

# Resting and Anergic B Cells Are Defective in CD28-dependent Costimulation of Naive CD4<sup>+</sup> T Cells

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## Summary

Successful antibody production *in vivo* depends on a number of cellular events, one of the most important of these being cognate B cell–T cell interaction. To examine this phenomenon *in vitro*, homogeneous populations of hen egg lysozyme (HEL)-specific small resting B cells and naive CD4<sup>+</sup> HEL-specific T cells (derived from immunoglobulin [Ig] and T cell receptor transgenic mice, respectively) were cultured together. On addition of intact HEL protein, HEL-specific B cells increase their expression of activation molecules, including a B7-related protein and CD44, and enlarge into blast cells. Within the same cultures, HEL-specific CD4<sup>+</sup> T cells also increase expression of the activation markers CD69 and CD44, enlarge, secrete lymphokines, and proliferate. This response is radiation sensitive, supporting the conclusion that HEL-specific B cells present antigen to and activate the naive T cells. By contrast, when a synthetic peptide fragment of HEL is used to bypass B cell antigen–receptor engagement, the naive T cells enlarge and display activation antigens, but fail to produce lymphokines, proliferate, or promote B cell blastogenesis. Presentation of HEL by tolerant B cells, which are no longer able to signal effectively through their antigen receptors, results in an identical pattern of incomplete T cell activation. Addition of a stimulating anti-CD28 antibody and blocking of CD28 signals with CTLA4/Ig fusion protein both show that complete activation of naive CD4<sup>+</sup> T cells depends on the initial induction of B7 and related costimulatory molecules after HEL binding to nontolerant HEL-specific B cells. Thus, in the absence of adequate costimulation from the B cell, naive CD4<sup>+</sup> T cells undergo a form of “partial activation” in which they upregulate surface expression of certain T cell activation antigens, but fail to efficiently produce lymphokine and proliferate. This may explain the different conclusions that have been reached regarding the consequences of B cell antigen presentation to T cells, in that the ability of B cells to activate naive CD4<sup>+</sup> T cells depends both on their specificity and their activation state.

Foreign antigens evoke an immune response by triggering an elaborate network of cell–cell interactions and activation events. For effective antibody production, antigen-specific B cells must be activated to clonally expand and differentiate into plasma cells, and this can depend on a variety of cells, including CD4<sup>+</sup> helper T cells (1), macrophages (2), dendritic cells (3–5), and follicular dendritic cells (6, 7). Many factors, such as the form and amount of antigen, route of administration, the presence of particular adjuvants, and prior exposure to the antigen, influence whether a foreign antigen will elicit an antibody response, and to what extent particular cell types are required. How each of these factors ultimately influences the immunogenicity or tolerogenicity of antigens is not well understood, in large part because of the many different cell interactions that may be affected. One

of the key cellular interactions in antibody responses is that between antigen-specific B cells and cognate helper T cells (1, 8, 9). A number of critical molecular events occurring during this interaction have been identified. First, helper T cell–dependent antibody production depends on MHC-restricted recognition of antigen presented by the B cell. In response to T cell recognition of presented antigens occurring through TCR, helper T cells can potentially display molecules such as CD40L (10–13) or membrane TNF (14) on their cell surface, or secrete the cytokines IL-2, IL-4, IL-5, or IL-10 (9). Each of these molecules has profound positive effects on B cell proliferation and differentiation into plasma cells. Selective delivery of T cell help is achieved because antigen-specific B cells are much more efficient at taking up antigen for presentation to helper T cells, using their surface

Ig (sIg)<sup>1</sup> antigen receptors to bind and internalize antigen for degradation into peptides that are then presented on the cell surface in association with class II MHC molecules (15). Presentation of antigen by B cells to helper T cells is nevertheless not a simple inductive event leading inevitably to antibody production, but may instead lead to tolerance or have no effect (1, 9, 16). Resolving what factors influence the outcome of this interaction is clearly important for understanding the immunogenicity or tolerogenicity of antigens, but has been limited by the need to use indirect *in vivo* assays or transformed or immortalized cell lines *in vitro*.

To directly visualize interactions between antigen-specific B and T cells, we describe below the use of transgenic mice carrying rearranged Ig and TCR genes as a source of many homogeneous resting B and T cells specific for a well-characterized protein antigen, hen egg lysozyme (HEL). Transgenic mice were produced on an inbred C57BL/6 background carrying transgenes encoding the TCR  $\alpha$  and  $\beta$  chains from the HEL-specific T cell hybridoma 3A9 (17, 18). Purified resting T cells from these mice were cultured *in vitro* with resting B cells from previously described transgenic mice expressing heavy and light chain genes encoding high-affinity HEL-specific IgM and IgD (19). On addition of HEL antigen, resting HEL-specific B cells could trigger a spectrum of responses in naive HEL-specific CD4<sup>+</sup> T cells. The form taken by the resulting T cell response is very much dependent on the activation state of the B cell and is determined at least in part by the induction of B7/B7-related molecules on the B cells after B cell antigen-receptor engagement, and their subsequent interactions with CD28 on the T cell.

## Materials and Methods

**Transgenic Mice.** Transgenic mice expressing HEL-specific IgM and IgD and soluble HEL/anti-HEL "double-transgenic" mice have been described elsewhere (19). Mice expressing the cytochrome C-specific TCR- $\alpha/\beta$  5C.C7 have also been described (20–22). Mice expressing the HEL-specific 3A9 TCR were derived by inserting the 3A9 VJ $\alpha$  and VDJ $\beta$  fragments into modified genomic shuttle vectors, the original forms of which have previously been described (23). The  $\beta$  shuttle vector was modified such that it carried 7.5 kb 3' to the constant region, including the TCR- $\beta$  enhancer region (24–26), instead of the Ig enhancer originally used in the shuttle vector. The  $\alpha$  shuttle also had its Ig enhancer removed and replaced with 3.5 kb 3' to the  $\alpha$  constant region (27). The VJ and VDJ regions of the  $\alpha$  and  $\beta$  chains of the 3A9 TCR were amplified via PCR from cDNA clones (generously provided by L. Glimcher, Harvard University, Cambridge, MA [18]) with oligo primers containing cDNA homology, splice acceptor and donor sites, segments of intron sequence, and restriction sites required for subcloning into the modified genomic shuttle vectors. Resulting VJ and VDJ cassettes were gel purified, restricted, and subcloned into the modified shuttle vectors. Linearized TCR- $\alpha$  and - $\beta$  genomic shuttle constructs were then purified away from vector sequences and coinjected into fertilized C57BL/6J eggs. Two female founders were

generated. T cells were obtained from mice derived from one founder that had been crossed onto the B10.BR/SgSnJ background such that MHC class II A<sup>k</sup> molecules were present during T cell development. C57BL/6J and B10.BR/SgSnJ inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

**Antiserum, Antibodies, and Flow Cytometry.** Antiserum specific for the V $\alpha$  chain of the 3A9 TCR was generated (Josman Laboratories, Napa, CA) by immunizing a rabbit with chimeric protein consisting of the 3A9 $\alpha$  VJ region plus the IgG2a heavy chain constant region (28). Antiserum was preabsorbed on a mouse IgG column before use. Antibody to the murine TCR V $\beta$ 8.2 region was derived from the hybridoma F23.2 (29) and biotinylated. Texas red-conjugated donkey anti-rabbit Ig was obtained from Jackson ImmunoResearch (West Grove, PA) and avidin-allophycocyanin from Biomedica (Foster City, CA). PE-conjugated anti-L3T4 and FITC-conjugated anti-Lyt2 were obtained from Becton Dickinson & Co. (San Jose, CA). Antibodies to murine Thy1.2, CD8, and rat IgG were obtained from CALTAG Laboratories (South San Francisco, CA). Antibodies to murine CD43, I-E<sup>k,r</sup>, CD44, and CD69 were obtained from Pharmingen (San Diego, CA). Avidin-FITC was obtained from Organon Teknika (West Chester, PA). The F4/80 hybridoma was obtained from the American Type Culture Collection (Rockville, MD). Anti-CD28 mAb was a generous gift of J. Allison (University of California at Berkeley). CTLA4/Ig fusion protein and control L6-Ig fusion protein were kindly provided by P. Linsley (Bristol-Myers Squibb, Pharmaceutical Research Institute, Seattle, WA). Staining with CTLA4/Ig was developed with anti-human FITC (Tago Inc., Burlingame, CA).

**Purification of T and B Cells.** Naive CD4<sup>+</sup> 3A9 T cells were derived based on the method of Sagerström et al. (30). Inguinal, popliteal, axillary, brachial, cervical, mesenteric, and periaortic lymph nodes were harvested from 3A9-transgenic mice, and single-cell suspensions were made by forcing the tissue through wire mesh. After washing in 5% FCS/RPMI 1640, cells were stained with anti-CD8-FITC, anti-E<sup>k</sup>-biotin (17.3.3), and anti-CD44-PE followed by avidin-FITC. Cell sorting was performed on a FACStar<sup>®</sup> (Becton Dickinson & Co.), gating for FITC<sup>neg</sup>/CD44<sup>lo</sup> cells with additional forward and side scatter gates for small lymphocytes. The resulting population was >98% Thy1<sup>+</sup>. Naive CD4<sup>+</sup> 5C.C7 T cells were purified in the same way from 5C.C7-transgenic mice.

Small resting B cells were purified from anti-HEL Ig-transgenic or HEL/anti-HEL double-transgenic mice by preparing splenocyte cell suspensions with wire mesh. Erythrocytes were removed by centrifugation over Lympholyte<sup>®</sup> medium (Accurate Chemical and Scientific Corp., Westbury, NY) and lymphocytes washed and stained with anti-Thy1.2-FITC, anti-CD43-FITC (antibody S7 [31]), and a rat anti-murine macrophage (F4/80) mAb (32) followed by anti-rat IgG-FITC. Cells were sorted as above, gating for FITC<sup>neg</sup> cells with additional forward and side scatter gates for small lymphocytes. The resulting population was >98% B220<sup>+</sup>.

**Cell Culture.** T and B cells were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, 2-ME, and penicillin/streptomycin in 96-well flat-bottom polystyrene tissue culture plates. Cultures contained 2 × 10<sup>5</sup> sorted B cells and 1–1.5 × 10<sup>5</sup> sorted T cells. To assay lymphokine production, cultures were incubated at 37°C in 5% CO<sub>2</sub> for 2 d, and supernatants were harvested and assayed with the IL-2/IL-4-dependent cell line, HT-2 (33, 34). For phenotypic analyses, cultured cells were stained with antibodies and analyzed via four-color flow cytometry, dead cells being gated out with propidium iodide.

**Proliferation Assay.** Sorted B cells were irradiated using a Cs<sup>137</sup> source. After culture with naive CD4<sup>+</sup> cells and antigen for 2.5 d, wells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine for 12 h, harvested,

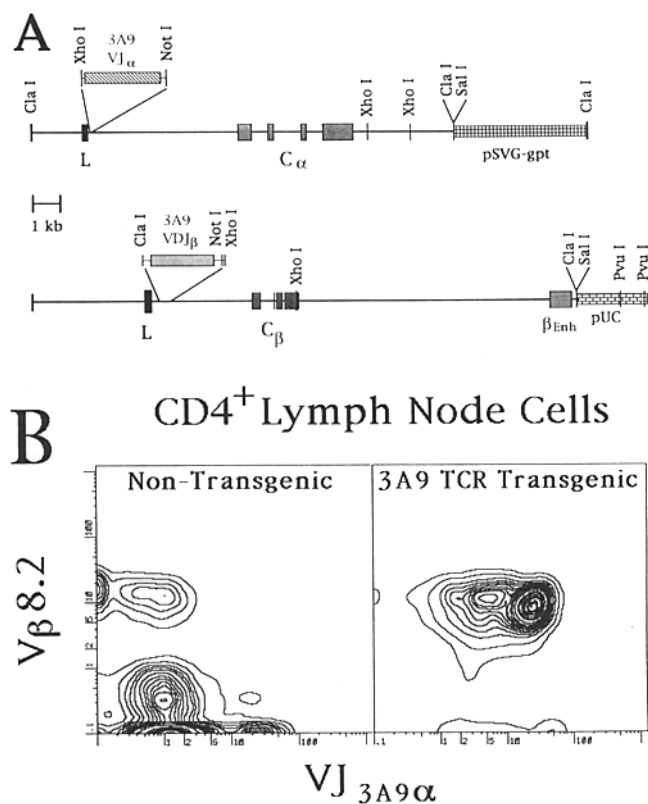
<sup>1</sup>Abbreviations used in this paper: HEL, hen egg lysozyme; MCC, moth cytokine C; sIg, surface Ig.

and incorporated [ $^3\text{H}$ ]thymidine counted on a Matrix 96 direct beta counter (Packard Instruments, Meriden, CT).

**Antigens.** HEL was from Sigma Chemical Co. (St. Louis, MO). Moth cytochrome C (MCC) 88-103 peptide (ANERADLIAYLK-QATK) was synthesized and HEL peptide 46-61 (NTDGSTDYGL-QINSR) was synthesized and HPLC-purified by the PAN facility of the Beckman Center for Molecular and Genetic Medicine (Stanford, CA).

## Results

**Activation of Antigen-Specific B and T Cells.** As described in Materials and Methods, HEL-specific T cells were derived from TCR-transgenic mice generated with a modified version of previously described (23) genomic TCR shuttle vectors (Fig. 1 A). The vast majority of  $\text{CD4}^+$  lymphocytes from these transgenic mice expressed both chains of the 3A9 TCR, as evidenced by staining of lymph node cells with mAb specific for V $\beta$ 8.2 and antiserum against the 3A9  $\alpha$  chain (Fig. 1 B).



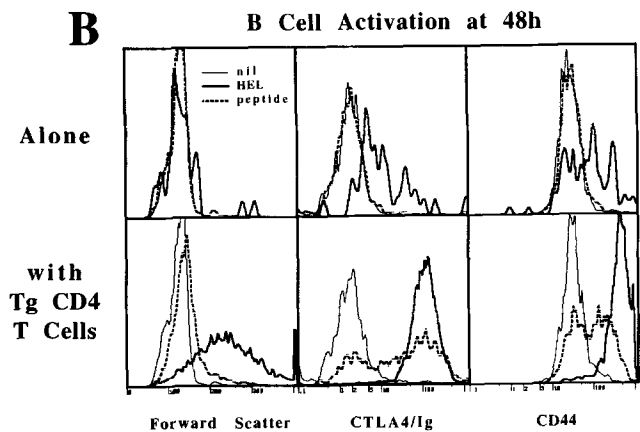
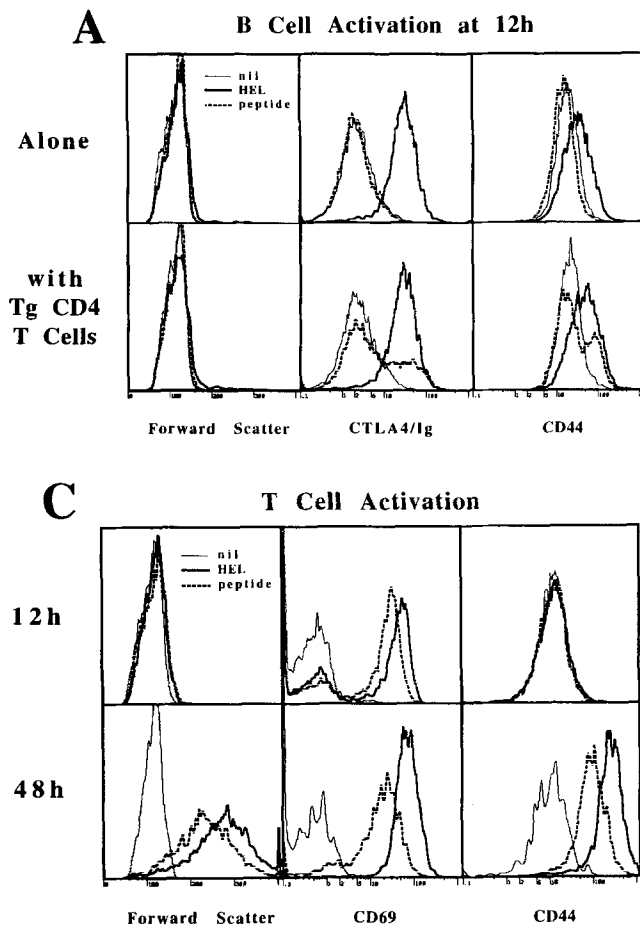
**Figure 1.** 3A9 TCR transgenic mice. (A) Modified TCR genomic shuttle vectors. Shuttle vectors derived from the genomic TCR- $\alpha$  and TCR- $\beta$  sequences of the hybridoma 2B4 were modified from the original (23), and 3A9 VJ $\alpha$  and VDJ $\beta$  sequences inserted, as described in Materials and Methods. (B) Expression of the 3A9 TCR on  $\text{CD4}^+$  lymphocytes. Cells were derived from the lymph nodes of 3A9 TCR transgenic mice ( $\text{H-2}^k$ ) and nontransgenic littermates and stained with anti-L3T4 (PE), anti-Lyt2 (FITC), biotinylated F23.2 (anti-V $\beta$ 8.2), avidin-allophycocyanin, rabbit anti-3A9 $\alpha$  antiserum, and anti-rabbit Ig (Texas red). Plots show levels of expression of the  $\alpha$  and  $\beta$  chains on  $\text{CD4}^+$  cells by use of 5% probability contour lines.

To examine cognate antigen recognition by both small resting B cells and naive  $\text{CD4}^+$  T cells, purified HEL-specific B and T cells were mixed and cultured either without antigen, with intact HEL, or with a synthetic HEL peptide (amino acids 46-61). In the cultures containing intact HEL, the HEL-specific B cells exhibited a rapid increase in cell surface expression of B7 and related molecules detected by staining with CTLA4/Ig (35) and an increase in expression of CD44 (36) (Fig. 2 A, bottom, solid lines). By 48 h of culture with HEL, expression of these markers had increased further, and the B cells were greatly enlarged, as indicated by the forward scatter (Fig. 2 B, bottom, solid lines). None of these changes occurred in cultures of T and B cells lacking HEL. Cultures of B cells with HEL but without T cells led to upregulation of CTLA4/Ig staining and CD44 expression at 12 h, but by 48 h most of the B cells had died, in contrast to the B cell blastogenesis that occurred in the presence of T cells (Fig. 2, A and B, top).

In cultures of T and B cells containing HEL peptide (which does not bind to the HEL-specific Ig molecules expressed by the B cells), a fraction of the B cells had increased the expression of B7/B7-related and CD44 molecules, but none of the B cells formed large blast cells. This HEL peptide-induced B cell activation is entirely T cell dependent, since no increase in CTLA4/Ig or CD44 staining occurred on B cells in peptide-stimulated cultures lacking HEL-specific T cells (Fig. 2, A and B, broken lines).

Parallel analysis of T cells in mixed B cell/T cell cultures showed that the presence of intact HEL induced the T cells to express high levels of CD69 (37) by 12 h, though there was no change in CD44 or in cell size (Fig. 2 C, top). By 48 h, all of the T cells had markedly elevated CD44 levels (38) and had enlarged into blasts (Fig. 2 C, bottom). Addition of HEL peptide instead of intact HEL also led to T cell activation, although induction of CD69, CD44, and cell enlargement was somewhat reduced (Fig. 2 C, broken lines).

**Lymphokine Production by Naive  $\text{CD4}^+$  T Cells.** Activation of naive  $\text{CD4}^+$  T cells in culture with antigen-specific B cells was further analyzed by measuring production of lymphokines with the IL-2/IL-4-dependent cell line HT-2. HEL-specific T cells produced significant amounts of lymphokine in culture with HEL-specific B cells and intact HEL (Fig. 3 A, open squares). A biphasic response was observed to increasing concentrations of added HEL. The first peak of lymphokine production occurred at 10-nM HEL, which corresponds to the  $K_d$  of the HEL-specific Ig expressed by the transgenic B cells (39). By contrast, much less lymphokine was produced by HEL-specific T cells cultured with sorted B cells from nontransgenic mice (which do not carry HEL-specific Ig molecules), and this low-level response required  $10^3$ - $10^5$ -fold higher concentrations of HEL (solid squares). As further controls, naive  $\text{CD4}^+$  T cells were sorted from nontransgenic littermates of the TCR-transgenic mice. When cultured with HEL and HEL-specific B cells, no detectable levels of lymphokine were produced (data not shown). In addition, sorted TCR-transgenic T cells incubated with the HEL antigen, but without added B cells, demonstrated no



**Figure 2.** Activation of HEL-specific B and T cells cultured with intact HEL or HEL peptide. Sorted small resting HEL-specific B cells from ( $B6 \times B10.BR$ ) $F_1$ -transgenic mice were cultured alone or mixed with sorted naive HEL-specific  $CD4^+$  T cells from TCR-transgenic mice. The cells were cultured in media alone,  $1 \mu M$  HEL, or  $1 \mu M$  HEL peptide (amino acids 46–61). At the indicated times of culture, cells were harvested, stained, and analyzed by flow cytometry. (A) Analysis of  $B220^+$  cells at 12 and (B) 48 h of culture, showing forward scatter as a measure of cell size and immunofluorescent staining with CTLA4/Ig or a monoclonal antibody to CD44. (C)  $CD4^+$  cells from the same cultures, showing cell size and immunofluorescent staining for CD69 or CD44. Note that histograms of HEL-exposed B cells cultured alone for 48 h are derived from very few cells, since most of the cells had died as a result of this treatment.

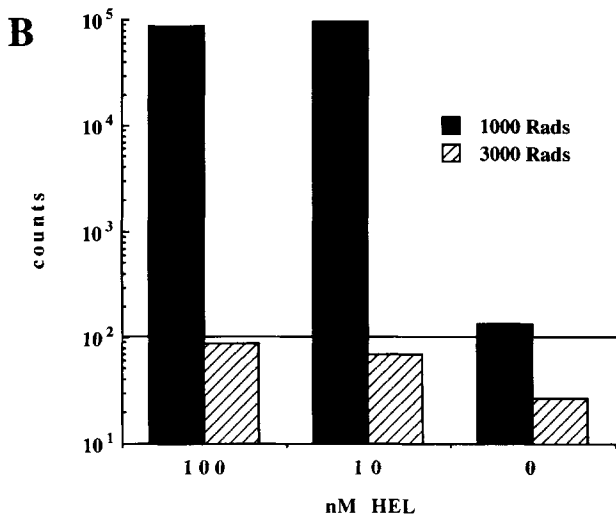
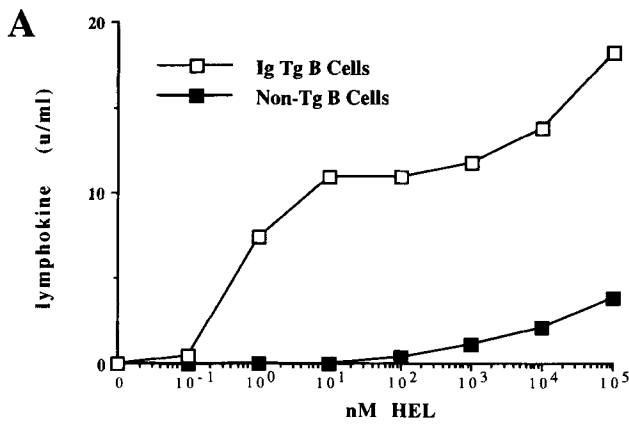
activation or lymphokine production (data not shown), indicating that the sorted T cell population had no intrinsic APC activity.

To address whether other APCs such as macrophages or dendritic cells might contaminate the sorted B cell population and account for the activation of the transgenic T cells, the HEL-specific B cells were irradiated before culture. Previous studies have established that APC activity of B cells is more radiosensitive than that of macrophages or dendritic cells (40, 41). As shown in Fig. 3 B, naive T cells proliferated efficiently in cultures with intact HEL and HEL-specific B cells pretreated with 1,000 rad, a dose that leaves the APC activity of B cells relatively intact while preventing them from dividing. [ $^3H$ ]Thymidine incorporation by T cells was reduced to background, however, when the B cells were pretreated with 3,000 rad, a dose that has been shown to abolish B cell APC activity but spare APC activity in macrophages and dendritic cells (40, 41). This finding indicates that the B cells are responsible for activating naive  $CD4^+$  T cells in these cultures.

**Anti-CD28 Antibody Mimics the Effect of Intact HEL on HEL-specific B Cell APC Function.** In contrast to the lymphokine production stimulated by intact HEL protein, little or no lymphokine was produced by cultures receiving the HEL pep-

ptide (Fig. 4, *solid triangles*). The possibility that the synthetic peptide was not taken up and presented by resting HEL-specific B cells was excluded, since induction of CD69, CD44, and blastogenesis occurred in the cultures receiving HEL peptide (Fig. 2 C). An alternative explanation for the failure to trigger lymphokine production was that the B cells lacked some costimulatory molecule that was induced when intact HEL bound to sIg (42). Cultures of HEL-specific  $CD4^+$  T cells and B cells were therefore stimulated with HEL peptide in the presence of anti-CD28 mAb (43) that would cross-link CD28 molecules on T cells and deliver a costimulatory signal (44–50). Lymphokine production by the T cells was indeed restored by this treatment (Fig. 4, *open triangles*), confirming that HEL peptide loading of I-A<sup>k</sup> had occurred on the B cells and implying that a costimulatory signal was lacking from the peptide-loaded-HEL-specific B cells.

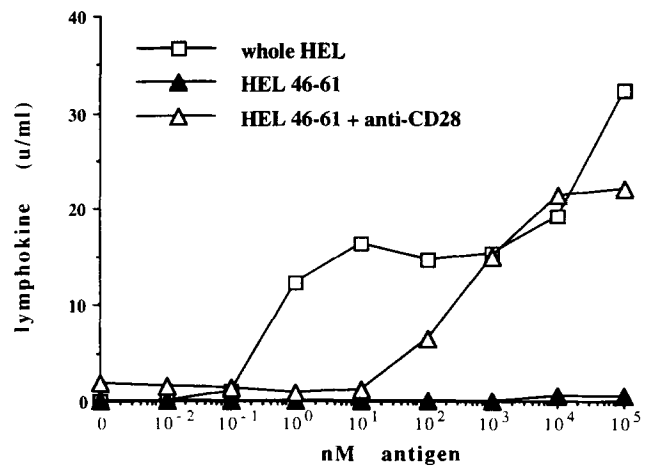
Although the above finding confirms that HEL peptide is being presented by peptide-pulsed HEL-specific B cells, the difference between stimulation with intact HEL and HEL peptide might still reflect some quantitative differences in the amount of HEL peptide being presented. To dissociate HEL binding from antigen presentation, naive  $CD4^+$  T cells were purified from mice transgenic for a different TCR, 5C.C7



**Figure 3.** Response of naive HEL-specific CD4<sup>+</sup> T cells to B cells and intact HEL. (A) Naive CD4<sup>+</sup> T cells sorted from lymph nodes of 3A9 TCR-transgenic mice were mixed with resting B cells sorted from spleens of anti-HEL Ig-transgenic mice (or nontransgenic littermates) and cultured for 48 h with varying amounts of intact HEL. Culture supernatants were assayed for lymphokines by measuring proliferation of HT-2, an IL-2/IL-4-dependent cell line. (B) Proliferative response in cultures of HEL-specific T and B cells measured by [<sup>3</sup>H]thymidine incorporation after 2.5-d culture with 100, 10, or 0 nM HEL. Before culture, the HEL-specific B cells were pretreated with either 1,000 or 3,000 rad irradiation. Horizontal line marks approximate upper boundary of background counts obtained.

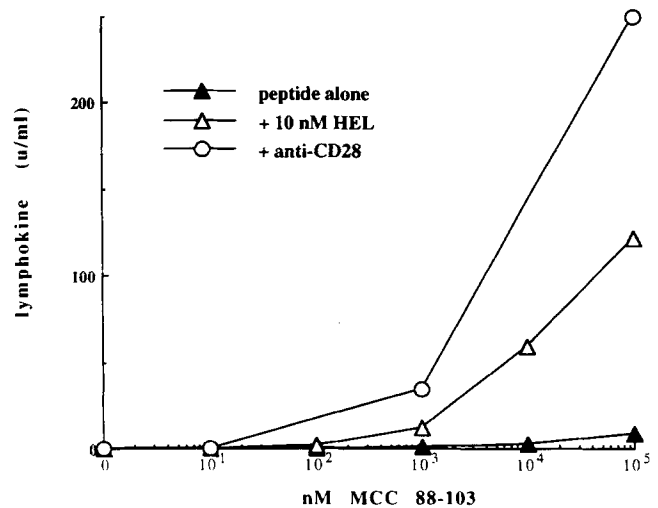
(21, 22), which is specific for the MCC peptide 88-103 bound to the I-E<sup>k</sup> molecule (20). Increasing concentrations of MCC peptide were added to cultures of naive 5C.C7 T cells and HEL-specific B cells, and lymphokine production was assayed as before. Again, the small resting B cells exposed to peptide induced only very modest lymphokine production by the CD4<sup>+</sup> T cells (Fig. 5, *solid triangles*). In contrast, addition of intact HEL to the cultures led to efficient lymphokine production by the cytochrome-reactive T cells (*open triangles*). Somewhat higher stimulation could also be achieved by addition of anti-CD28 antibody (*open circles*).

**CTLA4/Ig Blocking Studies.** It has been shown previously

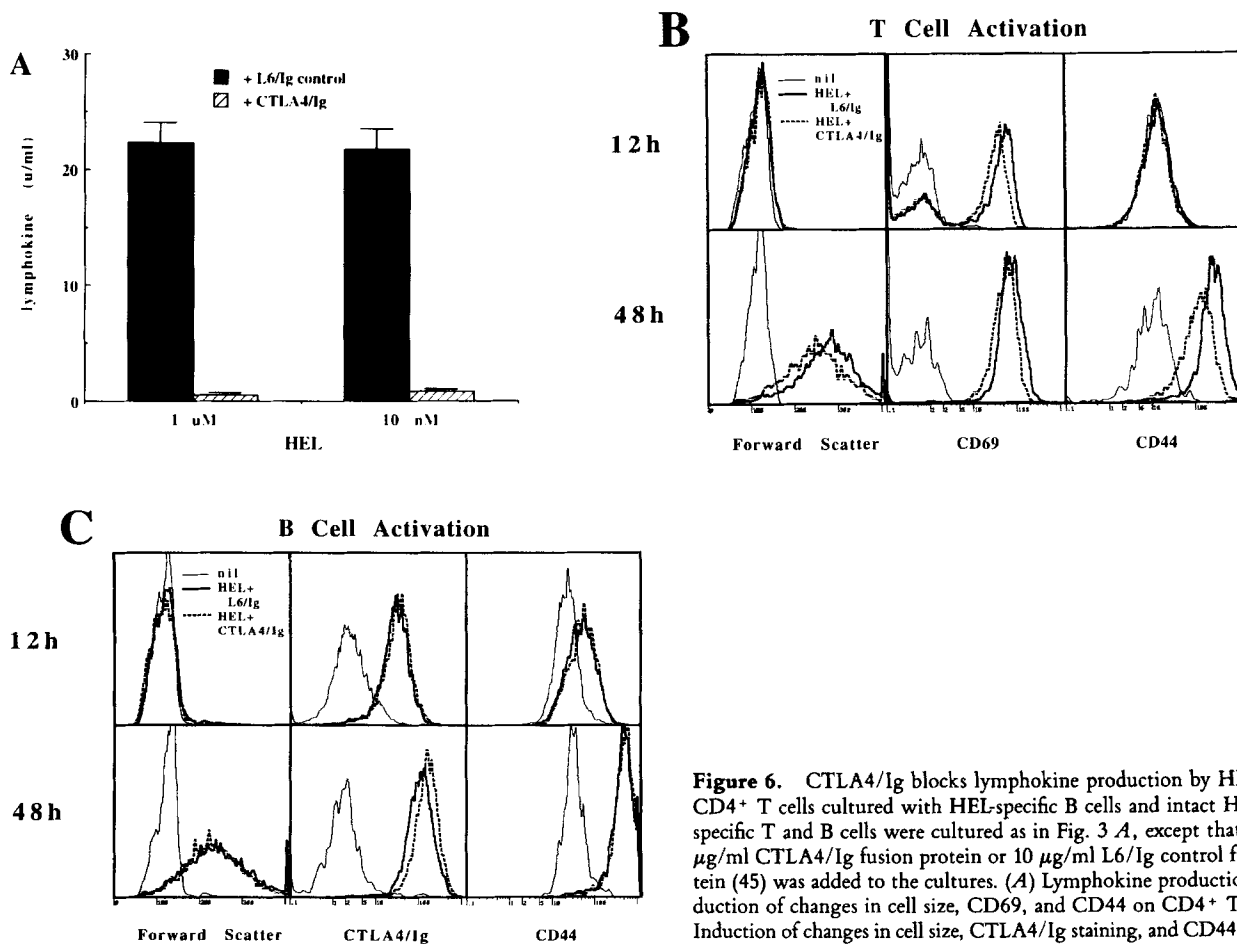


**Figure 4.** Absence of lymphokine production by HEL-specific naive CD4<sup>+</sup> cells cultured with HEL-specific B cells and HEL peptide and rescue with CD28 costimulation. HEL-specific T and B cells were cultured as in Fig. 3 A and lymphokine production measured after 48 h exposure to intact HEL, HEL peptide (46-61), or HEL peptide plus monoclonal antibody to CD28.

that intact HEL, like nonmitogenic anti-Ig antibodies, activates a tyrosine kinase signaling pathway via surface Ig in HEL-specific Ig-transgenic B cells, and triggers rapid cell surface expression of B7 and related molecules as detected by the CTLA4/Ig fusion protein (51). CTLA4/Ig binds to B7 and related molecules, and to test whether these were critical for lymphokine production, CTLA4/Ig was added to the



**Figure 5.** Presence of intact HEL or anti-CD28 antibody triggers lymphokine production by both cytochrome C (MCC)-specific naive CD4<sup>+</sup> T cells cultured with sorted HEL-specific B cells and MCC peptide. MCC-specific naive CD4<sup>+</sup> T cells were sorted from 5C.C7 TCR-transgenic mice and cultured with HEL-specific B cells sorted from Ig-transgenic mice. The indicated concentrations of MCC peptide 88-103 were added to the cultures together with 10 nM HEL, anti-CD28 mAb, or no additional stimulus. Lymphokines were assayed from culture supernatants after 48 h.



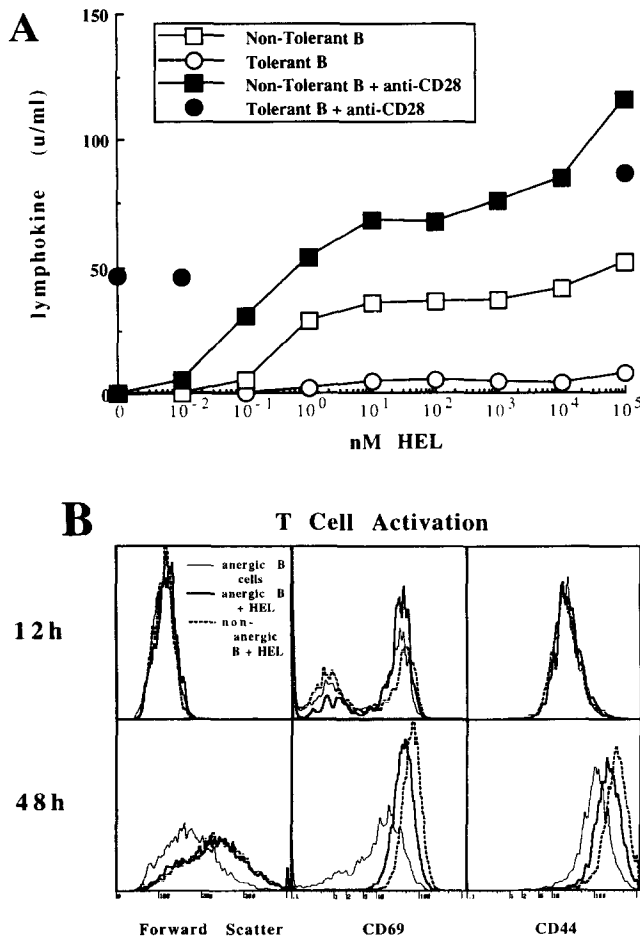
**Figure 6.** CTLA4/Ig blocks lymphokine production by HEL-specific CD4<sup>+</sup> T cells cultured with HEL-specific B cells and intact HEL. HEL-specific T and B cells were cultured as in Fig. 3 A, except that either 10  $\mu$ g/ml CTLA4/Ig fusion protein or 10  $\mu$ g/ml L6/Ig control fusion protein (45) was added to the cultures. (A) Lymphokine production. (B) Induction of changes in cell size, CD69, and CD44 on CD4<sup>+</sup> T cells. (C) Induction of changes in cell size, CTLA4/Ig staining, and CD44 on B cells.

cultures to block the binding of B7 and related molecules to CD28 on the T cells (35). As shown in Figure 6 A, addition of CTLA4/Ig abolished lymphokine production to background levels. Control cultures receiving an irrelevant fusion protein, L6/Ig (45), produced large amounts of lymphokine.

In contrast to the striking effect of CTLA4/Ig on lymphokine production, the induction of CD69, CD44, and blastogenesis is not abolished by this treatment (Fig. 6 B). Although the degree of T cell enlargement and levels of cell surface markers were somewhat reduced, these observations were consistent with the response observed in T/B cell cultures stimulated with HEL peptide (Fig. 2 C). (Unlike peptide-stimulated cultures, however, HEL-binding B cells stimulated with intact HEL but containing CTLA4/Ig continued to undergo blastogenesis [Fig. 6 C]). These data show that the lack of a costimulatory signal through CD28 (and possibly CTLA4) leads to a state of “partial activation,” in which T cells appear to be phenotypically activated but are profoundly deficient in lymphokine production and subsequent proliferation.

*Activation of Naive CD4<sup>+</sup> T Cells by Anergic B Cells.* It has previously been found that HEL-specific B cells developing

in “double-transgenic” mice that express HEL as a soluble self-antigen are rendered anergic because of a proximal block in the sIg-signaling pathway that prevents induction of B7 and related molecules after antigen binding (51). To examine further the relationship between sIg signaling, B cell activation, and T cell activation, anergic small resting B cells derived from double-transgenic mice were cultured with HEL-specific T cells. In contrast to nontolerant B cells from Ig-transgenic mice, anergic B cells stimulated with intact HEL remained unable to trigger efficient lymphokine production by naive HEL-specific T cells (Fig. 7 A, *open circles*). As with the HEL peptide, addition of the anti-CD28 antibody restores T cell stimulation (*solid circles*), consistent with the notion that the tolerant B cells are capable of presenting antigen but simply lack expression of the necessary costimulatory molecules. Analysis of these T cells cultured with tolerant double-transgenic B cells also shows that they are phenotypically activated even when HEL is not added to the cultures, implying that the tolerant B cells had already been loaded with HEL peptide during continuous exposure in vivo (Fig. 7 B, *thin lines*). Addition of exogenous HEL to the mixture of tolerant B cells and T cells triggered further induction of CD69 and CD44



**Figure 7.** T cell activation by tolerant HEL-specific B cells from HEL/anti-HEL double-transgenic mice. (A) Lymphokine production by naive CD4<sup>+</sup> HEL-specific T cells cultured for 2 d with either sorted nontolerant B cells from Ig-transgenic mice or tolerant B cells from HEL/anti-HEL double-transgenic mice. Anti-CD28 antibody was also added where indicated to provide exogenous costimulation. (B) Induction of changes in cell size, CD69, and CD44 in CD4<sup>+</sup> HEL-specific T cells after 12 or 48 h culture with tolerant double-transgenic B cells or nontolerant Ig-transgenic B cells, in the presence or absence of 10<sup>-6</sup>M intact HEL.

in the T cell population, but not to the same levels achieved in cultures with nontolerant HEL-specific B cells. Both tolerant and normal B cells were equally able to stimulate T cell enlargement (Fig. 7 B, left, 12 versus 48 h).

## Discussion

The findings discussed above demonstrate that antigen-specific B cells can trigger cognate T cells to become activated, secrete lymphokine, and proliferate. This ability to activate T cell effector functions is not constitutive, but depends on the specificity and activation state of the B cells. This varying competence of B cells to trigger different T cell effector functions may play an important role in determining the im-

munogenicity or tolerogenicity of antigens and may account for the contradictory findings in other studies (52–63).

Naive CD4<sup>+</sup> HEL-specific T cells display a spectrum of cellular responses when cocultured with nontolerant HEL-specific B cells and intact HEL antigen. This spectrum includes a marked increase in cell surface expression of CD69 and CD44, T cell enlargement into blast cells, lymphokine production, and T cell proliferation. In addition, T cell-dependent blastogenesis is also triggered in the cocultured B cells (Fig. 2 B), presumably in response to T cell-derived lymphokines or cell-bound signals such as CD40L (10, 11, 13) or membrane TNF (14). In contrast, only parts of this response spectrum are triggered in T cells when antigen is presented by HEL-specific B cells under conditions that preclude B cell activation via direct binding of intact HEL. Thus B cells exposed to the HEL peptide (which cannot engage the HEL-specific receptors on the B cells) trigger T cell enlargement and expression of CD69 and CD44, but no lymphokine production occurs and no blastogenesis is induced in the B cells. A similar outcome was observed for CD4<sup>+</sup> T cells in mixtures of T cells and tolerant HEL-specific B cells exposed to intact HEL. Rapid induction of CD69 on the cocultured T cells nevertheless confirms that the tolerant B cells are presenting intact HEL to T cells effectively, and that the B cells have in fact been loaded with HEL peptides during their prolonged exposure to HEL in vivo. Since tolerant B cells are unable to sustain sIg-mediated signaling and B cell activation after binding of intact HEL (51), their failure to trigger T cell lymphokine production also points to the importance of sIg-mediated B cell activation.

We also show here that costimulation through the CD28 molecule can account, at least in part, for the stimulatory competence conferred on HEL-specific B cells by binding of intact antigen and subsequent sIg signaling. Binding of HEL to nontolerant HEL-specific B cells triggers rapid induction of cell surface molecules detected by CTLA4/Ig. These B7-related molecules detected on HEL-activated B cells are likely to be B7-2/B70 (64–67), since they do not bind previously described B7-specific monoclonal antibodies, nor can their staining with CTLA4/Ig be blocked by these monoclonal antibodies (Lenschow, D., J. Bluestone, M. P. Cooke, and C. C. Goodnow, unpublished observations). B7 and related molecules were shown to be essential for induction of lymphokine production by the T cells, since the latter was abolished in cultures where the B7 and related molecules were blocked by soluble CTLA4/Ig (Fig. 6 A).

Several lines of evidence suggest that the induction of B7 and related molecules on B cells is not the sole determining factor rendering them competent to trigger the full spectrum of T cell responses. First, CTLA4/Ig-reactive molecules were rapidly induced to equivalent levels on a reasonable fraction of HEL-specific B cells cultured with T cells and the HEL peptide (Fig. 2 A, bottom center). Based on the studies of Nabavi et al. (68), it seems likely that the induction of these molecules occurs by signaling through I-A<sup>k</sup> molecules on the B cells that have been engaged by HEL/I-A<sup>k</sup>-specific antigen receptors on the T cells. Since no lymphokine production

or B cell blastogenesis resulted from these interactions, some additional event that occurs after binding of intact HEL must have been lacking in the peptide-stimulated cultures.

The second piece of evidence pointing to an additional costimulatory pathway induced after HEL binding to B cells is the fact that B cell blastogenesis continued to occur in cultures with intact HEL when B7 and related molecules were blocked by CTLA4/Ig (Fig. 6 C, *bottom left*). Since blastogenesis did not occur in B cells unless they were exposed to intact HEL and HEL-specific T cells, and T cell lymphokine production was blocked in CTLA4/Ig-treated cultures, other T cell effectors such as membrane-bound CD40L (10–13) or TNF (14) are presumably mediating this effect. Taken together with the lack of B cell blastogenesis in peptide-stimulated cultures, that which we see occurring in the CTLA4/Ig-treated cultures may depend on another molecule induced in HEL-exposed B cells that costimulates certain T cell effector functions. It will be important in future studies to identify the T cell molecules responsible for B cell blastogenesis in cultures where B7 and related molecules are blocked and determine if the additional costimulatory pathway involves known candidates such as IL-1, intercellular adhesion molecule 1, or heat-stable antigen (69–71).

The role of B cells during a response to antigen has long been an area of extensive investigation, leading to many significant—but seemingly contradictory—conclusions (1, 41). For example, experiments with B cell-depleted mice have indicated that B cells play a vital role in the priming of T cells (52–57), whereas work in a *scid* mouse transfer system has shown that B cells are not required for T cell priming (58). Within the question of whether antigen presentation by B cells is required for the activation of naive T cells is the point of whether B cells are even capable of activating naive T cells. A number of experimental approaches in chickens and mice have concluded that naive T cells cannot be activated by B cells. Non-B APCs were needed to fully reconstitute an antibody response in work by Lassila et al. (59), whereas Ronchese and Hausmann (60) were unable to prime class II-restricted T cell responses by B cell antigen presentation, even when antigen-specific activated B cells were present. It has also been suggested that B cell antigen presentation can be tolerogenic (61, 62). Both studies looking at CTL responses

in female mice immunized with either male B cells or splenocytes (61) and work involving mice immunized with anti-IgD Fab fragments (62) demonstrated tolerance induced by B cell antigen presentation. Collectively, the findings of the highly simplified system presented here emphasize a remarkable degree of molecular coordination regulating the outcome of interactions between naive helper T cells and antigen-presenting B cells. In our system, antigen-specific B cells can indeed fully activate naive CD4<sup>+</sup> T cells to upregulate activation molecules, secrete lymphokine, and proliferate, but only if the B cells have themselves been activated (by antigen) to provide crucial costimuli. These findings are consistent with the work of Croft et al. (63), which compared the relative capabilities of different types of APCs to present antigen to and activate transgenic naive CD4<sup>+</sup> T cells. Whether the “partial activation” of T cells demonstrated here might be related to T cell tolerance or anergy—as the work of others (72–80) suggests—remains an intriguing possibility, although recent experiments by St. Louis et al. (81) have demonstrated the “partial activation” of T cell clones by a “costimulator-deficient” endothelial cell line, without any apparent induction of T cell anergy (81).

From a broader perspective, the ability of B cells to stimulate particular T cell effector functions may play a decisive role in determining the immunogenicity or tolerogenicity of antigens. For example, small amounts of soluble foreign antigens administered intravenously to primed individuals are generally found to be potent immunogens in the absence of any accompanying adjuvant. In this case, immunogenicity may be ensured at least in part by efficient and selective uptake of antigen by antigen-specific memory B cells, which then become activated and competent to trigger helper T cells. In contrast, large amounts of soluble foreign antigen given intravenously to unprimed recipients are often tolerogenic (82). Under these conditions, antigen may be taken up and presented by many nonspecific resting B cells, which are unable to trigger the full spectrum of T cell effector functions, and may instead lead to T cell unresponsiveness. Further definition of the factors influencing the outcome of interactions between B cells, T cells, and other cells in the lymphoid microenvironment should ultimately lead to a rational basis for predicting the outcome of antigenic challenge.

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