Brief Definitive Report

Bone Marrow–derived Cells Fail to Induce Positive Selection in Thymus Reaggregation Cultures

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Summary

The requirements for inducing positive selection of T cells were examined in thymus reaggregation cultures, a system in which dispersed populations of immature $CD4^+8^+$ cells and purified thymic epithelial cells (TEC) are reaggregated in tissue culture. Studies with TEC from mice selectively lacking major histocompatibility complex (MHC) class I (I^-II^+), class II (I^+II^-), or both class I and II (I^-II^-) molecules showed that class II expression was essential for the differentiation of $CD4^+8^+$ cells into $CD4^+8^-$ cells. Unexpectedly, the generation of TCR^{hi} $CD4^-8^+$ cells from $CD4^+8^+$ cells was apparent with I^-II^+ TEC but not with I^-II^- TEC, perhaps reflecting cross-reactive specificity of $CD4^-8^+$ cells for class II molecules. Significantly, the failure of I^-II^- TEC to generate TCR^{hi} $CD4^+8^-$ or $CD4^-8^+$ cells could not be overcome by adding MHC^+ bone marrow–derived cells. These findings, together with experiments on purified subsets of TEC, suggest that positive selection in thymus reaggregation cultures is an exclusive property of cortical TEC.

Positive selection of T cells in the thymus causes preferential survival of a minority (<5%) population of cortical $CD4^+8^+$ (double positive [DP]) cells with TCR specificity for self peptides bound to MHC class I and II molecules (1, 2). In conjunction with CD4 or CD8 coreceptors, TCR recognition of cell-bound peptide/MHC complexes signals TCR^{lo} DP cells to differentiate into mature single-positive (SP) $CD4^+8^-$ or $CD4^-8^+$ cells; through their MHC class specificity for class I and class II molecules, respectively, CD8 coreceptors stimulate production of $CD4^-8^+$ cells, whereas CD4 molecules control the generation of $CD4^+8^-$ cells. The vast majority of DP cells lack specificity for thymic peptide–MHC complexes, and these cells die rapidly from "neglect" (3).

Early studies with bone marrow (BM) chimeras and thymus-grafted mice led to the conclusion that positive selection is controlled by a radioresistant component of the thymus, presumably epithelial cells in the cortex (4–7). This conclusion has since been supported by studies on transgenic mice expressing MHC molecules selectively on cortical epithelium (8) and by experiments with thymus reaggregation cultures prepared from purified epithelial cells (9, 10). Collectively, these findings provide strong evidence that positive selection is controlled by thymic epithelial cells (TEC). Nevertheless, there are a number of reports that other types of cells are capable of inducing positive selection under defined conditions. Thus, injecting mice intrathymically with MHC-incompatible fibroblasts was found to cause positive selection to the MHC molecules on the injected cells (11, 12). Likewise, evidence that BM-derived cells can induce positive selection is provided by the finding that class I-deficient (β_2-microglobulin [β_2m]-deficient) irradiated hosts reconstituted with class I–bearing normal BM cells generated low but significant numbers of donor MHC–restricted $CD8^+$ cells (13). Interestingly, generation of $CD4^+$ cells was not seen in reciprocal chimeras in which class II–deficient hosts were reconstituted with normal (class II^+) BM (14). This apparent difference could reflect that the BM-derived cells in the cortex (most DP thymocytes) show low but significant class I expression but virtually no class II expression. TEC, by contrast, express high levels of both class I and II molecules (15).

These data raise the question whether introducing MHC^{hi} BM-derived cells directly into the cortex would accentuate positive selection by these cells. This issue is difficult to address by intrathymic injection because in our experience BM-derived cells are cleared rapidly from the cortex after intrathymic injection (unpublished data of the authors). We have approached this question with the aid of thymus reaggregation cultures (TRC), a system in which immature thymocytes are aggregated with dispersed suspensions of TEC in a three-dimensional network in tissue culture (9, 10). In this model, culturing purified $TCR^{-/lo}$ DP cells with TEC for 4–5 d leads to efficient generation of $CD4^+$ and $CD8^+$ SP cells, indicative of positive selection. We show here that, in contrast to normal TEC, TEC from combined class I^-II^- TEC fail to induce positive selection of DP cells. Significantly, the inability of MHC^- TEC to

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induce positive selection cannot be overcome by supplementing these TEC with normal MHC$^+$ dendritic cells or B cells. These findings imply that the role of BM-derived cells in positive selection is extremely limited. Based on studies with purified cortical vs medullary TEC, we conclude that positive selection is controlled mainly, and perhaps exclusively, by cortical TEC.

Materials and Methods

Animals. C57Bl/6J (B6) mice and B6.PL-Thy-1.1^a/Cy mice were obtained from the breeding colony of The Scripps Research Institute (La Jolla, CA). Mice deficient in β_2m (16) and mice deficient in I-A (17), kindly provided by Dr. Laurie Glimcher, were bred into the B6 background. Mice deficient in both β_2m and I-A were obtained from F_2 crossbreeding.

Antibodies. mAbs reactive to the following markers were previously described (15, 18): CD3 (C363.29, rat IgG), CD4 (RL142, rat IgM), CD8 (3.168.8, rat IgM), Thy 1 (T24, rat IgG), K^bD^b (28-8-6s, mouse IgG), 6C3/BP-1 (6C3, rat IgG) and a mouse fibroblast antigen (2F7, hamster IgG). Biotinylated mAbs to TCR-β (H57-597, hamster IgG) and CD45 (30f11.1, rat IgG) were purchased from PharMingen (San Diego, CA). FITC-conjugated mAbs specific for CD4 (H129.19, rat IgG) (GIBCO BRL, Gaithersburg, MD), CD8 (53-6.7, rat IgG) (GIBCO BRL), K^b (AF6-88.5, mouse IgG) (PharMingen), and PE-conjugated anti-CD4 (GK1.5, rat IgG) (Becton Dickinson & Co., San Jose, CA), anti-I-A^b (AF6-120.1, mouse IgG) (PharMingen), streptavidin (GIBCO BRL), Red613-conjugated anti-CD8 (53-672, rat-IgG) (GIBCO BRL), and biotinylated mouse anti–rat IgG (H+L chains) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were also used.

Cell Purification. Methods for purification of TCR$^{-/lo}$ CD4$^+$ CD8$^+$ thymocytes and TEC have been described elsewhere (18). Briefly, TCR$^{-/lo}$ CD4$^+$CD8$^+$ thymocytes were purified from newborn (1–3 d) B6 or combined β_2m- and I-A–deficient mice by treatment with a mixture of cytotoxic anti-CD3 (C363.29B) and anti-K^bD^b (28-8-6s) mAbs + C followed by positive panning on anti-CD4 (RL172)–coated plates (18). TEC were obtained by treating day 15–16 fetal thymic lobes with deoxyguanosine for 5 d followed by trypsin digestion. TEC were depleted of fibroblasts and CD45$^+$ cells by using antifibroblast (2F7) and anti-CD45 mAbs and magnetic columns (18). 6C3$^+$ versus 6C3$^-$ TEC were obtained from CD45- and fibroblast-depleted TEC by first staining with 6C3 mAb followed by biotinylated anti–rat IgG and PE-conjugated streptavidin and sorting on a FACStar® Plus (Becton Dickinson & Co.). Purified TEC from normal and MHC-deficient B6 fetuses were stained with a mixture of FITC–anti-K^b (AF6-88.5) and PE–anti-I-A^b (AF6-120.1) mAbs and analyzed on a FACScan® to confirm the lack of MHC expression. T cell–depleted (T$^-$) LN cells were obtained from adult (6–8 wk) B6.PL mice by treatment with a mixture of anti-CD4 (RL172), anti-CD8 (3.168.8) plus anti-Thy 1 (T24) mAbs + C followed by removal of dead cells on a Ficoll gradient. Dendritic cells (DC) were prepared from adult B6.PL mice using established procedures (19) and were treated with anti-CD4 (RL172) plus anti-CD8 (3.168.8) mAbs + C before use.

TRC Conditions. As described elsewhere (9, 18), each TRC was established by centrifuging a mixture of 0.5×10^6 TEC and 10^6 TCR$^{-/lo}$ DP thymocytes and then reaggregating these cells on nucleopore filters (Costar Sci. Corp., Pleasanton, CA); some cultures were supplemented with either 0.25×10^6 T$^-$ LN cells or DC. Cultures were incubated for 5 d, and single-cell suspensions were obtained by teasing with needle points. Cell yields were estimated by counting viable lymphocytes under a phase contrast microscope.

Flow Cytometry Analysis. For two-color staining, cells were incubated with FITC-conjugated anti-CD8 (53-6.7) and PE-conjugated anti-CD4 (GK-1.5) mAbs. For three-color staining, cells were first incubated with biotinylated mAbs to TCR-β (H57-597), washed, and then incubated with FITC-conjugated anti-CD4 (H129.19), Red613-conjugated anti-CD8 (53-6.7) mAbs, and PE-conjugated streptavidin and analyzed on a FACScan® (Becton Dickinson & Co.) (18).

Results

Experimental Approach. T cell differentiation in TRC has been described elsewhere (18). For the experiments discussed below, purified TCR$^{-/lo}$ DP thymocytes were prepared from neonatal thymus by removing TCR^{hi} and class I^{hi} cells by mAb + C treatment followed by positive panning for CD4$^+$ cells (see Materials and Methods). TEC were purified by culturing fetal thymuses with deoxyguanosine, then dispersing the surviving cells with trypsin, followed by removal of contaminating fibroblasts and CD45$^+$ cells by magnetic bead separation. DP cells and TEC were cultured on nucleopore filters for 4–5 d. As exemplified in Fig. 1, aggregation of TCR$^{-/lo}$ DP cells (Fig. 1 *a*) with TEC for

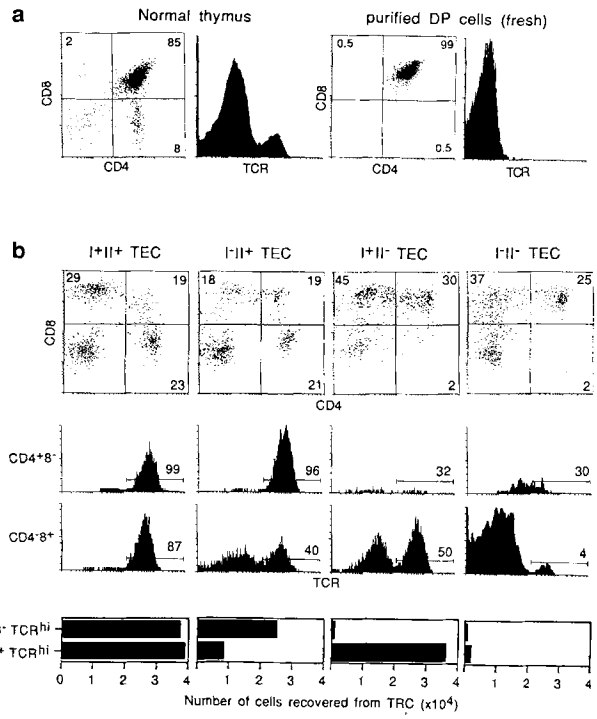

Figure 1. Capacity of MHC-deficient TEC to cause differentiation of DP thymocytes in TRC. Cells were three-color stained for CD4, CD8, and TCR-α/β and analyzed on a FACScan®. (*a*) Coexpression of CD4, CD8, and TCR-α/β on unseparated thymocytes vs purified DP thymocytes before culture with TEC. (*b*) Generation of TCR^{hi} SP cells after culturing DP thymocytes in TRC for 5 d with TEC from I$^+$II$^+$, I$^-$II$^+$, I$^+$II$^-$, or I$^-$II$^-$ mice; the data show CD4 vs CD8 expression (*top*), TCR-β expression on CD4$^+$8$^-$ versus CD4$^-$8$^+$ cells (*middle*), and total numbers of viable TCR^{hi} SP cells recovered from the cultures (*bottom*).

Table 1. *Generation of TCR^{hi} SP Cells from DP Thymocytes Cultured with MHC-deficient TEC*

TEC	Origin of input cells for TRC: DP thymocytes (number of experiments)	Origin of input cells for TRC: BM-derived cells (I^+II^+)	Total percent yield of lymphoid cells relative to input number (SD)		Generation of TCR^{hi} SP cells: Mean cell numbers recovered ($\times 10^{-4}$) $CD4^+$	Mean cell numbers recovered ($\times 10^{-4}$) $CD8^+$	Mean percent recovery relative to yields with I^+II^+ TEC $CD4^+$	Mean percent recovery relative to yields with I^+II^+ TEC $CD8^+$
I^+II^+	I^+II^+ (5), I^-II^- (4)	—	18.0	(8.7)	5.4	4.0	(100)	(100)
I^+II^-	I^+II^+ (1), I^+II^- (2)	—	19.6	(8.6)	0.1	3.0	1	74.3
I^-II^+	I^+II^+ (1), I^-II^+ (9)	—	12.6	(5.8)	2.0	0.6	37.4	14.8
I^-II^-	I^+II^+ (2), I^-II^- (6)	—	8.7	(3.1)	<0.1	0.1	<0.9	2.8
I^+II^+	I^-II^- (2)	DC	9.5	(0.7)	1.4	2.3	26.1	56.9
I^-II^-	I^-II^- (2)	DC	4.0	(1.4)	<0.1	<0.1	<0.7	<1.0
I^+II^+	I^-II^- (2)	B cells	12.5	(0.7)	1.3	1.5	23.3	37.1
I^-II^-	I^-II^- (2)	B cells	2.5	(0.7)	<0.1	<0.1	<0.2	<0.1

As described in Materials and Methods, TRC were prepared with 10^6 $TCR^{-/lo}$ DP cells and 0.5×10^6 TEC/culture; BM-derived cells added 0.25×10^6/ culture.

5 d leads to efficient positive selection, i.e., to generation of TCR^{hi} $CD4^-8^+$ and $CD4^+8^-$ SP cells (Fig. 1 *b*, *left*). As discussed elsewhere (18), cell division during the switch from DP to SP cells in TRC is quite limited.

Positive Selection with MHC$^-$ TEC. Using TRC as a model for positive selection rests on the assumption that differentiation of DP cells into SP cells reflects direct contact with the MHC class I and II molecules on TEC. If so, one would expect TEC expressing only class I and not class II molecules to generate $CD8^+$ cells but not $CD4^+$ cells, and vice versa. To test this prediction, purified $TCR^{-/lo}$ DP cells (Fig. 1 *a*) were cultured with TEC taken from β_2m-deficient mice (I^-II^+ TEC), I-A–deficient mice (I^+II^- TEC) or combined β_2m- and I-A–deficient mice (I^-II^- TEC); all of the TEC donors had a B6 ($H\text{-}2^b$) background. The results are illustrated in Fig. 1 *b* and summarized in Table 1. The DP cells used were either from normal B6 or MHC$^-$ mice; the source of DP cells seemed to be irrelevant and, for simplicity, the data for normal and MHC$^-$ DP cells have been pooled.

In the case of I^+II^- TEC, the results were clear-cut. Thus, in marked contrast to normal (I^+II^+) TEC, culturing DP cells with I^+II^- TEC led to selective generation of $CD4^-8^+$ cells, most of which were TCR^{hi}; $CD4^+8^-$ cells were barely detectable. The results with I^-II^+ TEC were different. The expectation here was that I^-II^+ TEC would generate large numbers of $CD4^+8^-$ cells but very few $CD4^-8^+$ cells. This was not the case. For obscure reasons, aggregate formation with I^-II^+ TEC was less efficient than with normal or I^+II^- TEC, and this was associated with an overall reduction in cell recovery. With I^-II^+ TEC, yields of $CD4^+8^-$ cells were generally two- to threefold lower than with normal TEC (Table 1). Surprisingly, I^-II^+ TEC led to significant generation of TCR^{hi} $CD4^-8^+$ cells. Total yields of these cells were much lower than with normal TEC, but the ratio of TCR^{hi} $CD4^-8^+$ to TCR^{hi} $CD4^+8^-$ cells was quite high, i.e., 0.3 for I^-II^+ TEC compared with 0.8 for normal TEC. Significantly, the generation of TCR^{hi} $CD4^-8^+$ cells was not seen with I^-II^- TEC; $CD4^-8^+$ cells were found (5–37%), but nearly all of these cells were $TCR^{-/lo}$. Relative to normal TEC, total yields of TCR^{hi} SP cells with I^-II^- TEC were reduced by a mean of 36-fold for $CD4^-8^+$ cells and by >100-fold for $CD4^+8^-$ cells.

These data support the view that generation of SP cells from DP cells in TRC reflects direct contact with MHC class I and II molecules. The production of TCR^{hi} $CD4^-8^+$ cells with I^-II^+ TEC but not I^-II^- TEC could reflect cross-reactivity for class II molecules (see Discussion).

Positive Selection with I$^-$II$^-$ TEC Supplemented with Normal BM-derived Cells. The obvious approach for determining whether BM-derived cells can induce positive selection in TRC is to culture DP cells with BM-derived cells, e.g., T^- spleen cells, in the absence of TEC and then search for a switch to SP cells. This approach was ineffective in our hands because placing a mixture of DP cells and T^- spleen cells on nucleopore filters without TEC failed to cause aggregate formation, and the DP cells died rapidly (data not shown). To avoid this problem, we established TRC at a 4:2:1 ratio of DP cells, I^-II^- TEC, and BM-derived cells. The results of two separate experiments with LN B cells (T^- LN) and purified DC as a source of BM-derived cells are illustrated in Fig. 2 and summarized in Table 1.

Aggregate formation was clearly apparent in TRC, prepared with a mixture of DP cells, TEC, and BM-derived cells, but the aggregates were somewhat less "tight" than in the absence of BM-derived cells; with control B6 (I^+II^+)

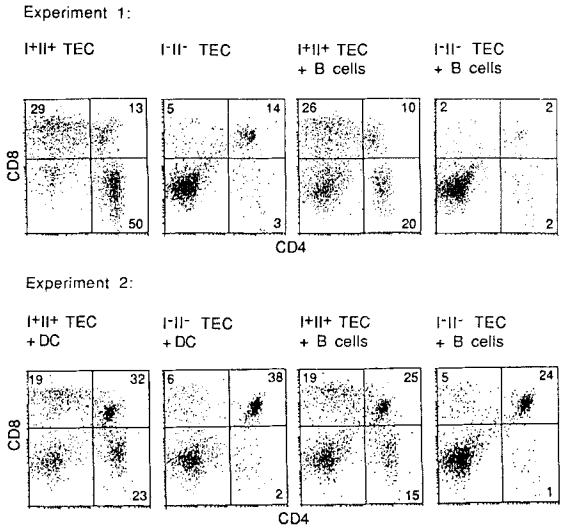

Figure 2. Failure of BM-derived cells to cause positive selection in TRC. TRC were prepared by culturing a 4:2:1 ratio of DP thymocytes, I^-II^- TEC, and either LN B cells (T^- LN) or splenic DC as sources of BM-derived cells. TRC were harvested and stained on day 5.

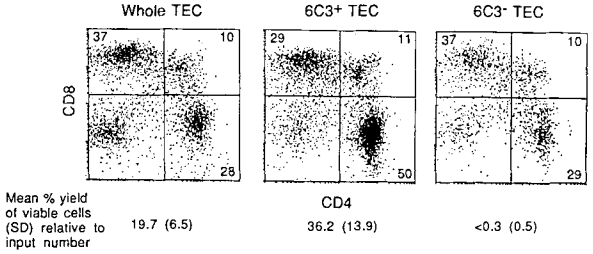

Figure 3. Positive selection of DP thymocytes in TRC prepared with purified cortical ($6C3^+$) versus medullary ($6C3^-$) TEC. Purified TEC were FACS® sorted after staining with 6C3 mAb (see Materials and Methods). Cells were harvested after 5 d and stained for expression of CD4 and CD8. Data on surface staining are from a single experiment. The data on percentage of cell yields (relative to the initial number of DP cells) represent the mean results of three experiments; SD are shown in parentheses. The higher yields with $6C3^+$ TEC relative to unseparated (whole) TEC was statistically significant ($P = 0.5$).

TEC, this was associated with a two- to fourfold reduction in SP cell yields (relative to B6 TEC without BM-derived cells). However, a high proportion of the cells recovered from these control cultures were typical TCR^{hi} SP cells. In marked contrast, generation of TCR^{hi} SP cells was almost undetectable in TRC prepared with a mixture of I^-II^- TEC and BM-derived cells. This finding applied to T^- spleen cells (data not shown), T^- LN (mostly B cells), and purified DC; all of these populations expressed a high density of MHC class I and II molecules. The failure to detect positive selection with I^-II^- TEC did not seem to reflect nonspecific suppression because TCR^{hi} SP cells were generated in TRC prepared with a mixture of normal TEC and I^-II^- TEC (data not shown). (As discussed elsewhere [18], the $CD4^-8^-$ cells in TRC comprise a heterogenous mixture of T cells [mostly nonviable], contaminating TEC, and [in Fig. 2] B cells and DC.)

These data indicate that positive selection in TRC cannot be induced by a mixture of MHC^+ BM-derived cells and MHC^- TEC. The implication therefore is that positive selection requires direct contact with MHC^+ TEC. Since TEC are a heterogeneous population, we studied positive selection with purified subsets of TEC.

Positive Selection with Cortical vs Medullary TEC. We used the cortical epithelial marker, 6C3, and FACS® sorting to prepare purified populations of cortical ($6C3^+$) and medullary ($6C3^-$) cells from normal B6 mice. In terms of SP yields, cortical TEC were about twofold more efficient at mediating positive selection in TRC than unseparated TEC (Fig. 3). By contrast, positive selection with medullary TEC was almost undetectable; a few SP cells were found, but total yields of these cells were extremely low. The medullary epithelial cells were not toxic because recombining these cells with cortical epithelial cells restored positive selection (data not shown).

Discussion

As discussed earlier, the apparent minimal role of BM-derived cells in inducing positive selection under in vivo conditions could simply reflect that BM-derived cells expressing a high density of MHC molecules are largely excluded from the cortex, i.e., from the presumed physiological site of positive selection. To investigate this question, we cultured DP thymocytes with a mixture of MHC^+ BM-derived cells and MHC^- TEC in TRC. The assumption here was that the presence of MHC^- TEC would promote aggregate formation and, in addition, provide appropriate bystander (non-MHC) signals required for positive selection of MHC-stimulated DP cells. As shown here, this approach failed to cause any detectable evidence of positive selection. The implication therefore is that the MHC and non-MHC ligands required for positive selection have to be coexpressed on the same cell. This conclusion is based on studies with a short-term (5-d) tissue culture system. Thus, our data do not rule out the possibility that bystander-positive selection by BM-derived cells can occur over an extended period of time in vivo, e.g., in normal BM→I^-II^+ chimeras (13).

It was mentioned earlier that bystander-positive selection is apparent after intrathymic injection of MHC^+ fibroblasts (11, 12). Although these data are convincing, it is worth noting that the fibroblasts were cell lines (L cells) and were maintained in vitro for prolonged periods before injection; these conditions may have caused the cells to change their normal phenotype and acquire some of the characteristics of TEC. We have searched for bystander-positive selection by fibroblast lines added to MHC^- TEC in TRC, but without success; in our hands, fibroblasts are toxic in TRC and lead to rapid death of thymocytes without proper aggregate formation.

The capacity of fibroblast lines to induce positive selection after intrathymic injection raises the question whether fibroblasts contribute to positive selection in the normal thymus. This possibility seems unlikely because MHC expression on resting fibroblasts is generally quite low. Fibro-

blasts are clearly not essential for positive selection because the TEC used in this work were routinely depleted of fibroblasts.

Several types of epithelial cell lines, including cells with features of medullary epithelium, have been found to mediate positive selection after intrathymic injection (20, 21). However, based on studies with $6C3^+$ and $6C3^-$ TEC (Fig. 3), we concur with Anderson et al. (10) that positive selection mediated by freshly isolated TEC in TRC is an exclusive property of cortical epithelial cells. In agreement with these workers we have been unable to demonstrate positive selection with other types of fetal epithelium, e.g., from gut or salivary gland (unpublished data). On a molecular level, however, the unique capacity of cortical epithelial cells to mediate positive selection is still a mystery.

Since the signaling events and phenotypic changes involved in positive selection are clearly complex (1, 2), it is crucial to know whether the type of positive selection seen in TRC is physiologically relevant. In this respect it was encouraging to find that, as in intact I^+II^- mice, the generation of TCR^{hi} SP cells in TRC prepared with I^+II^- TEC was skewed almost exclusively toward $CD8^+$ cells. However, the generation of appreciable numbers of TCR^{hi} $CD8^+$ cells with I^-II^+ TEC was unexpected. Since TCR^{hi} $CD8^+$ cells were very rare with combined I^-II^- TEC, the generation of class I-independent $CD8^+$ cells appeared to require class II expression. In normal I^-II^+ mice, it is well established that TCR^{hi} $CD8^+$ cells are quite rare (13, 16). Interestingly, however, studies with transgenic mice have shown that constitutive upregulation of Bcl-2 has no demonstrable effect on $CD4^+8^-$ cells but induces substantial production of $CD4^-8^+$ cells in I^-II^+ mice but not in I^-II^- mice (22). These findings thus correlate closely with the present data on TRC. The implication therefore is that $CD4^-8^+$ cells undergoing positive selection to class II are short lived in the normal thymus but can be rescued by high Bcl-2 expression and/or by the particular microenvironment found in TRC.

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