

## An Essential Role for gp39, the Ligand for CD40, in Thymic Selection

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### Summary

The interactions between CD40 on B cells and its ligand gp39 on activated T helper cells are known to be essential for the development of thymus-dependent humoral immunity. However, CD40 is also functionally expressed on thymic epithelial cells and dendritic cells, suggesting that gp39-CD40 interactions may also play a role in thymic education, the process by which self-reactive cells are deleted from the T cell repertoire. Six systems of negative selection were studied for their reliance on gp39-CD40 interactions to mediate negative selection. In all cases, when the antigen/superantigen was endogenously expressed (in contrast to exogenously administered), negative selection was blocked by loss of gp39 function. Specifically, blockade of gp39-CD40 interactions prevented the deletion of thymocytes expressing V $\beta$ 3, V $\beta$ 11, and V $\beta$ 12, specificities normally deleted in BALB/c mice because of the endogenous expression of minor lymphocyte-stimulating determinants. Independent verification of a role of gp39 in negative selection was provided by studies in gp39-deficient mice where alterations in T cell receptor (TCR) V $\beta$  expression were also observed. Studies were also performed in the AND TCR transgenic (Tg) mice, which bear the V $\alpha$ 11, V $\beta$ 3 TCR and recognize both pigeon cytochrome *c* (PCC)/IE<sup>k</sup> and H-2A<sup>s</sup>. Neonatal administration of anti-gp39 to AND TCR Tg mice that endogenously express H-2A<sup>s</sup> or endogenously produce PCC prevented the deletion of TCR Tg T cells. In contrast, deletion mediated by high-dose PCC peptide antigen (administered exogenously) in AND TCR mice was unaltered by administration of anti-gp39. In addition, deletion by *Staphylococcus* enterotoxin B in conventional mice was also unaffected by anti-gp39 administration. gp39 expression was induced on thymocytes by mitogens or by antigen on TCR Tg thymocytes. Immunohistochemical analysis of B7-2 expression in the thymus indicated that, in the absence of gp39, B7-2 expression was substantially reduced. Taken together, these data suggest that gp39 may influence negative selection through the regulation of costimulatory molecule expression. Moreover, the data support the hypothesis that, for negative selection to some endogenously produced antigens, negative selection may be dependent on TCR engagement and costimulation.

**A**lthough it is clear that both positive and negative selection in the thymus require recognition of peptide-MHC complexes by the TCR, the requirement for addi-

tional costimulatory molecules is unclear. This is in contrast to the activation of peripheral T cells, where it has repeatedly been demonstrated that recognition of peptide-MHC by TCR on mature peripheral T cells is insufficient for optimal T cell activation and that costimulatory molecules provided by APCs are necessary to achieve complete T cell activation (1).

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While most studies demonstrate that TCR signals alone are sufficient to induce deletion of thymocytes (2), two reports suggest that additional costimulatory signals are required (3, 4). Double-positive thymocytes express CD2, CD5, lymphocyte-function-associated antigen 1 (LFA-1), very late antigen 4 (VLA-4), and CD28, suggesting that any of these molecules could potentially be involved in interactions with thymic APC that mediate the processes of thymic selection (3). The requirement for CD28-B7 interactions in thymic selection has been examined in a number of distinct models. A causal role for B7 in negative selection is suggested by recent studies demonstrating that the expression of B7 within the thymus medullary epithelium was T dependent and correlated with epithelium-mediated deletion of V $\beta$ 5<sup>+</sup> thymocytes (5). However, other studies using in vitro or in vivo models of negative selection have provided conflicting results as to the requirement for B7 in negative selection (3-8). Thus, a clear role for the CD28/B7 signaling pathway in thymic selection has yet to be demonstrated.

Although the respective roles of a number of costimulatory molecules in thymic selection have been examined, the role of gp39-CD40 interactions remains unknown. The fact that CD40 is functionally expressed on epithelial cells (9) of the thymus as well as on other APCs (10) indicates that it may participate in thymic selection. The present study examines the role of gp39-CD40 interactions in a number of distinct models of negative selection. The results demonstrate that, in some models of negative selection, lack of gp39 function permits the escape of self-reactive T cells. Furthermore, gp39 expression was critical for the medullary expression of B7-2 within the thymus. Since B7-2 expression in the thymus is gp39 dependent, gp39 may impart its effects on negative selection by its capacity to regulate the expression of costimulatory molecules on thymic epithelium or thymic dendritic cells. These data suggest that gp39 plays an active role in shaping the T cell repertoire, most likely through signaling of CD40-bearing thymic APCs.

## Materials and Methods

**Mice.** BALB/c mice were bred in the Animal Resource Facility at Dartmouth Medical School (Lebanon, NH) using adult breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME). (CBA/J  $\times$  gp39 deficient)F<sub>1</sub> mice were obtained by mating CBA/J (H-2<sup>k/k</sup>) adult males (The Jackson Laboratory) with gp39-deficient (H-2<sup>b/b</sup>) homozygous (-/-) females. Because the gp39 gene is encoded on the X chromosome, female progeny of this mating were H-2<sup>b/k</sup> heterozygous (+/-) for gp39, whereas male F<sub>1</sub> progeny were H-2<sup>b/k</sup> gp39 deficient (-/0). H-2<sup>k/k</sup> AND<sup>1</sup> TCR transgenic (Tg) were bred and maintained at Dartmouth Medical School. H-2<sup>s/k</sup> AND TCR Tg mice were

generated by mating adult male H-2<sup>k/k</sup> AND TCR Tg with B10.S (H-2<sup>s/s</sup>) females. RO pigeon cytochrome *c* (PCC) Tg mice (designated PCC Tg) were generated as described (Oehen, S., and S. M. Hedrick, manuscript in preparation). These mice were generated using a construct consisting of PCC fused to the influenza neuraminidase signal anchor driven by the H-2K<sup>b</sup> promoter and Ig heavy chain enhancer. PCC expression was detectable in the spleen and thymus by Northern analysis. (AND TCR Tg  $\times$  PCC Tg)F<sub>1</sub> mice were obtained by mating homozygous AND TCR Tg (H-2<sup>b/b</sup>) and heterozygous PCC Tg (H-2<sup>s/a</sup>) adult breeding pairs. This mating generated H-2<sup>b/a</sup> progeny that expressed the AND TCR Tg, half of which also expressed the PCC Tg.

**Antibodies and Reagents.** The following mAbs, obtained from Pharmingen (San Diego, CA), were used in this study: RM4-4, anti-murine CD4; 53-6.72, anti-murine CD8; RR8-1, anti-murine V $\alpha$ 11; KJ-25, a hamster anti-murine V $\beta$ 3; MR9-4, a mouse anti-murine V $\beta$ 5.1, V $\beta$ 5.2; RR47, anti-murine V $\beta$ 6; TR310, anti-murine V $\beta$ 7; biotin-MR10-2, anti-murine V $\beta$ 9; RR3-15 anti-murine V $\beta$ 11; MR11-1, anti-murine V $\beta$ 12; and GL1, anti-murine B7-2. 16-10A1, hamster anti-murine B7-1, was kindly provided by Hans Reiser (Dana-Farber Cancer Institute, Boston, MA). F23.1, anti-murine V $\beta$ 8.1, 8.2 (11) and MR1, anti-murine gp39 (12), were purified by DEAE HPLC from ascites fluid. Purified, conjugated rat IgG or hamster Ig (HIg) were used as isotype controls in the flow cytometric assays. Control HIg was similarly purified from normal hamster serum. PCC peptide fragment (PCCF) corresponding to residues 88-104 of PCC (KAERADLIAYLKQATAK) was synthesized in the Peptide Synthesis Core Facility, Department of Pathology, Dartmouth Medical School.

**Anti-gp39 Treatment.** BALB/c, H-2<sup>s/k</sup> AND TCR Tg, and (AND TCR Tg  $\times$  PCC Tg)F<sub>1</sub> were treated every 2 d from birth with purified HIg or anti-gp39 (MR1; 100  $\mu$ g/injection). Adult mice were treated with 250  $\mu$ g/injection.

**Flow Cytometric Analysis.** Before staining, single-cell suspensions were prepared and spun through Ficoll-Hypaque, followed by washing in balanced salts solution. Thymocytes (0.5  $\times$  10<sup>6</sup>) were stained by incubation with primary antibodies for 30 min at 4°C. Rat serum and 25  $\mu$ g of the anti-Fc $\gamma$ RII antibody 2.4G2 were added to eliminate background staining. Cells were then washed with staining buffer and subsequently incubated with the PE-streptavidin for 30 min at 4°C. After a second wash, the samples were fixed in 1.25 $\times$  PBS, 1% formaldehyde and analyzed on a flow cytometer (FACS<sup>®</sup> 440; Becton Dickinson & Co., Cockeysville, MD). A minimum of 10,000 cells were collected for each sample. Two-color contours are represented as 5% probability plots.

**Antigen-induced Proliferation of T Cells.** CD4<sup>+</sup> T cells were obtained from thymus by complement-mediated lysis. Cells from control or anti-gp39-treated H-2<sup>b/s</sup> AND TCR Tg mice were stimulated with variable concentrations of moth cytochrome *c* (cross-reactive antigen) in the presence of APCs and were assessed for proliferation by [<sup>3</sup>H]thymidine incorporation after 48 h as described (13).

**Staphylococcus Enterotoxin B (SEB)-mediated Deletion of Thymocytes.** SEB (20  $\mu$ g/d) was administered three times weekly to BALB/c mice from birth. In addition, mice were similarly treated with control HIg or anti-gp39 (100  $\mu$ g/d). Deletion of thymocytes was assessed flow cytometrically by staining with anti-CD4, -CD8, and -V $\beta$ 8.

**Antigen/Mitogen-induced Expression of gp39 on Thymocytes.** Thymocytes (5  $\times$  10<sup>6</sup>/ml) from AND TCR Tg (for PCCF-induced

<sup>1</sup>Abbreviations used in this paper: CsA, cyclosporine A; DN, double negative; DP, double positive; HIg, hamster Ig; PCC, pigeon cytochrome *c*; PCCF, pigeon cytochrome *c* peptide fragment; RT, room temperature; SEB, *Staphylococcus enterotoxin B*; SP, single positive; Tg, transgenic; wt, wild type.

expression) or BALB/c mice (for mitogen-induced expression) were cultured for 12 h in the presence or absence of PCCF, Con A (5  $\mu$ g/ml), or PMA (10 ng/ml) and ionomycin (250 nM) to induce gp39. Expression of gp39 was detected flow cytometrically by staining with a combination of CD4-PE, CD8-Cy, and biotin anti-gp39.

**Immunohistochemical Analysis of B7-2 Expression in Thymus.** Fresh thymuses from 21-d-old wild-type (wt) or gp39-deficient mice were snap frozen in liquid nitrogen, and 8- $\mu$ m cryostat sections were cut. Sections were stored at room temperature (RT) overnight under humidified conditions. After 1 h of air drying, sections were fixed in fresh acetone for 10 min and incubated overnight with a biotinylated rat anti-murine B7-2 (PharMingen). Sections were rinsed three times with PBS and incubated for 1 h at RT with a biotinylated rabbit anti-rat IgG (H + L chain) (Vector Laboratories, Inc., Burlingame, CA). Sections were washed and incubated for 1 h at RT with PE-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL). Stained sections were scanned for fluorescence using a laser cytometer (ACAS Ultima; Meridian Instruments, Inc., Okemos, MI). The digital image created by laser scanning was further analyzed to quantitate the average fluorescence intensity of each section. The medullary region was gated for each section and analyzed to determine an average fluorescence intensity.

## Results

**gp39-dependent Mls-mediated Selection in BALB/c Mice.** To examine if gp39-CD40 interactions were critical in the development of mature T and/or B cells, mice were treated from birth with anti-gp39 or HIg, and T and B subsets were examined. As anticipated from the lymphocyte subset analysis from gp39- and CD40-deficient mice (14, 15) as well as hyper-IgM patients (16), no alterations in thymocyte, mature T cell, or mature B cell subsets were noted in mice chronically treated with anti-gp39 (data not shown). To determine if gp39-CD40 interactions influenced thymic selection, the deletion of T cells to a variety of antigens in both TCR Tg and non-Tg models of negative selection was investigated.

The deletion of T cells expressing TCRs reactive against endogenous retroviral Mls antigens was examined in mice chronically treated with anti-gp39. Due to the expression of Mls determinants encoded by endogenous mouse mammary tumor viruses, young adult BALB/c mice normally delete TCR expressing V $\beta$ 3, V $\beta$ 11, and V $\beta$ 12, but not TCR using V $\beta$ 8 family members (17). Mice treated from birth with anti-gp39 or HIg were analyzed for the presence of T cells expressing V $\beta$ 3, V $\beta$ 11, V $\beta$ 12, and V $\beta$ 8 TCR. T cell subset analysis was performed by correlated flow cytometric analysis of CD4, CD8, and TCR usage at 4–6 wk of age. Thymocytes expressing V $\beta$ 3, V $\beta$ 11, and V $\beta$ 12 were absent in the CD4<sup>+</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> single-positive (SP) thymocyte populations obtained from normal, untreated (data not shown) or control HIg-treated BALB/c mice, whereas the V $\beta$ 8 population was present (Fig. 1, left). However, analysis of V $\beta$ 3, V $\beta$ 11, and V $\beta$ 12 expression on SP thymocytes from animals treated in vivo with anti-gp39 demonstrated increases in the percentage of SP CD4<sup>+</sup> or CD8<sup>+</sup> cells expressing V $\beta$ 11<sup>+</sup> (three- to fivefold) or V $\beta$ 12<sup>+</sup>

(16–20-fold), although only modest increases in V $\beta$ 3-expressing thymocytes were observed (Fig. 1, right). The percentage of V $\beta$ 3-, V $\beta$ 11-, and V $\beta$ 12-expressing SP thymocytes in mice treated with anti-gp39 was similar to that in a nondeleting strain, C57BL/6 (18) (data not shown). V $\beta$ 3-, V $\beta$ 11-, and V $\beta$ 12-bearing thymocytes were detected primarily in the CD4 and CD8 SP subsets and were not present in the double-positive (DP) or double-negative (DN) thymocyte populations. These results demonstrated that Mls-mediated clonal deletion of self-reactive T cells was prevented as a result of anti-gp39 treatment.

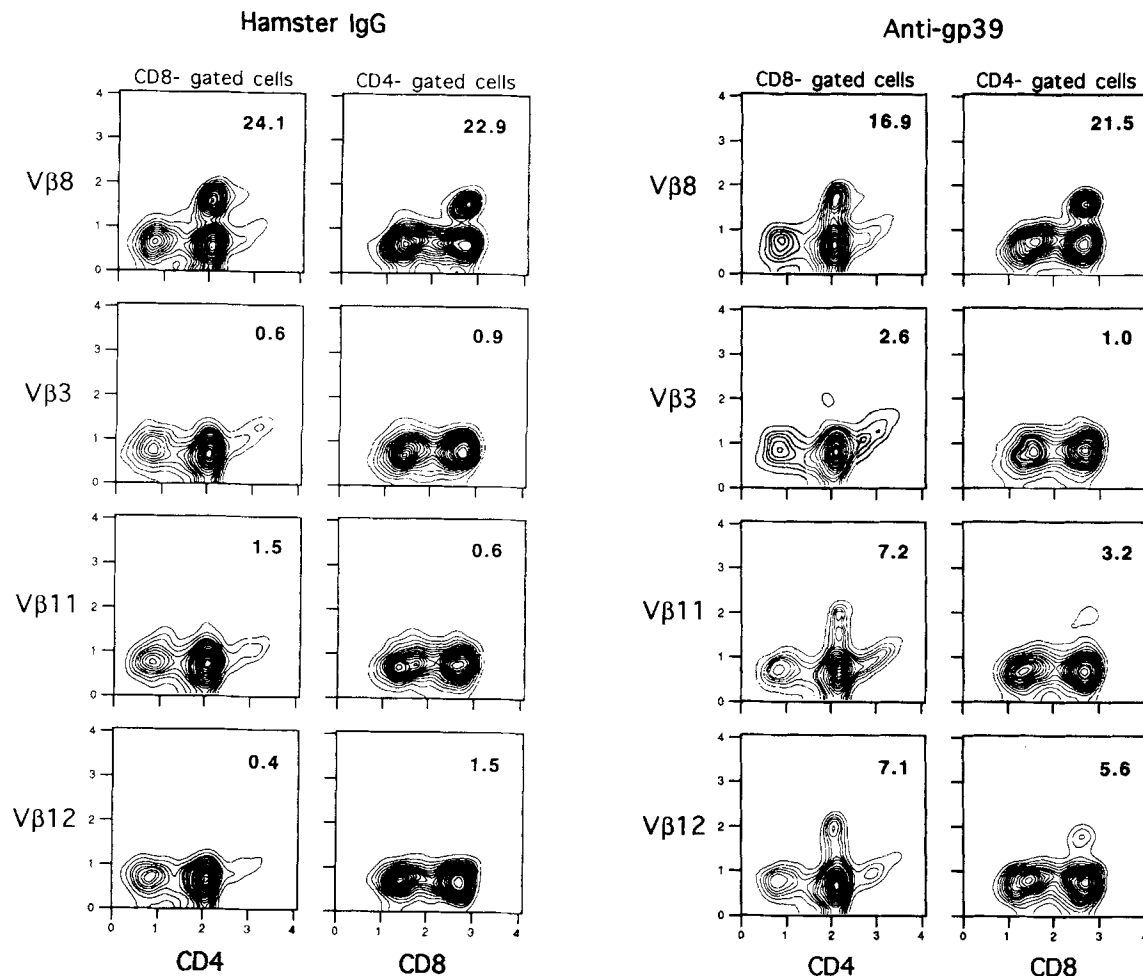
**Altered Mls-mediated Selection in gp39-deficient Mice.** To further confirm the participation of gp39 in Mls-mediated selection of thymocytes, analysis of TCR V $\beta$  usage in gp39-deficient mice was performed. Normal CBA/J mice (Mls<sup>a,c,f</sup>) (data not shown) and gp39 heterozygous CBA/J F<sub>1</sub> mice delete a host of V $\beta$ -bearing T cells, including V $\beta$ 3, V $\beta$ 5, V $\beta$ 6, V $\beta$ 7, V $\beta$ 9, V $\beta$ 11, and V $\beta$ 12 (Fig. 2). However, in CBA/J F<sub>1</sub> mice genetically deficient in gp39, there was an increase in CD4 and/or CD8 SP thymocytes bearing V $\beta$ 5, V $\beta$ 6, V $\beta$ 7, V $\beta$ 11, and V $\beta$ 12 TCRs with modest or no changes in the other V $\beta$ -expressing thymocyte populations. These results confirm those obtained from mice chronically treated with anti-gp39 and provide supportive evidence for the role of gp39 in Mls-mediated deletion of thymocytes.

**gp39-dependent Selection by H-2A<sup>s</sup> in the AND TCR Tg Mice.** To evaluate the role of gp39-CD40 interactions in the selection of T cells to other self antigens, a third in vivo system of negative selection involving TCR Tg mice (AND TCR Tg) expressing an  $\alpha/\beta$  TCR specific for pigeon or moth cytochrome *c* in association with H-2E<sup>k</sup> class II MHC molecules was used (19). Thymocytes from these mice undergo efficient maturation in the presence of H-2E<sup>k</sup>; however, in the presence of H-2A<sup>s</sup>, the vast majority of Tg thymocytes undergo negative selection (13). Negative selection mediated by H-2A<sup>s</sup> results in the deletion of late-stage CD4/CD8 DP thymocytes, leading to a virtual absence of TCR Tg CD4<sup>+</sup> SP thymocytes (13). To determine whether gp39-CD40 interactions play a role in this in vivo model of negative selection, H-2<sup>s/k</sup> AND TCR Tg mice were treated in vivo with anti-gp39 or control Ig from birth. Analysis of thymocytes from untreated or HIg-treated mice revealed a large percentage of DP (40%) and DN (43%) immature thymocytes and an almost complete absence of CD4 SP (9%) thymocytes, indicating that negative selection of thymocytes bearing the Tg TCR had occurred in these mice (Fig. 3, top). The majority of thymocytes in these mice expressed low to moderate levels of the V $\alpha$ 11 and V $\beta$ 3 TCR transgene components, characteristic of the DP population (data not shown). Negative selection of a large number of thymocytes in H-2<sup>s/k</sup> mice was also reflected by a 50–65% decrease in the total number of thymocytes as well as in the total number of mature CD4 SP thymocytes (Table 1). In contrast, H-2<sup>s/k</sup> mice treated with anti-gp39 contained a large number of CD4 SP thymocytes (26%; Fig. 3, bottom) and fewer immature DN cells (24%). Although the levels of CD4 SP thy-

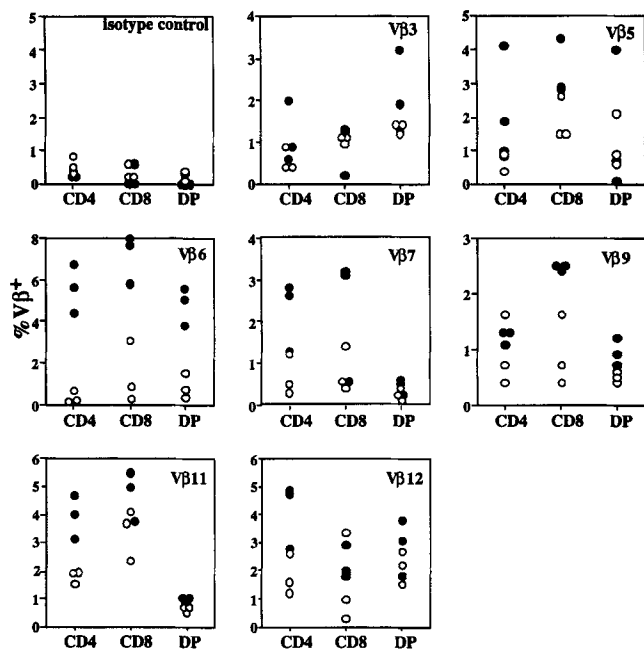
mocytes observed in normal H-2<sup>k/k</sup> Tg mice are generally higher (60–70%; see Fig. 4) than those seen in H-2<sup>s/k</sup> mice treated with anti-gp39, blockade of gp39 function does result in a dramatic increase in both the percentage and the total number of CD4 SP thymocytes compared with untreated or HIg-treated H-2<sup>s/k</sup> mice. The heightened levels of Vβ3<sup>hi</sup> (data not shown) and Vα11<sup>hi</sup> thymocytes representing the CD4 SP population in anti-gp39-treated mice are also indicative of an absence of negative selection, as are the relatively higher numbers of total and CD4 SP thymocytes (Table 1). Collectively, these data demonstrate that blockade of gp39–CD40 interactions in the thymus interferes with this alloantigen-mediated model of negative selection.

*gp39-dependent Selection by Endogenous PCC Antigen in the AND TCR Tg Mice.* The generation of Tg mice endogenously expressing PCC in the thymus provides a system in which to examine the selection of Tg thymocytes to an en-

dogenously encoded “self antigen.” Mating the AND TCR Tg mice with Tg mice expressing PCC results in the recognition of PCC self antigen by thymocytes bearing the TCR specific for PCC and results in their deletion (Oehen, S., and S. M. Hedrick, manuscript in preparation). To determine whether gp39 was required for the deletion of mature Tg thymocytes in this model of negative selection, AND TCR Tg or (AND TCR Tg × PCC Tg)F<sub>1</sub> mice were treated from birth with anti-gp39, and the thymocyte populations were examined 4 wk later. The results, summarized in Table 2, demonstrate a dramatic decrease (55–65%) in the percentage of CD4<sup>+</sup> Vα11<sup>hi</sup> thymocytes and an even greater decrease (56–83%) in the total number of mature CD4<sup>+</sup> Vα11<sup>hi</sup> thymocytes in (AND TCR Tg × PCC Tg)F<sub>1</sub> mice compared with AND TCR Tg. The administration of anti-gp39 completely restores the CD4 SP thymocyte compartment in the (AND TCR Tg × PCC Tg)F<sub>1</sub> mice, as can be seen by the increase in both the total



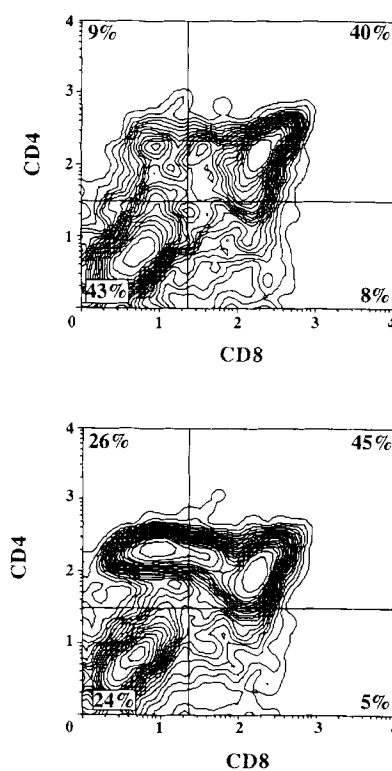
**Figure 1.** Anti-gp39 treatment prevents Mls<sup>sf</sup>-mediated deletion of thymocytes in BALB/c mice. Mice were treated every 2 d from birth with purified HIg or anti-gp39 (100 μg/injection). 4–6 wk later, the thymus was removed and stained with a combination of PE anti-CD4, cyanine anti-CD8, and FITC-conjugated anti-TCR Vβ mAbs Vβ8.1, 8.2, Vβ3, Vβ11, or Vβ12. Purified rat IgG was used as an isotype control. The contour plots were first gated on the CD8<sup>−</sup> or CD4<sup>−</sup> T cell populations (to eliminate DPs), and then represented as CD4<sup>+</sup> or CD8<sup>+</sup> in combination with Vβ3, Vβ8, Vβ11, or Vβ12. Numbers in the upper right corners represent the percentages of Vβ<sup>+</sup> cells in the CD4<sup>+</sup> or CD8<sup>+</sup> thymocyte subsets. A single animal from one of four representative experiments is shown for each treatment group.



**Figure 2.** Mls-mediated deletion of thymocytes is impaired in gp39-deficient mice. Thymocytes from 4–6-wk-old (CBA/J × gp39 deficient)<sub>F1</sub> heterozygous wt (open circles) or gp39-deficient (closed circles) mice (three mice per group) were stained as described in Materials and Methods with FITC-conjugated anti-murine CD4, Cy-chrome-labeled antimurine CD8, and one of the following biotin-conjugated anti-TCR Vβ mAbs followed by PE-streptavidin: Vβ3, Vβ5.1, Vβ5.2, Vβ6, Vβ7, Vβ9, Vβ11, or Vβ12. Background staining using a biotin-conjugated isotype control mAb is also shown for each subset. A minimum of 20,000 cells were collected for each sample. Data are expressed as the percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, or DP thymocytes bearing each particular Vβ TCR. One of three representative experiments is shown.

number and percentage of CD4<sup>+</sup> Vα11<sup>hi</sup> thymocytes in mice receiving anti-gp39 from birth. These results demonstrate that blockade of gp39 function interferes with antigen-mediated deletion of thymocytes in this Tg model of negative selection, and provide further support for the hypothesis that gp39-CD40 interactions play an essential role in negative selection of self-reactive thymocytes.

**gp39-independent Selection by Peptide Antigen in the AND TCR Tg Mice.** Deletion of antigen-reactive, immature thymocytes in TCR Tg mice has also been described as a result of injection of high doses of soluble, relevant peptide (20). DP thymocytes from AND TCR Tg mice undergo apoptosis after administration of high-dose, exogenous PCCF (Oehen, S., and S. M. Hedrick, unpublished observations). To determine whether administration of anti-gp39 interfered with high-dose PCCF-mediated deletion of thymocytes, AND TCR Tg mice were injected with PCCF (200 μg three times) or PBS, with or without coadministration of anti-gp39. The total number of thymocytes and DP thymocytes recovered was assessed 24 h after the last administration of peptide. The results, shown in Fig. 4, demonstrate that, although administration of PCCF resulted in profound loss of DP thymocytes (1.1% DP in mice receiving PCCF compared with 19.9% DP in mice receiving PBS; Fig. 4, left panels), coadministration of anti-



**Figure 3.** Anti-gp39 treatment prevents H-2A<sup>b</sup>-mediated negative selection of thymocytes in AND TCR Tg mice. H-2<sup>b/k</sup> AND TCR Tg mice were treated from birth with either control HIg (top) or anti-gp39 (bottom) antibody as described in Materials and Methods. Thymocytes obtained from 4-wk-old mice were double stained for CD4 and CD8. Numbers indicate percent thymocytes in each quadrant.

gp39 did not prevent this deletion (Fig. 4, lower right). It is also noteworthy that the prominent effect of exogenous PCCF is the loss of DP thymocytes, whereas endogenous production of PCCF leads to loss of the SP CD4<sup>+</sup> thymocytes. Mice receiving anti-gp39 alone had levels of DP thymocytes similar to those of mice receiving PBS (Fig. 4, upper right). These results demonstrate that deletion of DP TCR Tg thymocytes by high concentrations of antigen does not appear to be dependent upon gp39, as DP thymocytes were not “rescued” by blockade of gp39 function.

**gp39-independent Selection to SEB.** Deletion of thymocytes by exogenous superantigens has also been used extensively as a model for negative selection in the thymus. The gp39 dependency of negative selection mediated by exogenous superantigens was also investigated. Administration of SEB to neonatal BALB/c mice causes the selective loss of Vβ8-bearing T cells. To determine whether gp39 was required for deletion of thymocytes mediated by SEB, neonatal BALB/c mice were administered SEB alone or in combination with HIg or anti-gp39 from birth. Administration of SEB caused a dramatic loss in the percentage of Vβ8-bearing T cells in the CD4 (73–82% decrease) and CD8 (53–83% decrease) SP thymocyte compartments (Table 3). Coadministration of SEB and anti-gp39 did not alter selection of Vβ8-bearing T cells (Table 3), but did result in selective survival of Vβ11- and Vβ12-bearing SP thymocytes (data

**Table 1.** Anti-gp39 Treatment Partially Blocks Negative Selection in H-2<sup>s/k</sup> AND TCR Tg Mice and Results in an Increase of CD4<sup>+</sup> Vα11<sup>hi</sup> Mature Thymocytes

In vivo anti-gp39 treatment*	Total thymocytes	% CD4 <sup>+</sup>	Total CD4 <sup>+</sup> thymocytes	% Vα11 <sup>hi</sup> CD4 <sup>+</sup>	Total Vα11 <sup>hi</sup> CD4 <sup>+</sup> thymocytes
	× 10 <sup>6</sup>		× 10 <sup>6</sup>		× 10 <sup>6</sup>
–	66.4 <sup>‡</sup>	9.2 <sup>§</sup>	6.1	7.1 <sup>§</sup>	4.7
–	63.2	9.0	5.7	5.2	3.3
+	131.8	25.5	33.6	19.1	29.8
+	131.8	24.9	32.8	18.9	30.4

\*H-2<sup>s/k</sup> AND TCR Tg F1 mice were treated from birth with HIg or anti-gp39.

<sup>‡</sup>At 4 wk of age the total number of thymocytes recovered from each animal was determined.

<sup>§</sup>Flow cytometric analysis was performed to determine the number of mature thymocytes. Values represent the percentage of total thymocytes which are CD4<sup>+</sup> or CD4<sup>+</sup>Vα11<sup>hi</sup>. One of six representative experiments is shown.

not shown), suggesting that SEB-mediated deletion occurs independently of gp39.

**The Response of T Cells Rescued from Deletion.** To determine if anti-gp39 administration altered the functional capacity of the rescued T cells, the proliferative response of rescued T cells was tested. Unfortunately, it was not possible to measure the proliferative response of the BALB/c mice to Mls<sup>c,f</sup> nor the AND TCR Tg mice to H-2A<sup>s</sup>. In both of these cases, it has been shown that the response of mature T cells to Mls<sup>c,f</sup> (21) and H-2A<sup>s</sup> (13) is extremely weak and poorly detectable. In spite of these limitations, the proliferative response to Ag of AND TCR Tg T cells tolerized or rescued in the H-2A<sup>s</sup> system was measured. CD4<sup>+</sup> cells from AND TCR Tg, H-2<sup>s/k</sup> F<sub>1</sub> mice that were treated with or without anti-gp39 were stimulated in vitro with varying concentrations of MCC presented by APCs from H-2<sup>k/k</sup>-bearing mice. Measurement of MCC proliferative responses (Fig. 5) demonstrated that CD4<sup>+</sup> thymocytes from anti-gp39-treated mice were reactive with antigen, as demonstrated by proliferative responses two to threefold higher than those observed with CD4<sup>+</sup> T cells from control mice that have undergone negative selection. Similar results were obtained using CD4<sup>+</sup> T cells from spleen and lymph node (data not shown). The finding that CD4<sup>+</sup> T cells from control-treated H-2<sup>s/k</sup> mice are somewhat responsive to stimulation with cytochrome *c* is consistent with previous reports demonstrating that deletion of Tg T cells, although profound, is not complete (13). These results indicate that the blockade of negative selection allows for the accumulation of increased numbers of mature, self-reactive thymocytes in this model system. Similar studies performed in the (AND TCR Tg × PCC Tg)F<sub>1</sub> mice revealed that mature thymocytes that were rescued from deletion as a result of anti-gp39 treatment mounted strong proliferative responses to Ag in vitro, yet mature Tg T cells were not visible in the spleen, indicating that some mecha-

mocytes from anti-gp39-treated mice were reactive with antigen, as demonstrated by proliferative responses two to threefold higher than those observed with CD4<sup>+</sup> T cells from control mice that have undergone negative selection. Similar results were obtained using CD4<sup>+</sup> T cells from spleen and lymph node (data not shown). The finding that CD4<sup>+</sup> T cells from control-treated H-2<sup>s/k</sup> mice are somewhat responsive to stimulation with cytochrome *c* is consistent with previous reports demonstrating that deletion of Tg T cells, although profound, is not complete (13). These results indicate that the blockade of negative selection allows for the accumulation of increased numbers of mature, self-reactive thymocytes in this model system. Similar studies performed in the (AND TCR Tg × PCC Tg)F<sub>1</sub> mice revealed that mature thymocytes that were rescued from deletion as a result of anti-gp39 treatment mounted strong proliferative responses to Ag in vitro, yet mature Tg T cells were not visible in the spleen, indicating that some mecha-

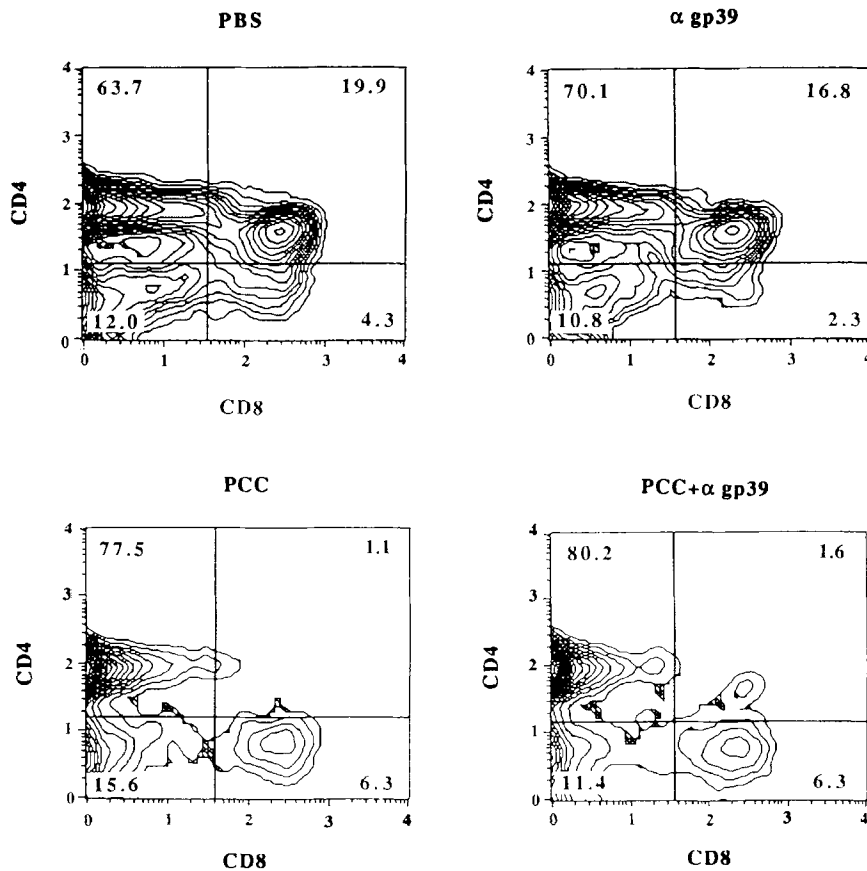
**Table 2.** Anti-gp39 Treatment Prevents the Deletion of Mature CD4<sup>+</sup> Vα11<sup>hi</sup> Tg Thymocytes in (AND TCR Tg × PCC Tg)F<sub>1</sub> Mice

Strain*	In vivo anti-gp39 treatment <sup>‡</sup>	% CD4 <sup>+</sup> Vα11 <sup>hi</sup>	Total No. CD4 <sup>+</sup> Vα11 <sup>hi</sup>	% DP	Total No. DP
			× 10 <sup>6</sup>		× 10 <sup>6</sup>
AND TCR Tg	–	49–51 <sup>§</sup>	16.3–20.0	34–37	11.2–14.4
AND TCR Tg	+	38–44	13.7–15.4	26–47	10.5–15.9
(AND TCR Tg × PCC Tg)F <sub>1</sub>	–	18–22	3.5–5.9	26–60	9.6–16.8
(AND TCR Tg × PCC Tg)F <sub>1</sub>	+	38–45	12.8–18.3	41–45	13.8–17.1

\*AND TCR Tg or (AND TCR Tg × PCC Tg)F<sub>1</sub> mice were treated with PBS or anti-gp39.

<sup>‡</sup>Mice (three to four per group) were treated from birth with anti-gp39 (100 μg/injection, three times weekly) or PBS (100 μl/injection, three times weekly).

<sup>§</sup>15–18 d after birth, thymi were removed and stained for CD4, CD8, and Vα11 expression as described in Materials and Methods. A summary of two experiments is shown.

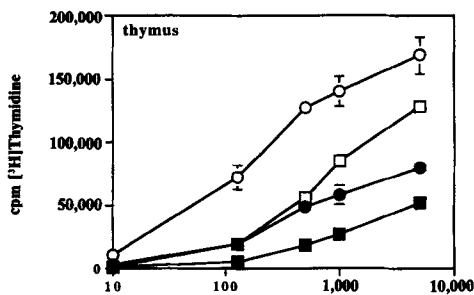


**Figure 4.** Anti-gp39 treatment does not prevent PCCF-mediated negative selection of DP thymocytes in AND TCR Tg mice. Adult AND TCR Tg mice were administered 200  $\mu$ g PCCF (bottom panels) or 0.2 PBS (top panels) daily for 3 d. In addition, mice were either treated with anti-gp39 (250  $\mu$ g/d for 3 d; right panels) or PBS (left panels). On day 4, thymocytes were obtained and double stained for CD4 and CD8. Injection of exogenous PCCF results in deletion of DP thymocytes in AND TCR Tg mice (lower left). Coadministration of anti-gp39 with PCCF did not prevent PCCF-mediated deletion (lower right). The numbers indicate percent positive thymocytes in each quadrant. One of three representative experiments is shown.

nism of peripheral tolerance prevented the survival of escaped, PCC-reactive T cells in these mice (data not shown).

**Antigen- and Mitogen-induced Expression of gp39 on Thymocytes.** The hypothesis that gp39 is involved in negative selection implies its expression in the thymus. To determine whether gp39 could be expressed on cells in the thymus, thymocytes from AND TCR Tg mice were cultured with antigen and analyzed for expression of gp39. As

shown in Table 4, thymocytes cultured with medium alone did not express gp39. However, the addition of antigen (200 nM of PCCF) to the cultures resulted in the induction of gp39 on  $\sim$ 5% of thymocytes. Three-color analysis of gp39 expression on the various thymocyte subsets indicated that the 94% of gp39<sup>+</sup> cells were contained in the CD4 SP population. Pharmacological (PMA/ionomycin) or mitogen (Con A) activation of thymocytes from normal mice resulted in the induction of gp39 on 54–75% of CD3<sup>+</sup> thy-



**Figure 5.** Anti-gp39 treatment allows for the survival of antigen-reactive CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells were obtained from thymus by complement-mediated lysis. Cells from control or anti-gp39-treated AND H-2<sup>b/s</sup> Tg mice were stimulated with various concentrations of moth cytochrome c (cross-reactive antigen) in the presence of APCs and were assessed for proliferation by [<sup>3</sup>H]thymidine incorporation after 48 h. Two individual animals (represented by squares or circles) were assessed in each group. Open symbols represent anti-gp39-treated animals and closed symbols represent control-treated animals.

**Table 3.** SEB-mediated Deletion of V $\beta$ 8<sup>+</sup> Thymocytes Is Not Blocked by the Administration of Anti-gp39

In vivo anti-gp39 treatment*	SEB <sup>†</sup>	% V $\beta$ 8 <sup>‡</sup>	
		CD4 <sup>+</sup>	CD8 <sup>+</sup>
–	–	22.5–24.7	45.0–52.6
–	+	4.5–6.0	9.0–21.0
+	+	2.5–5.2	5.0–7.5

\*Mice (three per group) were treated with control HIg or anti-gp39 (100  $\mu$ g/d, three times weekly) for 4 wk.

<sup>†</sup>SEB (20  $\mu$ g/d) was administered on alternating days.

<sup>‡</sup>Thymocytes were stained for CD4, CD8, and V $\beta$ 8 as described in Materials and Methods. The number of V $\beta$ 8<sup>+</sup> cells is expressed as a percentage of each SP subset. One of three representative experiments is shown.

mocytes, indicating that DP thymocytes must also have the capacity to express gp39. From these studies, it is clear that, given the appropriate signals, gp39 expression can be induced on both mature and immature thymocytes.

*Expression of B7-2 in the Thymus of gp39-deficient Mice.* The expression of B7-2 in the thymus has been implicated in influencing the process of negative selection, and numerous studies have shown that gp39 regulates the expression of B7-2 on B cells (22–24). Therefore, it was of interest to determine if the absence of gp39 in the thymus would alter B7-2 expression. The results of quantitative immunohistochemical analysis revealed that, in the absence of gp39 compared with wt control mice (6 A), the expression of B7-2 in the medullary region of the thymus was substantially reduced in both the gp39-deficient mice (Fig. 6 B) and in mice chronically treated with anti-gp39 (data not shown). Quantitation of B7-2 expression on gp39-deficient (red bars) and wt (green bars) mice is depicted in Fig. 6 C. These data show that gp39 expression in the thymus is critical for B7-2 expression and that the reduced expression of B7-2 (or other CD40-regulated costimulatory molecules) may contribute to the defects in negative selection observed in the absence of gp39.

## Discussion

Although early studies of thymic selection suggested that engagement of self-reactive TCR was necessary and sufficient for the elimination of both DP and mature thymocytes (2), recent studies indicate that factors other than the engagement of TCR alone, including costimulatory molecules (3, 4), the nature of the Ag (25–27), and the timing and distribution of Ag (28), may influence negative selection. The data presented in this study indicate that negative selection mediated by MIs (in BALB/c and CBA/J mice), by H-2A<sup>s</sup> (in AND TCR Tg mice), and by endogenously expressed PCC in AND TCR Tg × PCC Tg mice requires the interaction of gp39 with its receptor, CD40. Studies in gp39-deficient mice demonstrating that some MIs-specific Vβ-bearing T cells are spared from deletion provide supportive evidence that gp39 plays an essential role in negative selection. In contrast to these findings, negative selection induced by SEB or by high doses of exogenously administered antigen (PCCF in the AND TCR Tg mice) occurred in the absence of gp39–CD40 interactions. The data also demonstrate that thymocytes can be induced to express gp39, and the expression of gp39 is required for the expression of costimulatory molecules (e.g., B7-2) on cells within the thymus. Taken together, these data are consistent with the hypothesis that gp39-regulated expression of costimulatory molecules in the thymus may be critical for the negative selection of T cells to low-dose, endogenously expressed antigens/superantigens. Finally, the observation that, in some models of negative selection, functional blockade or deletion of gp39 results in the survival of DP as well as SP thymocytes, coupled with the observation that gp39 expression can be induced on immature CD3<sup>+</sup> thymocytes, suggests that gp39-dependent thy-

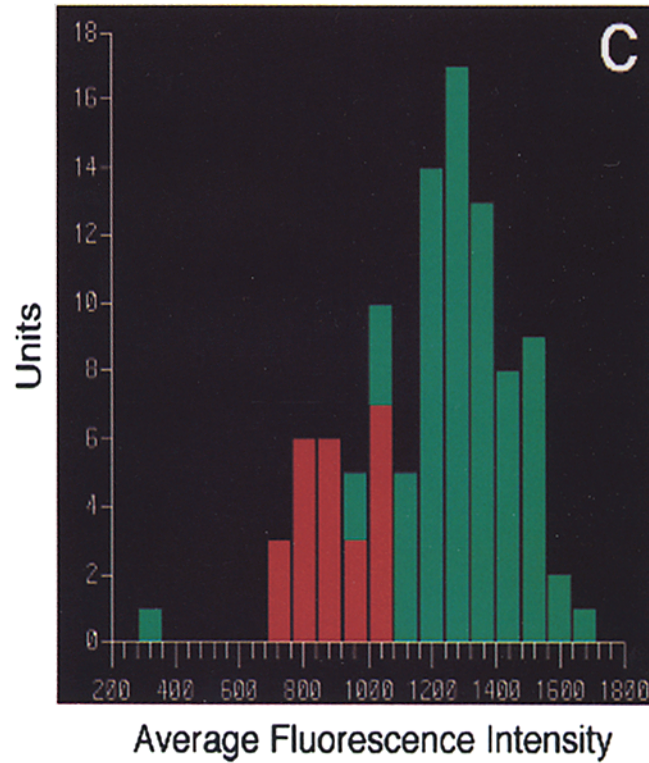
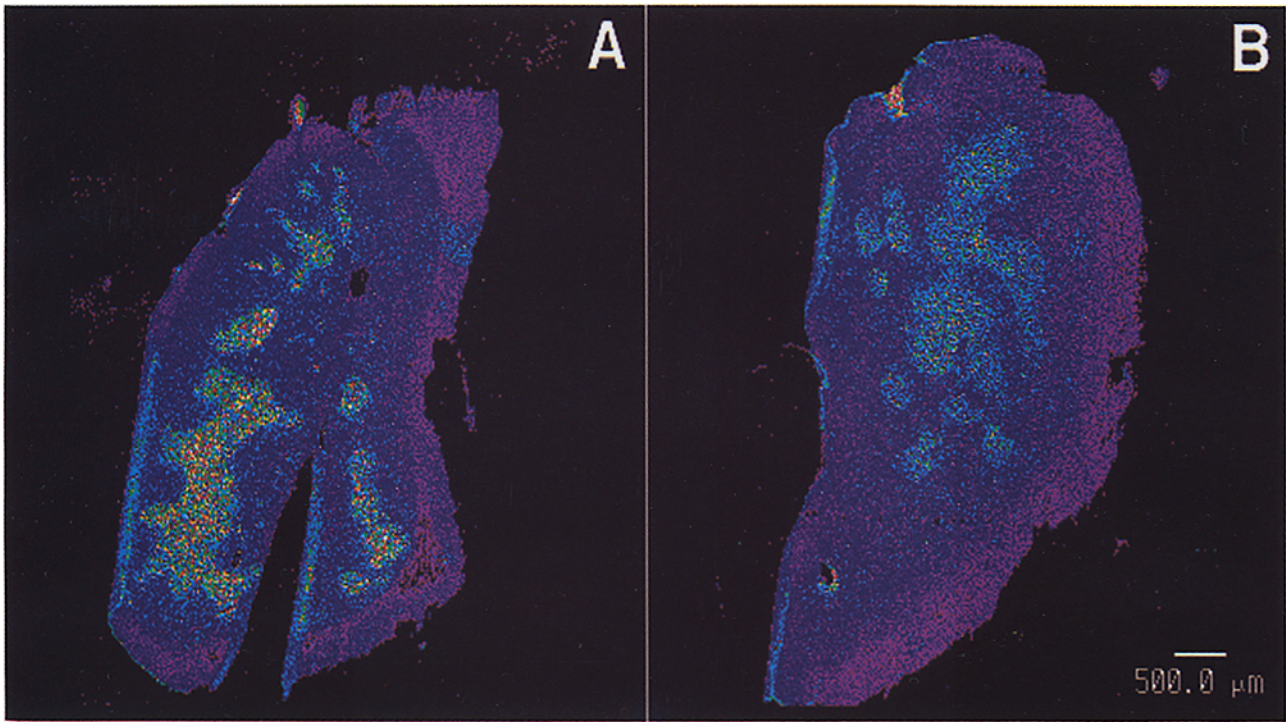
mocyte deletion may not be limited to cells of the mature SP subset.

Of the six models of negative selection examined, some common characteristics exist amongst models that are dependent on gp39 for deletion. First, in all models where the production of the antigen or superantigen was endogenous, negative selection was influenced by anti-gp39 administration or by the absence of gp39 expression. That is, anti-gp39 blocked the deletion to T cells reactive to MIs<sup>c,f</sup> and altered the deletion of AND TCR Tg T cells in mice endogenously expressing H-2A<sup>s</sup> or PCC. Furthermore, deletion to MIs in the gp39-deficient × CBA/J mice was significantly skewed. In two models in which exogenous antigen or superantigen was administered (SEB or exogenous PCCF), no effect of anti-gp39 on negative selection was observed. Based on these results, it appears that anti-gp39 interferes with antigen/superantigen-driven selection when antigen is produced under conditions that approximate physiologic conditions. In contrast, supraphysiological concentrations of antigen/superantigen (such as that used in the exogenous PCCF and SEB models) may override the requirements for costimulation and allow thymocyte deletion to occur in the absence of CD40-mediated costimulation. Attempts were made to evaluate whether negative selection to low doses of PCCF (exogenously administered) was gp39 dependent; however, the level of PCCF-induced deletion was too low to provide definitive results (data not shown). It is possible that deletion of thymocytes by high doses of PCCF or SEB may be the result of nonspecific deletion due to a gp39-independent corticosteroid stress response elicited by administration of exogenous Ag rather than Ag-specific deletion. Finally, it is also clear from the data that the mechanisms of deletion induced by exogenous versus endogenous PCC are different, since the former caused deletion of the DP, whereas the latter only caused alterations in the SP population.

Low affinity/avidity interactions between TCR and Ag/MHC may be another hallmark for gp39-dependent selection. Mature AND TCR Tg T cells do not proliferate in response to H-2A<sup>s</sup> (13), and Vβ3, 11-, and 12-bearing mature T cells proliferate poorly in response to MIs<sup>c,f</sup> (29, 30), suggesting that the avidity of the TCR for H-2A<sup>s</sup> or MIs<sup>c,f</sup>/MHC is weak. Therefore, thymocytes bearing these TCRs most likely interact with these antigens/superantigens in a low affinity/avidity fashion. As such, deletion in the thymus requires the contribution of CD40-derived signals. Although it is not clear that deletion mediated by endogenously expressed PCC in the (AND TCR Tg × PCC Tg)F<sub>1</sub> mice is the result of low-affinity TCR–Ag interactions, the fact that PCC is believed to be expressed at only low levels on the cell surface of these Tg mice (Oehen, S., and S. M. Hedrick, manuscript in preparation) supports the observation that deletion in this model also requires gp39-dependent costimulatory signals. In contrast, two gp39-independent models of selection are mediated by high-affinity TCR ligands (high-dose PCCF and SEB) that elicit robust proliferative responses from mature T cells.

Much of the data presented in this report were generated





**Figure 6.** B7-2 expression is decreased in the thymus of mice deficient for gp39. (A) Expression of B7-2-bearing cells in cryostat sections of wt thymus is prominent in medullary regions but undetectable in the cortex or paracortex. (B) The level of B7-2 expression is decreased in the thymus of gp39-deficient mice. Images show digital pictures of the thymus after laser scanning sections stained with B7-2 as described in Materials and Methods. (C) Quantification of the fluorescence signal is depicted in the histograms. The average fluorescence intensity of B7-2 expression in wt thymus is shown in the green bars, and gp39-deficient thymus is shown in the red bars. The histograms represent the overall average fluorescence intensity for all sections scanned in a single experiment. A total of 34 sections from each wt and gp39-deficient thymus were analyzed in two separate experiments.

**Table 4.** Induction of gp39 on Thymocytes

Stimulus*	gp39 <sup>+</sup> as % of each thymocyte subset <sup>‡</sup>					
	Total	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	DP	DN
None	0.1	—	—	—	—	—
200 nM PCC	5.2	ND	93.9	0.4	5.7	0
Con A	16.3	54.0	ND	ND	ND	ND
PMA+						
ionomycin	25.0	72.0	ND	ND	ND	ND

\*Thymocytes ( $5 \times 10^6$ /ml) from AND TCR Tg mice (for Ag-induced expression) or normal B10.BR mice (for Con A- or PMA/ionomycin-induced expression) were cultured for 12 h in the presence or absence of PCC, Con A (5  $\mu$ g/ml), or PMA (10 ng/ml) and ionomycin (250 nM).

<sup>‡</sup>Thymocytes expressing gp39 were detected flow cytometrically by staining with a combination of CD4-PE, CD8-Cy, and biotin anti-gp39. A minimum of 10,000 cells were analyzed for each sample. One of three representative experiments is shown.

in mice in which the administration of anti-gp39 was used to block the function of the molecule. While it is clear that anti-gp39 can block the binding of CD40 to gp39, it is also possible that the antibody may directly transduce signals to the thymocyte via gp39. It is conceivable that the binding of anti-gp39 to activated, gp39-expressing thymocytes induces a positive signal to rescue them from negative selection. However, we have never documented any positive effects of anti-gp39 on thymocyte or mature T cell functions (data not shown). Nonetheless, one report does indicate that gp39 engagement can costimulate the activation of mature T cells to anti-CD3 (31). The fact that genetic deletion of gp39 also alters the selection of specific V $\beta$ -bearing thymocytes strongly argues that the absence of gp39 function, and not positive signaling via gp39, alters negative selection. Finally, although CD40 is the only identified receptor for gp39 to date, our studies do not rule out the possibility that gp39 may be mediating negative selection in the thymus through the interaction with another, as yet unidentified, gp39-binding molecule.

Based on the arguments presented above, it appears that the loss of gp39 function permits the escape of self-reactive thymocytes. Although much of the literature examining thymocyte selection suggests that TCR-derived signals alone are sufficient to induce death, there is precedence that additional costimulatory molecules are required for negative selection (3, 32, 33). Because a major function of gp39 is the regulation of costimulatory molecules (23, 24, 34, 35), it is reasonable to speculate that gp39 regulates negative selection via its influence on the expression of costimulatory molecules within the thymic environment. Triggering via CD40 has been shown to up-regulate the expression of costimulatory molecules on B cells, dendritic cells, and Langerhans cells (22–24). Furthermore, CD40 has been shown to be functionally expressed on thymic epithelial

cells (9). Whether CD40 signaling directly up-regulates costimulatory molecule expression on thymic epithelial cells or thymic dendritic cells has not yet been critically tested. Data presented in this study show that the genetic deletion of gp39 or the administration of anti-gp39 to neonatal mice (data not shown) significantly reduces the medullary expression of B7-2. Unfortunately, B7-1 expression in the thymus was not detectable in our hands. Supportive evidence for a role of gp39 in thymic expression of B7 also comes from studies that have shown that B7 expression in the thymus is T cell dependent (5).

Evidence that CD28–B7 interactions play a role in negative selection comes from *in vitro* studies (4) showing that the induction of apoptosis of DP thymocytes by anti-CD3 required simultaneous engagement of CD28. Moreover, the observation that the restricted expression of B7 on thymic medullary cells correlates well with the preferential deletion of self-reactive thymocytes in the medulla is suggestive of a causal role for B7 in negative selection (5). Although these studies build a strong circumstantial case for B7 playing a causal role in negative selection, definitive proof of a role for B7 negative selection has not been readily obtained. Direct evidence for the lack of CD28 involvement in selection is provided by studies in CD28-deficient mice in which the deletion of Mls<sup>1a</sup>-reactive V $\beta$ 6- or H-2E<sup>k</sup>-reactive V $\beta$ 11-bearing T cells appeared unaltered (36). In addition, studies by Kruisbeek and co-workers (8) have shown that the administration of human CTLA4-Ig did not alter the deletion of V $\beta$ 5- or V $\beta$ 6-bearing thymocytes to Mls in (C57BL/6  $\times$  DBA/2)F<sub>1</sub> (BDF<sub>1</sub>) mice. The authors did not exclude a role for CD28–B7 interactions in thymocyte death but concluded that CD28–B7 interactions did not perform a role in thymocyte deletion. In addition to these *in vivo* studies, several *in vitro* studies do not support the conclusion that costimulation is involved in negative selection. Using an *in vitro* culture system that included CD4<sup>+</sup>CD8<sup>+</sup> (DP) thymocytes from Tg mice expressing the AND TCR, investigators demonstrated that B7 was not required to mediate negative selection (3). However, results from these studies did confirm that an APC-derived costimulatory signal was required for negative selection. In a separate study, using an *in vitro* deletion assay involving thymocytes expressing the Tg H-Y antigen-specific TCR cultured with H-Y<sup>+</sup> dendritic cells, Teh and co-workers demonstrated that neither CTLA-4 Ig nor B7 Ig fusion proteins block negative selection of DP thymocytes (6). In this study, the authors suggested that the level of B7 expressed on thymic epithelial cells is inadequate to provide a costimulatory signal (6). Given that many factors may contribute to the requirements for costimulation in negative selection, the role of B7-1 and B7-2 in the process of negative selection must be revisited in a more comprehensive manner. Finally, the data presented in this study do not provide any proof of B7 involvement in negative selection; they prove only that gp39–CD40 interactions are important. It is possible that gp39-induced expression of cytokines or costimulatory molecules other than B7 is important for the selection process.

There are many genetic loci that contribute to an increased risk of autoimmune disease. Mutations in genetic loci that alter the function of the costimulatory "loop" identified in this study may alter the efficiency with which self-reactive T cells are centrally deleted and may permit the escape of autoreactive T cells to the periphery. Based on the findings presented, low-to-moderate-affinity, self-reactive T cells may most easily escape deletion when this costimulatory loop is incapacitated by mutation. However, in the context of the same mutations, high-affinity, self-reactive T cells may be effectively deleted by a gp39-independent mechanism. It would be predicted that hyper-IgM patients (who lack functional gp39 [37]) would have a high frequency of autoreactive T cells in the periphery. While this may be the case, the absence of gp39 expression on

mature T cells would most likely limit the ability of those T cells to mediate autoimmune disease (38). On the other hand, if there were mutations that interfered with CD40-dependent deletion within the thymus, then functional-, low-, or moderate-affinity autoreactive T cells may escape selection. In addition to genetic mutations, xenobiotics that alter this loop may also be autoimmunogenic. For example, it has been shown that cyclosporine A (CsA), an inhibitor of gp39 expression (12), can block negative selection (39, 40). The fact that negative selection is blocked by CsA may be the basis for the increased incidence of autoimmune disease in CsA-treated patients (41–43). Support for this hypothesis is currently being sought through studies in strains of mice that are prone to the development of autoimmune disease.

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