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# Activated T Cells Induce Expression of B7/BB1 on Normal or Leukemic B Cells through a CD40-dependent Signal

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## Summary

Cognate interactions between antigen-presenting B and T cells play crucial roles in immunologic responses. T cells that have been activated via the crosslinking of CD3 are able to induce B cell proliferation and immunoglobulin secretion in a major histocompatibility complex-unrestricted and contact-dependent manner. We find that such activated human CD4<sup>+</sup> T cells, but not control Ig-treated T cells, may induce normal or leukemic B cells to express B7/BB1 and significantly higher levels of CD54 intercellular adhesion molecule 1 via a process that also requires direct cell-cell contact. To discern what cell surface molecule(s) may be responsible for signalling B cells to express B7/BB1, we added various monoclonal antibodies (mAbs) specific for T or B cell accessory molecules or control mAbs to cocultures of  $\alpha$ -CD3-activated T cells and resting B cells. We find that only  $\alpha$ -CD40 mAbs can significantly inhibit the increased expression of B7/BB1, suggesting that the ligand for CD40 expressed on activated T cells may be an important inducer of B7/BB1 expression. Subsequent experiments in fact demonstrate that  $\alpha$ -CD40 mAbs, but not control mAbs, induce changes in B cell phenotype similar to those induced by activated T cells when the mAbs are presented on Fc $\gamma$ RII (CDw32)-expressing L cells. These phenotypic changes have significant effects on B cell function. Whereas chronic lymphocytic leukemia (CLL) B cells normally are very poor stimulators in allogeneic mixed lymphocyte reactions (MLRs), CLL-B cells preactivated via CD40 crosslinking are significantly better presenters of alloantigen, affecting up to 30-fold-greater stimulation of T cell proliferation than that induced by control treated or nontreated CLL-B cells. Similarly, the MLR of T cells stimulated by allogeneic nonleukemic B cells can be enhanced significantly if the stimulator B cells are preactivated via CD40 crosslinking. The enhanced MLR generated by such preactivated B cells may be inhibited by blocking B7/BB1-CD28 interaction with CTLA4Ig. These studies demonstrate a novel, CD40-dependent pathway for inducing B cell expression of B7/BB1 and enhancing B cell antigen-presenting cell activity that can be initiated via cell-cell contact with  $\alpha$ -CD3-stimulated CD4<sup>+</sup> T cells.

**B** cells may play an important role in antigen presentation (1-5). Cognate interactions between T and B cells may result in bidirectional stimulatory signals, allowing properly activated B cells both to induce T cell proliferation and to elicit helper activity from T cells that recognize antigen presented by B cell MHC molecules. It is important that the B cell phenotype may influence whether such cognate interactions result in either T cell activation or anergy (6). For example, resting B cells generally are ineffective stimulators of allogeneic T cells in the mixed lymphocyte reaction (MLR)<sup>1</sup> unless preactivated by  $\alpha$ -IgM antibodies, PMA or LPS (7-9). In addition, soluble monomeric antigen directed

to B cells for presentation in vivo may result in specific T cell anergy (10). However, that same antigen may stimulate a vigorous antigen-specific immune response when delivered as a divalent molecule that can crosslink B cell surface Ig. Recent studies have indicated that the interactions between B and T cell surface molecules, such as intercellular adhesion molecule 1 (ICAM-1) (CD54) and LFA-1 (CD11a/CD18), LFA-3 (CD58) and CD2, very late antigen 4 (VLA-4) (CDw49d) and vascular cell adhesion molecule 1 (VCAM-1), and B7/BB1 and CD28, play important roles in B-T cell conjugate formation and in patterning the response to B cell-presented antigen (11-15). In particular, B cell expression of B7/BB1, induced by sIg crosslinking (16, 17), may activate antigen-specific T cells to secrete IL-2 and to proliferate in response to B cell-presented antigen, thereby possibly preventing the induction of antigen-specific T cell anergy (6, 18-21).

<sup>1</sup> Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; MFIR, mean fluorescence intensity ratio; MLR, mixed lymphocyte reaction; PI, propidium iodide; RPMI-10, penicillin-streptomycin; SM, staining media; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4.

Little is known about how activated T cells may affect expression of these important B cell accessory molecules. Prior studies demonstrated that T cells, activated by TCR cross-linking with  $\alpha$ -CD3 mAb or Con A may induce B cell proliferation, differentiation, and Ig secretion in an MHC and antigen-independent manner that requires direct T-B cell contact (22–28). These studies primarily addressed how such activated T cells may induce B cells to differentiate into Ig-secreting cells. However, because of the pivotal role that surface phenotype may play in determining whether a B cell can function as a stimulatory APC, we assayed for changes in the levels of accessory molecules expressed by B cells after coculture with control Ig-treated or  $\alpha$ -CD3-activated T cells.

To examine the changes induced by activated T cells on relatively homogeneous populations of resting B cells, we performed initial studies using chronic lymphocytic leukemia (CLL) B cells. CLL-B cells coexpress CD19, CD20, CD40, CD45, and class I and II MHC antigens, and, like a subset of normal B cells, are CD5<sup>+</sup> (29–32). The leukemia cells from most patients express surface IgD and/or IgM and have levels of CD54 and CD58 that are comparable with those of normal resting B cells (33). CLL-B cells are poor stimulators in autologous or allogeneic MLR (34, 35). Arguably consistent with this, CLL-B cells express low levels of CD11a and VLA-4 (36, 37), and little to no B7/BB1 (16). Although CLL-B cells are similar in many respects to normal resting B cells, we performed parallel experiments with normal peripheral blood and tonsillar B cells to examine whether any noted effects were peculiar to leukemic B cells.

## Materials and Methods

**Antibodies.** 64.1, a murine IgG2a  $\alpha$ -CD3 mAb, was provided by Bristol-Myers Squibb (Seattle, WA). G28-5 (a murine IgG1  $\alpha$ -CD40 mAb, [38]) was a gift from Dr. Edward Clark (University of Washington, Seattle, WA). Murine mAbs specific for CD11a (IgG1 anti-LFA-1), CD54 (IgG1 anti-ICAM-1), CD58 (IgG2a anti-LFA-3), HLA-DR (IgG2b), or CDw49d (IgG1 anti-VLA-4) were purchased from AMAC, Inc. (Westbrook, ME). PE-conjugated  $\alpha$ -CD19 (B4, a murine IgG1) was purchased from Coulter Immunology (Hialeah, FL). MOPC21 (murine IgG1) was purchased from Caltag, Inc. (South San Francisco, CA). RPC-5 (murine IgG2a) was purchased from Calbiochem Novabiochem Corp. (La Jolla, CA).  $\alpha$ -CD14 (clone LeuM3, a murine IgG2b) and  $\alpha$ -CD16 (clone Leu11a, a murine IgG1) were purchased from Becton Dickinson & Co. (San Jose, CA).  $\alpha$ -B7/BB-1 (clone L307.4, a murine IgG1) was purchased from the Advanced Cellular Biology Division of Becton Dickinson & Co. (San Jose, CA) and conjugated to FITC as described (39). HB57 (murine IgG1  $\alpha$ -human IgM) and W6/32 (murine IgG1  $\alpha$ -HLA class I) producing hybridomas were obtained from American Type Culture Collection (Rockville, MD), and mAbs were purified from ascites as described (40). The CTLA4Ig human Ig-recombinant molecule (41), was a gift from Dr. Peter Linsley (Oncogen Division, Bristol-Myers Squibb). Goat  $\alpha$ -human IL-4 was purchased from R & D Systems, Inc. (Minneapolis, MN).

**Cell Purification.** Human T and B cells were purified from buffy coats obtained from normal donors at the San Diego Blood Bank (San Diego, CA). PBMC were isolated by centrifuging over Histopaque 1077 (Sigma Immunochemicals, St. Louis, MO) and monocytes depleted by adherence to plastic culture flasks for 2 h in a

37°C, 5% CO<sub>2</sub> incubator in RPMI-1640 media plus 10% FCS, 4 mM L-glutamine, and 100 U/ml penicillin–streptomycin (hereafter referred to as RPMI-10). Monocyte-depleted PBL were incubated with antibodies directed at B cells ( $\alpha$ -CD19), NK cells ( $\alpha$ -CD16), and monocytes ( $\alpha$ -CD14), at 1  $\mu$ g of mAb per 10<sup>6</sup> estimated target cells, for 30 min at 4°C, washed, and incubated with sheep  $\alpha$ -mouse IgG-conjugated magnetic beads (Dynal, Inc., Great Neck, NY) at a bead to target cell ratio of 7:1 for 1 h, rocking, at 4°C. Magnetic depletion of bead-bound cells resulted in >95% pure CD3<sup>+</sup> T cells, as determined by FACS<sup>®</sup> analysis (Becton Dickinson & Co.). Normal B cells were purified from tonsil samples and peripheral blood with magnetic beads as described above, substituting  $\alpha$ -CD3 mAb for  $\alpha$ -CD19. CLL PBMC samples were purified only if B cells constituted <95% of the total cells. In normal cell samples, we generally obtained populations consisting of 80–95% CD19<sup>+</sup> B cells. Cells were used fresh or viably frozen in FCS plus 10% DMSO in liquid nitrogen before use.

**64.1 Activated T Cell Assay.** T-B cocultures were performed using a protocol modified from that described by Tohma and Lipsky (26). 12-well culture plates (Corning Inc., Corning, NY) were coated with  $\alpha$ -CD3 mAb (64.1) or control IgG2a mouse myeloma protein (RPC-5) at 100 ng/ml in 50 mM Tris buffer, pH 8.5, at room temperature for 2–4 h (0.5 ml/well). Wells were washed thoroughly with sterile PBS followed by RPMI-10. Purified normal T cells were suspended at 5  $\times$  10<sup>6</sup>/ml in RPMI-10 and treated with mitomycin C (Sigma Immunochemicals) at a concentration of 50  $\mu$ g/ml for 1 h at 37°C to prevent proliferation. T cells were then washed 4 times with RPMI-10, plated onto mAb-coated wells at 6  $\times$  10<sup>6</sup>/well, and allowed to adhere for 1 h at 37°C. CLL or normal B cells were added in RPMI-10 at 2  $\times$  10<sup>6</sup> cells/well. Cells were cultured at 37°C in 5% CO<sub>2</sub>, and then harvested after 48–72 h for flow cytometric analysis.

**Multiparameter Flow Cytometric Analysis.** Cells were washed and then suspended in staining media (SM; consisting of 1 $\times$  HBSS, 3% BSA, .01% NaN<sub>3</sub>, and 1  $\mu$ g/ml propidium iodide [PI]) with saturating amounts of FITC- or PE-conjugated mAbs against various cell surface antigens or isotype-specific control mAbs. After 30 min at 4°C, cells were washed in PI-deficient SM and analyzed on a flow cytometer (FACSscan<sup>®</sup>; Becton Dickinson & Co.). Dead cells staining with PI were excluded from analysis. B cells were distinguished from cocultured T cells by electronic gating on cells staining with CD19-PE. The expression of all other cell surface markers was determined by staining with FITC-conjugated mAbs, as described above.

**CDW32-L Cell Assay.** We employed a modified version of the B cell in vitro culture system described by Bancheureau et al. (42). Briefly, CDW32-expressing murine fibroblast cells (CDW32-L cells) obtained from the American Type Culture Collection (Rockville, MD), were suspended at 5  $\times$  10<sup>6</sup>/ml and then treated with 100  $\mu$ g/ml mitomycin C in RPMI-10 for 1 h at 37°C. These cells then were washed four times in PBS plus 50% RPMI-10. In assays for FACS<sup>®</sup> analysis of surface phenotype changes, 5  $\times$  10<sup>5</sup> CDW32-L cells in 0.5 ml RPMI-10 per well were plated into a 24-well culture plate (Corning Inc.) and allowed to adhere for  $\geq$ 1 h at 37°C. B cells then were added at 2  $\times$  10<sup>6</sup> per well in final volume of 1 ml. Various mAbs and/or recombinant human IL-4 (rhIL-4; Biosource International, Camarillo, CA) were added at various concentrations. Cultures were incubated 48–72 h before harvesting for FACS<sup>®</sup> analysis.

**MLR.** CLL-B cells were stimulated for 48 h with  $\alpha$ -CD40 or control mAb plus IL-4 as above, but using 6-well culture plates with 1.5  $\times$  10<sup>6</sup> CDW32-L cells/well and 6  $\times$  10<sup>6</sup> B cells/well in a total volume of 4 ml RPMI-10. B cells were separated from ad-

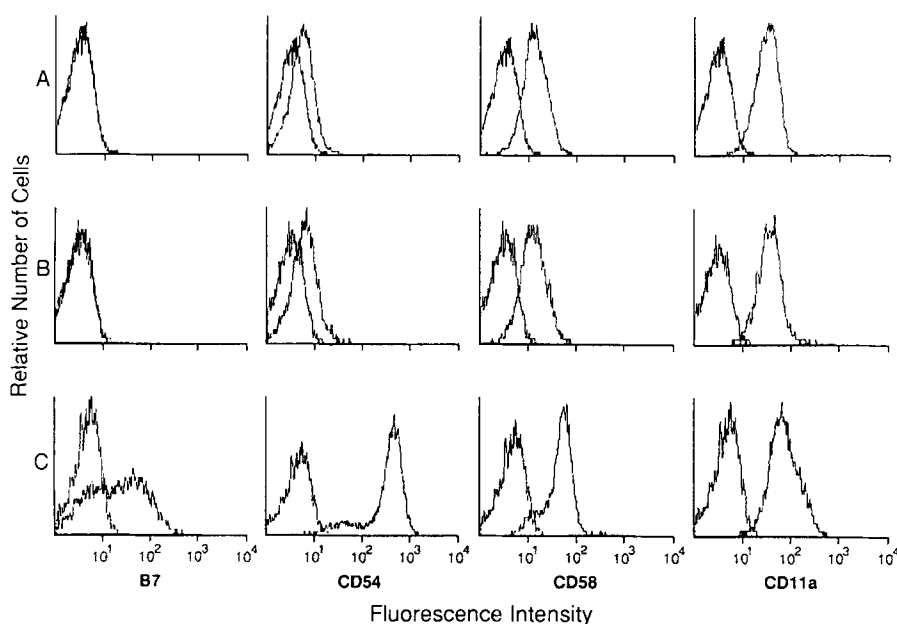
herent CDw32-L cells by gently rinsing wells with RPMI-10, resulting in >99% pure leukemic B cells as assessed by FACS<sup>®</sup> analysis. B cells were treated with mitomycin C as above, washed four times in RPMI-10, and then suspended in RPMI plus 10% pooled human AB serum (RPMI-10 hAB; GIBCO BRL; Gaithersburg, MD). B cells were plated with various blocking or control mAbs in 150  $\mu$ l RPMI-10 hAB in triplicate in 96-well round-bottomed plates (Corning Inc.) at  $10^5$  to 400 cells/well to give final responder T cell to stimulator B cell (R/S) ratios of between 1:1 and 25:1. After allowing mAbs to bind for 1 h at 37°C, responder allogeneic normal T cells, isolated as described above, were added at  $10^5$ /well in a final total volume of 200  $\mu$ l, and plates were incubated at 37°C, 5% CO<sub>2</sub> for 5 d. Proliferative response was measured by pulsing the cultures with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (ICN Biomedicals, Inc., Costa Mesa, CA) 18 h before harvesting onto glass fiber filters (PHD Cell Harvester; Cambridge Technology, Inc., Watertown, MA). Filter-bound [<sup>3</sup>H]thymidine that had been incorporated into newly synthesized DNA was measured using a scintillation counter (model LS1 801; Beckman Instruments, Inc., Fullerton, CA).

## Results

**B Cell Surface Phenotype Changes Induced by Activated Cells.** CLL-B cells or normal human B cells were cultured alone or with allogeneic, mitomycin C-treated T cells previously plated in culture wells coated with either  $\alpha$ -CD3 mAb (64.1) or control mouse IgG of the same isotype. As described (26, 43),  $\alpha$ -CD3 mAb-coated plates may induce T lymphocytes to become "activated T cells" (T<sub>a</sub> cells), affecting increases in T cell intravascular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>), phosphatidylinositol turnover, protein tyrosine kinase activity, proliferation, and expression of HLA-DR and CD25 (44–48). The term "resting T cells" (T<sub>r</sub> cells) is used to describe mitomycin C-treated T lymphocytes cultured in control Ig-coated wells. Flow cytometric analyses indicated that, whereas  $\leq 10\%$  of

T<sub>r</sub> cells expressed CD25 and/or HLA-DR, >50% of the T<sub>a</sub> cells used in these studies expressed these T cell activation antigens (data not shown). B cells cultured either alone or in control Ig-coated wells or with T<sub>r</sub> or T<sub>a</sub> cells were examined by flow cytometry after 48 h in culture. We find that B cells cocultured with T<sub>a</sub> cells dramatically increase their expression of CD54 (ICAM-1) and B7/BB1 (Fig. 1 C). B cells cocultured with T<sub>a</sub> cells also manifest moderate increases in surface expression of CD11a and CD58, compared with B cells cultured alone (Fig. 1 A). On the other hand, B cells cocultured with T<sub>r</sub> cells displayed minimal changes in their expression levels of B7/BB1, CD54, CD11a or CD58 (Fig. 1 B), as did B cells cultured in  $\alpha$ -CD3 mAb-coated wells without added T cells (data not shown).

To compare changes in expression levels of these B cell antigens within and across experiments, we computed the mean fluorescence intensity ratio (MFIR) in each staining experiment for each antigen. The MFIR for a given antigen is defined as the mean fluorescence intensity (MFI) of gated B cells stained with an antigen-specific FITC-conjugated mAb divided by the MFI of such cells stained with a FITC-conjugated isotype control mAb of irrelevant specificity. For example, B cells cocultured with T<sub>a</sub> cells in the experiment depicted in Fig. 1 C had a MFIR for B7/BB1 of 6.1, and a MFIR for CD54 of 55.7. On the other hand, B cells cocultured with T<sub>r</sub> cells (Fig. 1 B) had a B7/BB1 MFIR of 1.0, identical to that noted for B cells cultured alone. To compare the MFIRs observed in separate experiments, we calculated the ratio of the MFIR for a given antigen observed on B cells cocultured with T<sub>a</sub> or T<sub>r</sub> cells over the MFIR for that same antigen observed on B cells cultured alone. We find that, on average, CLL-B cells cocultured with T<sub>a</sub> cells have B7/BB1 MFIRs and CD54 MFIRs that are, respectively, 4.8- and 23.5-fold greater than that of B cells cultured alone ( $n = 7$ ). In con-



**Figure 1.** Changes in surface antigen phenotype of B cells cocultured with T<sub>r</sub> or T<sub>a</sub> cells. CLL-B cells were cultured alone in control IgG2a-coated wells (A), or with allogeneic mitomycin C-treated T cells in wells coated with control IgG2a mAb (T<sub>r</sub> cells) (B), or with the  $\alpha$ -CD3 mAb 64.1 (T<sub>a</sub> cells) (C). After 48 h of coculture, B cell accessory molecule expression was assessed by FACS<sup>®</sup> analysis. Each histogram depicts the logarithmic green fluorescence intensity of live-gated CD19<sup>+</sup> B cells stained with either a FITC-conjugated mAb specific for a cell surface antigen (listed below each column) or FITC-conjugated mouse isotype control Ig. The histogram that is nearest the ordinate in each figure represents the fluorescence of B cells stained with an isotype control Ig.

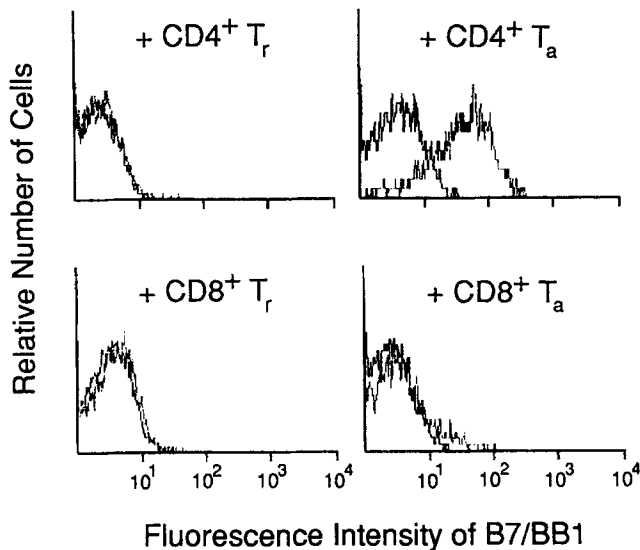
trast,  $T_a$  cells mediated only 2.7- and 2.1-fold increases in CD11a ( $n = 3$ ) and CD58 ( $n = 4$ ) MFIRs, respectively, over those measured in B cells cultured alone. Studies employing normal tonsillar and peripheral blood B cells revealed very similar responses to those obtained with CLL-B cells. Coculture of normal B cells with allogeneic  $T_a$  cells induced 3–10-fold increases in B7/BB1 expression and 15–32-fold increases in the expression of CD54 as compared with levels observed on B cells cultured alone. As with CLL-B cells, coculture with  $T_r$  cells was without effect.

***$T_a$  Cells that Induce B Cell Expression B7/BB1 Are  $CD4^+$***  To further characterize the  $T_a$  cells that may induce B cells to express B7/BB1, isolated normal peripheral blood T cells were separated into  $CD4^+$  or  $CD8^+$  cell populations by magnetic bead depletion of  $CD8^+$  cells or  $CD4^+$  cells, respectively.  $CD4^+$   $T_a$  induced an 8.3-fold increase in B7/BB1 MFIR on cocultured CLL-B cells compared with that on CLL-B cells cultured alone (Fig. 2). In contrast,  $CD8^+$   $T_a$  cells induced minimal changes in B cell expression of B7/BB1, similar to that induced by  $CD8^+$  or  $CD4^+$   $T_r$  cells (Fig. 2).

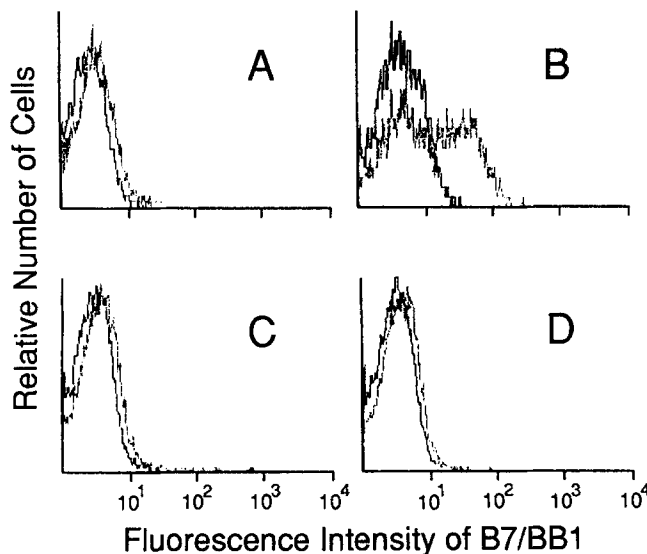
***Induction of B7/BB1 Requires Direct T-B Cell Contact.*** Previous studies demonstrated that the stimulatory effects of  $\alpha$ -CD3-activated T cells on B cell proliferation and Ig production were dependent on direct cell–cell contact (25, 26). To examine whether the increased expression of B7/BB1 was mediated by soluble factors or required cell–cell contact, we cultured CLL-B cells together with  $T_a$  or  $T_r$  cells, or in

chambers separated from the T cells by membranes that are permeable to particles  $\leq 0.4 \mu\text{m}$  in diameter (Transwell® inserts; Costar Corp., Cambridge, MA). We find that direct contact with  $T_a$  cells results in a B7/BB1 MFIR of 3.3, as compared to B7/BB1 MFIRs of 1.4, 1.2, and 1.5 induced by contact with  $T_a$  supernatant,  $T_r$  cells or  $T_r$  supernatant, respectively (average of two experiments) (Fig. 3). The suboptimal  $T_a$ -mediated induction of B7/BB1 in these experiments may be due to dilution of cells into the larger tissue culture wells necessary to accommodate the Transwells®. Maximal increases in CD54 expression are also dependent on direct  $T_a$ -B cell contact. Whereas contact with  $T_r$  cells or supernatants of T-B coculture increased CD54 MFIRs two- to fourfold, direct contact with  $T_a$  cells induced 20-fold increases in CD54 MFIR. These results suggest that a cell surface molecule(s) is expressed on T cells upon  $\alpha$ -CD3 crosslinking which may induce B cells to express B7/BB1 and other surface molecules upon cell–cell contact.

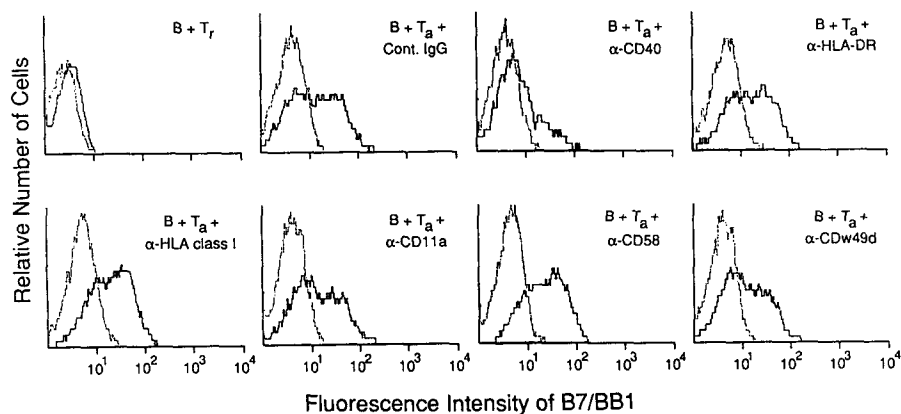
***The Induction of B7/BB1 Is Blocked by  $\alpha$ -CD40 mAbs.*** To discern what cell surface molecule(s) may be responsible for  $T_a$ -B cell interaction, we added each of several mAbs specific for T and/or B cell accessory molecules to cocultures of B and  $T_a$  cells. We find that soluble  $\alpha$ -CD40 mAb (G28-5), but not mAbs specific for HLA class I or II, CD11a (LFA-1), or CD58 (LFA-3), can significantly inhibit the induction of CLL-B cell surface B7/BB1 expression by  $T_a$  cells (Fig. 4). In five experiments, we noted that  $\alpha$ -CD40 mAb affected



**Figure 2.** Only  $CD4^+$   $T_a$  cells are able to induce CLL-B cell expression of B7/BB1. Normal peripheral blood T cells were isolated and enriched for either  $CD4^+$  or  $CD8^+$  cells by magnetic bead depletion of the alternate subset. CLL-B cells were cocultured with  $CD4^+$  or  $CD8^+$   $T_r$  or  $T_a$  cells for 48 h and assessed for B7/BB1 expression by FACS® analysis. Each histogram depicts the logarithmic green fluorescence intensity of live-gated  $CD19^+$  B cells stained with either FITC-conjugated  $\alpha$ -B7/BB1 or FITC-conjugated isotype control Ig. The histogram that is nearest the ordinate in each figure represents the fluorescence of B cells stained with an isotype control Ig.



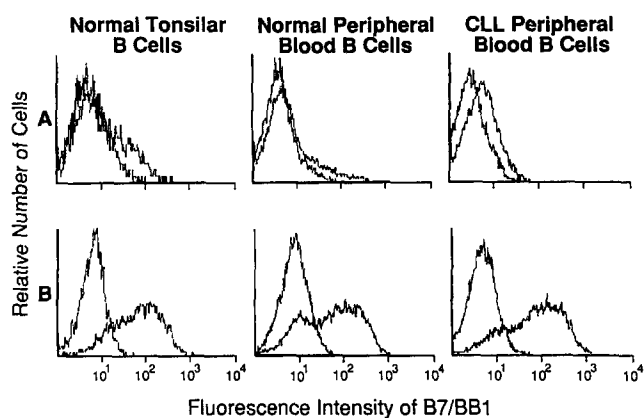
**Figure 3.**  $T_a$ -induced increases in B7/BB1 expression require direct  $T_a$ -B contact. CLL-B cells were cultured in direct contact with  $T_r$  (A) or  $T_a$  cells (B), or in Transwell® inserts contacting the supernatants of  $T_r$ -B cell (C) or  $T_a$ -B cell (D) cocultures, but separated from direct T cell contact by membranes permeable only to particles  $\leq 0.4 \mu\text{m}$  in diameter. After 48 h, B cells were assessed for B7/BB1 expression by FACS® analysis. Each histogram depicts the logarithmic green fluorescence intensity of live-gated  $CD19^+$  B cells stained with either FITC-conjugated  $\alpha$ -B7/BB1 or FITC-conjugated isotype control Ig. The histogram that is nearest the ordinate in each figure represents the fluorescence of B cells stained with an isotype control Ig. One representative experiment of two.



**Figure 4.**  $T_a$ -induced increases in B7/BB1 expression are inhibited by  $\alpha$ -CD40 mAbs. CLL-B cells were cocultured with  $T_r$  or  $T_a$  cells for 48 h with or without one of several mAbs specific for cell surface antigens or control Ig (Cont. IgG), as indicated in the top right corner of each histogram. Each histogram depicts the logarithmic green fluorescence intensity of live-gated CD19<sup>+</sup> B cells stained with either FITC-conjugated mAbs specific for B7/BB1 or FITC-conjugated mouse isotype control Ig. The histogram that is nearest the ordinate in each figure represents the fluorescence of B cells stained with an isotype control Ig.

a 55% ( $\pm$  16%, SD) mean reduction of  $T_a$ -induced B cell B7/BB1 MFIR compared with that of B cells cocultured with  $T_a$  cells and a control mouse IgG1 ( $p < 0.001$ ). Addition of  $\alpha$ -CD40 mAbs also reduced the induced level of CD54 by 37% ( $\pm$  7%, SD) ( $p = .001$ ). A modest reduction ( $13 \pm 6\%$ ) in the level of  $T_a$  cell-induced B7/BB1 also was noted when saturating amounts of mAbs specific for CDw49d (VLA-4) were added to the cultures (mean of three experiments) (Fig. 4). However, this inhibition was not significant ( $p > .05$ ). In contrast, saturating amounts of  $\alpha$ -CD11a or  $\alpha$ -HLA-DR mAbs, even at concentrations 20 times higher than that needed for maximal inhibition by  $\alpha$ -CD40, had no effect on  $T_a$ -cell-induced expression of B7/BB1 or CD54 (Fig. 4).

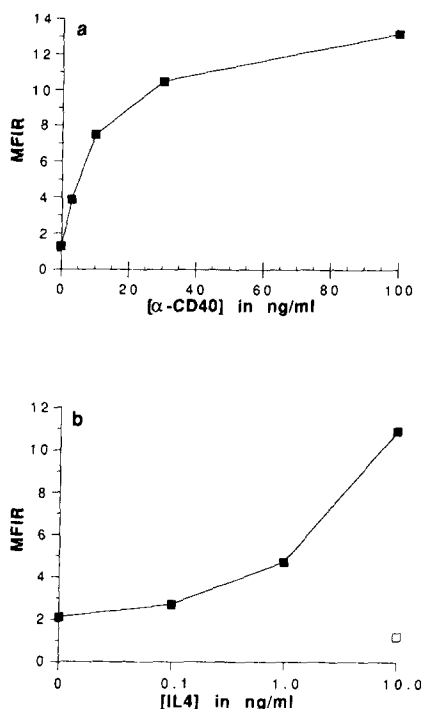
*Crosslinking CD40 Induces Changes in Surface Phenotype Similar to Those Induced by  $T_a$  Cells.* We used Fc $\gamma$ RII



**Figure 5.** Expression of B7/BB1 on B cells cultured with CDw32-L cells and mAbs specific for CD40. Normal or CLL-B cells enriched from tonsil or peripheral blood and mitomycin C-treated CDw32-L cells were cultured with rIL-4 (10 ng/ml) and control mouse IgG (A) or  $\alpha$ -CD40 mAb (B) for 48 h and assessed for B7/BB1 expression by FACS analysis. Each histogram depicts the logarithmic green fluorescence intensity of live-gated CD19<sup>+</sup> B cells stained with either FITC-conjugated  $\alpha$ -B7/BB1 or FITC-conjugated isotype control Ig. The histogram that is nearest the ordinate in each figure represents the fluorescence of B cells stained with an isotype control Ig.

(CDw32)-expressing murine fibroblast cells (CDw32-L cells) to present a cell surface array of mAbs specific for B cell surface molecules, as described by Banchereau et al. (42). Normal tonsillar or peripheral blood B cells, or CLL-B cells cultured with  $\alpha$ -CD40 mAbs and irradiated CDw32-L cells exhibit changes in surface phenotype that closely resemble those induced by  $T_a$  cells, including induced expression of B7/BB1 (Fig. 5). We note that  $\alpha$ -CD40 mAbs appear to be more effective in inducing B7/BB1 and CD54 on CLL-B cells than  $\alpha$ -IgM or  $\alpha$ -HLA-DR, two stimuli previously shown to increase B7/BB1 expression (16–18). Crosslinking sIgM with mAbs presented on CDw32-L cells induces a 1.6-fold or 2.7-fold increase in B7/BB1 or CD54 MFIRs, respectively (mean of four experiments). 72 h culture with  $\alpha$ -HLA-DR mAbs and irradiated CDw32-L cells resulted in similar increases in B7/BB1, but no detectable increase in CD54 (data not shown). In contrast, 72-h culture with  $\alpha$ -CD40 mAbs in the presence of IL-4 induced an average 7.2-fold increase in B7/BB1 MFIR ( $n = 15$ ), and an average 54.2-fold increase in CD54 MFIR ( $n = 6$ ) in a dose-responsive manner (Fig. 6 a, and data not shown). Subsequent experiments revealed that normal peripheral blood and tonsillar B cells responded similarly to CLL-B cells after CD40 crosslinking. Normal B cells from three different peripheral blood and three different tonsillar samples increased their B7/BB1 MFIR an average of 4.9-fold ( $n = 6$ ), and CD54 MFIR, an average of 5.5-fold ( $n = 5$ ) after 48-h culture with  $\alpha$ -CD40 mAbs, CDw32-L cells, and exogenous rIL-4. The final CD54 MFIRs achieved in  $\alpha$ -CD40-stimulated normal B cell cultures were similar to those observed on  $\alpha$ -CD40-stimulated CLL B cells. However, since nonstimulated normal B cells expressed higher levels of CD54 than nonstimulated CLL B cells, the fold increases in CD54 MFIR achieved with  $\alpha$ -CD40 crosslinking were less for normal B cells than for CLL B cells.

Although not necessary for the  $\alpha$ -CD40-induced phenotypic changes, exogenous rIL-4 augmented the effect of CD40 crosslinking in a dose-responsive manner (Fig. 6 b, filled squares). IL-4, alone or in combination with control mAbs, did not change the level of B7/BB1 expressed by CLL-B cells when cultured without  $T_a$  cells or  $\alpha$ -CD40 mAbs and CDw32-L cells (Fig. 6 b, open square). Furthermore,  $\alpha$ -hIL-4 neutralizing antibody at  $\leq 5 \mu\text{g/ml}$  did not reduce the

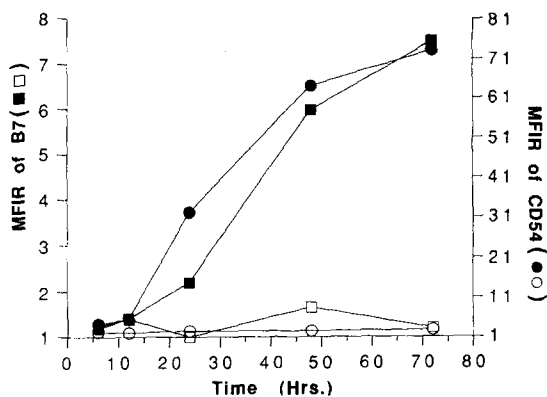


**Figure 6.** Dose-response increase in B7/BB1 MFIR on B cells cultured with CDw32-L cells and increasing concentrations of  $\alpha$ -CD40 mAb or IL-4. CLL-B cells and mitomycin C-treated CDw32-L cells were cultured with varying concentrations of  $\alpha$ -CD40 mAb in the presence of a fixed concentration of exogenous rhIL-4 (10 ng/ml) (a), or with varying concentrations of exogenous rhIL-4 and a fixed concentration of  $\alpha$ -CD40 mAb (100 ng/ml) (b). The B7/BB1 MFIRs of such cultured B cells (■) are plotted versus the concentration of added  $\alpha$ -CD40 mAb (a) or exogenous rhIL-4 (b). (□) The B7/BB1 MFIR of B cells cultured with CDw32-L cells and a mouse isotype control Ig in lieu of the  $\alpha$ -CD40 mAb.

B7/BB1 MFIR of CLL-B cells cultured with either  $T_h$  cells or  $\alpha$ -CD40 mAbs and CDw32-L cells, further arguing that IL-4 is not required (data not shown).

Changes in the expression levels of other surface antigens also are observed in  $\alpha$ -CD40-stimulated CLL or normal B cells. The CD58 MFIR increased  $\sim$ 14-fold, whereas the CD11a MFIR increased only 1–1.8-fold over that seen in control-treated cells (data not shown). In addition, the CD23 MFIR increased up to 80-fold, similar to the increase observed for CD54 (data not shown). However, the normally high expression levels of class I and II MHC and CD45 on CLL-B cells were not affected by CD40 crosslinking (data not shown).

The kinetics of  $\alpha$ -CD40 mAb or  $T_h$  cell-contact-mediated induction of CD54 or B7/BB1 expression were similar. As with  $T_h$  cell-mediated stimulation, B7/BB1 increases were noticeable only after 18–24 h of stimulation with  $\alpha$ -CD40 plus IL-4, reaching a maximum sevenfold increase in expression level after  $\sim$ 72-h culture (Fig. 7). The increase in B cell expression of CD54 followed similar kinetics, increasing nearly twofold at 6 h, fivefold at 12 h, and achieving a maximum 70-fold increase after  $\sim$ 70 h of culture. B cells cocultured with  $\alpha$ -CD40 and CDw32-L cells for prolonged

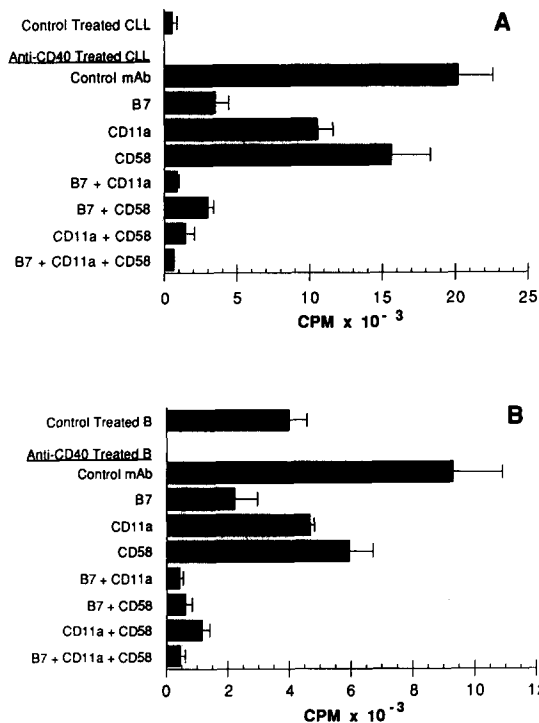


**Figure 7.** Time course of  $\alpha$ -CD40-induced expression of B7/BB1 or CD54. CLL-B cells and CDw32-L cells were cultured with rhIL-4 (10 ng/ml) and 100 ng/ml of  $\alpha$ -CD40 mAb (filled symbols) or mouse isotype control Ig (open symbols). Parallel cultures were harvested at various times for FACS<sup>®</sup> analysis of the live gated CD19<sup>+</sup> B cells. The B7/BB1 MFIRs (squares) are scaled on the left ordinate and the CD54 MFIRs (circles) are scaled on the right ordinate. Each value is plotted versus the number of hours in culture.

periods ( $\geq$ 120 h) did not manifest any further increases in expression of B7/BB1 or CD54 (data not shown). Finally, cell proliferation was not required, as mitomycin C did not affect the ability of B cells to manifest increases in B7/BB1 or CD54 MFIRs upon coculture with  $\alpha$ -CD40 mAbs and CDw32-L cells (data not shown).

**Enhanced Expression of B7/BB1 and other Accessory Molecules Has Functional Significance.** To determine whether the expression of B7/BB1 and other surface molecules induced by CD40 crosslinking enhanced the capacity of B cells to act as stimulatory APCs, we examined resting and  $\alpha$ -CD40-activated CLL- or normal B cells for their ability to present alloantigen to normal T cells derived from unrelated donors. Control Ig-treated or nontreated CLL-B cells are very poor stimulators of allogeneic normal T cells in the MLR (Fig. 8A). Upon coculture with  $\alpha$ -CD40 plus IL-4 and CDw32-L cells, however, CLL-B cells became significantly better presenters of alloantigen, affecting a significant increase in T cell [<sup>3</sup>H]thymidine uptake over that observed in T cells cocultured with control-treated CLL-B cells (Fig. 8A, top two bars,  $p = .002$ ). The ability of normal peripheral blood B cells to stimulate allogeneic T cells also was enhanced significantly by treatment with  $\alpha$ -CD40 presented on CDw32-L cells in the presence of exogenous IL-4 (Fig. 8B, top two bars,  $p = .02$ ).

Various mAbs were added at the beginning of the MLRs to determine whether they could inhibit the APC function of  $\alpha$ -CD40-stimulated allogeneic B cells (Fig. 8, A and B). All blocking reagents were used at concentrations that gave maximal inhibition and/or cell surface staining in preliminary experiments. The proliferation of T cells in response to  $\alpha$ -CD40-stimulated B cells was not affected significantly by addition of control mouse or human IgG of irrelevant specificity (data not shown). However, addition of CTLA4Ig, a recombinant human Ig capable of blocking B7/BB1/CD28



**Figure 8.** Inhibition of allogeneic MLR against  $\alpha$ -CD40-treated leukemic and normal stimulator B cells. CLL (A) or normal (B) peripheral blood B cells were cultured with CDw32-L cells, rhIL-4 (10 ng/ml), and  $\alpha$ -CD40 mAb (100 ng/ml) or mouse isotype control Ig. After 48 h in culture, B cells were separated from adherent CDw32-L cells, treated with mitomycin C and then used as stimulator cells at a 3:1 (responder/stimulator) ratio in an allogeneic MLR. T cell proliferation was measured by the uptake of [<sup>3</sup>H]thymidine into DNA, expressed as cpm  $\times$  10<sup>-3</sup> (*abscissa*). Various culture conditions are listed on the ordinate. Control treated CLL (A) and control treated B (B) represent the T cell proliferation to allogeneic CLL-B or normal B stimulator cells, respectively, previously cultured with CDw32-L cells and control Ig. All other groups represent T cell proliferation affected by  $\alpha$ -CD40-stimulated CLL or normal B cells in the presence of control mAb, CTLA4Ig (B7),  $\alpha$ -CD11a mAb (CD11a),  $\alpha$ -CD58 mAb (CD58), CTLA4Ig and  $\alpha$ -CD11a mAb (B7 + CD11a), CTLA4Ig and  $\alpha$ -CD58 mAb (B7 + CD58),  $\alpha$ -CD11a mAb and  $\alpha$ -CD58 mAb (CD11a + CD58) or CTLA4Ig,  $\alpha$ -CD11a mAb, and  $\alpha$ -CD58 mAb (B7 + CD11a + CD58). There were no significant differences between responses to  $\alpha$ -CD40-stimulated B cells alone or in the presence of control human or mouse IgG (data not shown). Results are reported as the mean cpm of triplicate wells for each condition (error bars = SD).

interactions (41), inhibited 82% of the MLR against stimulated CLL-B cells (Fig. 8 A) and 76% of the MLR against stimulated normal B cells (Fig. 8 B) ( $p < 0.01$ ). Addition of  $\alpha$ -CD11a inhibited T cell proliferation in response to stimulated leukemic or normal B cells by 46 or 50%, respectively ( $p < 0.05$ ) (Fig. 8). In contrast,  $\alpha$ -CD58 mAbs did not significantly inhibit the MLRs, affecting only a 20 or 36% reduction in [<sup>3</sup>H]thymidine uptake by T cells cocultured with stimulated leukemic or normal B cells, respectively. The combination of  $\alpha$ -CD11a together with  $\alpha$ -CD58, however, was strongly inhibitory ( $p < .01$ ), affecting a  $\geq 88\%$  inhibition of MLRs (Fig. 8). Even more effective were combinations of CTLA4Ig and  $\alpha$ -CD11a, with or without  $\alpha$ -CD58

(Fig. 8). Such combinations completely abrogated the allo-APC activity of stimulated leukemic or normal B cells.

We also find that CLL-B cells stimulated by contact with T<sub>a</sub> cells, but not with T<sub>r</sub> cells, exhibit enhanced MLR stimulatory capacity, similar to what was observed with  $\alpha$ -CD40-stimulated CLL-B cells. CLL-B cells were cultured alone or with T<sub>a</sub> or T<sub>r</sub> cells. After 48 h, B cells were isolated to  $>95\%$  purity by magnetic bead depletion of T cells. In MLRs, B cells previously cultured with T<sub>a</sub> cells stimulated allogeneic T cells to incorporate significantly more [<sup>3</sup>H]thymidine ( $9,546 \pm 383$  cpm) than did B cells previously cultured alone ( $2,480 \pm 1,579$ ;  $p = 0.02$ ) or with T<sub>r</sub> cells ( $1,424 \pm 598$ ;  $p < 0.01$ ). It is unlikely that residual T<sub>a</sub> cells were responsible for inducing this increase in MLR stimulatory activity, as the responder T cells in these MLRs were autologous to the T<sub>a</sub>/T<sub>r</sub> cells used to stimulate CLL-B cells in culture. Also, the MLR response against unseparated B-T<sub>a</sub> cocultured cells was less than that against purified B cells alone. As in MLRs using  $\alpha$ -CD40-stimulated B cells, the measured T cell proliferation against T<sub>a</sub> cell-stimulated B cells was reduced by  $\geq 75\%$  in MLR culture wells containing CTLA4Ig (data not shown).

## Discussion

We demonstrate that normal human CD4<sup>+</sup> T cells which are activated by mAb-mediated crosslinking of CD3, but not T cells stimulated with nonspecific control Ig, may induce normal or leukemic B cells to express substantially higher levels of B7/BB1 and CD54, two accessory molecules that are important in T-B conjugate interactions. Such changes are not secondary to the outgrowth of B7/BB1- and CD54-expressing B cells, as mitomycin C-treated B cells that are unable to divide also may be induced to express higher levels of these surface antigens. Also, the changes in B cell phenotype were not secondary to a direct effect of FcR crosslinking due to the solid phase 64.1 mAb, as control Ig of the same isotype as 64.1 (IgG2a) failed to stimulate B cells in T-B cocultures. Furthermore, even the 64.1 mAb did not induce surface phenotype changes in B cells unless CD4<sup>+</sup> T cells also were present. Finally, to affect substantial changes in phenotype, the B cells must be cultured in direct contact with T<sub>a</sub> cells, as evidenced by the failure of soluble factors from the supernatants of T<sub>a</sub> cells or T<sub>a</sub>-B cocultures to induce B cell expression of B7/BB1. Previous studies demonstrated that T<sub>a</sub> cell-induced B cell proliferation and Ig secretion also were contact dependent (22-28). Collectively, these findings indicate that a cell surface molecule(s) may be induced on CD4<sup>+</sup> T cells that can stimulate resting B cells to undergo a variety of phenotypic and/or functional changes.

Crosslinking CD40 induces B cell phenotypic changes similar to those caused by contact with T<sub>a</sub> cells. We find that soluble mAbs against CD40, but not mAbs against any one of a variety of other B cell accessory molecules, can inhibit the T<sub>a</sub> cell-induced increase in expression of B7/BB1 or CD54 on CLL-B cells. On the other hand,  $\alpha$ -CD40 mAbs, when presented on a membrane matrix of CDw32-L cells,



can affect changes in the expression of B cell accessory molecules that mimic those induced by coculture with T<sub>a</sub> cells. Collectively, these studies suggest that CD40 crosslinking may be necessary and sufficient for T<sub>a</sub> cell-induced expression of B7/BB1 and other accessory molecules on resting B cells.

Prior studies also noted that B cell expression of CD54 may be enhanced by treatment with  $\alpha$ -CD40 mAbs (49). Similar to what we observed in the current study, these investigators noted that normal B cells cocultured with soluble  $\alpha$ -CD40 mAbs may express approximately threefold greater levels of CD54 than those of control or untreated B cells. However, we find that  $\alpha$ -CD40 mAbs in the presence of CDw32-transfected L cells may affect a greater than 50-fold increase in normal or leukemic B cell expression of CD54. This augmented effect likely is secondary to the crosslinking of CD40 that is achieved by the  $\alpha$ -CD40 mAb when delivered on a membrane matrix of the CDw32-L cells. This crosslinking may more closely mimic the signal achieved through cognate T<sub>a</sub>-B cell interactions.

Earlier studies demonstrated that crosslinking surface Ig or class II MHC molecules induced resting B cells to express B7/BB1 (16-18). However, the greater than seven or fivefold increase in the expression of B7/BB1 that we observed upon crosslinking CD40 either with  $\alpha$ -CD40 mAb or T<sub>a</sub> cells, respectively, are greater than three times the maximum increase in expression of this surface antigen that we could affect by crosslinking sIgM or HLA-DR. Our results demonstrate the existence of a novel, CD40-dependent mechanism for inducing B7/BB1 expression on B cells that appears more effective than these previously reported stimuli.

Other investigators who studied B cell proliferation and Ig secretion induced by activated T cells also identified surface molecule(s) that may be involved in T<sub>a</sub>-B cell contact-dependent activation. Tohma and Lipsky (26) noted that mAbs that block CD11a/CD54 interactions could inhibit T<sub>a</sub> cell-induced B cell proliferation, with the caveat that T<sub>a</sub> cells unable to express CD11a retained the ability to stimulate B cells. In contrast, we find that  $\alpha$ -CD11a mAb, even at high concentrations, cannot inhibit T<sub>a</sub> cell-induced B7/BB1 expression. Our results are consistent, however, with more recent studies by Noelle et al. (50), who demonstrated that blocking CD40 interaction with its coligand, a type II membrane glycoprotein recently identified on activated T cells (51), could inhibit T<sub>a</sub> cell-mediated B cell DNA, RNA, and Ig synthesis. These results support the hypothesis that the B cell stimulation induced by activated T cells, whether measured as proliferation, Ig synthesis, or changes in surface phenotype, is dependent primarily on a CD40-transduced signal(s).

Previous work demonstrated that  $\alpha$ -CD40 mAb presented by CDw32-L cells could induce either normal or CLL-B cells to proliferate and secrete Ig in the presence of exogenous IL-4 (42, 52, 53). IL-4 is noted to increase B cell expression of CD40 and to act as a costimulatory cytokine for anti-Ig-induced increases in [Ca<sup>2+</sup>], (54). Although this cytokine may enhance the effects of CD40 crosslinking, we find that exogenous IL-4 is not necessary for  $\alpha$ -CD40-induced B cell

expression of B7/BB1. Moreover, neutralizing  $\alpha$ -IL-4 antibody could not inhibit B cells from expressing increased levels of B7/BB1 or CD54 when cultured with either T<sub>a</sub> cells, or CDw32-L cells and  $\alpha$ -CD40 mAbs. Collectively, these studies indicate that IL-4, although capable of augmenting the signalling induced by CD40 crosslinking, is neither necessary nor sufficient to affect the phenotypic changes that we observed on B cells exposed to activated T cells.

In addition to the increased expression of B cell accessory molecules induced by CD40 signalling, a crucial finding of the present work is that such T<sub>a</sub> cell-induced changes have functional significance for B cell APC capacity. Previous studies indicated that small resting B cells are poor APCs for primary responses, but may be effective after the responding T cell population has been activated (7, 55). We find that T<sub>a</sub> cells (or  $\alpha$ -CD40 mAbs and CDw32-L cells) in fact may induce changes in CLL cells or normal resting B cells that make these cells significantly better presenters of alloantigen. In this regard, induced expression of B7/BB1 appears to be a crucial factor. CTLA4Ig, a hybrid recombinant molecule that can bind B7/BB1 and thereby block B7/BB1-CD28 interactions (41, 56), can affect  $\geq 75\%$  inhibition of the MLR between stimulated CLL or normal B cells and allogeneic T cells. Increased expression of CD54, or its coligand CD11a, also may be important, as  $\alpha$ -CD11a mAbs inhibited the MLR  $>45\%$  when used alone, but  $>95\%$  when combined with CTLA4Ig. This conclusion is in agreement with other studies demonstrating that expression of B7/BB1 or CD54 is important in effecting allogeneic T cell proliferation (18, 19, 41, 49, 57). Collectively, these functional studies support the general hypothesis that activated T cells that express the CD40 ligand may not only "help" B cells differentiate into Ig secreting cells, but also may induce B cells into becoming stimulatory APCs of other resting T cells.

In this regard, it is noteworthy that CD40 also may be expressed on thymic epithelial cells (58) and dendritic cells (59), either of which may be involved in T cell development and/or activation. Already, CD40 crosslinking on thymic epithelial cells has been noted to induce production of GM-CSF (58). Conceivably, these cells also may receive inductive signals from CD40 ligand-expressing T cells that could enhance their capacity to act as stimulatory APCs.

That T cells activated by TCR crosslinking acquire the capacity to induce B7/BB1 expression on resting B cells may have implications for the generation of normal immune responses, the maintenance of self-tolerance, and the initiation and/or progression of autoimmune disease. Activated T cells may induce expression of B7/BB1 and other accessory molecules on resting B cells in an antigen-independent manner. Such stimulated B cells may engage nonactivated T cells specific for an antigen(s) presented by such cells. This, in turn, may induce CD40 ligand expression on these previously nonactivated antigen-specific T cells, allowing for an amplification of both the T and B cell immune response. However, should autoantibody-producing B cells interact with activated T cells, then such cells may be able to stimulate T cells that are reactive to B cell-presented self-antigens, similar to B7/BB1-

expressing APCs that encounter T cells specific for non-self antigens (18, 20, 21). Indeed, Lin et al., and Mamula et al. have noted that activation of autoreactive B cells may break T cell tolerance to self-antigens. This mechanism also may contribute to the loss of self-tolerance that sometimes accompanies persistent and/or repeated challenge with antigen, such as in adjuvant- or collagen-induced arthritis (62, 63). In this scenario, not only may autoantibodies be produced, but also, self-reactive T cells may become activated, thus potentially fueling a sustained autoimmune response.

Eynon and Parker (10) noted that *in vivo* challenge with the soluble, monomeric antigen (F[ab] fragments of rabbit- $\alpha$ -mouse IgD) resulted in antigen-specific T cell anergy, whereas challenge with divalent F(ab')<sub>2</sub> rabbit- $\alpha$ -mouse IgD antigen resulted in a vigorous immune response to rabbit Ig. In the absence of sIg crosslinking, they propose that B cells may present antigen in a fashion that induces antigen-specific

T cell anergy. Whatever TCR crosslinking occurs on antigen-specific T cells as a consequence of their interaction with such APCs may not be sufficient to induce these cells into becoming like T<sub>h</sub> cells that are capable of stimulating expression of accessory molecules on neighboring B cells. This is similar to what we noted with B cells cocultured with allogeneic T<sub>r</sub> cells. Even though many T<sub>r</sub> cells may undergo TCR crosslinking upon encounter with alloantigen, these T<sub>r</sub> cells do not induce the B cells to express substantially higher levels of B7/BB1 or CD54. Either allostimulation is not sufficient to induce expression of CD40 ligand on such T cells, or other factors may interfere with the signals transduced by CD40 crosslinking on bystander B cells. Further investigation of the kinetics of B7/BB1 and CD40 ligand-induced expression are required to determine what role this bidirectional signalling pathway might play in the initiation and/or amplification of normal or autoreactive immune responses.

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