B cell APCs to Ag-specific T cells through a mechanism that has been believed to involve receptor binding and uptake (56–59). Although B7-1 expression on human B cells was studied in one model (57, 59), its relevance and that of B7-2, especially compared with the independent effects of human CD21 on Ag binding and uptake, were not clearly distinguished. In addition, it was hypothesized in that study that simultaneous CD21 and FcγRII (CD32) binding on human B cells mediated enhanced B7-1 expression. In the other studies (56, 58), B-7 expression was not evaluated. In contrast, in mice that have been treated with anti-CD21/CD35 mAb (60) and in the CD21/CD35-deficient mouse (13), T cell priming and the ability to provide help following T-dependent Ag immunization were not found to be altered using adoptive transfer techniques.

It is also possible that the increased B7-1 and B7-2 expression mediated by CD21 primarily affects endogenous B cell activation through B7-mediated signal transduction events, a hypothesis that has been previously presented (61). Indeed, recent studies have suggested that human B cell activation is enhanced by anti-B7-2 mAb (62). Interestingly, this increase in activation is seen in a model of CD23-mediated enhancement of IgE synthesis that is a result of CD23 binding to human CD21 in the presence of IL-4 and CD40L. Clearly further analysis is necessary to understand these specific issues.

B7-1 and B7-2 have been hypothesized to manifest differential effects on lymphocyte activation. Therefore, we examined several additional features following CD21/CD35-mediated enhanced expression of these molecules. Specifically, we studied the ability of B cells upon which CD21/CD35 and slgM had been co-cross-linked (thus expressing both B7-1 and B7-2), compared with slgM cross-linking alone (expressing B7-2 alone), to secrete differential IgG isotypes in an allogeneic MLR, or, using OVA peptide and T cells from the OVA TCR transgenic DO11 (63), to manifest Th1 vs Th2 dissociation, with or without adding exogenous IL-4 or CD40L. No differences were found in any of these analyses (data not shown). We also examined, using the allogeneic MLR system described above, whether B7-2 more effectively costimulates CD4+ T cells and B7-1 more effectively costimulates CD8+ T cells, but again found no differences (data not shown).

Finally, it is interesting to consider why B7 molecules are up-regulated with similar and rapid kinetics following CD21/CD35 ligation. We believe this is so because the immune response is poised to efficiently respond to infection by pathogens (17). When complement is activated on a viral or bacterial Ag by the classical and/or alternative pathway, this target is marked as foreign. In addition, complement can identify many infectious organisms as non-self via the alternative pathway without using Ab (64). C3b-bound Ag will then co-cross-link Ag-specific slgM with CD21 on a subset of B cells. It would make teleologic sense if this co-cross-linking should result in a rapid initiation of the immune response that involved costimulator expression and potentially rapid recruitment of T cell help. This CD21/CD35-mediated response would complement that induced by LPS, a product of many infectious organisms, which also activates B cells to express B7 molecules. Indeed, p38 was found to be the major tyrosine-phosphorylated protein following LPS treatment (65). However, the CD21/CD35 effect is different in that B7-1 expression occurs relatively late following LPS stimulation compared with rapidly following CD21/CD35. This hypothesis is also consistent with recent results using single and double B7-1 and B7-2 knockout mice, which demonstrated unique, but necessary, roles for each protein in B cell.
responses and germinal center formation (41) and suggest that altered B7 expression may mediate part of the defects seen in mice in which CD21/CD35 and CD19 expression has been eliminated.

In summary, previous studies using inhibitors and knockouts have shown that the CD21/CD35/CD19 complex plays an important role in the initiation and amplification of the cellular immune response. We propose that regulation of B7-1 and B7-2 expression is an important function of the CD21/CD35/CD19 complex. In particular, the ability to mediate rapid increases in B7-1 expression may allow this receptor complex to make a unique contribution to the immune response.

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References


