

## Immunoglobulin Signal Transduction Guides the Specificity of B Cell-T Cell Interactions and Is Blocked in Tolerant Self-reactive B Cells

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### Summary

The specificity of antibody (Ab) responses depends on focusing helper T (Th) lymphocyte signals to suitable B lymphocytes capable of binding foreign antigens (Ags), and away from nonspecific or self-reactive B cells. To investigate the molecular mechanisms that prevent the activation of self-reactive B lymphocytes, the activation requirements of B cells specific for the Ag hen egg lysozyme (HEL) obtained from immunoglobulin (Ig)-transgenic mice were compared with those of functionally tolerant B cells isolated from Ig-transgenic mice which also express soluble HEL. To eliminate the need for surface (s)Ig-mediated Ag uptake and presentation and allow the effects of sIg signaling to be studied in isolation, we assessed the ability of allogeneic T cells from bm12 strain mice to provide *in vivo* help to C57BL/6 strain-transgenic B cells. Interestingly, non-tolerant Ig-transgenic B cells required both allogeneic Th cells and binding of soluble HEL for efficient activation and Ab production. By contrast, tolerant self-reactive B cells from Ig/HEL double transgenic mice responded poorly to the same combination of allogeneic T cells and soluble HEL. The tolerant B cells were nevertheless normally responsive to stimulation with interleukin 4 and anti-CD40 Abs *in vitro*, suggesting that they retained the capacity to respond to mediators of T cell help. However, the tolerant B cells exhibited a proximal block in the sIg signaling pathway which prevented activation of receptor-associated tyrosine kinases in response to the binding of soluble HEL. The functional significance of this sIg signaling defect was confirmed by using a more potent membrane-bound form of HEL capable of triggering sIg signaling in tolerant B cells, which markedly restored their ability to collaborate with allogeneic Th cells and produce Ab. These findings indicate that Ag-specific B cells require two signals for mounting a T cell-dependent Ab response and identify regulation of sIg signaling as a mechanism for controlling self-reactive B cells.

The ability to distinguish between self and foreign Ags is a central feature of immune recognition, allowing immunity to be acquired against foreign organisms while avoiding destructive autoimmunity. Studies of self-nonsel discrimination in recent years (for reviews see references 1–3) have confirmed the clonal selection theory proposed by Burnet (4), which hypothesized that foreign Ags provoke immunity by triggering clonal expansion and differentiation of Ag-binding B and T lymphocytes, whereas self-Ags induce tolerance by triggering elimination or inactivation of self-reactive cells. Although these advances provide a cellular framework for understanding self-nonsel discrimination, the molecular mechanisms that dictate the choice between lymphocyte ac-

tivation or tolerance nevertheless remain to be defined. Understanding the molecular basis for these cellular decisions will be important for controlling the immunogenicity or tolerogenicity of vaccines, tumors, and tissue transplants, and for understanding the breakdown of self-tolerance in autoimmune diseases.

The decision between lymphocyte activation and tolerance seems particularly approachable in B lymphocytes, since much is already known about the cellular and molecular events causing B cells to proliferate and differentiate into Ab-secreting plasma cells in response to foreign Ags. For many foreign Ags, Th cells play a key role in promoting B cell proliferation and Ab production, by adhering to suitable B cells and

transmitting a set of B cell growth and differentiation signals. One of the most important signals is an integral membrane protein, CD40-ligand (CD40L)<sup>1</sup>, that is transiently displayed on the surface of activated Th cells and triggers B cell proliferation through a receptor, CD40, constitutively expressed on B cells (5, 6). Other molecules secreted by activated Th cells, such as IL-4 and -10, synergize with CD40L in promoting B cell proliferation and in triggering isotype switching and differentiation into plasma cells (for a recent review see reference 7).

Since the receptors for CD40L, IL-4, and IL-10 are constitutively expressed on all B cells, development of an effective Ab response depends on preferential delivery of these helper signals to useful B cells whose surface Ig (sIg) bind foreign Ag, and not to useless B cells that do not bind Ag or to potentially harmful B cells that bind self-Ags. The specificity of T cell help is directed, at least in part, by the requirement that Th cells recognize a complex composed of foreign Ag peptide fragments and class II MHC molecules, that is displayed on the surface of potential B cell targets (8–12). Recognition of peptide–MHC complexes by the TCR strengthens the interaction between T and B cells (13), triggers CD40L expression (6, 14), and polarizes IL secretion to the point of synapsis between T and B cells (15, 16). Since B cells that bind foreign Ags are more efficient at internalizing these Ags than other nonbinding B cells, Ag-specific B cells are most likely to present foreign peptide fragments and trigger delivery of T cell help (for a review see reference 12). Other receptor/ligand pairs also contribute to the synapsis of T and B cells, such as the binding of LFA-1 on both T and B cell to intercellular adhesion molecule 1 (ICAM-1) on the opposing cell (13, 17, 18) or the binding of CD28 on the T cell to B7 on the B cell (19), but their role in guiding the specificity of T–B cell interaction is not known.

The molecular mechanisms responsible for B cell tolerance are not well understood, especially in situations where self-reactive B cells become functionally inactivated (anergic) but are not physically eliminated. In this paper, we have explored the basis for tolerance in anergic self-reactive B cells by using a model developed previously (20) in which Ig gene transgenic mice, whose B lymphocytes express surface IgM and IgD specific for the Ag hen-egg lysozyme (HEL), were mated with transgenic mice expressing the soluble form of HEL. In the resulting soluble lysozyme/anti-lysozyme double (Dbl)-transgenic mice, self-reactive anti-lysozyme B cells developed normally and populated the peripheral lymphoid organs but were functionally tolerant to lysozyme. Since the tolerant B cells were present in normal numbers and expressed all of the developmentally regulated cell surface molecules thought to contribute to B cell activation, the explanation for tolerance was obscure. Here, we describe that tolerant B cells from these mice remain responsive to T cell–derived signals, but

exhibit a proximal block in B cell sIg signaling after Ag binding that precludes effective collaboration with T cells. Moreover, the sIg signaling block can be partially overcome by very extensive receptor cross-linking, and this markedly restored collaboration with Th cells. These findings demonstrate a pivotal role for sIg signaling in guiding T cell–dependent Ab responses and that regulation of sIg signaling provides a mechanism for controlling self-reactive B cells.

## Materials and Methods

**Mice.** Anti-HEL/sHEL Dbl-transgenic animals were obtained by mating transgenic animals from the MD4 anti-HEL IgM + IgD line and the ML5 soluble HEL line, which were produced and maintained on a C57BL/6J (B6) background as described (20). To mark the Ig- or Dbl-transgenic cells with Ly5<sup>+</sup>, B6 MD4 × ML5 Dbl-transgenic mice were mated with C57BL/6-Ly5<sup>+</sup> congenic mice (the generous gift of Dr. I. Weissman, Stanford University, Stanford, CA), and the F<sub>1</sub>-hybrid offspring used as spleen cell donors. Membrane (m)HEL transgenic mice were from the line KLK4 expressing lysozyme fused to class I transmembrane and cytoplasmic regions (21). B6 and bm12 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used were between 8 and 16 wk of age.

**Adoptive Transfers.** Recipient mice were age matched or littermate C57BL/6J nontransgenic, sHEL transgenic (ML5 line), or mHEL transgenic (KLK4 line) mice, sublethally irradiated with 750 rad  $\gamma$ -irradiation and injected intravenously with 10<sup>7</sup> transgenic splenocytes and 5 × 10<sup>7</sup> splenocytes from B6 or bm12 mice. Cotransfer experiments (e.g., see Fig. 2, A–C) used the same protocol except that 10<sup>7</sup> B6-Dbl-transgenic splenocytes were injected along with 10<sup>7</sup> Ig/Ly5<sup>+</sup> splenocytes and 5 × 10<sup>7</sup> splenocytes from B6 or bm12 mice. 5 d after transfer, spleen cells from the recipients were harvested, counted by hemocytometer, stained for FACS<sup>®</sup> analysis as described below, and the number of anti-HEL IgM<sup>+</sup> Ab-secreting cells enumerated by spot ELISA (22).

**In Vitro Cultures.** For MLCs (see Fig. 2 D), 10<sup>5</sup> Ly5<sup>+</sup>-bearing splenocytes from either Ig-, Dbl-, or non-transgenic mice were cultured with 5 × 10<sup>5</sup> splenocytes from B6 mice and 5 × 10<sup>5</sup> splenocytes from bm12 mice. Cells were cultured in round-bottomed 96-well plates (Falcon) at 37°C, in 5% CO<sub>2</sub> in 0.2 ml of RPMI media supplemented with 10% FCS, 2 mM glutamine, 50  $\mu$ M 2-MER, with or without 100 ng/ml HEL (Sigma Chemical Co., St. Louis, MO). After 4 d of culture, cells were washed free of residual HEL and the number of anti-HEL IgM<sup>+</sup> secreting cells determined by spot ELISA. For LPS stimulation, 20  $\mu$ g/ml LPS (*Escherichia coli* 0111:B4; Difco Laboratories, Detroit, MI) was added to parallel cultures containing 10<sup>5</sup> Ig- or Dbl-transgenic spleen cells. Proliferation assays used 10<sup>5</sup> splenocytes cultured in flat-bottomed 96-well plates (Linbro) in a final volume of 0.1 ml RPMI media (see Fig. 3) or 0.2 ml (see Figs. 4 and 6) supplemented with the indicated concentrations of rat antisera to recombinant extracellular domain of mouse CD40 (Heath, A., W. Wu, and M. Howard, manuscript submitted for publication), HEL, LPS, recombinant mouse IL-4 (DNAX), polyclonal goat anti-mouse IgD Ab (23), or ionomycin (Calbiochem-Novabiochem Corp., La Jolla, CA) and PMA (Sigma Chemical Co.). To assess proliferative responses to ionomycin and PMA, splenocytes were depleted of T cells using sheep anti-FITC coated magnetic beads (Advanced Magnetics, Cambridge, MA) after staining with FITC-conjugated Abs to CD4 (clone GK1.5, Becton Dickinson & Co., Mountain View, CA) and CD8 (clone TYS169.4, Becton Dickinson & Co.). T cell-depleted

<sup>1</sup> Abbreviations used in this paper: CD40L, CD40-ligand; Dbl, double; HEL, hen-egg lysozyme; ICAM-1, intercellular adhesion molecule 1; sIg, surface Ig; SA-PE, streptavidin-PE.

splenocytes contained <1% CD4- or CD8-positive cells. Proliferating cells were detected by a 12–16 h pulse with [ $^3$ H]thymidine 48 h after stimulation as described (24). [ $^3$ H]uridine incorporation was determined by pulsing cells from 0–24 h of culture with 1  $\mu$ Ci/well [ $^3$ H]uridine (Amersham Corp., Arlington Heights, IL) and harvesting the cells for scintillation counting. Values represent the means of triplicate determinations. For FACS<sup>®</sup> analysis (Becton Dickinson & Co.) of stimulated cells, splenocytes from Ig or Dbl-transgenic mice were cultured at  $10^6$ /ml in 1 ml for 12 or 24 h at 37°C in medium alone or with rIL-4 (100 U/ml), anti-CD40 (final dilution 1:10<sup>4</sup>), lysozyme (100 ng/ml), or goat anti-IgD (10  $\mu$ g/ml).

**Flow Cytometry Staining and Analysis.** Cell staining and three-color FACS<sup>®</sup> analysis were conducted as described (25). Ly5<sup>a</sup> was detected using FITC-conjugated AS20 (25) and HEL-binding cells were revealed by incubating with 100 ng/ml HEL followed by HyHEL5-biotin and streptavidin-Tricolor (Caltag Laboratories, San Francisco, CA) as described (25). Syndecan was detected with mAb 284.1 (26) followed by F(ab)<sub>2</sub> anti-rat IgG conjugated to PE (Caltag Laboratories). Class II was detected using mAb 7-16.17 conjugated to biotin (PharMingen, San Diego, CA) followed by streptavidin-PE (SA-PE; Caltag Laboratories). CTLA4-Ig ligand was detected using 10  $\mu$ g/ml CTLA4-Ig fusion protein (27) followed by anti-human IgG-FITC (Tago Inc., Burlingame, CA). ICAM-1 was detected using mAb 3E2 conjugated to biotin (PharMingen) followed by SA-PE. Staining for B220 used Ab RA3-6B2 conjugated to either FITC or PE (Caltag Laboratories).

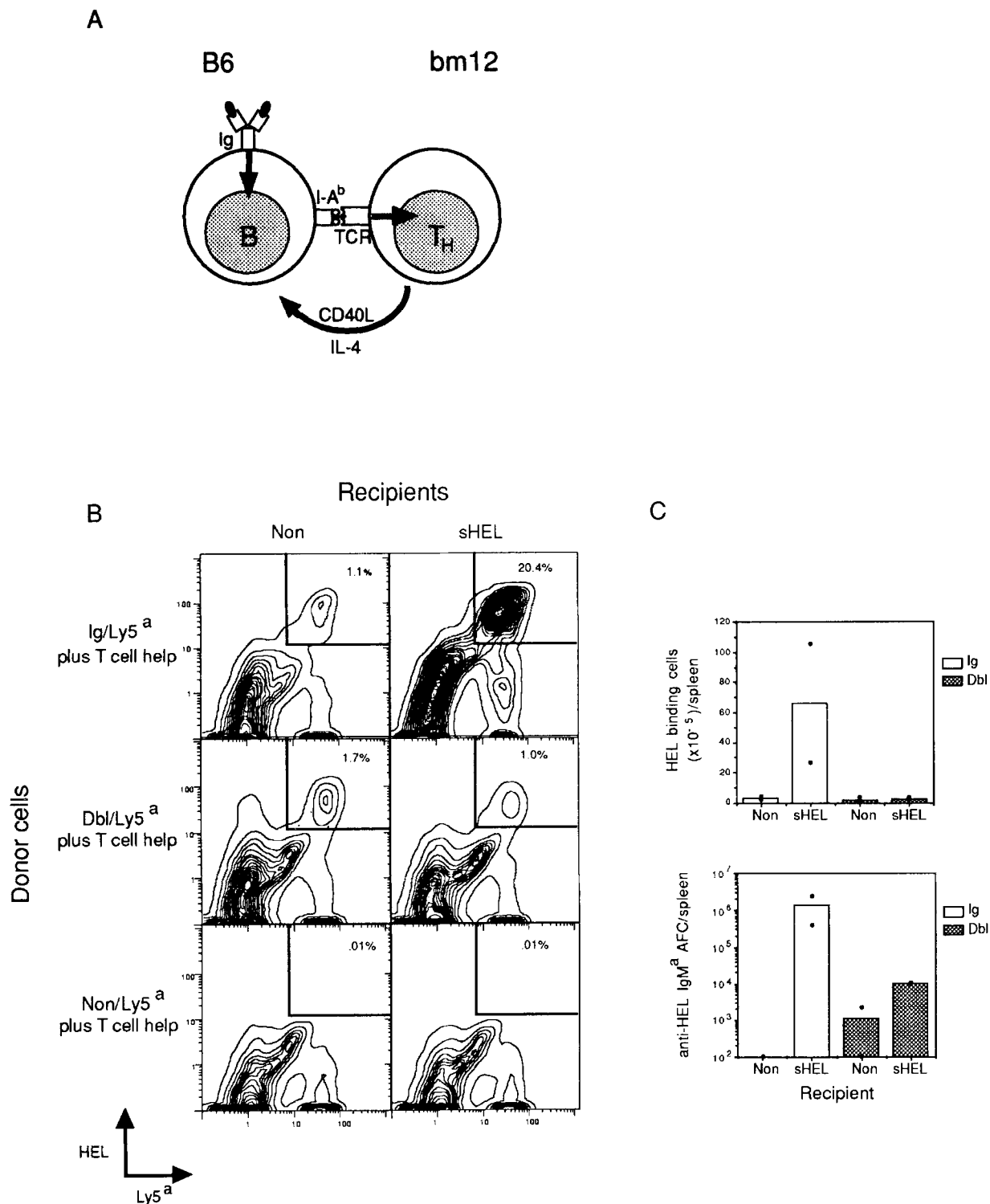
**Calcium Analysis.** Calcium analysis was conducted essentially as described (24). Briefly, splenocytes were isolated, washed, and resuspended at  $10^7$  cells/ml in 10% FCS/RPMI and loaded with the calcium indicator Indo-1AM (1  $\mu$ M, final concentration; Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C. Cells were subsequently washed and stained with FITC-conjugated Abs to CD4 and CD8. Indo-1 loaded cells were suspended at  $5 \times 10^6$ /ml and prewarmed to 37°C immediately before analysis. Analysis was conducted at 37°C, unstimulated cells were collected for 45 s, Ag added at 1 min, and data collection continued for a total of 7 min, with a flow rate of 200–400 cells/s. For mHEL stimulation (see Fig. 7 A) cells were collected for 45 s to establish basal calcium levels, cells removed, and mHEL-bearing or control thymocytes were added to a concentration of  $10^7$ /ml. The mixture was pelleted for 5 s using a microfuge, resuspended using a micropipette, and data collection resumed. Thymocytes were excluded from analysis by electronically gating on Indo-1 loaded cells. CD4- or CD8-positive cells were excluded from analysis by electronic gating on FITC-negative cells. Detection of Indo-1 and FITC fluorescence used a dual laser FACStar Plus<sup>®</sup> flow cytometer (Becton Dickinson & Co.). Conversion of Indo-1 violet-blue fluorescence ratios to calcium levels was determined as described (28, 29). Transfer of Ig or Dbl-transgenic B cells into nontransgenic recipients was performed by intravenous injection of  $10^7$  spleen cells into 750 rad  $\chi$ -irradiated recipients. "Parked" cells were recovered from the spleen 36 h after transfer, loaded with Indo-1, and analyzed as described above.

**Phosphotyrosine Immunoblotting.** For analysis of phosphotyrosine levels, freshly isolated splenocytes were washed, resuspended at  $10^7$ /ml in serum-free RPMI, and stimulated with the indicated reagents for 5 min at 37°C. Cells were then pelleted, lysed, and lysates from  $10^6$  cell equivalents were resolved on an 11% SDS-PAGE gel and immunoblotted with antiphosphotyrosine mAb 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) (final concentration 1:2,000), using enhanced chemiluminescent detection as described previously (24).

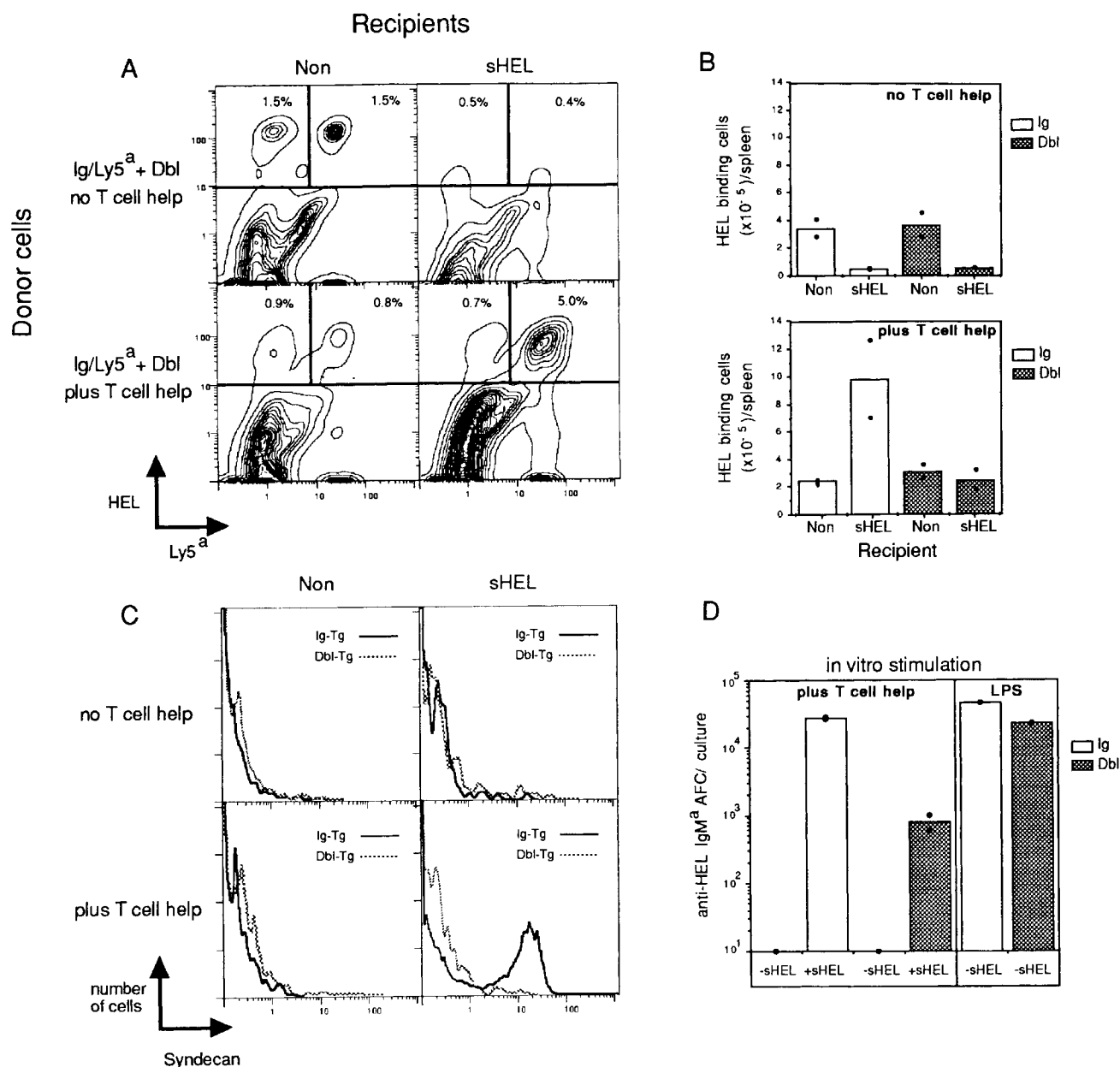
## Results

**Tolerant B Cells Fail to Respond to T Cells Plus Ag.** In previous experiments, self-reactive B cells from the lysozyme/anti-lysozyme Dbl-transgenic mice produced 10–50-fold less Ab than nontolerant B cells from Ig-transgenic mice lacking lysozyme, when assayed in cell transfer experiments after stimulation with Th cells primed to SRBC Ags and an antigenic conjugate of lysozyme and SRBC (20, 21, 30). The failure of self-reactive B cells from Dbl-transgenic mice to collaborate with SRBC-specific T cells could have reflected either a defect in their ability to take up and present lysozyme-SRBC Ag conjugates to Th cells, or a defect in the subsequent ability to interact with and receive growth and differentiation factors from T cells. To explore which of these steps was altered, the requirement for Ag binding and processing into MHC-associated peptides was bypassed by providing a constitutively expressed TCR-ligand on the B cells, using unique determinants on class II MHC molecules themselves. B cells in C57BL/6 (B6) strain mice display I-A<sup>b</sup> MHC molecules that differ at three residues from I-A molecules in the coisogenic strain, bm12 (31). Because of this allelic difference, a proportion of Th cells in bm12 mice carry Ag receptors that are triggered by I-A<sup>b</sup> (Fig. 1 A; 31). Since I-A<sup>b</sup> molecules are displayed at normal levels on tolerant and nontolerant B cells from B6 strain-transgenic mice (see Fig. 2 A), successful collaboration between tolerant B6 B cells and bm12 Th cells would be expected if the B cell defect lay in the ability to take up and present Ags, but not if the defect lay elsewhere.

Following the approach diagrammed in Fig. 1 A, spleen cells from B6 strain Dbl- and Ig-transgenic mice were marked with an allelic Ly5<sup>a</sup> cell surface marker and an IgM<sup>a</sup> Ig allotype marker to allow unequivocal identification, and were transferred to recipient animals of the B6 strain. Spleen cells from bm12 mice, including anti I-A<sup>b</sup> Th cells, were cotransferred in numbers determined to cause optimal B cell proliferation and Ab production. In the absence of any cotransferred B cells, activation of the bm12-derived T cells occurred in response to I-A<sup>b</sup> radioresistant cells in the recipients, producing marked splenomegaly and other symptoms of GVHD within 5–7 d (data not shown). When bm12 T cells and nontolerant Ig-transgenic B cells were cotransferred into mice expressing lysozyme, the lysozyme-binding B cells were transformed into large blast cells (data not shown) and increased their numbers 10–50-fold (Fig. 1, B top right, and C top). The apparent proliferation of lysozyme-binding B cells was accompanied by differentiation into Ab-secreting plasma cells, detected by ELISA-spot assay of spleen cells (Fig. 1 C, bottom). The presence of both lysozyme and I-A<sup>b</sup>-reactive T cells was required for proliferation and differentiation of the nontolerant lysozyme-binding B cells, since neither occurred when (a) the same cell mixtures were transferred into nontransgenic recipients lacking lysozyme (Fig. 1, B, top left, and C); or (b) Ig-transgenic cells were transferred to lysozyme-expressing mice without bm12 T cells (Fig. 2, A–C, top, and additional data not shown). By contrast with the response of nontolerant



**Figure 1.** In vivo collaboration between nontolerant or tolerant lysozyme-binding B cells expressing I-A<sup>b</sup> and I-A<sup>b</sup>-specific helper T cells ( $T_H$ ) from bm12 strain mice. (A) Experimental design to dissociate B cell Ag binding from T cell stimulation, using constitutively expressed I-A<sup>b</sup> molecules to engage the TCR on bm12  $T_H$  cells. (B and C) Ly5<sup>a</sup>-marked splenocytes from B6-Ly5<sup>a</sup> Ig-transgenic (nontolerant; Ig/Ly5<sup>a</sup>), double-transgenic (tolerant; Dbl/Ly5<sup>a</sup>), or nontransgenic (Non/Ly5<sup>a</sup>) mice were transferred with splenocytes from bm12 mice (plus T cell help) into irradiated B6-strain nontransgenic mice (Non) or transgenic mice expressing soluble HEL (sHEL). 5 d after transfer, spleen cells were stained for HEL binding and Ly5<sup>a</sup> expression and the frequency of positively stained cells measured by FACS<sup>®</sup> (B). The number of HEL-binding cells (C, top) and anti-HEL IgM<sup>+</sup> Ab-secreting cells (C, bottom) in the spleen of each recipient was determined. (Dots) The number of cells in individual recipients; (bars) arithmetic means. The data shown are from a single experiment and are representative of three independent experiments involving a total of 10 separate Ig- and 10 separate Dbl-transgenic donors.



**Figure 2.** Ag and bm12 Th cells are both required for expansion and differentiation of nontolerant lysozyme-binding B cells, but remain unable to trigger tolerant B cells. (A–C) Equal numbers of Ig/Ly5<sup>+</sup> and Dbl/Ly5<sup>-</sup> splenocytes were mixed and transferred with B6-strain splenocytes (*no T cell help*) or bm12 splenocytes (*plus T cell help*) into irradiated nontransgenic or sHEL-transgenic recipients. (A) 5 d after transfer, splenocytes were stained for HEL-binding and Ly5<sup>+</sup> expression to distinguish nontolerant (Ly5<sup>+</sup>) from tolerant (Ly5<sup>-</sup>) B cells. (B) The number of HEL-binding Ig/Ly5<sup>+</sup> (open bars) or Dbl/Ly5<sup>-</sup> (stippled bars) cells per spleen was determined for all the recipients. (C) Differentiation of B cells in the recipients was measured in parallel by a third-color immunofluorescent stain for the plasma cell marker, sydecane, on HEL-binding Ig/Ly5<sup>+</sup> (solid line) or Dbl/Ly5<sup>-</sup> (dashed line) B cells. Histograms were derived from three-color analysis after gating on the HEL-binding Ly5<sup>+</sup> or Ly5<sup>-</sup> cells shown in B. The data shown are from one experiment and are representative of three independent experiments involving a total of six separate Ig- and six separate Dbl-transgenic donors. (D) Numbers of anti-HEL IgM<sup>a</sup>-secreting cells generated after 4 d in vitro culture of Ig- or Dbl-transgenic splenocytes with an excess of B6-strain and bm12-strain spleen cells (*plus T cell help*) or with lipopolysaccharide (LPS). Cultures were performed in the presence (+sHEL) or absence (-sHEL) of 100 ng/ml HEL. The experiment shown is representative of six independent in vitro culture experiments.

cells, tolerant lysozyme-binding B cells from Dbl-transgenic mice showed little proliferation or Ab production in the presence of both bm12 T cells and lysozyme (Fig. 1, B and C).

To confirm that activated I-A<sup>b</sup>-reactive Th cells were indeed being generated in recipients of tolerant B cells, Dbl- and Ig-transgenic B cells were mixed and cotransferred with

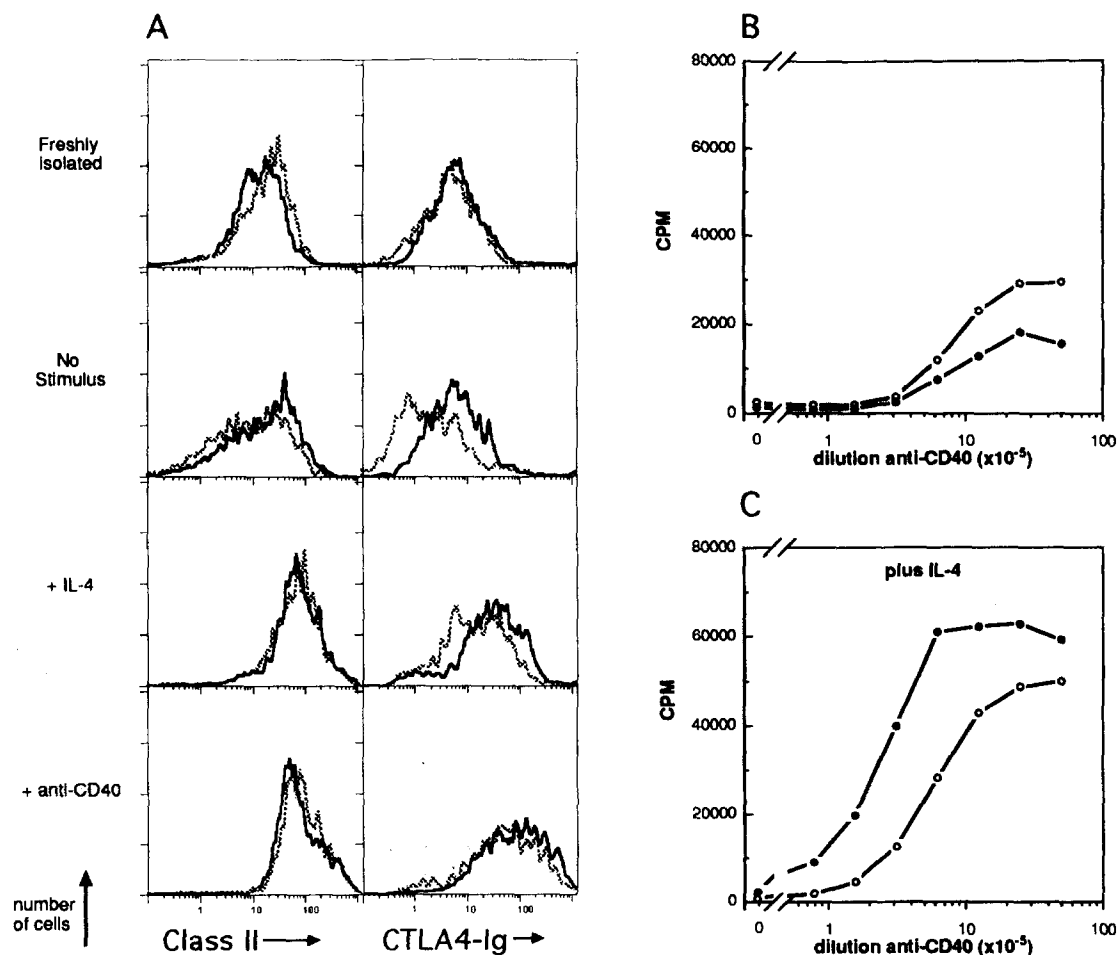
bm12 T cells into recipient animals. Nontolerant lysozyme-binding cells from the Ig-transgenic mice were in this case distinguished from tolerant Dbl-transgenic cells by the presence of the Ly5<sup>a</sup> allelic marker only on the former (e.g., Fig. 2 A, top). Differentiation of lysozyme-binding Ly5<sup>+</sup> (Ig-transgenic) or Ly5<sup>-</sup> (Dbl-transgenic) cells into Ab-secreting

cells was measured in parallel by staining with an Ab to the plasma cell marker, syndecan (26 and Fig. 2 C). Nontolerant  $\text{Ly5}^{+}$  B cells proliferated and differentiated efficiently in the presence of lysozyme and bm12 T cells (Fig. 2, A–C, *bottom right*), confirming that activated I-A<sup>b</sup>-specific Th cells were indeed generated in these recipients. By contrast, the tolerant  $\text{Ly5}^{+}$  B cells in the same hosts failed to undergo blastogenesis, increase in cell number or differentiate into plasma cells (Fig. 2, A–C).

The possibility that the tolerant B cells were capable of interacting with T cells but failed to migrate to the appropriate *in vivo* microenvironment was tested by performing equivalent experiments with bm12 T cells in suspension cultures *in vitro*. Small numbers of  $\text{Ly5}^{+}$ -marked spleen cells from B6 Ig- or Dbl-transgenic mice were added to two-way mixed lymphocyte reactions between nontransgenic B6 and bm12 spleen cells. Again, lysozyme-binding B cells from Ig-transgenic mice proliferated, as detected by blastogenesis and

BrdU incorporation (data not shown) and differentiated into Ab-secreting cells (Fig. 2 D) only when lysozyme was added to the cultures. Dbl-transgenic cells, by contrast, showed little proliferation and generated 50–100-fold fewer Ab-secreting cells (Fig. 2 D and data not shown). Parallel cultures stimulated with the nonspecific B cell mitogen, LPS, nevertheless confirmed that the tolerant B cells were potentially capable of efficient proliferation and differentiation into Ab-secreting cells (Fig. 2 D, *right columns*) as previously described (32).

**Tolerant B Cells Respond to T Cell-derived Mediators.** Two signals appeared necessary for efficient proliferation and differentiation of nontolerant lysozyme-binding B cells under the conditions of I-A<sup>b</sup>-specific T cell help used above: one provided by the bm12 T cells, and one resulting from the binding of lysozyme to the B cell (see Fig. 1 A). Unresponsiveness in the tolerant B cells could therefore have reflected a defective response to either of these signals. To test the response to T cell-derived signals, membrane fractions from



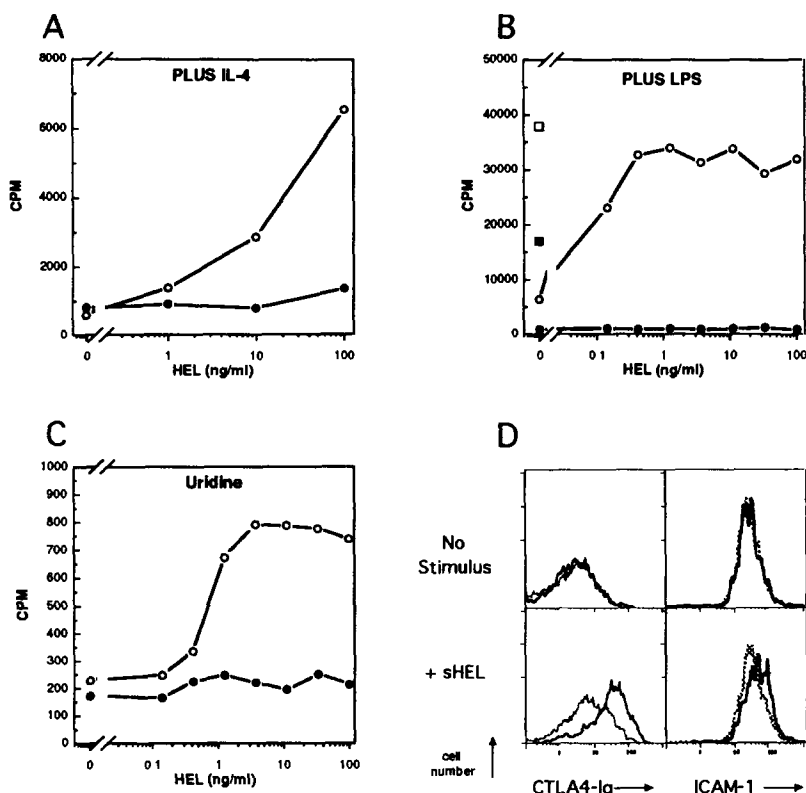
**Figure 3.** Response of tolerant and nontolerant B cells to Th cells signals. (A) Immunofluorescent staining with CTLA4-Ig to detect B7 and B7-related molecules (*right*) and class II MHC molecules (*left*) on Ig-transgenic (*solid line*) and Dbl-transgenic (*dashed line*) splenic B cells immediately after removal from the animal (*Freshly isolated*) or after 24 h culture with medium alone (*No stimulus*) or medium containing IL-4 or anti-CD40 Abs (*bottom*). The histograms displayed are gated on B220<sup>+</sup> cells after excluding dead cells by staining with propidium iodide. (B and C) Proliferation of Ig-transgenic (*open circles*) or Dbl-transgenic (*filled circles*) B cells, assessed by  $^3\text{H}$ thymidine incorporation after stimulation with anti-CD40 (B) or anti-CD40 and IL-4 (C). Data displayed represent means of triplicate cultures and are representative of four independent experiments.

activated T cell hybridomas that contain CD40L (14, 33) were initially tested and found to trigger equivalent proliferation in tolerant and nontolerant B cells, and staining with Abs to CD40 showed equivalent expression on both types of B cells (data not shown). More specifically, treatment with a polyclonal antiserum to recombinant mouse CD40 that mimics the action of membrane-bound CD40L (Heath, A. et al., manuscript submitted for publication), or stimulation with IL-4, each induced tolerant and nontolerant B cells to increase cell surface expression of class II MHC (Fig. 3 A). Additionally, these stimuli also increased the immunofluorescent staining of both cell types with CTLA4-Ig fusion protein (Fig. 3 A) which recognizes B7 and B7-related molecules expressed on activated B cells (hereafter referred to CTLA4-Ig ligand; 34; see Discussion). Vigorous proliferation was induced in both cell types by antisera to CD40 (Fig. 3 B), and this was augmented in both tolerant and nontolerant B cells by addition of IL4 (Fig. 3 C). Taken together, these findings indicate that the tolerant B cells were fully responsive to key signals mediating T cell help.

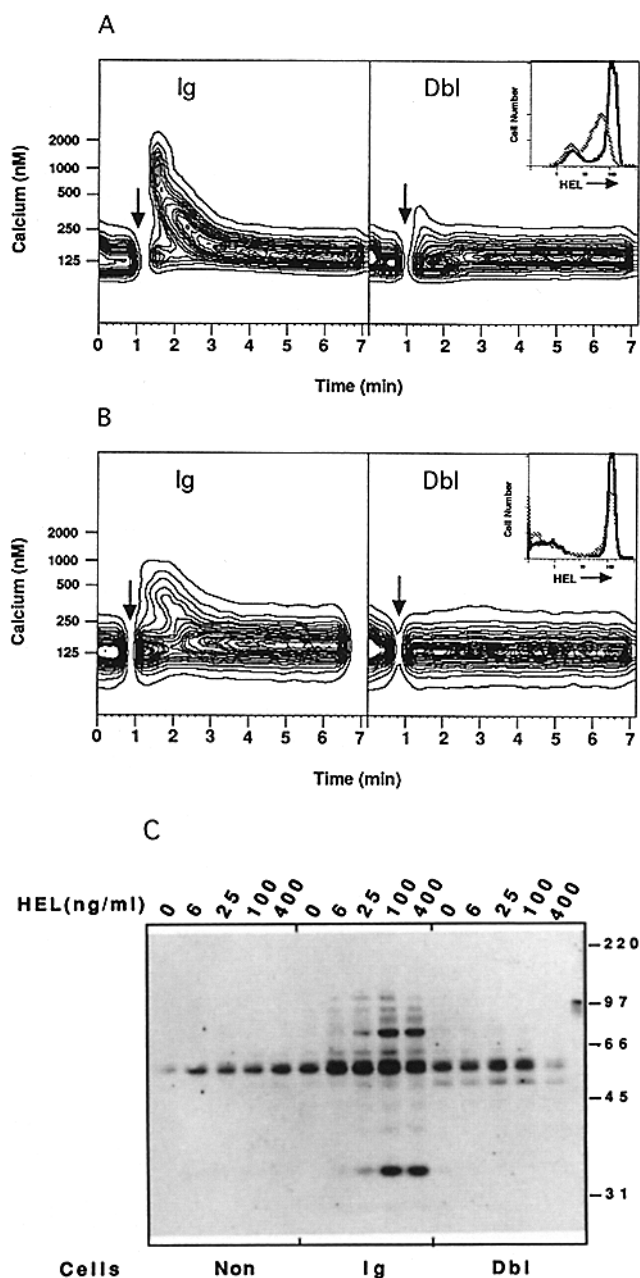
**Tolerant B Cells Do Not Respond to Ag.** In contrast to the normal response to T cell-derived signals, many cellular responses normally triggered by Ag binding to sIg (for review see references 35 and 36) were absent in tolerant B cells. Binding of lysozyme at concentrations as low as 1 ng/ml, which engages <5% of the sIg receptors (22), triggered vigorous proliferation of nontolerant Ig-transgenic B cells in the presence of either IL-4 (Fig. 4 A), submitogenic concentrations of LPS (Fig. 4 B), or suboptimal concentrations of

anti-CD40 antiserum (data not shown). However, binding of lysozyme to tolerant B cells had no mitogenic effects in any of these assays (Figs. 4, A and B and not shown). Similarly, whereas binding of lysozyme alone was not mitogenic, it promoted activation of nontolerant Ig-transgenic B cells from G<sub>0</sub> to G<sub>1</sub> of the cell cycle, as assessed by increased RNA synthesis (Fig. 4 C) or by cell enlargement detected flow cytometrically (data not shown). In contrast, lysozyme binding failed to induce cell enlargement or increased RNA synthesis in tolerant B cells (Fig. 4 C, and data not shown). Finally, lysozyme binding triggered increased expression of CTLA4-Ig ligand and ICAM-1 molecules on nontolerant Ig-transgenic cells, but induced little increase in the expression of these molecules on tolerant Dbl-transgenic cells (Fig. 4 D).

The absence of many cellular responses to Ag binding in the tolerant cells was further explored by following early signal-transduction events normally triggered by sIg engagement. One of the earliest biochemical events, an increase in the level of intracellular calcium ([Ca]<sub>i</sub>; 35, 36), was measured by four-color flow cytometry after loading spleen cells with the calcium indicator, Indo-1. Binding of lysozyme to receptors on nontolerant Ig-transgenic B cells induced a rapid rise in the level of [Ca]<sub>i</sub> that peaked within 1 min and included nearly all of the HEL-specific B cells (Fig. 5 A). By contrast, stimulation of tolerant Dbl-transgenic B cells with a range of concentrations of lysozyme from 20 to 500 ng/ml failed to induce a detectable increase in [Ca]<sub>i</sub> (Fig. 5 A, and data not shown). Activation of protein tyrosine kinases is an integral event in signal transduction by sIg that lies immediately



**Figure 4.** Early cellular responses after Ag binding to tolerant and nontolerant B cells. (A) Proliferation of B cells from Ig-transgenic (open symbols) or Dbl-transgenic (filled symbols) mice as measured by [<sup>3</sup>H]thymidine incorporation after 48 h of culture with the indicated amounts of HEL in the presence of IL-4. (B) Proliferation in response to HEL in the presence of submitogenic concentrations of LPS (1 µg/ml). For comparison, square symbols show proliferation to optimal concentrations of LPS (20 µg/ml). (C) Transition from G<sub>0</sub> to G<sub>1</sub> of the cell cycle as measured by incorporation of [<sup>3</sup>H]uridine into cellular RNA in response to the indicated concentrations of HEL. (D) Expression of CTLA4-Ig ligand and ICAM-1 after Ag stimulation of Ig-transgenic (solid line) or Dbl-transgenic (dotted line) B cells after 12 h (CTLA4-Ig) or 24 h (ICAM-1) of culture with 100 ng/ml lysozyme (bottom) or medium alone (top).



**Figure 5.** Signaling events triggered by HEL-binding in tolerant and nontolerant B cells. (A) Intracellular calcium levels as a function of time in freshly isolated Ig- or Dbl-transgenic splenic B cells before and after the addition of lysozyme (arrows). (Inset) Relative number of free HEL binding sites on Ig (solid line)- or Dbl (dotted line)-transgenic B cells before stimulation, as detected using fluorescently labeled HEL. (B) Intracellular calcium levels of Ig- or Dbl-transgenic B cells that had been parked in nontransgenic mice for 36 h before harvesting and in vitro stimulation with HEL (arrows). Note (inset) that the number of available HEL-binding sites is nearly identical on parked Ig- and Dbl-transgenic B cells. (C) Anti-phosphotyrosine immunoblotting, showing induction of phosphotyrosine-containing proteins 5 min after addition of lysozyme to nontransgenic, Ig-transgenic, and Dbl-transgenic spleen cells. Note that the reduced level of phosphotyrosine-containing proteins in unstimulated nontransgenic cells (lane 1) and in Dbl-transgenic cells stimulated with 400 ng/ml HEL (lane 15) results from poor transfer and is not representative (see Fig. 6 A).

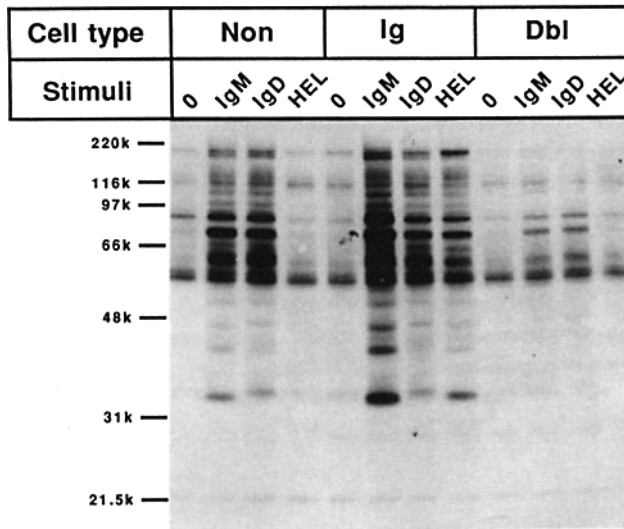
upstream to the initiation of increased  $[Ca]_i$  (37–40). To measure tyrosine kinase activation, Dbl- and Ig-transgenic spleen cells were stimulated with lysozyme in vitro, and cell lysates were then prepared and probed by immunoblot using Abs specific for phosphotyrosine. Lysates from unstimulated tolerant or nontolerant cells had few phosphotyrosine-containing proteins (Fig. 5 C). Stimulation with concentrations of lysozyme as low as 6 ng/ml resulted in rapid appearance of many phosphotyrosine-containing proteins within the non-tolerant Ig-transgenic B cells. By contrast, even saturating concentrations of lysozyme failed to induce a detectable increase in phosphotyrosine species in the tolerant B cells (Fig. 5 C).

Because of modulation of IgM Ag receptors and in vivo occupancy of many IgD receptors with lysozyme, B cells from the Dbl-transgenic mice display 3–4-fold fewer available receptors for binding additional exogenous lysozyme molecules than nontolerant B cells from Ig-transgenic mice (Fig. 5 A inset). To test whether the signaling defects in the Dbl-transgenic B cells were simply a result of fewer receptors, or alternatively, a short-term refractory phenomenon due to recent in vivo exposure to lysozyme, B cells were removed from the Dbl-transgenic mice and “parked” in irradiated non-transgenic mice to allow bound lysozyme to dissociate. 36 h after transfer, the parked Dbl-transgenic B cells displayed a nearly equivalent capacity to bind fluorescently labeled lysozyme as did parked Ig-transgenic B cells (Fig. 5 B, inset). Despite this, the tolerant cells remained unable to increase  $[Ca]_i$  after stimulation with lysozyme (Fig. 5 B), indicating that the defect in signaling was not due simply to receptor modulation. Whereas  $[Ca]_i$  increase is an immediate consequence of tyrosine kinase activation (37–40), parked B cells could not be recovered in sufficient numbers or purity to measure protein tyrosine phosphorylation directly.

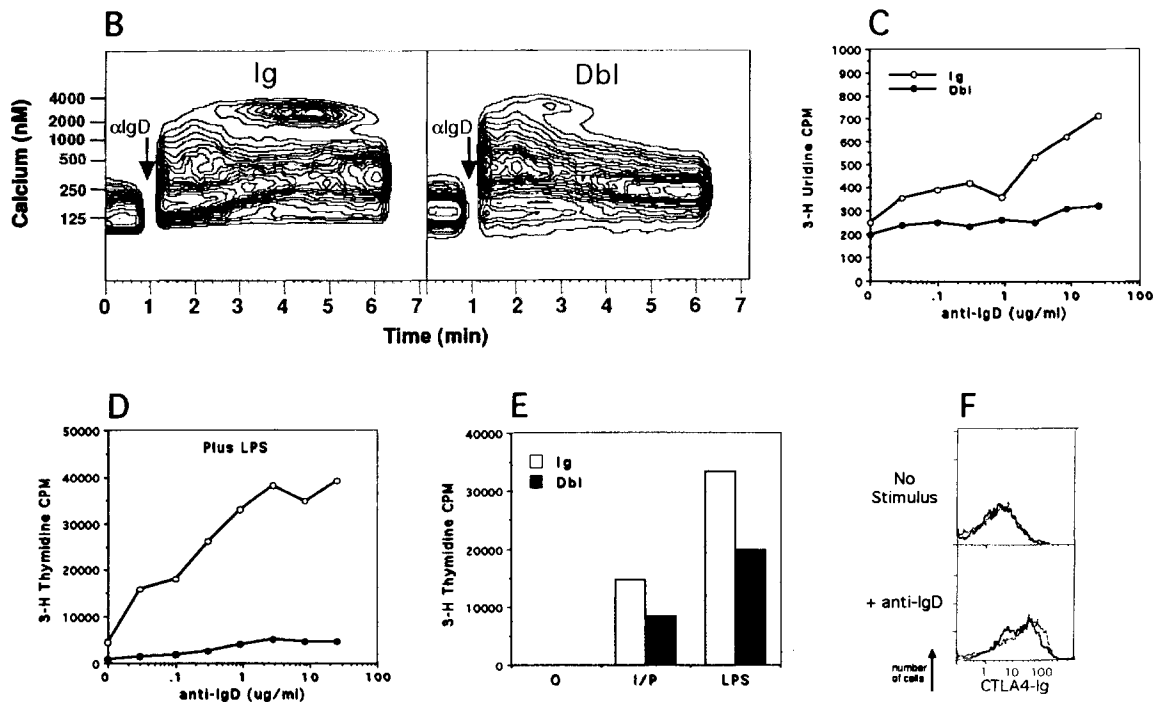
**Defective Response of Tolerant B Cells to anti-IgD Abs.** The absence of intracellular signaling events after HEL binding to tolerant B cells was further explored by stimulating tolerant and nontolerant B cells with affinity-purified Abs to the C regions of IgD. Stimulation by anti-IgD differed from lysozyme stimulation in that IgD is expressed at identical levels on tolerant and nontolerant B cells, is equally accessible to anti-C region Abs, and therefore should be cross-linked equally well on the two cell types. Additionally, the polyclonal anti-IgD Abs should promote extensive receptor cross-linking and therefore would test whether tolerant B cells retained any capacity to signal through sIg. In contrast to stimulation with lysozyme, extensive cross-linking of sIgD receptors elicited some early signaling events in the tolerant B cells, although the magnitude and kinetics of the response remained altered compared with nontolerant B cells. Thus, treatment with anti-IgD antisera induced an identical pattern of phosphotyrosine-containing proteins in both Dbl- and Ig-transgenic B cells, although the relative abundance of labeled proteins was markedly reduced in tolerant cells (Fig. 6 A). Similarly, anti-IgD stimulation triggered a prolonged increase in  $[Ca]_i$  in nontolerant Ig-transgenic cells, with the fraction of B cells containing micromolar concentrations of  $[Ca]_i$  remaining at



A



**Figure 6.** Response of tolerant and nontolerant B cells to receptor cross-linking by polyclonal anti-IgD Abs. (A) Phosphotyrosine-containing cellular proteins 5 min after stimulation of Ig-, Dbl-, or non-transgenic splenocytes with media (0), 10  $\mu$ g/ml goat anti-IgM Abs (IgM), 10  $\mu$ g/ml goat anti-IgD Abs (IgD), or 100 ng/ml HEL (HEL). Note that the prominent 32- and 33-kD phosphotyrosine species induced by anti-IgM and anti-IgD, respectively, are likely to represent the Ig-associated molecules IgM $\alpha$  and IgD $\alpha$  (77). (B) Intracellular calcium levels after stimulation of Ig- and Dbl-transgenic B cells with goat anti-IgD Abs (10  $\mu$ g/ml). (C) Activation from G<sub>0</sub> to G<sub>1</sub> of the cell cycle, as measured by [<sup>3</sup>H]uridine incorporation induced by goat anti-IgD stimulation of Ig (open symbols)- and Dbl (filled symbols)-transgenic splenocytes. (D) Proliferation of Ig (open symbols)- and Dbl (filled symbols)-transgenic B cells induced by goat anti-IgD in the presence of submitogenic concentrations of LPS (1  $\mu$ g/ml). (E) Mitogenic response of Ig (open bars)- and Dbl (filled bars)-transgenic B cells after stimulation with media alone (0), Ionomycin (1  $\mu$ g/ml), and PMA (1 ng/ml) (I/P) or LPS (20  $\mu$ g/ml). (F) Induction of CTLA4-Ig ligand on Ig (solid line)- and Dbl (dotted line)-transgenic B cells 12 h after stimulation with goat anti-IgD (10  $\mu$ g/ml).



10–15% for up to 4 min (Fig. 6 B, left). In contrast, stimulation of tolerant B cells with anti-IgD induced a more rapid initial  $[Ca]_i$  response, but this response diminished much more quickly and lacked the sustained phase observed in the nontolerant B cells (Fig. 6 B, right).

Whereas the ability of anti-IgD antisera to evoke proximal signaling events suggested that the necessary receptor-associated components were at least partially present and activatable in the tolerant B cells, more distal events such as cell cycle entry still failed to occur. Thus, stimulation with the same anti-IgD antiserum failed to trigger G<sub>0</sub>-G<sub>1</sub> transition in Dbl-transgenic cells as measured by induction of RNA

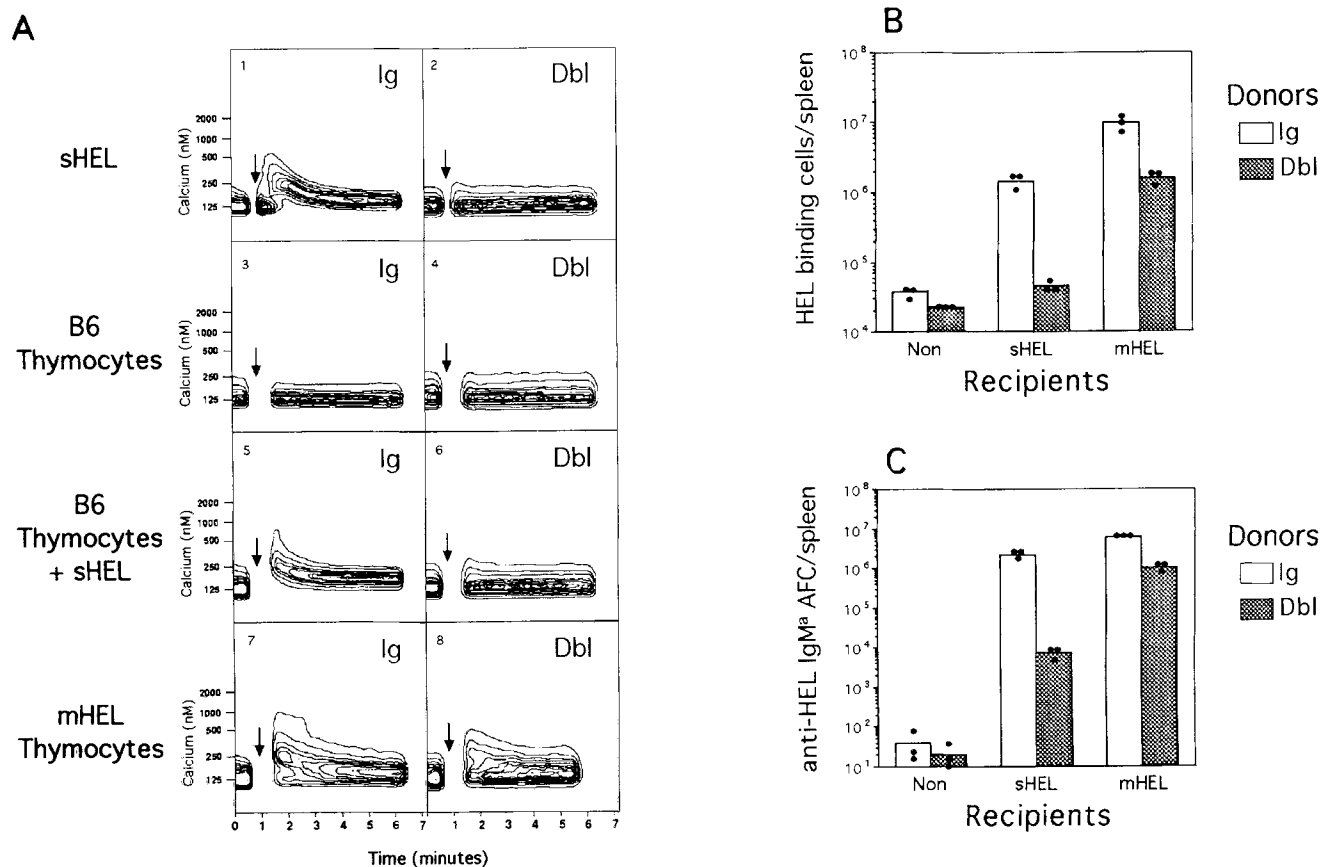
synthesis (Fig. 6 C), and promoted little DNA synthesis in the tolerant cells in combination with submitogenic concentrations of LPS (Fig. 6 D). Similar results were obtained with mAbs to IgD coupled to dextran (41), which also cross-link Ig receptors extensively and promoted vigorous proliferation in the nontolerant Ig-transgenic B cells, but induced little or no mitogenic response in tolerant B cells (data not shown). Proliferation in response to calcium ionophore and phorbol ester, which bypass the requirement for sIg signaling, remained intact in the tolerant cells (Fig. 6 E). Whereas extensive receptor cross-linking with anti-IgD Abs was unable to promote cell cycle entry, this stimulus was nevertheless sufficient

to induce high expression of CTLA4-Ig ligand on the tolerant cells (Fig. 6 F).

**Tolerant B Cells Respond to Membrane-bound Lysozyme** Since early signaling events could be partially restored and CTLA4-Ig ligand induced on the tolerant B cells after extensive receptor cross-linking with Abs to IgD, we tested whether a highly multivalent form of lysozyme expressed on cell surfaces as an integral membrane protein (mHEL) would also restore sIg signal transduction. Thymocytes from transgenic mice expressing mHEL on cell surfaces (21) were therefore mixed with Indo-1 loaded B cells and changes in  $[Ca]_i$  measured as previously. In contrast to stimulation with soluble lysozyme which remained unable to elicit a  $[Ca]_i$  response in tolerant B cells (Fig. 7 A, 2), stimulation with membrane lysozyme triggered a clear  $[Ca]_i$  response in both tolerant- and nontolerant-transgenic B cells although the response in the latter was somewhat weaker (Fig. 7 A, 7 and 8). Control B6 thymocytes lacking mHEL elicited no response in either cell type (Fig. 7 A, 3 and 4). Combined stimulation with

control B6 thymocytes and soluble HEL remained unable to elicit a calcium response in tolerant B cells (Fig. 7 R, 6), indicating that Ag display on membranes was required and that costimulatory molecules on thymocytes were not sufficient.

To test whether the partial restoration of sIg signaling induced by displaying lysozyme in multimeric cell-bound form was sufficient to restore collaboration with I-A<sup>b</sup>-specific Th cells, transgenic B cells, and bm12 T cells were transferred into recipient mice expressing mHEL. Tolerant B cells from Dbl-transgenic mice remained poorly responsive to soluble lysozyme (sHEL) and bm12 helper cells (Fig. 7, B and C, middle columns), but their helper cell-induced proliferation and differentiation into Ab-secreting plasma cells increased 100-fold in recipients expressing mHEL (Fig. 7, B and C, right columns). The magnitude of the response mounted by tolerant B cells in the presence of mHEL was equivalent to that mounted by nontolerant Ig-transgenic B cells in the presence of sHEL, and only fivefold less than the response of the latter in mHEL recipients (Fig. 7, B and C). Thus, display



**Figure 7.** Membrane-bound lysozyme can trigger sIg signaling in tolerant B cells and promote effective collaboration with I-A<sup>b</sup>-specific helper T cells. (A) Intracellular calcium levels in Ig (left)- and Dbl (right)-transgenic B cells after stimulation with soluble HEL (1 and 2), B6 thymocytes (3 and 4), B6 thymocytes plus soluble HEL (5 and 6), or thymocytes bearing membrane-bound lysozyme (mHEL; 7 and 8). At the arrow, B cells were removed and stimulated as indicated (see Materials and Methods for details). (B and C) In vivo collaboration between Ig- and Dbl-transgenic B cells and bm12 Th cells. (B) Enumeration of HEL-binding B cells and (C) numbers of anti-HEL-specific plasma cells was determined as in Fig. 1, after transfer of Ig-transgenic (open bars) or Dbl-transgenic (filled bars) splenocytes with bm12 splenocytes into irradiated B6 strain mice (Non), or into transgenic mice expressing soluble lysozyme (sHEL) or membrane-bound lysozyme (mHEL). Shown are the arithmetic averages (bars) and individual values (dots) for cells from three separate Ig- or Dbl-transgenic donors. The experiment shown is representative of three independent experiments involving seven separate Ig- and seven separate Dbl-transgenic donors.

of membrane-bound Ag could partially restore sIg signaling in tolerant B cells and effective collaboration with I-A<sup>b</sup>-specific Th cells.

## Discussion

The studies above localize the molecular mechanism accounting for B cell clonal anergy to a proximal step in the sIg signal transduction pathway. Moreover, the findings indicate that Ag-specific B cells require signals from T cells and from Ag binding to sIg for efficient proliferation and differentiation into Ab-secreting cells, as originally proposed by Bretscher and Cohn (42). The conclusion that Ag-derived signals through sIg are required was based on three observations: (a) in the presence of activated I-A<sup>b</sup>-reactive T cells, nontolerant, lysozyme-specific B cells expressing I-A<sup>b</sup> made no Ab response unless they were also exposed to antigen; (b) under the same conditions, tolerant, lysozyme-specific B cells failed to respond even when exposed to lysozyme, and these cells lacked lysozyme-induced sIg signaling; (c) a more potent multivalent form of lysozyme, membrane lysozyme, was able to trigger some signaling through sIg in tolerant cells, and this markedly restored their ability to mount an Ab response in the presence of activated Th cells.

**Basis for Blocked Signaling in Tolerant B Cells.** The absence of sIg signaling after Ag binding to the tolerant B cells could in principle reflect either a biochemical block in signal transduction or simply the presence of fewer available receptors on the Dbl-transgenic B cells. Three findings establish that the latter cannot account for the signaling defect. Firstly, the tolerant B cells display 30% of the number of available HEL-binding receptors on nontolerant B cells, yet failed to respond to any concentration of HEL in assays where nontolerant B cells responded optimally to concentrations that engage <5% of available receptors (Figs. 3, B and C, and 4 C). Second, Dbl-transgenic B cells that had been parked in nontransgenic mice and that had recovered comparable HEL-binding capacity remained unable to increase [Ca]<sub>i</sub> after HEL binding (Fig. 4 B). Third, IgD receptors are expressed at identical levels on tolerant and nontolerant B cells and should be cross-linked identically after treatment with polyclonal antisera to IgD or anti-IgD coupled to dextran. These stimuli nevertheless elicited markedly reduced proximal signaling and failed to induce mitogenesis in tolerant B cells (Fig. 5 C and D, and data not shown).

Given the evidence for a signaling block, the failure of anti-IgD stimulation to induce G<sub>0</sub>-G<sub>1</sub> transition or cell cycle entry in the tolerant B cells despite activating some tyrosine phosphorylation and calcium influx (Fig. 5), could have implied a block downstream of these initial events. The fact that the tolerant B cells proliferated normally after stimulation with calcium ionophore and phorbol ester (Fig. 6 E) nevertheless argues against a downstream defect. Moreover, the short-lived calcium response induced by anti-IgD in the tolerant cells (Fig. 6 A) is most consistent with a receptor-proximal defect, since coupling of [Ca]<sub>i</sub> stores to cellular influx, as assessed by exposure to thapsigargin occurs normally in the tolerant B cells (Cooke, M.P., unpublished ob-

servations). Sustained Ag receptor signaling is necessary for activation of both B (43) and T cells. Removal or blocking of TCR stimuli any time during the first 2 h of stimulation blocks early cellular responses such as IL-2 gene induction (44) and similarly, removal of anti-IgM Abs any time during the first 16 h of B cell stimulation effectively halts cell cycle progression (45). Indeed, the lack of response to weak stimuli (sHEL) and transient responses to strong stimuli (polyclonal anti-IgD) resembles the response to different anti-TCR Abs in mutant T cell lymphomas that lack a single component of the TCR signal-transduction complex, such as the tyrosine kinase p56<sup>lck</sup> (46).

Exactly how Ag binding induces sIg signaling remains to be fully elucidated, although Ag-driven receptor dimerization or multimerization is thought to play an important role (for a review see reference 47). The ability of sHEL to trigger sIg signaling is in this respect surprising, since sHEL is known to bind the transgene-encoded Ig as a monomer (48). Many of the signaling events induced by sHEL (Figs. 4 and 5) nevertheless appear equivalent to those described for nonmitogenic monoclonal IgG Abs to IgM or IgD (for reviews see references 35 and 36). We therefore favor the view that sHEL, like anti-Ig Abs, induces sIg signaling by receptor dimerization. HEL-mediated dimerization could occur via preformed HEL dimers in solution or be driven by high local concentrations of receptor-bound HEL on the cell surface, since HEL dimerizes in solution at higher concentrations (49). By comparison, the ability of polyvalent ligands such as polyclonal anti-IgD and membrane-bound HEL to partially overcome the sHEL-induced signaling blockade may simply reflect more extensive sIg multimerization induced by these ligands. Consistent with this notion, increasing the valency of initially oligovalent Ags or divalent anti-Ig Abs has been found to markedly increase their potency for B cell activation (39, 50).

Since other B cell surface proteins such as CD19, CD21 (for reviews see references 51 and 52), and CD22 (53) may also participate in signaling by sIg, it will be important to determine whether differences in their activity may contribute to the sIg signaling defect found in tolerant B cells. It will also be important to relate the regulation of sIg signaling in tolerant B cells to the interruption of sIg signaling induced in mature B cells by acute sIg cross-linking (54, 55) or by coligation with the Fcγ receptor (56, 57), particularly since the latter is thought to provide an important negative feedback during Ab responses to foreign Ags (for a review see reference 58).

**Role of sIg Signaling in B-T Cell Interaction.** Whereas the role of sIg in mediating Ag uptake for subsequent processing and presentation to Th cells is well established (9-12), the role of sIg signal transduction in T cell-dependent Ab responses has remained unclear (59-62). In particular, it is difficult to reconcile the need for both MHC-restricted B cell Ag presentation and the need for sIg signaling with recent studies demonstrating that, in the absence of both events, membrane CD40L together with T cell-derived lymphokines can nevertheless trigger resting B cells to proliferate and produce Ab efficiently (for a review see reference 7).

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