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The costimulatory molecule B7 is expressed in the medullary region of the murine thymus

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SUMMARY

Several laboratories, including our own, have previously shown that the B7 antigen, a glycoprotein expressed on activated B cells, macrophages and dendritic cells, can provide a potent costimulatory signal for peripheral murine T lymphocytes. In the present report we have analysed the expression and function of B7 in the murine thymus. The expression of B7 was demonstrated histochemically. B7-expressing cells were present in the thymic medulla but virtually absent from the cortex. Further analysis by immunofluorescence and flow fluorocytometry revealed that the B7-positive cells also expressed major histocompatibility complex (MHC) class II molecules. Both epithelial and dendritic cells expressed the B7 antigen. Finally, although we have demonstrated expression of mB7 in the murine thymus, we have been unable to detect a function for this antigen in this organ. The implications of these findings are discussed.

INTRODUCTION

Activation of mature T cells occurs upon T-cell receptor (TcR) cross-linking, induced by peptides bound by major histocompatibility complex (MHC) molecules which are expressed on the surface of an antigen-presenting cell (APC). TcR stimulation, however, is not sufficient to impart complete T-cell activation. The induction of lymphokine secretion and cell proliferation requires a second, costimulatory signal. This costimulatory signal is also provided by an APC and may play a central role in the regulation of the immune response. The role of costimulatory signals in T-cell development, if any, is currently unknown.[1,2]

The best-characterized costimulatory ligand to date is the B7 antigen.[3-6] We and others have shown that this molecule, a 45,000–60,000 MW cell-surface glycoprotein, expressed on activated B cells,[3,6] monocytes/macrophages[6,7] and dendritic cells[8,9] can provide a potent costimulatory signal for interleukin-2 (IL-2)-secreting CD4$^+$ T cells. First, transfected cell lines which express human[10,11] or murine B7 (mB7)[12] or recombinant B7–Ig fusion protein [10,13] can synergize with anti-CD3 monoclonal antibody (mAb) or lectins to induce T-cell activation. Second, we and others have shown that B7 is a sufficient signal for antigen-dependent, MHC-restricted activation of CD4$^+$ T cells. Chinese hamster ovary (CHO) cell lines which express the I-A^d molecule in context with mB7, were able to stimulate primary mixed lymphocyte reactions and to present peptide antigens to *in vivo*-primed, antigen-specific, MHC-restricted T cells.[14] Similarly, expression of human B7 in a human MHC class II$^+$ leukaemia cell line conferred the capacity to stimulate allo-mixed lymphocyte responses (MLR).[15] Finally, recombinant B7–Ig fusion protein is a sufficient signal for the activation of human T lymphocytes by bacterial superantigens.[13]

There are at least two receptors on the T-cell surface with affinity for the B7 antigen. Binding of mAb to CD28,[16] a 44,000 MW homodimeric T-cell glycoprotein that is a receptor for B7,[17] can augment the proliferation or lymphokine secretion of T cells stimulated with anti-CD3 mAb or with antigen.[18-21] A second receptor, CTLA-4, has been described but its function remains to be determined.[22-25] It is well established that at least the CD28 costimulatory receptor is expressed in the thymus.[20,26] In this report, we have analysed the expression and function of the costimulatory ligand mB7 in the murine thymus.

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Abbreviations: FTOC, fetal thymic organ culture; mB7, murine B7.

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MATERIALS AND METHODS

Animals

BALB/c mice (H-2^d, Thy-1.2, CD8.2) were purchased from the Jackson Laboratory (Bar Harbor, ME) or from Taconic Laboratories (Germantown, NY) and maintained in accordance with the regulations of the Committees on Animals of Harvard Medical School and of the Dana-Farber Cancer Institute.

Monoclonal antibodies and lectins

The following mAb were used in this study: unconjugated, biotinylated or fluorescein isothiocyanate (FITC)-conjugated mAb 16-10A1 = anti-mB7;[6] FITC-conjugated anti-CD8 mAb

(Becton Dickinson, San Jose, CA); phycoerythrin (PE)-conjugated anti-L3T4 mAb (Becton Dickinson); biotin- or FITC-conjugated anti-K^d mAb (Pharmingen, San Diego, CA); biotin- or FITC-conjugated anti-I-$A^{k,s}$ mAb (Gibco BRL, Gaithersburg, MD); biotinylated N418 mAb[27] (hybridoma obtained from the ATCC); mAb 2.4G2 = anti-mouse Fc receptor;[28] biotinylated Ulex europeus agglutinin (Vector, Burlingame, CA); HP-25, a hamster control mAb, kindly provided by Dr K. L. Rock (Boston, MA).

Fractionation of thymocytes

To prepare a single-cell suspension of thymocytes, lobes were gently squeezed between the frosted ends of microscope slides in a small amount of ice-cold medium [RPMI-1640, supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol (2-ME), 10 mM HEPES, 4 mM L-glutamine and non-essential amino acids]. The obtained cell suspension was filtered through nylon mesh and depleted of erythrocytes with Tris-NH_4Cl. Thymic APC were purified by T-cell depletion with a cocktail of anti-Thy-1.2, anti-CD4 and anti-CD8.2 mAb and C'.[29]

Immunohistochemistry

Frozen sections, 4 μm thick, were air dried and fixed in acetone for 10 min. Sections were incubated with 2% normal goat serum and 0·2% gelatin followed by a 60-min incubation with hamster anti-mouse B7 (2·5 μg/ml). This and all subsequent incubations were followed by a wash in PBS. Endogenous peroxidase was blocked by a 30-min incubation in 0·3% H_2O_2. Sections were then incubated and biotinylated goat $F(ab')_2$ anti-hamster IgG (1:800–1:1600) for 40 min followed by a 30-min incubation in avidin–biotin–horseradish peroxidase (ABC Kit, Vector Labs). After washing, the sections were incubated for 10 min in 0·01% H_2O_2 and biotinylated tyramine, 10 μl/ml. The stock of biotinylated tyramine[30] was prepared by dissolving 25 mg sulphosuccinimidyl-6-(biotinamido) hexanoate (Pierce, Rockford, IL) in 10 ml 50 mM borate buffer (pH 8·0), to which 7·8 mg tyramine was added (Sigma Chemical Co., St Louis, MO). The solution was mixed overnight at room temperature and filtered through a 0·45-μm filter. Incubation in H_2O_2-biotinylated tyramine was followed by another 30-min incubation in avidin–biotin–horseradish peroxidase (ABC Kit). Reaction product was generated with 0·03% 3-amino-9-ethylcarbazole, 0·03% H_2O_2 in 0·1 M acetate buffer, pH 5·5. Sections were counterstained with haematoxylin and coverslipped with glycergel (Dako, Carpinteria, CA).

Fluorescence staining

Single-cell suspensions, obtained as detailed above, were spun through a Ficoll–Hypaque gradient in order to remove small particles of connective tissue and dead cells.[29] Cells were preincubated for 30 min on ice with anti-Fc receptor mAb 2.4G2, followed by a second incubation on ice for 30 min with the appropriate mAb and, when necessary, subsequently with a second-step reagent (Phycoprobe PE–Strepavidin, Biømeda Corp., Foster City, CA). The cells were washed three times with staining solution [PBS, pH 7·3, supplemented with 2 mg/ml bovine serum albumin (BSA)] after each incubation. The samples were analysed on a FACScan® (Becton Dickinson). Control stainings including stainings with second-step reagents only and stainings with irrelevant mAb in the first step were used to obtain background fluorescence values. Five thousand cells were analysed per sample.

Fetal thymic organ cultures

Sterile 20 × 20 × 7 mm gelatin sponges (Gelfoam® gelatin sponge; The Upjohn Company, Kalamazoo, MI) were placed individually in 4-cm diameter wells (Cell Wells®, 6 wells/plate; Corning Glass Works, Corning, NY) containing 3·5 ml culture medium (RPMI-1640, supplemented with 10% FCS, 50 μM 2-ME, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, antibiotics and antimycotics; in cultures where the effect of an antibody was tested 20 μg/ml of the respective antibody was added to the culture medium). Sterile polycarbonate filters (Nuclepore®, 0·8 μm, 13 mm diameter; Costar, Cambridge, MA) were put onto the gelatin sponges and the assemblies then left to soak overnight. The next day, intact thymic lobes removed from 14-day-old embryos were placed on the surface of the filters (five to six thymic lobes per assembly) and incubated for 6 days at 37° in a humidified incubator containing 5% CO_2. Where the effect of an antibody was tested 25 μl of a 2·4 mg/ml stock solution of purified mAb (mAb 16-10A1 = anti-mB7; mAb HP-25) were applied daily per well on top of the lobes. Control groups received equivalent amounts of culture medium.

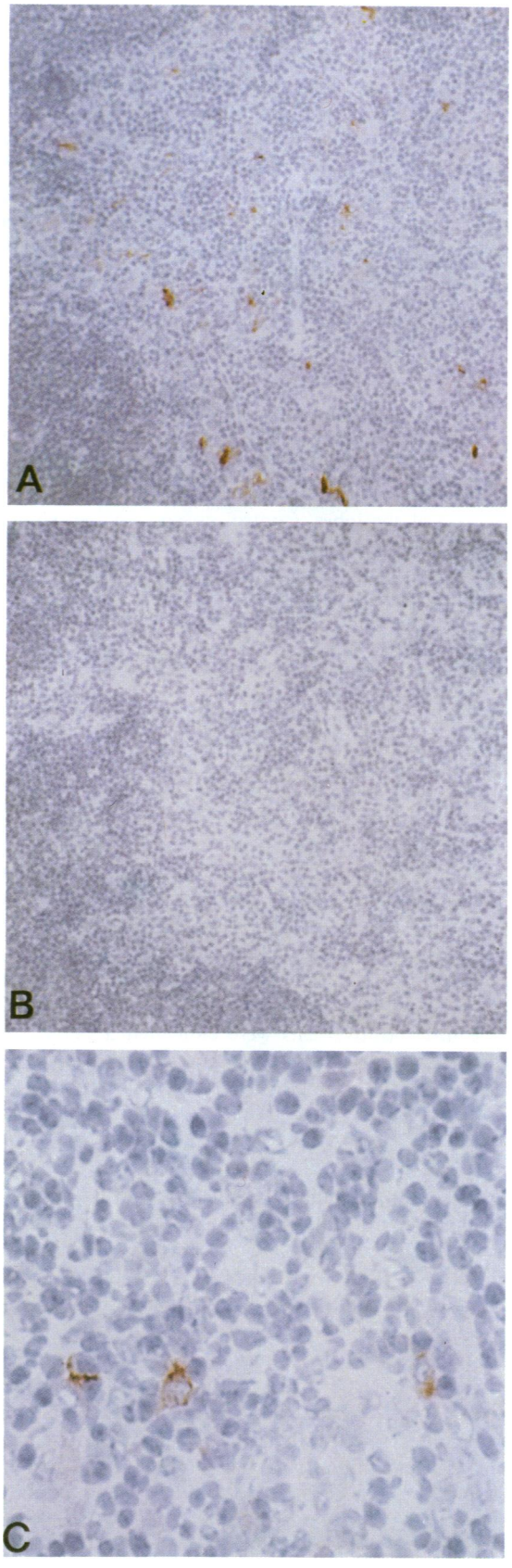
RESULTS

The B7 molecule is expressed in the thymic medulla

The expression of mB7 in the thymus was initially analysed *in situ* using a histochemical method. For this purpose, we employed the anti-mB7 mAb 16-10A1 which we described recently[6] and an immunoperoxidase technique.[30] Using this approach, mB7-expressing thymocytes were easily detected. Reactivity of the anti-mB7 mAb was largely confined to the medullary region of the thymus and virtually absent from the cortex (Fig. 1A). The staining with anti-mB7 mAb was specific as no staining was observed with second-step reagent in the absence of specific anti-mB7 mAb (Fig. 1B) or with an isotype control antibody (data not shown). Analysis of thymic tissues stained with anti-mB7 mAb at a higher magnification revealed that the mB7-positive cells were elongated and displayed a morphology which most closely resembled spatulate medullary epithelial cells or dendritic cells (Fig. 1C).

Phenotype of the B7-positive cell

We subsequently determined the phenotype of B7-positive cells in the thymus. For this purpose, single-cell suspensions of thymocytes were prepared and thymic APC were purified by three cycles of T-cell depletion with a cocktail of anti-T-cell mAb and complement.[29] The expression of mB7 and MHC class II molecules was subsequently examined by dual-colour immunofluorescence and flow fluorocytometry. A representative experiment is shown in Fig. 2. Murine B7 expression is largely restricted to cells which also express MHC class II molecules. These stainings were specific as no staining was observed with various control antibodies (data not shown). As mentioned above, mB7-positive cells displayed a non-lymphoid morphology which most closely resembled medullary epithelial cells or dendritic cells. Consistent with their morphology, the mB7-



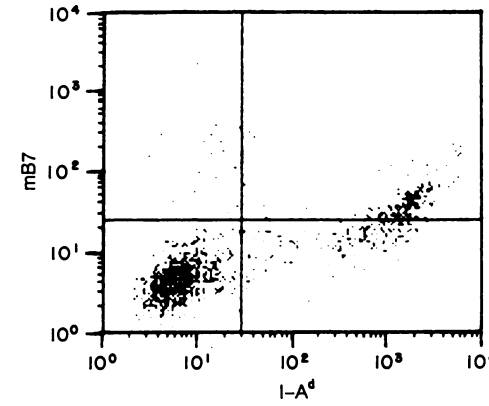


Figure 2. Cells expressing mB7 are also MHC class II-positive. Thymic APC were purified by T-cell depletion with a cocktail of anti-T-cell mAb. The expression of mB7 and MHC class II molecules was subsequently examined by dual-colour immunofluorescence and flow fluorocytometry. Expression of mB7 was analysed using biotinylated anti-mB7 mAb 16-10A1 and Phycoprobe PE–Strepavidin (*y*-axis). Expression of MHC class II molecules (I-A^d) was examined using a FITC-labelled anti-I-A^d mAb (Becton Dickinson). Samples were analysed on a FACScan®. Five thousand cells were analysed per sample. Data are presented as dot plots using logarithmic scales on both *x*- and *y*-axes. Control stainings to obtain background fluorescence values were performed using a biotin-conjugated anti-I-$A^{k,s}$ mAb in the first step, developed with Phycoprobe PE–Strepavidin (*y*-axis) or FITC-conjugated anti-I-$A^{k,s}$ mAb (*x*-axis). Compensation was set using biotin-conjugated anti-K^d mAb developed with Phycoprobe PE–Strepavidin (*y*-axis), and with FITC-conjugated anti-K^d mAb (*x*-axis).

expressing thymocytes were still detectable after depletion of thymic B cells[31] with anti-CD5 (Ly-1) mAb and C′ (Fig. 3).

To investigate the nature of the cells expressing mB7 further, we purified thymic APC by depletion of T lymphocytes expressing the Thy-1, CD4 and CD8 markers. Thymic dendritic and epithelial cells were subsequently sorted using the N418[27] and UEA[32] markers respectively. The sorted populations were analysed for mB7 expression by immunofluorescence and flow cytometry. As shown in Fig. 4A, a subpopulation of epithelial cells, sorted on the basis of reactivity with UEA, expressed mB7. Similarly, a subpopulation of $N418^+$ thymic dendritic cells was mB7-positive (Fig. 4B).

Anti-mAb does not block the development of the major T-cell subsets in fetal thymic organ cultures (FTOC)

Given the expression of mB7 in the thymus, it was of interest to determine whether the molecule was functional in this organ.

Figure 1. Expression of mB7 in the thymic medulla. (A) Frozen sections of BALB/c thymus were stained with purified anti-mB7 mAb followed by biotinylated goat $F(ab')_2$ anti-hamster IgG and a subsequent incubation with avidin–biotin–horseradish peroxidase.[30] Reaction product was generated with 3-amino-9-ethylcarbazole. Sections were counterstained with haematoxylin. (B) Staining with second-step reagent only, omitting the primary reagent. (C) Higher magnification of a portion of the section in (A) reveals the morphology of the cells stained by the anti-mB7 mAb. Photographs were taken using a magnification of ×99 (A and B) and ×396 (C).

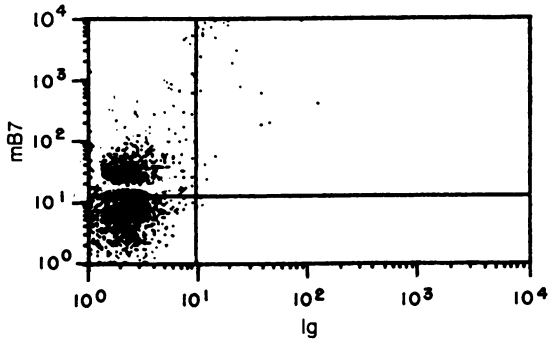


Figure 3. Murine B7-expressing thymocytes are detectable after B-cell depletion. Thymic APC were depleted of T and B cells with a cocktail of mAb [anti-Thy-1, anti-CD4, anti-CD5 (Ly-1) anti-CD8] and C′. The expression of mB7 and Ig molecules was examined by dual-colour immunofluorescence and flow fluorocytometry. Expression of mB7 was analysed using biotinylated anti-mB7 mAb 16-10A1 and Phycoprobe PE–Strepavidin (*y*-axis). Cells were analysed for Ig expression using a FITC-labelled $(Fab')_2$ goat anti-mouse (IgM + IgG) reagent. Samples were analysed on a FACScan®. Five thousand cells were analysed per sample. Data are presented as dot plots using logarithmic scales on both *x*- and *y*-axes. Control stainings to obtain background fluorescence values were performed using a biotin-conjugated anti-I-$A^{k,s}$ mAb in the first step, developed with Phycoprobe PE–Strepavidin (*y*-axis) or FITC-conjugated anti-I-$A^{k,s}$ mAb (*x*-axis). Compensation was set using biotin-conjugated anti-K^d mAb developed with Phycoprobe PE–Strepavidin (*y*-axis), and with FITC-conjugated anti-K^d mAb (*x*-axis).

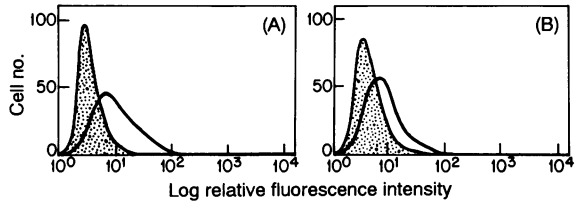


Figure 4. Murine B7 is expressed by thymic epithelial and dendritic cells. Thymic APC were purified by T-cell depletion with a cocktail of anti-T-cell mAb. Thymic epithelial (A) and dendritic (B) cells were subsequently sorted using biotinylated UEA[30] (A) and N418[27] (B) markers and Phycoprobe PE–Strepavidin. The sorted populations were analysed for mB7 expression by immunofluorescence and flow cytometry using FITC-labelled anti-mB7 (——). Control stainings to obtain background fluorescence values were performed using FITC-conjugated anti-I-$A^{k,s}$ mAb OX6 (▨). Samples were analysed on a FACScan®. Five thousand cells were analysed per sample.

Initial experiments revealed that addition of anti-mB7 to thymocyte cultures did not significantly inhibit the proliferation of T cells induced by thymic APC. At the concentration employed, the anti-mB7 mAb efficiently blocked T-cell proliferative responses induced by mB7 transfectants as expected[6] (data not shown). To analyse further whether mB7 plays a role in T-cell development, we set up FTOC in the presence of either medium or anti-mB7. After 7 days, thymic lobes were harvested and teased. Single cells were prepared for immunofluorescence. The relative distribution of CD4- and CD8-positive cells was assessed in dual-colour staining experiments, using PE-labelled anti-CD4 mAb and FITC-labelled anti-CD8 mAb. Our hypothesis was that if mB7 triggering is crucial for the development for

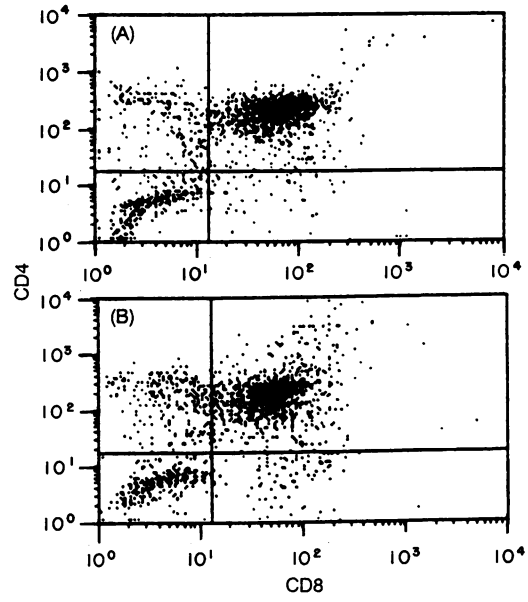


Figure 5. Effect of anti-mB7 mAb on the development of the major T-cell subsets in FTOC. Intact thymic lobes, removed from embryos at day 14 of gestation, were incubated for 6 days at 37° in a humidified incubator containing 5% CO_2. Cultures treated with anti-mB7 mAb received daily additions of 25 μl of a stock solution of purified anti-mB7 mAb (2·4 mg/ml) (B); control cultures received equivalent amounts of culture medium (A). After an incubation period of 6 days single-cell suspensions were prepared and analysed by indirect immunofluorescence and dual-colour flow fluorocytometry for the expression of CD4 and CD8 molecules. Expression of these markers was analysed using a FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb plus Phycoprobe PE–Streptavidin as well as control antibodies. Negative and positive controls for this staining were carried out as detailed in the legend to Fig. 2. Samples were analysed on a FACScan®. Data are presented as dot plots using logarithmic scales on the *x*- and *y*-axes displaying CD8 on the *x*-axis versus CD4 on the *y*-axis. (A) $CD4^-$ $CD8^-$, 10%; $CD4^+$ $CD8^+$, 76%; $CD4^+$ $CD8^-$, 10%; $CD4^-$ $CD8^+$, 4%. (B) $CD4^-$ $CD8^-$, 10%; $CD4^+$ $CD8^+$, 72%; $CD4^+$ $CD8^-$, 13%; $CD4^-$ $CD8^+$, 5%.

a subpopulation of T cells (e.g. the $CD4^+$ $CD8^-$ cells), then this population should be missing. On the other hand, if mB7 does not play a major role in the expansion of T cells, we would not expect either the total number of lymphocytes or the ratio of lymphoid subsets to be affected in anti-mB7-treated FTOC. In several independent experiments, the number of thymocytes recovered from FTOC treated with anti-mB7 mAb was similar to the number of thymocytes recovered from FTOC treated with a control mAb (data not shown). Furthermore, when the expression of the CD4 and CD8 markers was analysed on the treated versus untreated populations no significant difference between the groups was observed (Fig. 5, data not shown). Taken together, these experiments suggest that the addition of anti-mB7 mAb to FTOC does not interfere with the development of the major T-cell subsets.

DISCUSSION

In the present report we demonstrate that the costimulatory molecule mB7 is expressed on a subset of MHC class II-positive cells in the medullary region of the murine thymus. Morphologically, the mB7-positive thymocytes resemble most closely either

medullary epithelial or dendritic cells. This interpretation was further supported by immunofluorescence and flow cytometry analysis.

Since submission of this manuscript, a study has been published which documents reactivity of the CTLA4 fusion protein with thymic tissue.[33] The CTLA4 reagent recognizes mB7 and a second, novel ligand for the CTLA-4 receptor.[34-36] In contrast, the anti-mB7 mAb is specific for the mB7 antigen.[36] Thus the experiments reported here provide definite evidence for the expression of mB7 in the murine thymus. Consistent with our findings, the expression of mB7 in the murine thymus has been recently documented by RNA analysis.[36]

Our study also complements a previous report in the human system by Turka *et al.*[26] These authors demonstrated binding of an anti-B7/BB-1 mAb to human thymic tissues, although the pattern of expression they observed appears different from our result.

Although we have demonstrated expression of mB7 in the murine thymus, we have been unable to detect a function for this antigen in this organ. The anti-mB7 mAb 16-10A1, which abrogates the major functional determinant of mB7 on transfected CHO cell lines,[6,14] did not greatly alter thymic development in FTOC as both the number and the relative ratio of thymocyte subset appear unaffected by treatment with anti-mB7 mAb. These findings, which are consistent with recent experiments using CTLA4Ig by Nelson *et al.*[33] and with recent studies on the CD28 knock-out mice by Shahinian *et al*,[37] argue against a role for mB7 in the positive selection of the major thymocyte subsets. It should be noted, however, that mB7 may have more subtle effects. For example, it is conceivable that triggering with mB7 *in vivo* affects selection of the TcR repertoire by preferentially selecting for particular Vβ TcR. This possibility remains to be tested. Our data are also consistent with the recent data of Tan *et al.*[38] who have shown in a Tc$R\alpha/\beta$ transgenic mouse model that B7 neither induces nor prevents negative selection. It is therefore possible that negative or positive selection of $\alpha\beta$ T cells does not depend on costimulatory molecules at all, or that these differentiation steps depend on costimulatory ligands distinct from mB7.

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