Mutually antagonistic signals regulate selection of the T cell repertoire

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Abstract

The sensitivity of T cells to agonist-induced death during development contrasts with their proliferative responses after agonist challenge in the periphery. The means by which TCR engagement results in these distinct outcomes is incompletely understood. It has been previously hypothesized that glucocorticoids (GC) modulate the threshold for thymocyte activation by blunting the consequences of TCR engagement. In support of this possibility, inhibition of GC production in fetal thymic organ culture was shown to result in CD4+CD8+ thymocyte apoptosis. This was dependent upon MHC diversity, implying that endogenous GC might regulate antigen-specific selection. Similarly, mice expressing reduced GC receptor (GR) levels due to the presence of an antisense transgene have fewer CD4+CD8+ thymocytes, which was due to an impaired transition from CD4±CD8± precursors and increased apoptosis. Here we ask how manipulating peptide diversity in the context of reduced GC signaling might affect T cell development and function. In mice with impaired GR expression there was a rescue of thymocyte cellularity and proportions as the diversity of peptides presented by self-MHC was reduced. Furthermore, whereas more CD4+ T cells survived the selection process in mice expressing single-peptide–MHC class II complexes and reduced GR levels, these cells largely failed to recognize the same MHC molecules bound with foreign peptides. Together, these results support a role for endogenous GC in balancing TCR-mediated signals during thymic selection.

Introduction

Selection processes in the thymus ensure that peripheral T cells fulfill two essential prerequisites: activation by foreign peptides bound to host MHC molecules, but tolerance to self-derived peptides presented in the same context. Fulfillment of the former occurs passively in the thymus, by tying thymocyte survival and subsequent differentiation to self-restriction (1,2). In contrast, the latter requires the active elimination of thymocytes that react strongly with self-peptide–MHC molecules (1,3,4). It is thought that the strength of the TCR-mediated signal received during selection ultimately distinguishes T cells that survive from those that do not. Selection occurs only if signals received through the TCR fall between two thresholds; signals below one are incompatible with survival (neglected death), while signals above another result in deletion (5,6).

By influencing the strength of TCR engagement, coreceptors and adhesion molecules can modify the outcome of thymocyte selection (7–10). Soluble factors (e.g. IL-7, IL-4) present in the thymic milieu provide additional cues for thymocytes that may influence differentiation, division or death (11,12). In addition, glucocorticoids (GC) produced by thymic epithelial cells can modify the biological consequences of TCR-mediated signaling (13). While signaling through either the TCR or GC receptor (GR) individually results in apoptosis, simultaneous exposure to both antagonizes the death pathway (13,14). Thus, GC, by modulating the signaling threshold during thymic selection, may influence the range of selectable avidities for a particular TCR, thereby directly influencing the antigen-specific T cell repertoire (15–18). This phenomenon of ‘mutual antagonism’ potentially provides an...
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explanation to a long-standing paradox—how do thymocytes distinguish between death and survival signals originating from the same receptor (14)? Implicit in the mutual antagonism model is the idea that a reduction in GR activity should increase the thymocyte response depending on the degree of TCR occupancy (19). In this model, thymocytes bearing TCR with avidities appropriate for selection undergo deletion, while those bearing TCR with inadequate avidities undergo selection (20,21).

The role of endogenous GC in thymocyte selection has been examined previously in transgenic mice that express an antigen-specific GR transcript specifically in the thymus (homozygous transgenic mice are referred to as TKO), but not in the periphery. These mice displayed a hypoplastic thymus, hyporesponsiveness to GC and increased sensitivity to apoptosis induced by anti-TCR antibodies (22). Other studies using GR ‘knockout’ mice have cast doubt on the inferences made using pharmacologic inhibitors of GC synthesis, GC antagonists and TKO mice (23).

In light of this, we have re-examined the possibility that GC influence the consequences of TCR-mediated signaling during thymocyte development. To do so, we bred TKO mice with strains differing in their ability to present peptides in the context of MHC-encoded molecules. We have tested the prediction that an altered antigen-specific repertoire is a consequence of reduced thymic GC responsiveness by comparing the repertoires and reactivities of CD4+ T cells selected on a single-peptide–MHC complex. Our data indicates that GC affect T cell repertoire formation by restricting the development of T cells to those that recognize host MHC. Thus, by setting a threshold for thymocyte selection, GC inhibit the development of useless T cells and consequently enforce MHC restriction.

Methods

Animals

Mice engineered to express transgenic AβEp complexes in the absence of endogenous Aββi and invariant chain (Ii) were generated at the National Jewish Medical and Research Center (Denver, CO) as previously described (24). TKO mice that express a fragment of the 3'-untranslated region of the rat GR under the control of the proximal lck promoter were described elsewhere (22). TKO mice were backcrossed with the AβEpIi0 strain to generate mice homozygous for the TKO transgene, which expressed the AβEp transgene, but were devoid of the Ii and endogenous Aββi (TKO.AβEpIi0). Some TKO.AβEpIi0 mice were further crossed with AβEpIi0βm1 mice. TKO mice deficient in both Aββi and βm were generated by backcrosses with doubly deficient Aββiβm1 mice. TKO mice singly deficient for Ii, Aββi or βm resulted from the crosses mentioned above. All mice used in experiments were 6–8 weeks old and were on the H-2b (C57BL/6) genetic background. Mice deficient in βm were originally purchased from the Jackson Laboratory (Bar Harbor, ME).

AutoMACS sorting of cells

Cells were then incubated in the presence of biotinylated monoclonal antibody for 15 min at 4°C and rinsed with MACS running buffer (PBS containing 1 mM EDTA). Cells were then incubated for an additional 15 min at 4°C in the presence of MACS streptavidin beads, rinsed and sorted with an AutoMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol.

Preparation of cells and flow cytometric analysis

Tissues were harvested and dissociated into single-cell suspensions. The following antibodies were used for flow cytometric analysis: allophycocyanin–anti-CD4 (clone GK1.5), perCP–anti-CD8α (clone 53-6.7), PE–anti-CD69 (clone H1.2F3) and FITC–anti-CD5 (clone 53-7.3) (purchased from PharMingen, San Diego, CA). Samples were stained in BSS wash buffer (BSS containing 2% FCS and 0.1%NaCl) after pre-incubation for 5 min with anti-FCαRI II/III (clone 2.4G2) antibody. Samples were acquired using a dual-laser FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA) with CellQuest software (Becton Dickinson). Analysis of flow cytometry data was carried out using WinMDI (Joseph Trotter, La Jolla, CA) or WinList (Verity Software House, Topsham, ME) software. Dead cells were excluded from analysis by gating appropriate forward and side scatter.

Analysis of apoptosis

Samples were first stained with antibodies as described above. To measure apoptotic cell death, following staining, cells were rinsed and resuspended in 1 × Annexin binding buffer (0.01 HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM CaCl2). Annexin V (PharMingen) was added to samples according to the manufacturer’s recommendations and subsequently analyzed by flow cytometry.

Generation of T cell hybridomas

Plastic 75-cm² tissue culture flasks were coated with purified anti-CD3 (clone 145-2C11) at a concentration of 25 µg/ml overnight at 4°C and vigorously rinsed at least 5 times with PBS before use. Pooled mesenteric, axillary and inguinal lymph nodes were isolated and prepared for MACS sorting as described above. Purified CD4+ T cells from control and TKO

Fig. 1. The rescue of CD4+CD8+ thymocytes is inversely proportional to the diversity of peptides presented by MHC-encoded molecules. (A) Representative two-color flow cytometry analysis after staining cells with allophycocyanin-labeled anti-CD4 and perCP-labeled anti-CD8 antibodies. The phenotype of each pair of mice is shown above and below the middle table. Proportion of positive cells present in the thymus (TKO+ shown in upper panels; A–F and M–R) and lymph nodes (TKO– shown in lower panels; G–L and S–X) of indicated mice. Numbers in quadrants represent the corresponding percentages of each respective population. Bar graphs representing absolute (B) thymocyte and (C) CD4+ lymph node cell numbers of the indicated populations were calculated from the percentage of cells determined by flow cytometry shown above. Results, represented as means ± SEM, were from four to seven independent experiments. Mice were sacrificed between 4 and 6 weeks of age. Significant differences between TKO mice of different genotypes were determined by the Mann–Whitney U-test. *P < 0.05.
mice were then added to the culture flasks and incubated for 6 days. On the third day of incubation, IL-2 was added to the culture flasks. At the end of the culture period, the resulting T cell blasts were fused with the thymoma cell line BW5147 $\alpha\beta$ to generate hybridomas as described previously (24).

**Analysis of the reactivities of T cell hybridomas**

Uncloned T cell hybridomas from A\textsuperscript{b}EpI\textsuperscript{i}0 and TKO.A\textsuperscript{b}EpI\textsuperscript{i}0 mice were briefly expanded on individual wells of 24-well plates, and assessed for expression of CD4 and TCR by flow cytometry. Hybridomas expressing high levels of CD4 were tested for IL-2 production in response to spleen cells from $\beta$\textsubscript{2}m\textsuperscript{0} and Ii\textsuperscript{0} mice. In parallel, hybridomas were checked for their ability to produce IL-2 after overnight incubation on anti-CD3-coated 96-well plates. To assess the frequency of responders from each type of mouse, only hybridomas responding to anti-CD3 cross-linking were included in the analysis. Measurement of IL-2 production was accomplished by overnight incubation of the IL-2-dependent cell line HT-2 with supernatants harvested from the responders. HT-2 cells were incubated for 5 h at 37°C in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). Viability was determined by measuring absorbance at 590 nm in each well containing HT-2 cells using an ELISA plate reader.

**Results**

**Rescue of thymocytes in TKO mice correlates with the diversity of MHC-bound peptides**

If the increased death of double-positive thymocytes in TKO mice were due to unopposed TCR occupancy, one would predict that it would vary as a function of antigen/MHC diversity. To test this, we examined the CD4/CD8 profile in thymocytes and lymph nodes from the indicated strains of TKO mice that differ in their abilities to present self-peptides (Fig. 1A). In the presence of a diverse array of peptides, TKO mice displayed a sharp reduction in thymic cellularity and proportion of CD4+$\beta$+$\delta$+ thymocytes and lymph nodes from the indicated strains of TKO mice displayed a sharp reduction in thymic cellularity and proportion of CD4+$\beta$+$\delta$+ thymocytes and lymph nodes from the indicated strains of TKO mice. In parallel, hybridomas were checked for their ability to produce IL-2 after overnight incubation on anti-CD3-coated 96-well plates. To assess the frequency of responders from each type of mouse, only hybridomas responding to anti-CD3 cross-linking were included in the analysis. Measurement of IL-2 production was accomplished by overnight incubation of the IL-2-dependent cell line HT-2 with supernatants harvested from the responders. HT-2 cells were incubated for 5 h at 37°C in the presence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). Viability was determined by measuring absorbance at 590 nm in each well containing HT-2 cells using an ELISA plate reader.

**Rescue of thymocytes proportions and cellularity depends on the diversity of peptides presented by A\textsuperscript{b}, not its expression level**

In addition to reducing the number of peptides presented during selection, the absence of II results in a reduction in the surface expression of A\textsuperscript{b} by bone marrow-derived antigen-presenting cells (APC) and thymic epithelial cells. Therefore, the rescue of thymocyte numbers observed in TKO mice may be a consequence of decreased expression of A\textsuperscript{b}, as all of the ‘low peptide diversity’ mice tested were II deficient. Mice presenting a diverse array of peptides in the context of reduced numbers of surface A\textsuperscript{b} (designated A\textsuperscript{a}EpI\textsuperscript{i}) have been described (26). The A\textsuperscript{a}EpI\textsuperscript{i} strain expresses A\textsuperscript{b} covalently linked to Ep52-68 as a transgene and in the absence of endogenous A\textsuperscript{b}. Due to the presence of the II, the covalently attached Ep52-68 peptide is cleaved from the binding groove of transgenic A\textsuperscript{b} and subsequently replaced by a diverse array of self-peptides (27,28). Due to low expression of the

**Fig. 2.** Rescue of thymocytes in TKO mice is dependent on reduced peptide diversity and is not related to expression level of A\textsuperscript{b}. (A) Expression of CD4 and CD8 in TKO.A\textsuperscript{a}EpI\textsuperscript{i}+ and A\textsuperscript{a}EpI\textsuperscript{i}+ mice was determined by flow cytometry after staining single-cell thymocyte (top panels) and lymph node (lower panels) suspensions with allophycocyanin-labeled anti-CD4 and perCP-labeled anti-CD8 antibodies. Numbers in quadrants represent the corresponding percentages of each respective population. (B and C) Absolute cell numbers were calculated from the percentage of cells recovered as determined by the flow cytometric analysis. Results are represented as means ± SEM calculated from three independent experiments (TKO.A\textsuperscript{a}EpI\textsuperscript{i}+, n = 5; A\textsuperscript{a}EpI\textsuperscript{i}+, n = 4) from mice sacrificed between 4 and 6 weeks of age.
transgene-encoded Ab, thymic epithelial cells express 2-fold fewer Ab complexes while APC express ~10-fold less. These mice have ~2 times more CD4+ T cells and CD4+ SP thymocytes compared to wild-type mice due to inefficient negative selection, while the efficiency of positive selection is unperturbed (26). The inefficient clonal deletion of CD4+ T cells in these mice results in an autoimmune syndrome characterized by an activation of T and B cells, which leads to progressive lymphoadenopathy and minor hypergamma-globulinemia (26).

We crossed TKO transgenics onto the A<sup>b</sup>EpI<sup>i</sup>+ background to distinguish between the effects of decreased peptide diversity and reduced Ab expression. Flow cytometric analysis of total splenocytes from both A<sup>b</sup>EpI<sup>i</sup>+ and I<sup>i</sup>0 mice previously demonstrated a similar 10-fold reduction in the surface levels of Ab in each strain, consistent with previous findings (26). The reduced expression of Ab, however, had little impact on restoring the cellularity and proportions of thymocytes in TKO mice, which remained well below non-transgenic levels (Fig. 2A, top left). The proportion of CD4+CD8+ thymocytes was decreased in TKO.A<sup>b</sup>EpI<sup>i</sup>+ mice in comparison to TKO.A<sup>b</sup>wt mice (Fig. 2B). No significant difference was observed in either the absolute number of CD4+CD8+ thymocytes (Fig. 2B, right graph), suggesting that the decrease occurred passively due to an increase in CD4+ SP thymocytes in TKO.A<sup>b</sup>EpI<sup>i</sup>+ mice. Furthermore, an increase in the CD4/CD8 ratio in the lymph nodes of TKO.A<sup>b</sup>EpI<sup>i</sup>+ mice to ~1.4:1 from the 1:1 ratio observed in TKO.A<sup>b</sup>wt mice further validates this point (Fig. 2C). Thus, the diversity of TCR ligands available for selection ultimately determines the extent of thymocyte deletion in TKO mice.

Fig. 3. Reducing thymocyte exposure to GC increases sensitivity to TCR engagement. (A) Expression of CD5 and (C) CD69 on gated CD4+CD8+ thymocytes correlates with increased sensitivity to TCR stimuli and is inversely related to the availability of TCR ligands. Histograms show analysis of CD5 expression by flow cytometry after surface staining with anti-CD5-FITC antibodies. Histograms are shown after gating on the populations indicated at the top of each column. (B) Bar graphs of CD5 expression represent the mean fluorescence intensities ± SEM. (C) Flow cytometric analyses of CD69 expression were accomplished by staining single-cell thymocyte suspensions. Histograms shown are gated on CD4+CD8+ thymocytes in mice with reduced (TKO; solid line) or normal (Non-TKO; broken line) levels of GR on thymocytes. The genotype of each mouse is indicated to the left of the panel. Results are represented as means ± SEM and significant differences between TKO and non-Tg mice were determined by the Mann-Whitney U-test. Results were obtained from four independent experiments (TKO.MHC II<sup>i</sup>, n = 5; MHC II<sup>i</sup>, n = 4; TKO.I<sup>i</sup>, n = 9; I<sup>i</sup>, n = 9; TKO.A<sup>b</sup>wt, n = 5; A<sup>b</sup>wt, n = 4). *P < 0.010, **P < 0.005.

Thymocyte resistance to GC leads to MHC-dependent increases in the expression of activation markers

Expression of CD5 and CD69 on immature thymocytes is regulated by the avidity of the selecting interaction (29–31). Previous work demonstrated the level of CD5 on CD4+CD8<sup>+</sup> thymocytes was increased in TKO mice (16). To determine if reduced thymocyte exposure to GC increased their sensitivity to TCR engagement in vivo, we quantitated the expression of the activation markers CD5 and CD69 in a panel of TKO mice that differed in the diversity of peptides presented during selection. The level of CD5 on CD4+CD8<sup>+</sup> thymocytes was increased over the control levels in all mice expressing the TKO transgene (Fig. 3A, left column). This difference was significant as calculated from the mean fluorescence intensity values (Fig. 3B, left graph; *P < 0.005, **P < 0.010). A corresponding increase in the level of CD69 on CD4+CD8<sup>+</sup> thymocytes accompanied an increase in peptide diversity in both control and TKO mice, suggesting that CD5 expression reflects the availability of TCR ligands (Fig. 3A, left most column). SP CD4 thymocytes from TKO.MHC II<sup>i</sup> and non-transgenic MHC II<sup>i</sup> mice expressed indistinguishable levels of
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**Fig. 4.** The extent of thymocyte apoptosis in TKO mice is related to the diversity of MHC-bound peptides. (A) Flow cytometric analysis of apoptosis by Annexin V–FITC was accomplished by staining freshly prepared thymocyte single-cell suspensions. Histograms shown are gated on CD4+CD8+ thymocytes in mice with reduced (TKO; solid line) or normal (non-Tg; broken line) thymic levels of the GR. Text at the right indicates the genotype of the respective mice. (B) Histograms represent CD4+CD8+ thymocytes stained with Annexin V–FITC from mice of the indicated genotypes. Results are represented as means ± SEM, and significant differences between TKO and non-Tg mice were determined by the Mann-Whitney U-test. Results were obtained from four independent experiments (TKO.MHC II0, n = 5; MHC II0, n = 4; TKO.II0, n = 9; II0, n = 9; TKO.Abwt, n = 5; Abwt, n = 4). *P < 0.005.

CD5 (Fig. 3A and B, middle columns), indicating that the increased level seen in TKO mice depends on TCR engagement and not an intrinsic property of the transgene. The expression of CD5 on CD4+SP thymocytes on both A wt and II0 backgrounds was also higher in TKO mice than in non-transgenic, and again its level correlated with the diversity of self-peptides. Consistent with the unvarying level of MHC class I on all of the mice tested, the CD5 expression on CD8 SP thymocytes mice was unchanged among TKO mice crossed on MHC II0, II0 and A wt genetic backgrounds. However, the level of CD5 on CD8+ T cells was on average increased compared to the corresponding non-transgenic control population, although less dramatically than in CD4+ T cells (Fig. 3A and B, last column). The expression of CD69 was also higher on the bulk of CD4+CD8+ thymocytes in mice bearing the TKO transgene (Fig. 3C). Together, these data further support the notion that a reduction in GC blunting of TCR-mediated signals alters the perceived avidity of TCR–MHC engagement.

**Increased thymocyte apoptosis in TKO mice depends on recognition of self-peptide–MHC**

Thymocytes bearing TCR with high avidity for self-peptide–MHC undergo apoptosis during thymic selection (1). In previous reports, inhibition of GC synthesis with metyrapone in fetal thymic organ culture enhanced the TCR-dependent apoptosis of thymocytes (21). To examine this issue in a more physiologic setting, we asked if decreasing the diversity of selecting peptides during thymic selection correlated with a reduction in the apoptotic index of thymocytes. Apoptosis was measured in fresh thymocytes by assaying for phosphatidylserine exposure using Annexin V (Fig. 4A). As the proportion of

| Table 1. Absence of T cells from TKO mice selected on a single AEp complex that respond to other peptides bound to Ab |

<table>
<thead>
<tr>
<th>T cell hybridoma source</th>
<th>Frequency responding to APC from the following</th>
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<tbody>
<tr>
<td>A wt</td>
<td>Ii0</td>
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<tr>
<td>10</td>
<td>0</td>
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<tr>
<td>AEpII0</td>
<td>49</td>
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*T cell blasts from each type of mouse were fused with the thymoma cell line BW5147α-β- as described in Methods. Individual hybridoma clones were screened for expression of CD4 and TCR before further use. In addition, hybridomas were incubated on anti-CD3 were excluded from the above analysis.

**Significant difference between groups with Fisher’s exact probability, P = 0.036.**

MHC class II-bound peptides decreased, a significant decrease in CD4+CD8+ thymocyte apoptosis was apparent (Fig. 4B). Therefore, the extent of apoptosis due to reduced GC exposure during thymocyte development is dependent upon cognate interactions with a diverse array of self-peptides.

**Thymocyte development in an environment of reduced GC signaling impacts the repertoire of selected T cells**

We hypothesized that if GC shift the avidity ‘window’ for thymic selection, the peripheral repertoire of T cells selected in TKO mice might have a different capacity to recognize foreign peptides bound to host MHC. Previously, mice have been engineered that express a single (32) peptide tethered to MHC class II molecules. In these mice, the β chain of AEp is covalently linked to a peptide derived from the α chain of the EII0 MHC class II protein (Ep52–69) which, in the absence of the II and endogenous Aβ0, covalently bound Ep is the only detectable peptide presented to MHC class II-restricted T cells (33). As a single peptide bound to MHC class II mediates both positive and negative selection in this model, many CD4+ T cells are selected to mature that would be deleted in wild-type mice because of their strong cross-reactivity with other self-peptides (32,34).

To examine how reduced GC signaling during thymocyte development affects the peripheral T cell repertoire, we took advantage of this cross-reactivity by breeding TKO mice onto the AEpII0 background. Hybridomas prepared from lymph node CD4+ T cells isolated from TKO.AEpII0 and AEpII0 mice were compared for their responses to APC from βmII0 mice (to exclude any responses of CD4+ T cells selected on non-classical MHC molecules). As a control, the same hybridomas were incubated in parallel on 96-well plates coated with anti-CD3 antibody and only CD3-responsive hybridomas were included in the study (supplemental data). Consistent with previous results (32), a large fraction of hybridomas from AEpII0 responded to splenocytes from Awt mice and to a lesser extent splenocytes from II-deficient mice (Table 1). Strikingly, however, few or no hybridomas from TKO.AEpII0 mice responded to APC from βmII0 or II0 mice respectively. Thus, consistent with the possibility that endogenous GC
with low avidity for self-peptide–MHC, the repertoire of CD4+ T cells selected from TKO.AbEpIi0 mice failed to respond upon encounter with foreign peptides.

**Discussion**

Several observations led to the hypothesis that locally produced GC regulate the signaling threshold for thymocytes undergoing positive and negative selection. Initial studies of GC function using T cell hybridomas demonstrated that stimulation through either the GR or TCR individually led to apoptosis, while simultaneous occupancy of both receptors antagonized the death pathway (14,35). In addition, treatment of fetal thymic organ culture with reagents that inhibit either GC production or GR binding enhanced TCR-induced apoptosis (13). The observation that GC antagonize TCR-triggered signaling events formed the foundation of the mutual antagonism model of thymocyte development (14). A testable prediction stemming from this model is that altering the responses of pre-selection thymocytes to GC will result in a corresponding change in the mature T cell repertoire.

By varying the level of available TCR ligands and, consequently, the probability of TCR occupancy, a direct correlation between the restoration of the CD4+CD8+ thymocyte numbers and the occupancy of GC and TCR was most apparent in TKO mice doubly deficient in the expression of MHC class I and II molecules. The restoration of the CD4+CD8+ thymocyte proportions was accompanied by a substantial, albeit incomplete, restoration of CD4+CD8+ thymocyte numbers. The lack of a complete rescue of thymic cellularity indicates that the observed reduction in thymus size cannot be due entirely to enhanced TCR-mediated deletion of CD4+CD8+ thymocytes, consistent with the observation that there is a partial block in the double-negative to double-positive transition in these animals (22).

The low frequency of T cell hybridomas generated from TKO.AbEpIi0 mice that recognize syngeneic Abwt cells might reflect an enhanced deletion of the cohort of T cells that normally respond to other self-peptides bound to Ab. This would be consistent with an overall lowered threshold required for negative selection in the absence of the appropriate amount of GC blunting of TCR-mediated signals. In this scenario, the cells that survive selection might be skewed towards those with low functional avidities, having been primarily selected on conserved regions of MHC molecules independent of their ability to scan peptides. It is plausible that such ‘framework’ selected cells could develop if one considers a two-step mechanism for TCR recognition (36). In this model, an initial TCR contact with MHC is established with little contribution from the peptide. Once settled on the MHC, the TCR senses the peptide with its two CDR3 loops, only considering a two-step mechanism for TCR recognition (36). In this model, an initial TCR contact with MHC is established with little contribution from the peptide. Once settled on the MHC, the TCR senses the peptide with its two CDR3 loops, only becoming activated if a stable interaction is established. Therefore, within the context of this model it is conceivable that when GC-mediated signaling is reduced, a subsequent increase in the perceived TCR avidity may allow MHC binding in the first step to support positive selection, independent of recognition of MHC-bound peptides.

It is also possible that many CD4+ T cells that normally respond to foreign peptides in AbEpIi0 mice are present in an anergized state in TKO.AbEpIi0 mice and are consequently not likely to be included among the hybridomas generated, as fusion strongly favors those cells that are actively dividing. Thus, the hybridomas from these mice might simply reflect an over-representation of those CD4+ T cells selected on non-classical MHC molecules, which are passively over-represented among total CD4+ T cells in AbEpIi0 mice (25). However, this seems unlikely given the paucity of these cells in lymph nodes (37) and the failure of hybridomas from TKO.AbEpIi0 mice to respond to Ii0 splenocytes (Table 1), which were βm sufficient. We are currently investigating the presence of anergized T cells in TKO mice, in particular those with reportedly suppressive capabilities.

Alternatively, a reduced GC environment may sensitize thymocytes to TCR engagement revealing an otherwise undetectable leak of low-abundance self-peptides in AbEpIi0 mice, upon which TKO thymocytes undergo negative selection. In fact, a predominant involvement of low-abundance peptides in the selection of CD4+ T cells was proposed by another group using a different ‘single-peptide’ model (38). They suggested that the predominately expressed AbEp complexes likely had little impact on the appearance of CD4+ T cells and therefore, an undetectable leak of other peptides must be mediating the selection process. However, the same group more recently reported that many T cells are indeed selected on abundant peptide(s) (39). Notwithstanding, while a leak of endogenous peptides in other single peptide systems is readily detectable, several reports have failed to detect any such leak in AbEpIi0 mice (33,38,40).

A considerable body of evidence suggests that GC affect thymocyte development (13,15–18,21,22,41). One study has reported that thymocyte development is grossly normal in mice deficient in GR (23). The extrapolation of that observation to the present study is problematic, however. First, neither antigen-specific selection nor TCR repertoire was analyzed in those mice and therefore those data do not bear upon the present analysis of antigen-specific thymocyte development. Second, the same authors have recently reported that the animals are not true knockout mice, but express a truncated GR lacking exon 2-encoded residues (the N-terminal half of the molecule) but containing the C-terminal half of the molecule, which includes DNA-binding, ligand-binding and transactivation domains (42). These truncated GR are able to bind GC with the same affinity as wild-type mice and cDNA microarray analysis of GC-treated thymocytes has shown that this receptor is capable of modulating the expression of a large number of genes (J. D. Ashwell, manuscript submitted). Therefore, one cannot conclude that the GR is dispensable for any aspects of thymocyte development based on the analysis of these animals.

In conclusion, an assessment of the impact of thymic GC hypo responsiveness was carried out in mice, which present many, few, one or no peptides in the context of MHC class II molecules. Our results are consistent with the hypothesis that endogenous GC bias the selection of TCR towards those with a refined capacity to scan various peptides presented by...
MHC-encoded molecules. The partial rescue of CD4+CD8+ thymocytes observed in TKO mice, when the probability of TCR occupancy was reduced, further supports the idea that signals derived from the GR oppose TCR-mediated signaling during thymocyte development.

**Abbreviations**

\[ \beta_m \] \( \beta_m \)-microglobulin  
APC antigen-presenting cell  
GC glucocorticoid  
GR glucocorticoid receptor  
II invariant chain  
SP single positive

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