CD54/Intercellular Adhesion Molecule 1 and Major Histocompatibility Complex II Signaling Induces B Cells to Express Interleukin 2 Receptors and Complements Help Provided through CD40 Ligation

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Summary

We have examined signaling roles for CD54 intercellular adhesion molecule 1 and major histocompatibility complex (MHC) II as contact ligands during T help for B cell activation. We used a T helper 1 (Th1)-dependent helper system that was previously shown to be contact as well as interleukin 2 (IL-2) dependent to demonstrate the relative roles of CD54, MHC II, and CD40 signaling in the events leading to the induction of B cell proliferation and responsiveness to IL-2. Paraformaldehyde-fixed activated Th1-induced expression of IL-2Rα, IL-2Rβ, and B7, and upregulated MHC II and CD54 on B cells. Anti-CD54 and MHC II mAbs as well as a CD8α-CD40 ligand (L) soluble construct inhibited both the T-dependent induction of Ig secretion, and B cell phenotypic changes. We then compared the effects of activated Th1 cells with that of cross-linking these molecules. Cross-linking of CD54 and MHC II resulted in the upregulated expression of MHC II and of CD54 and B7, respectively, analogous to the effect of fixed activated Th1 cells. B7 expression was further enhanced by co-cross-linking CD54 and MHC II. Cross-linking of CD40 achieved comparable effects. Strikingly, cross-linking ligation of CD54 and MHC II in the presence of IL-5 induced expression of a functional IL-2R on small resting B cells. By contrast CD40 ligation, which induced B cell proliferation, did not induce IL-2 responsiveness. These data show that CD40 ligation is necessary but may not be sufficient for B cell differentiation and identify CD54 and MHC II as contact ligands that can complement CD40 signaling in the generation of T-dependent B cell responses to IL-2.

The induction of Ab responses towards the majority of Ags requires help from Th lymphocytes. B cells present antigenic peptides to CD4+ Th cells whose TCR specificity is directed towards such peptides in the context of class II MHC (1–3). Cell contact occurs during MHC-cognate B-T interactions, and signaling events resulting from this contact have been shown to promote B cell entry into cycle and responsiveness to T-derived cytokines (4–8). The role of cell contact has been extensively studied (reviewed in 6–8), using allogeneic T cells (9–13), Th membranes (14–16), and Th cells whose cytokine production was inhibited (17). The dependence on antigen recognition by the T cell confers antigen specificity on otherwise nonspecific helper signals (6).

The nature of the receptor/ligand pairs that mediate contact signaling during T help for B cells has been the focus of extensive research. Recent cloning of a T cell ligand for CD40 by a number of laboratories demonstrated two characteristics of this molecule that satisfied requirements for its being critical to signal transduction in contact help (6, 18–20).

First, the CD40-ligand (CD40-L)1 molecule is not expressed by resting T cells, whether they be cloned or ex vivo, but is transiently induced by TCR/CD3 signaling (6, 18–21). Second, antibodies against CD40-L, or constructs of soluble CD40 with IgG constant region domains inhibited T-dependent B cell help (18–21), and individuals who genetically lack expression of the CD40-L molecule suffer an immunodeficiency in which antibody responses do not mature to IgG production (22–25). Demonstrations that anti-CD40 mAbs and cells transfected with the CD40-L induce B cell proliferation (6, 20, 26), and that upregulation of B7 expression on B cells can be induced through a CD40-dependent signal (27) and that anti-CD40 mAbs rescued germinal center B cells from apoptosis (28) all contribute to acceptance of CD40-L

1 Abbreviations used in this paper: CD40-ligand, CD40-L; LFA-1, lymphocyte function-associated antigen 1; SI, stimulation index.
as a major ligand for delivery of help (6, 29). These observations support a model for B cell activation whereby antigen presentation to T cells induces CD40-L expression, which then signals B cells through CD40. Experimentally, the requirement for antigen presentation can be bypassed through activation of T cells by anti-TCR/CD3, thereby facilitating direct application of T helper effector functions (6-8). In these systems, contact help suffices to induce high rate Ig secretion. Resting T cells that do not express CD40-L are ineffective as contact helpers (6). However, expression of CD40-L is not by itself sufficient to confer helper activity, at least on T cells immediately isolated to culture (30).

The above model does not include a signaling role for other adhesion molecules. However, under circumstances where CD40-L levels are low (e.g., on primary or ex vivo T cells [30]) one might envisage an accessory role for other receptor/ligand pairs. Candidate ligands on murine B cells include CD54 and MHC II (6, 31, 32). Although levels of expression of adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) are not profoundly affected by T cell activation, there is inferential evidence for conformational changes that may modify their biological activity e.g., through altered avidity (33, 34). There is, therefore, an a priori case for consideration of LFA-1 as a helper ligand on T cells, at least on the basis of its activation dependence for effect. Also, a T-dependent rise in intracellular calcium that was induced in a human B cell line during peptide presentation to a T cell clone was inhibited by mAb to CD18 on the T cell or CD54 on the B cell, further demonstrating signaling through this ligand pair during T-B interactions (35). Ligation of MHC II is intimately associated with T cell activation, and numerous reports have described signaling via MHC II on B cells (36, 37). In previous work we described a Th1 clone that induced a high frequency of small resting allogeneic B cells to enter cycle and secrete Ig (9, 10). The T-B interaction was contact dependent, and did not involve either Ag or MHC recognition by the T cells. Significantly, this response was inhibited by anti-LFA-1 and CD54 mAbs that did not affect B cell responses to mitogens, T cell activation to cytokine secretion, or the formation of T-B conjugates (31).

We have now further examined the role of CD54 and MHC II in contact help and have compared it with the effects generated by the ligation of CD40. We show a signaling role for CD54 and MHC II in the induction of cytokine receptors. This may complement help provided to B cells through CD40 ligation, or independently of the latter, might constitute a possible alternative pathway of B cell activation.

Materials and Methods

mAbs and Soluble Constructs. mAbs used were F23.1 (mouse IgG2a, anti-TCR Vβ8) (38), 30H12 (rat IgG2a, anti-Thy-1.2) (39), F7D5 (mouse IgM anti-Thy-1.2) (40), KT4 (rat IgG2a, anti-TCR Vβ4) (41), TRFK-5 (rat IgG1, antimouse IL-5) (42), 172.4 (rat IgM, antimouse CD4) (43), HO2.2 (mouse IgM anti-Lyt-2.2) (44), PC61 (rat IgG1, antimouse IL-2Rα) (43), TM81 (rat IgG2a, antimouse IL-2Rα) (45), 145-2C11 (hamster IgG antimouse CD3ε) (46), S4B6 (rat IgG2a, antimouse IL-2) (47, 53-6.7.2 (rat IgG2b, antimouse Lyt-2 [CD8ε]) (39), GK1.5 (rat IgG2b, antimouse CD4) (48), P7/7.1 (rat IgG2b, antimouse I-A, I-E) (49), YN1/1.7.4.1 (rat IgG2b, antimouse CD54 intercellular adhesion molecule 1 [ICAM-1]) (50). Antibodies were either used as supernatants or were affinity-purified from culture supernatants using Protein G-Sepharose (Pharcma LKB, Montréal, Québec, Canada). A hybridoma secreting soluble mCD40-L-mCD8α chimeric protein (26) was kindly provided by Dr. P. Lane (Basil Institute for Immunology, Basel, Switzerland), and chimeric molecules expressing human-ICAM constant domains and extracellular portions of the mouse CTLA4 gene (51) or mouse CD40 gene were kindly provided by Dr. P. Linsley (Bristol Myer Squibb, Seattle, WA) and by Dr. M. Kehry (Boehringer Ingelheim Corp., CT), respectively.

B Cells. Spleen cells from C57BL/6 female mice (Charles River Laboratories, St-Constant, Québec, Canada) were depleted of red blood cells by hypotonic lysis, then depleted of T cells by two rounds of treatment with anti-T cell mAb (172.4 + F7D5 + HO2.2) and rabbit complement (Cedarlane, Hornby, Ontario, Canada). Cells were then fractionated on discontinuous Percoll (Pharcma LKB) gradients. Cells that sedimented between 1.08 and 1.09 g/ml were used in all assays. They were <1% Thy-1.2+, >90% surface Ig+, and homogeneously small.

T Cells. The CBA-derived hapten-specific CD4+ Vβ8+ Th1 cell clone E9.D4 (9) was used after periods of 2-5 d resting culture, in the absence of Ag, filler cells, or IL-2. Cells were mycoplasma-free and irradiated (2,000 rad) before use in experiments. Screening for mycoplasma was carried out by 6-methylpurindeoxyriboside cytotoxicity (Mycotect; GIBCO BRL, Burlington, Ontario, Canada). When needed, cells were fixed using 0.8% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) followed by 0.2 M lysine (Sigma Chemical Co.).

B Cell Activation. B-T cocultures were established in RPMI 1640 (Flow Laboratories, Mississauga, Ontario, Canada) supplemented with 5% FCS (Flow), 2 mM l-glutamine (GIBCO BRL), 5 x 10^-5 M 2-ME (Sigma Chemical Co.), and penicillin (100 U/ml)-streptomycin (100 μg/ml) (GIBCO BRL), in triplicate, by the addition of 10^6 B cells to 6 x 10^4 irradiated E9.D4 cells in F23.1-coated microwells (10 μg/ml) in PBS with or without cytokines. When using fixed T cells, B-T cocultures were set up in round-bottom microplates. In blocking assays, soluble mAbs or constructs were added at the onset of the B-T coculture. LPS (Salmonella arora; Difco, Detroit, Michigan) was added at 20 μg/ml. Purified anti-CD4 (NYT/1.7.1.1), anti-MHC II (P7/7.1), or anti-CD8α (53-6.7.2) were either coupled to biotin using biotinamidocaproyl hydroy-succinimide ester (Sigma Chemical Co.) or coupled to CNBr-activated Sepharose 4B beads (Pharmacia LKB) at 1 mg/ml. Biotinylated mAb (10 μg/ml) and streptavidin (Sigma Chemical Co.) or coupled beads (20 μl from a 20% suspension) were added to 10^6 B cells, in triplicate cultures, with or without cytokines or CD8α-CD40-L soluble construct.

Cytokines. Supernatants from the X63-Ag8.653-mIgL2 and -mIL-5 plasmacytomas were used as sources of IL-2 and IL-5, respectively (52). The IL-2 content of supernatants was calibrated by bioassay using the CTLL-6 line. To assess IL-5 activity, the induction of Ig secretion by resting B cells was measured by ELISA. Supernatants were compared to recombinant murine IL-5 (Genzyme, Markham, Ontario, Canada) and contained ~500 U/ml IL-5. The addition of the anti-IL-5 mAb TRFK-5 confirmed the specificity of these responses.

Assessment of B Cell Activation. Entry into cell cycle was assessed by measurement of incorporation of [3H]thymidine (specific activity 6.7 μCi/mM; ICN Biomedicals Canada Inc., Mississauga, Ontario, Canada) after a 6-h pulse of 0.5 μCi/microculture at 2 d of culture. Ig secretion was assessed using a modified ELISA assay.
Briefly, cultures were washed, then cultured for 4 h in 100 μl total volume, after which the total Ig content of 50 μl aliquots of supernatant was measured by ELISA, using peroxidase-coupled sheep anti-mouse Ig (Bio/Can Scientific, Toronto, Ontario, Canada), and goat anti-mouse Ig (Antibodies, Inc., Davis, CA) as a capture reagent. This ELISA was equivalently sensitive to IgM, IgG1, IgG2, IgG3. Conversion of optical densities (405 nm) to Ig concentrations was by SLT 400 AT ELISA software (Fisher Scientific, Montréal, Québec, Canada) using a titration of affinity-purified F23.1 mAb as reference. The sensitivity range for this assay was between 250 and 17 ng/ml. This ELISA measures levels of Ig secretion equivalent to those detected in a Protein A plaque assay (9).

**Flow Cytometry.** Cells were collected from culture, washed, and filtered through a nylon mesh (B. & S.H. Thompson, Ville Mont-Royal, Québeck, Canada) to remove Sepharose beads where appropriate. Cells were then stained by successive 20-min incubations at 4°C with biotinylated mAb or mAb directly coupled to FITC, at concentrations ranging between 5 and 20 μg/ml. Secondary reagents used were PE (Southern Biotechnology Associates, Birmingham, AL) or FITC-coupled streptavidin (Bio/Can Scientific). mAb were fluorescinized using FITC-Celite (Sigma Chemical Co.). Fluorescence was analyzed using a FACSscan® (Becton-Dickinson Canada Inc., Mississauga, Ontario, Canada). Dead cells were excluded on acquisition by a combination of propidium iodide staining and side scatter gating.

**Results**

Activated Th1 cells induce B cell proliferation and high rate Ig secretion. This is dependent on contact between T and B cells, and the induction of Ig secretion is IL-2 dependent (9, 10). In previous studies, anti-CD54 and anti-MHC II mAbs partially inhibited T-dependent B cell proliferation, and strongly inhibited Ig secretion (31). We have previously shown that small resting B cells do not respond to IL-2 and that neither mRNA for or surface expression of IL-2R proteins are detectable on the small resting B cells that we study (reference 33 and Fig. 1, A and B). This Th1 system therefore provides us the opportunity to examine induction rather than upregulation of a cytokine receptor; that is to say, a qualitative rather than quantitative effect.

We first characterized B cell phenotype changes induced by T contact, and the effects of CD54, MHC II, and CD40 ligation on these.

**Contact with Activated T Cells Upregulates IL-2 Receptors and Other Surface Markers on B Cells.** Fig. 1 shows the effect of 2 d T-B coculture on the expression of IL-2 receptor and other molecules by B cells. Paraformaldehyde-fixed activated T cells induced upregulation of both IL-2α and β chains (Fig. 1, A, B, F, and G), peak fluorescence increasing by 1 and 1/2 log over background, respectively. The level of expression of IL-2α was equivalent to that induced by LPS (53, 54).

Both MHC II and CD54 were also upregulated. In Fig. 1 the level of expression of MHC II increased from a peak fluorescence intensity of 30 to 200, and the peak fluorescence intensity for CD54 increased from 350 to 900. Comparable upregulations were observed in other experiments. Expression of the costimulator molecule B7 on B cells was induced from undetectable levels by coculture with activated T cells (Fig. 1). Supernatants from activated Th1 cells did not induce either chain of the IL-2R on B cells, and it was previously shown that such supernatant did not induce MHC II upregulation (9). Upregulation of these molecules was detectable after overnight coculture, usually as strongly as at 2 d as in Fig. 1. For comparison of surface phenotype changes with proliferative responses and responses to cytokines we routinely assessed phenotype changes at 2 d.

**T-dependent Induction of Activation Markers on B Cells Is Inhibited by Blocking CD54, MHC II, and CD40.** We then assessed the effects of reagents directed at CD54 and MHC II on the B cell surface phenotype changes induced by T contact. Both mAbs abrogated IL-2Rβ expression. The upregulation of IL-2Rα was inhibited by both mAbs and there was...
IL-2R BETA IL-2R ALPHA CD54 MHC II B7

Figure 2. CD54, MHC II, and CD40 blockade inhibits B cell activation. B cells were cultured with fixed Th1 cells either alone (control), or with the addition of anti-MHC II or anti-CD54 (10 μg/ml), or with the CD8α-CD40-L soluble construct (25% supernatant), and stained after 2 d in culture as described for Fig. 1. The effects of anti-MHC II and anti-CD54 being the same, only those generated by the anti-MHC II mAb are shown. The presence of anti-MHC II or -CD54 mAbs in culture impeded subsequent visualization of these molecules, and are therefore not shown.

no significant effect of either mAb on MHC II or B7 expression (Fig. 2). However, anti-MHC II almost completely inhibited the upregulation of CD54. Isotype-matched control mAbs (anti-Vβ4) did not inhibit. The combination of both mAb induced the same effect as each mAb alone (not shown).

The effects of anti-CD40 reagents were similar, with a few exceptions. The CD8α-CD40-L construct inhibited to background levels the upregulation of IL-2Rβ and IL-2Rα induced by activated Th1 on B cells. Expression of CD54 was reduced by half a log by CD8α-CD40-L, and B7 expression was slightly inhibited. MHC II fluorescence was not affected by CD8α-CD40-L. A control plasmacytoma (SP2/0) supernatant had no effect.

T-dependent B Cell Entry into Cycle and Differentiation Is Inhibited by Reagents against CD54, MHC II, and CD40. Fig. 3 A confirms previous results showing that anti-CD54 and anti-MHC II inhibit T-dependent B cell activation. Whereas an isotype-matched control mAb (anti-Thy-1.2) reduced Ig secretion from 220 ± 10 ng/ml to 140 ± 50 ng/ml, anti-MHC II reduced the response to 60 ± 1 ng/ml and anti-CD54 to 40 ± 10 ng/ml. In a separate experiment, the induction of Ig secretion was reduced by 65% when both mAb were used in combination, compared with 25-40% reduction by each mAb alone. In agreement with previous results, proliferative responses to optimally activated T cells were less strongly and more variably affected. Addition of an isotype matched anti-CD4 mAb gave similar results to the effect produced by anti-MHC II (not shown) when using fixed activated T cells. None of the mAb used had any effect on responses to LPS.

Inclusion of CD8α-CD40-L in T-B cocultures strongly inhibited B cell responses (Fig. 3, B and C). There was no

Figure 3. T-dependent induction of B cell entry into cycle and Ig secretion is inhibited by CD54, MHC II, and CD40 blockade. B cells were cocultured with activated Th1 cells. Controls included T cells alone (T), B cells alone (B) and LPS (20 μg/ml). For the Ig secretion data, LPS responses are shown as 50% of the final value. Incorporation of [3H]Tdr was measured at 2 d (6) and high rate Ig secretion by ELISA at 4-5 d (A and C). In A, purified anti-CD54, anti-MHC II, or anti-Thy 1.2 mAbs were added at 10 μg/ml at the initiation of culture and Ig secretion measured at 4 d. CD8α-CD40-L was added at the initiation of culture at 25% hybridoma supernatant, with supernatant from the SP2/0 plasmacytoma as control (SP2/0 Sn). Data in all panels represent means of triplicate cultures. Bars show SD.
effect of a control plasmacytoma (SP2/0) supernatant (Fig. 3), and the construct did not inhibit the response of B cells to LPS (not shown). This construct, as well as the other reagents used in blocking assays, did not inhibit T cell activation, as assessed by lack of effect on IL-2 secretion in response to anti-TCR stimulation. Proliferative responses were not totally inhibited by CD40 blocking, raising the possibility that other molecules are also implicated in the induction by T cells of this B cell response.

Induction of Functional IL-2R by CD54 and MHC II Ligation. When CD54 and MHC II were co-cross-linked by mAbs coupled to Sepharose beads, immunoglobulin secretion was induced by mixtures of IL-2 and IL-5 (Fig. 4 A). There was no augmentation of the usual low-level response to IL-5 alone, nor any response to IL-2 alone (Fig. 4 A). CD54 and MHC II co-cross-linking with Sepharose-coupled mAbs enhanced expression of IL-2Rα over that induced by IL-5 and induced IL-2Rβ (Fig. 4 B). These responses were therefore IL-2-driven. IL-5 by itself induces IL-2Rα on B cells, but does not induce IL-2 responsiveness, and was previously shown to synergize with LPS in the induction of functional IL-2 receptors on B cells (53, 55). By contrast to CD54 and MHC II, cross-linking of CD40 using the CD8α-CD40-L construct (26) and anti-CD8–coupled Sepharose beads, did not induce responsiveness to IL-2 either alone or in presence of IL-5 (not shown). The cross-linking ability of anti-CD8–coupled beads was independently confirmed by immunoprecipitation of CD8 from lymph node T cells. Cross-linking CD40 using CD8α–CD40-L and biotinylated anti-CD8 with streptavidin was similarly ineffective at inducing IL-2 responses.

IL-2Rα was slightly upregulated by CD40 ligation (not as strongly as IL-5, LPS, or activated Th1 cells) but this was not sufficient to induce responsiveness to IL-2 (see Fig. 4). The combination of CD40 ligation with IL-5 did not augment IL-2Rα induction by IL-5, and there was no detectable effect of either treatment on levels of expression of IL-2Rβ. Cross-linking CD54 or MHC II using biotinylated mAb and streptavidin did not affect expression of IL-2Rα or IL-2Rβ.

Effects of CD54, MHC II, and CD40 Ligation on B Cell Surface Phenotype. We also analyzed the effect of contact ligand cross-linking on the expression of MHC II, B7, and CD54. We used biotinylated reagents and streptavidin as a cross-linker both for comparison between treatments and to facilitate coaggregation of B cell ligands. Cross-linking of CD54 upregulated MHC II expression on B cells (Fig. 5 A), but did not affect B7. MHC II cross-linking induced small increases in CD54 and B7. Neither cross-linking of CD11a or CD18, both isotype-matched mAbs, induced these effects. Cross-linking of CD40 upregulated both MHC II and B7 on B cells, as has previously been described (27) (Fig. 5 B). The upregulation of B7 was enhanced by the inclusion of IL-5 (Fig. 5 B), which by itself did not affect B7 expression. IL-5 also synergized with CD40 cross-linking to increase CD54 upregulation. Effects of CD40 cross-linking were not reproduced by SP2/0 supernatant, or by anti-CD8α, with or without streptavidin.

We then further examined the effects of coaggregating pairs of contact ligands. Co–cross-linking CD54 with MHC II induced a greater increase in B7 level than MHC II alone, and this was enhanced by IL-5 (Fig. 5 A). These levels were comparable to those obtained by cross-linking CD40 and were not further enhanced by co–cross-linking CD54 and MHC II with CD40. Coaggregation of CD40 with CD54 or MHC II did not potentiate the phenotypic changes induced by CD40 ligation. The inclusion of IL-5 in these incubations had no effect beyond that of CD40 ligation alone.

Although CD40 seemed more potent than CD54 or MHC II alone at inducing activation-related B cell phenotype changes, coligation of CD54 with MHC II amplified the effects of these ligands and generated signals comparable to IL-2Rα and IL-2Rβ.
those induced by CD40. Furthermore, coligation of CD54 and MHC II delivers signals that are very distinct from that generated through CD40, in that functional IL-2R could not be upregulated through binding of this ligand.

Entry of B Cells into Cycle Induced through CD40 Cross-linking. To further examine the relative roles of CD54, MHC II and CD40 in B cell activation, we assessed their involvement in inducing B cell entry into cycle. Cross-linking CD40 using CD8α-CD40-L and biotinylated anti-CD8 with streptavidin induced significant thymidine incorporation. Stimulation indices of 15–20 were obtained, over background responses of <200 cpm. This is consistent with results of others who have used the same construct (26) (Table 1). Coaggregation of CD54 and MHC II only induced a 1.5–3-fold increase in thymidine incorporation. Other modes of cross-linking the molecules (plastic-adsorbed or Sepharose-coupled mAbs) were no more effective than using biotinylated mAbs.

These results indicate a primary signaling role for CD54 and MHC II in contact help, in the induction of a functional IL-2R on B cells. There is a fundamental distinction between CD54, MHC II, and CD40, in that the latter on its own induced entry into cycle but did not appear to influence IL-2
Table 1. 

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<th>Experiment</th>
<th>No addition</th>
<th>+ CD8α–CD40–L</th>
<th>SI*</th>
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<tr>
<td>1</td>
<td>240 ± 30</td>
<td>3,530 ± 270</td>
<td>15</td>
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<td>190 ± 5</td>
<td>3,280 ± 130</td>
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<td>4</td>
<td>60 ± 10</td>
<td>905 ± 65</td>
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Small resting B cells were either cultured alone (No addition) or with the CD8α–CD40–L soluble construct in presence of biotinylated anti-CD8α and streptavidin (+CD8α–CD40–L). [3H]Thymidine incorporation was measured at 2 d. Results are shown as means of triplicate cultures (cpm ± SD).

* SI, Stimulation index.

responsiveness. These results therefore demonstrate complementarity between contact ligands in signaling during T help for B cell activation.

Discussion
We have shown that MHC II and CD54 ligation induces IL-2R expression on B cells and responsiveness to IL-2. Our results indicate interdependence between contact signaling molecules that play a role in T-dependent B cell activation. Whereas cross-linking ligation of CD40 induced thymidine incorporation, and blocking CD40 strongly inhibited a wide range of B cell responses, MHC II and CD54 ligation had weak effects on thymidine incorporation. Coligation of MHC II with CD54 induced surface phenotype changes similar to those induced through CD40, consistent with B cell activation. This shows a signaling role for CD54 and MHC II that could complement help provided through CD40 ligation. Although the evidence to date (including this study) identifies CD40 as a critical contact ligand, our results show that CD40 blockade did not inhibit MHC II upregulation by activated Th contact, and did not totally inhibit proliferation, from which it can be inferred that CD40 is not the sole ligand for B cell activation. It was on this basis and because of our own previous results showing a role for CD54 and MHC II in T help that we compared CD54 and MHC II with CD40 as ligands in contact help.

The system that we have studied, in which a Th1 clone induces Ig secretion by resting B cells, may be considered to mimic the cellular contact events that occur during cognate T–B interaction. A role for Th1 helpers in protective antibody responses has been shown in a variety of in vitro and in vivo systems (56). In our system, induction of B cell proliferation occurs independently of cytokines, whereas Ig secretion is both contact and cytokine (IL-2) dependent (6–8). This allows us to study the induction of IL-2 responsiveness by B cells, given the inability of resting B cells to respond to this cytokine (53). Our own studies have shown upregulation of IL-2R proteins during B cell activation, and we have also shown a requirement either for synergy with Th2 cytokines or for continued contact for the maintenance of a functional receptor (reference 53, and Poudrier, J., and T. Owens, unpublished observations). The present study identifies the B cell surface molecules CD54 and MHC II, both ligated during contact with activated Th1, as signal transducing ligands for IL-2R upregulation.

It is of particular interest that CD40 ligation did not induce functional IL-2R, whereas MHC II and CD54 did. Technical reasons for this discrepancy are unlikely, since CD40 ligation by a number of approaches was equally unsuccessful, including using anti-CD8 coupled to Sepharose beads to cross-link CD8α–CD80–L, thereby mimicking the optimal conditions for MHC II and CD54 effect. In a separate study we have shown that very low levels of IL-2Rβ were sufficient to induce responses to IL-2, provided that IL-2Rα was adequately induced (53). In this study, IL-2Rα was weakly induced by CD40 cross-linking, but IL-2Rβ was undetectable. The resultant level of heterodimeric IL-2 receptor was obviously insufficient for response. CD40 appears most potent at transducing signals for proliferation, as indicated by its unique ability to induce thymidine incorporation, but other signals are required for IL-2-dependent B cell differentiation into an Ig-secreting cell. Since B cell proliferative responses do not require a functional IL-2R, the inability of CD40 to induce this molecule would not impinge on its role in inducing proliferation. MHC II and CD54 may therefore be distinguished from CD40 on the basis of their involvement in IL-2-dependent differentiation to Ig secretion.

In studies with human B cells, signaling through CD40 synergized with a number of cytokines, including IL-2, IL-4, and IL-10, to induce IgG secretion (57). However, it is noteworthy that neither IL-2 nor IL-10 synergized with CD40 when murine B cells were studied (58), consistent with our observations. We have confirmed that IL-4 promotes Ig secretion by murine B cells that had been stimulated with CD8α–CD40–L (Poudrier, J., unpublished observations). There are two points that require emphasis. The first is the fundamental distinction between Th1 and Th2 cytokines, whereby resting B cells express receptors for and respond to IL-4 and IL-5, but show no response to IL-2. Our study exploits this all-or-none situation to identify contact ligands that are involved in the IL-2-dependent IgG production that is a feature of this Th1 helper system. The second point concerns the mode of presentation of CD40-Ls to B cells. In the majority of studies, CD40-L and anti-CD40 mAbs have been presented either via transfection of cell lines (20), or through association with FeRα cells (27, 59). This has the potential to profoundly influence B cell response. We have shown that murine L cells secrete soluble factor(s) that synergize with IL-2 to induce murine B cells to express IL-2R and to produce IgG, although supernatants had no effect by themselves (32). Analogous effects of CV1-EBNA or L cells may have contributed to CD40-dependent IgG secretion in studies where these were used (20, 59).

Related to this, when CD40-Ls are presented on a cell surface, other ligands might also contribute to intercellular signaling. The proliferative responses that we obtained with
CD8α–CD40-L were similar (equivalent stimulation indices [SIs] with lower backgrounds) to those obtained by Lane et al. (26) who used the same construct. In their study, anti-Ig–Sepharose further amplified responses by 10–20-fold. Significantly, the same construct only induced threefold proliferative responses by human B cells, which were enhanced to sixfold by anti-IgD (26). This contrasts with results from Armitage et al. (57), who induced 20-fold or greater proliferative responses in human B cells by CV1-EBNA cells transfected with either human or mouse CD40-L. It becomes important to consider reasons for such discrepancy in the proliferative responses of human B cells. Our results show soluble CD40-L to be insufficient to induce cytokine-dependent progression to Ig secretion. The ability of CD40 signaling to synergize with cytokines for differentiation to Ig secretion in other studies may reflect the complex plethora of ligand-counter-ligand interactions that accompany CD40 ligation by transfected or FcR+ cells. Our previous results (31, 32) argue for a complementary role for adhesion molecules and MHC II in intercellular contact signaling. Signaling through these and other nonpolymorphic molecules that have ligands on CV1-EBNA cells could account for the greater responses to CD40-L–transfected cells. Our demonstration of induction of functional IL-2R expression through MHC II and CD54 ligation lends weight to and provides a mechanistic basis for such interpretation.

Finally, it should be kept in mind that both the absolute responses and the SI's obtained in thymidine incorporation assays using soluble CD40-L constructs and murine B cells by Lane et al. (26) and by ourselves were the same as or greater than those obtained by Armitage et al. (57). This might argue that CD40 signals are sufficient for proliferative responses of murine B cells, independent of other ligands. Nevertheless, synergy between ligands may contribute to the overall level of signaling during T help.

The lack of consensus as to whether CD54 and MHC II can transduce signals during T help for murine B cell activation may relate in part to the assays used. Noelle et al. (19) found no inhibition by antibodies against CD54 or MHC II when RNA synthesis induced by Th contact was assayed. We have shown strong inhibition of Ig secretion, but only partial inhibition of thymidine incorporation (31), and in the present study, there was again disparity between effects of these mAbs in different assays. The fact that anti-CD54 and anti-MHC II inhibited the induction of IL-2R proteins is consistent with their strong effect on Ig secretion and their relative lack of effect on proliferation. The profound inhibition by CD8α–CD40-L of IL-2R upregulation is not discordant with its inability to induce a functional IL-2R through CD40 ligation. CD40 is probably necessary but not sufficient for IL-2 responses. Because cross-linking of every ligand tested had a stimulatory effect of one kind or another, mAb inhibitions can be taken to reflect blockade of signaling interactions. The cell clustering that is a hallmark of T-B coculture systems was not inhibited in our experiments, arguing against gross steric effects. Negative signaling through MHC II was excluded by the fact that anti-CD4 mAbs were inhibitory.

The latter observation implicates CD4 as the ligand for MHC II, as predicted from studies showing adhesion mediated by this ligand–receptor pair (60). Effects of anti-CD4 or anti-MHC II on T cell activation could be unequivocally excluded because T cells were paraformaldehyde-fixed, in contrast to previous studies using live T cells.

Our data extend observations by Watts et al. (61) that MHC-restricted T-B interactions induced B7 expression by transformed B cell lines and also confirm observations of MHC II induction of B7 by Kouvelova et al. (62). We show that B7 was induced in nontransformed B cells through non-MHC-restricted T-B interaction, and by CD40 ligation, thus supporting Watts et al.'s (61) prediction that the CD40-L might contribute to B7 induction. The level of B7 upregulation on B cells was directly proportional to concentrations of anti-Vβ8 used to activate the T cells (Poudrier, J., unpublished observations). We have thus shown that T–B contact can induce B7 via at least two signaling pathways. CD40 ligation was also implicated in B7 induction by Ranheim and Kipps (27). We have further resolved the question of whether MHC II signals for B7 induction, by dissociating a role for MHC II in induction of T cell activation vs. a signaling role for B cells. More directly, we have been able to induce B7 by cross-linking MHC II with biotinylated mAb and streptavidin. Our own and Watts et al.'s data argue that only activated T cells should be effective in B7 induction, so that T-dependent induction of B7 will occur relatively late in the response. This is consistent with a model for T–B interaction in which the primary signal for B7 induction on B cells is surface Ig (sIg) ligation (63), which facilitates the initial B-dependent T cell response. Primary T cell activation probably occurs through recognition of antigen on non-B cell APC (7), which may reduce the threshold T cell dependence on B7 costimulation for subsequent response. During T–B contact, TCR–CD4 interaction with MHC II then delivers signals that simultaneously upregulate CD40-L on the T cell, and through MHC II/CD54 co-cross-linking upregulate B7 and MHC II on the B cell. The CD40-L–CD40 interaction further enhances B7 upregulation. Cross-talk between ligands is indicated by the fact that ligation of CD40 and CD54 upregulated MHC II, CD40 and MHC II ligation upregulated CD54, and CD54 and MHC II coligation upregulated B7 as strongly as did CD40. These molecular upregulations serve to strengthen the cellular interaction and so augment subsequent signaling.

Can these noncognate ligands bypass a role for antigen in B cell activation? Although CD54 ligation could in principle occur independently of antigen recognition, LFA-1 on T cells may require activation for effective signaling (34). Furthermore, our data show that CD54 ligation alone only upregulates MHC II. Similarly, MHC II ligation may occur whenever B cells encounter a CD4-bearing cell. But, our use of cross-linking mAbs might have amplified MHC II signaling beyond what is normally achieved through CD4 ligation, and TCR–MHC II interactions are probably more effective. Also, as for CD54, MHC II cross-linking alone does not itself drive functional responses, but induces B7 upregu-
ulation, and so only predisposes B cells to interact with T cells. B7 expression by B cells is not sufficient to upregulate CD40-L expression by T cells, either alone or in conjunction with suboptimal anti-TeR stimulation (Poudrier, J., unpublished observations). Coaggregation of these ligands induces functional cytokine receptors, but only through synergy with other cytokines, and so is dependent on T cell activation for signaling, and effectively predisposes to receipt of help. T cells that are sufficiently activated to produce cytokines would also express CD40-L, whose effects would overshadow those of MHC II and CD54. Effective cytokine production probably only occurs at short range, e.g., through cognate T-B interaction. Bystander activation may occur by this complex of signals, and this in fact is what our in vitro experimental system induces.

The signaling role that MHC II and CD54 play is analogous to but distinct from the central role played by CD40. This raises the possibility that CD54 and MHC II could constitute a CD40-independent pathway of B cell activation. This might operate during interaction with cells that do not express the CD40 ligand, such as FDC. We have shown the ability of CD54 to synergize with cytokines. Both of these stimuli are present in the germinal center and may contribute to the generation of high affinity Ab responses and B cell memory.

A synthesis of our and other results would suggest that proliferation in the germinal center (64) is primarily a slg-dependent event, which precedes and is necessary for subsequent affinity maturation and generation of memory. This is dependent on T-B interaction during which the contact ligands that we have described contribute to further proliferation, as well as induction and maintenance of cytokine responsiveness. By linking levels of functional expression of adhesion/accessory molecules to the state of T and B cell activation the immune response has exploited lymphocyte cell biology to control the antigen specificity of the antibody response.

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