

A T Cell Receptor-specific Blockade of Positive Selection

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Summary

To investigate the influence of endogenous peptides on the developmental processes that occur during thymocyte selection, we have used monoclonal antibodies that preferentially recognize the major histocompatibility complex (MHC) molecule I-E^k when it is bound to the moth cytochrome *c* peptide (88–103). One of these antibodies (G35) specifically blocks the positive selection of transgenic thymocytes expressing a T cell receptor that is reactive to this peptide–MHC complex. Furthermore, G35 does not block the differentiation of transgenic T cells bearing receptors for a different I-E^k–peptide complex. This antibody recognizes a subset of endogenous I-E^k–peptide complexes found on a significant fraction of thymic antigen-presenting cells, including cortical and medullary epithelial cells. The sensitivity of G35 to minor alterations in peptide sequence suggests that the thymic peptide–MHC complexes that mediate the positive selection of a particular class II MHC–restricted thymocyte are structurally related to the complexes that can activate it in the periphery.

Key words: thymic selection • peptides • CD4⁺ T cell • thymocyte • monoclonal antibodies

T cell maturation depends on the specificity of thymocyte TCRs for peptide–MHC complexes that are expressed in the thymus (1–3). Thymocytes that fail to transduce a signal in response to thymic MHC do not mature and instead die in the thymus. The surviving T cells that may eventually emigrate to the periphery are said to be positively selected. The result of this positive selection is a repertoire of thymocytes bearing TCRs that are reactive to self-MHC when it is bound to both foreign and endogenous peptides. Before leaving the thymus cells that react strongly with thymic peptide–MHC complexes are eliminated by activation-induced cell death or negative selection (1). Together, these processes select a peripheral TCR repertoire composed of cells that preferentially recognize self-MHC molecules bound to foreign peptides.

The molecular basis by which the same MHC molecule can mediate these different developmental decisions remains unclear (for review see reference 4). Several factors may influence the fate of a given thymocyte. These include its developmental stage at the time it receives a signal, the characteristics of the APCs that interact with it, and the na-

ture of the peptide–MHC complexes that are displayed by the APCs. Here we employ TCR transgenic mice in which all T cells express the same TCR to examine the influence of peptides on the positive selection of a particular TCR specificity.

Peptides are clearly required for the positive selection of a complete T cell repertoire, yet the precise role of peptide sequence in these processes is not well understood (for review see reference 4). Positive selection by both class I and class II MHC molecules is inhibited by mutations that diminish peptide loading and/or surface expression of MHC molecules (5–10). Addition of certain peptides to fetal thymic organ cultures restores the positive selection of class I–restricted TCR transgenic thymocytes (11–15). Although the peptides that can positively select a given TCR are varied, many resemble (or in one case are identical to) the peptides that can stimulate the TCR transgenic T cells in the periphery. More recently, endogenous peptides that induce the positive selection of CD8⁺ class I–restricted T cells have been identified. Surprisingly, these self-peptides bear little resemblance (in sequence) to the peptides that are known to activate the mature TCR transgenic cells (16, 17).

Because in vitro rescue of positive selection has not been possible for CD4⁺ T cells, studies of class II–restricted T cells have examined class II–mediated positive selection in

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vivo. Mice that have been genetically modified so that they express class II MHC molecules bound to a single peptide generate a large peripheral CD4⁺ T cell population and an unperturbed repertoire of TCR β chains (8–10, 18, 19). However, these “single-complex mice” do not select all TCR specificities and fail to support the development of several specific transgenic TCRs (20–22). Thus, although a peptide’s sequence and/or concentration may direct an individual thymocyte toward either positive or negative selection, current models still fail to predict the selective properties of an individual peptide.

To refine these models, several questions remain. In particular, what are the endogenous peptides that normally induce positive selection of a particular class II–restricted TCR specificity? How are these peptides related to the nominal peptide(s) to which a mature T cell responds? And finally, what is the expression level and tissue distribution of these endogenously expressed peptides? Without such information, it is difficult to assess how other components of thymocyte recognition, such as affinity and coreceptor stimulation, contribute to the distinction between positive and negative selection.

To investigate these aspects of the role of peptide in the class II system, we have used mAbs that recognize the class II MHC molecule I-E^k only when it is bound to peptides that resemble the COOH-terminal portion of moth cytochrome *c* (MCC 88–103).¹ One of these antibodies specifically blocks positive selection of TCR transgenic thymocytes that bear a receptor specific for this same peptide–MHC combination. The distribution of this antibody epitope in the thymus indicates that this endogenous complex is present in both the thymic cortex and the medulla. Thus, not only are a subset of endogenous I-E^k–associated peptides essential for positive selection, but the sequence or structure of these endogenous peptides must be related to the peptides that can stimulate the selected T cell in the periphery.

Materials and Methods

Antibodies

mAbs were generated by immunizing mice with soluble MCC–I-E^k complexes and purified with a 1:1 mixture of protein A– and protein G–sepharose (Pharmacia) from tissue culture supernatants derived from bulk culture of hybridomas grown in roller bottles, or on a CellPharm (Unisyn Technologies) using RPMI supplemented with penicillin–streptomycin and 10% fetal calf serum (Gemini). Purified antibodies were concentrated by ultrafiltration, and yields were quantified by absorbance. Where necessary, antibodies were conjugated to FITC (Molecular Probes), biotin (Pierce), or digoxigenin (Boehringer Mannheim) using standard protocols.

Mice

Specific pathogen-free B10.Br, C57BL/6, and AKR/J mice were obtained from The Jackson Laboratory and crossed to mice transgenic for the TCR derived from the T cell clone 5C.C7 (23)

or the T cell hybridoma A18 (24). All mice were 4–8 wk of age or 1–3-d-old neonates. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Antibody Administration In Vivo

Male 5C.C7 TCR transgenic mice were crossed under specific pathogen-free conditions with B10.Br females (The Jackson Laboratory). Pregnant mice were removed on the day after impregnation (day 0), and were injected intraperitoneally with 1 mg per day of the indicated antibodies in 100 μ l sterile PBS.

Preparation of Stromal Cells

Thymuses were digested for 45 min at 37°C with collagenase type IV (Sigma Chemical Co.) and dissociated by vigorous pipetting. Stromal cell cultures were then grown for 5–8 d in selective media (25) and either incubated with thymocytes for the *in vitro* positive selection assays or removed from the plate by a combination of collagenase treatment and vigorous pipetting for FACS[®] analysis.

In Vitro Positive Selection Assays

5C.C7 Only Assays. Unsorted 5C.C7 cells from 5C.C7/C57BL/6 TCR transgenic, 4-wk-old females were isolated by passing the cells through nylon mesh and washing. These cells were then incubated with thymic stromal cell cultures at 37°C for the indicated times.

A18/5C.C7 Assays. Purified unselected CD4⁺8⁺ thymocytes were isolated from positively selecting strains by four-color flow cytometry. Dead thymocytes and non-T cells were excluded by gating out dead cells staining positive for propidium iodide or thymic APCs that were positive for class II MHC or B220. 2×10^6 CD4^{lo/+}CD8^{lo/+}CD69[–] cells were sorted from each transgenic mouse. 2×10^5 immature thymocytes per well were incubated with 5–8-d-old stromal cell cultures for 2.5 d. Cells were removed by incubation at 4°C in PBS/2% FCS/2.5 mM EDTA followed by vigorous pipetting. Thymocytes were subsequently stained for the transgenic α or β chain, CD4, CD8, and class II MHC/B220.

FACS[®] Analyses

In Vivo Positive Selection Assay. Cells from neonatal thymuses were isolated by pushing whole thymuses through metal screens. Cells were stained with CD4-PE, CD8-APC (Caltag), KJ25, followed by anti–hamster Texas Red, and rabbit anti-5C.C7 V α followed by anti–rabbit-FITC. Dead cells were excluded from analyses by size and failure to exclude propidium iodide.

In Vitro Positive Selection Assay. Cells were stained with anti-V α 11-FITC (clone RR8.1; PharMingen) or anti V β 8 (clone F23.1-FITC; gift from Dr. Irving Weissman, Stanford University, Stanford, CA), anti-I-A^{k/b}-biotin, B220-biotin followed by streptavidin-PE (all from PharMingen), CD4-APC (clone GK1.5; Caltag Labs.), and CD8-Texas Red (Elizabeth Kerr, Stanford University) before analysis on a modified FACS[®] II dual laser flow cytometer (Becton Dickinson) and analyzed using FACS[®] Desk software (Beckman Center Shared FACS Facility, Stanford, CA). Where necessary, stromal cells and dead cells were excluded from analysis by gating out propidium iodide– and class II MHC/B220–positive cells. Stromal cells were stained with anti I-E^k (14.4.4S-biotin) followed by avidin–Texas Red, B220-APC (clone RA3-6B2), CD3-PE (clone 145-2C11) (all PharMingen, San Diego, CA), and G35 or D4 directly conjugated to FITC.

¹Abbreviation used in this paper: MCC, moth cytochrome *c*.

ELISA

Plates were coated with 10 $\mu\text{g}/\text{ml}$ of the anti-I-E^k antibody G14 (Reay, P.A., unpublished data). Soluble propidium iodide-linked I-E^k (26) was incubated for 3 d at 37°C in citrate phosphate buffer, pH 5.0, in the presence of a 50-fold molar excess of each of the 220 single amino acid substituted peptide variants (27). After neutralization, 50 μl of a 10 μM solution of each variant-complex was captured in the presence of 2% BSA. After extensive washing, biotinylated G35 was titrated on each complex, and the amount bound was assessed using alkaline phosphatase-conjugated avidin (Biomedica). The amount of G35 resulting in one-half maximal signal was determined by curve fitting and extrapolation.

Immunohistochemistry

Light and electron microscopic immunohistochemical studies were performed as described previously (28, 29). Digoxigenin-conjugated mAbs were detected with horseradish peroxidase-conjugated Fab fragments of goat anti-digoxigenin antibodies (Boehringer Mannheim). Light microscopic studies were done with fresh frozen sections fixed by immersion in cold acetone. Ultrastructural immunohistochemistry was performed on thin slices of thymus tissue previously fixed by vascular perfusion with 4% paraformaldehyde. After demonstration of peroxidase activity with hydrogen peroxide and 3,3' diaminobenzidine, these tissue sections were treated with OsO₄, and then processed for conventional electron microscopy.

Purification of Thymic I-E^k

Thymuses were removed from 327 B10.Br mice and placed on ice in PBS. Thymuses were dissociated by dounce homogenization and lysed in PBS containing protease inhibitors (2 mM PMSF, 100 μM iodoacetamide, 5 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 3 ng/ml EDTA, and 0.2% sodium azide) and 2% Mega-9 detergent (Sigma Chemical Co.) for 1 h. The detergent lysate was spun at 100,000 g for 1 h to remove nuclei and insoluble protein. The lysate was then cleared by filtration through 0.22- μm filters (Corning) and run serially over five cyanogen bromide-conjugated antibody columns in the following order: CN-Br conjugated to total mouse IgG, CN-Br D4, CN-Br-G35A, CN-Br G35 B, and CN-Br 14.4.4. Each column was washed extensively with detergent containing Tris, pH 8.0, plus 150 mM NaCl, then Tris, pH 8.0, plus 150 mM NaCl, Tris, pH 8.0, plus 0.5 M NaCl, Tris, pH 8.0, and finally eluted with acetic acid, pH 2.7, containing 10 ng/ml leupeptin as a carrier. Fractions were acidified to 2.1 with glacial acetic acid and boiled for 10 min to release peptides. Fractions were then separated on a Millipore Ultrafree CL device with a 5,000 dalton cut-off (Millipore UFC4LCC25).

Western Blotting Analysis/SDS-PAGE

SDS-PAGE was performed according to standard methods. Samples were collected from the top (>5 kD) portion of Millipore Ultrafree CL devices and resuspended in running buffer containing SDS and the reducing agent β -ME. The 12% gels with the 14.4.4 and G35 samples were prepared and run in parallel. Proteins were transferred overnight at 4°C onto Hybond filter paper that was then blocked with 2% dehydrated milk/PBS/0.1% Tween solution. The blots were incubated with polyclonal rabbit anti-I-E^k serum in blocking solution, washed, and incubated with goat anti-rabbit HRP (Biomedica). Horseradish peroxidase activity was visualized with the ECL detection kit (Amersham).

Microbore HPLC Analysis of Peptides

Fractions were concentrated by lyophilization and peptides from equivalent amounts of total I-E^k were separated by reverse-phase HPLC. Samples were separated on a C18 column (1 \times 150 mm; Reliosil) with a linear water/acetonitrile gradient in 0.1% TFA (0–45% acetonitrile, 30 min) at a flow rate of 75 $\mu\text{l}/\text{min}$. Chromatograms are displayed as absorbance at 209 nm versus retention time in minutes.

Results

G35 Blocks Positive Selection of I-E^k/MCC-reactive T Cells In Vivo. We have generated several mAbs that specifically recognize the class II MHC molecule I-E^k bound to a peptide derived from MCC 88–103. Two of these antibodies (D4 and G35) bind to the same region of this complex as T cells and inhibit the activation of T cells that recognize this complex (Reay, P.A., unpublished data). We wondered if these antibodies might also inhibit the activation events involved in thymic selection.

To address this question, we injected mice with G35, D4, and PBS and analyzed the effects of these antibodies on the development of I-E^k-MCC-specific thymocytes that express the TCR transgene 5C.C7. To provide nontransgenic internal controls, 5C.C7 TCR transgenic males were crossed to nontransgenic females to generate litters in which 50% of the progeny were transgenic. The pregnant females were injected intraperitoneally each day (from day 15 of gestation until birth) with either 1 mg of antibody or PBS. On day 21, thymuses were harvested from the neonates and four-color flow cytometry was used to determine the transgenic status and CD4 versus CD8 profile of each mouse (Fig. 1 A). In transgenic pups, the percentage of identified transgene-positive cells was ~80% in all treatment groups; the rest of the cells were presumably immature cells that lacked detectable TCR expression or thymic APCs.

Analysis of several pups from each mother showed that the G35-treated transgenic mice are almost devoid of

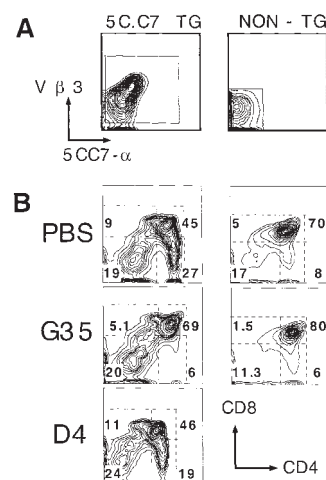


Figure 1. Effect of G35 on the selection of 5C.C7 transgenic thymocytes. (A) Distribution of 5C.C7- α chain and KJ25 (V β 3) determinants on thymocytes derived from transgenic (left) or nontransgenic (right) pups. (B) Distribution of CD4 and CD8 on separate populations of cells derived from transgenic or nontransgenic pups exposed in utero to G35, PBS, or D4 as indicated. Numbers refer to the percentage of total live transgenic or nontransgenic thymocytes that fall within each boxed population.

CD4⁺8⁻ cells, whereas the nontransgenic pups produce normal numbers of CD4⁺8⁻ cells. Representative plots of CD4 versus CD8 from one nontransgenic and one transgenic neonate from mothers that were injected with PBS or the relevant mAb are displayed in Fig. 1 B. Because even identically treated littermates often exhibit substantial variation in the percentage of cells in each thymic subset, the average percentage of total live cells that fell into the CD4⁺ or CD4⁺8⁺ gates in each treatment group was computed and plotted (Fig. 2). G35 treatment of transgenic mice causes a fourfold reduction in the percentage of positively selected CD4⁺8⁻ thymocytes present at day 21, while modestly increasing the relative proportion of CD4⁺8⁺ thymocytes. Notably, treatment with G35 antibody has no reproducible or significant effect on the thymocyte profiles of the nontransgenic littermates of the transgenic mice. Similarly, injections with PBS or the isotype-matched control antibody, D4, did not perturb thymic development in transgenic or nontransgenic mice. All transgenic and nontransgenic pups had equivalent circulating levels of G35 (45–68 μg/ml) and D4 (88–113 μg/ml).

G35 Blocks Positive Selection of I-E^k/MCC-reactive T Cells In Vitro. In previous studies, injection of anti-class II MHC mAbs that are peptide independent inhibited positive selection of CD4⁺ T cells (30, 31). This effect may have resulted from a simple blockade of TCR access to MHC or, alternatively, long-term antibody treatment may have caused general downregulation of MHC or killed the MHC-bearing cells required for positive selection. Because we were interested in peptide-specific effects of antibody treatment we wanted to exclude the possibility of complete ablation of positive selection. Thus, we examined the effect of anti-MHC antibodies in vitro by using short-term thymic stromal cell explant cultures (25).

Thymic epithelial cells were isolated from thymuses taken from 3–4-wk-old mice and grown in media that selectively kills fibroblast-derived cells to enrich for thymic stromal epithelial cells (32). To provide unmanipulated immature CD4⁺8⁺ cells bearing the 5C.C7 receptor, TCR transgenic mice were crossed to a nonselecting background (C57BL/6). The majority of the immature thymocytes from these mice are immature CD4⁺8⁺CD69⁻TCR^{low/intermediate} cells that fail to initiate CD8 downregulation even after 52 h in culture (Baldwin, K.K., unpublished data and reference 33). In contrast, when these immature thymocytes are cocultured with thymic stromal cells that express I-E^k, they begin to downregulate CD8 after 26 h (Fig. 3, a and d). After 52 h,

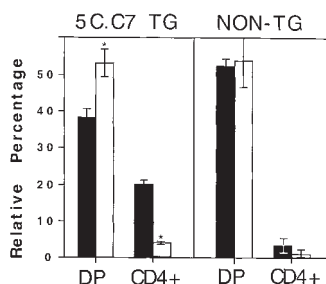


Figure 2. Effects of antibody treatment on thymocyte subpopulations. The figure shows the average percentage of total live cells with the indicated CD4/CD8 expression patterns from three to five transgenic or nontransgenic neonates born to mothers treated with G35 (white bars) or PBS (black bars). Error bars represent 1 SD.

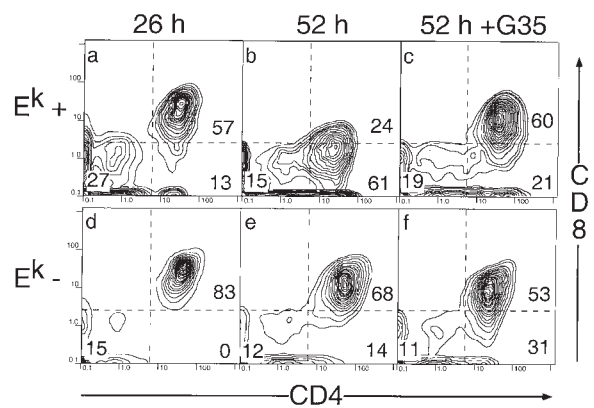


Figure 3. G35 blocks 5C.C7 transgenic T cell differentiation in vitro. Analysis of CD4/8 expression on thymocytes removed from stromal cell cultures derived from B10.Br (top) or C57BL/6 (bottom) mice after 26 (a and d) or 52 h (b, c, e, and f). G35 was added to a final concentration of 50 μg/ml in c and f. Numbers on plots refer to the percent of total live transgenic thymocytes that fall within each boxed population.

the thymocytes still attached to the I-E^k-positive stromal cells are almost entirely mature CD4⁺8⁻ cells (Fig. 3, b and e). Additional FACS[®] analyses show that these cells express the high levels of CD69 and low levels of HSA that are characteristic of differentiated CD4⁺8⁻ thymocytes (Baldwin, K.K., unpublished data).

Importantly, as in the in vivo experiments, the presence of 50 μg/ml of G35 antibody inhibits the positive selection of the transgenic thymocytes but does not affect the thymocytes on nonselecting stromal cells (Fig. 3, c and f). The effects of G35 on selection do not increase at antibody concentrations of 100 μg/ml, are more modest at 33 μg/ml, and are undetectable at 10 μg/ml (data not shown). Furthermore, G35 treatment does not affect the viability or MHC expression levels of the cells in the culture (data not shown). We conclude that G35 inhibits positive selection of thymocytes in vitro by blocking their access to I-E^k-peptide complexes rather than by killing APCs or inducing MHC downregulation.

Blockade by G35 Is Specific to TCRs That Recognize MCC-I-E^k Complexes. The fact that G35 does not influence the development of nontransgenic CD4 thymocytes suggests that it may recognize only the subset of thymic peptides that is responsible for selecting this particular range of TCR specificity. Alternatively, G35 may block selection by binding to all or most thymic I-E^k molecules regardless of their bound peptides. In this model, nontransgenic thymocytes would not be visibly affected because they could mature on non-I-E^k class II MHC molecules such as I-A^k. To distinguish between these models, we examined the influence of G35 on the development of thymocytes from mice transgenic for a different TCR that requires I-E^k for positive selection yet does not cross-react with MCC (24). These mice express a TCR derived from the T cell clone A18, which is specific for a peptide derived from the murine C5 complement protein that is present in the B10.Br strain. Since the C5 protein is deleted in the

I-E^k-bearing strain AKR/J, A18⁺ thymocytes are positively selected in AKR/J backgrounds but negatively selected on B10.Br. Rather than crossing these mice to a nonselecting background (C57BL/6) that would also introduce a potential source of the negatively selecting C5 protein, we modified the *in vitro* positive selection assay.

Instead of taking immature thymocytes from nonselecting strains, we isolated immature (CD4⁺8⁺) unactivated (CD69^{lo}) thymocytes from positively selecting strains by FACS[®]. To standardize the results between the two transgenic mouse strains, and to test the validity of the modified *in vitro* system, this sorting procedure was performed on cells from both the 5C.C7 × B10.Br and A18 × AKR/J positively selecting lines. As shown in the previous experiments, the immature thymocytes from both strains down-regulate CD8 only in the presence of I-E^k (Fig. 4 A). Furthermore, G35 efficiently blocks 5C.C7 thymocyte differentiation at the CD4⁺8⁺ stage. In contrast, although A18 thymocyte development depends on I-E^k expression, G35 does not influence A18 T cell development (Fig. 4 A).

The results of three independent experiments are plotted in Fig. 4 B. Because the ability of the stroma to support differentiation varies among experiments, the data are presented as the percentage of live, transgene-positive, CD4⁺8⁻ cells relative to CD4⁺8⁺ cells (termed selective index), normalized to the average CD4⁺8⁻/CD4⁺8⁺ ratio for the untreated I-E^k-positive samples. These data show that G35 blocks development in a TCR-specific manner.

G35 Stains a Subset of Class II MHC Positive Cells. To conclude that the TCR-specific nature of the G35 blockade results from recognition of the self-peptide-MHC complexes that mediate positive selection of thymocytes bearing the 5C.C7 but not the A18 TCRs, it is necessary to show that G35 recognizes only a subset of thymic peptide-MHC complexes. To determine the localization and expression level of the G35 epitope, we examined the distribution of G35, D4, or 14.4.4S antibodies on frozen sections of thymus, spleen, and lymph node derived from B10.Br mice (Fig. 5 A). As shown in Fig. 5 A, a, 14.4.4S, which detects I-E^k in a peptide-independent manner, reacts

homogeneously with both the cortical and medullary areas, although it exhibits a more confluent pattern within the medulla. In contrast, the determinant for G35 is clearly expressed at higher levels by a subset of cells within the thymic medulla, although a lower level of G35 staining of cortical stromal processes and the subcapsular region was routinely observed (Fig 5 A, b). The 14.4.4S antibody exhibits widespread reactivity in lymph node and spleen, labeling both B and T cell-dependent areas (Fig 5 A, d and g). Labeling of these tissues with G35 was more heterogeneous; staining was restricted to follicular structures in the lymph node cortex, and to B cell-dependent areas of splenic white pulp. Reactivity of G35 with T cell-dependent areas of lymph node or spleen was weak and restricted to a few scattered cells. B10.Br thymic tissue failed to react with the D4 mAb (Fig. 5 A, c) and no G35 reactivity with tissues from C57BL/6 mice was detected (data not shown).

G35 staining is more intense in some regions of the thymus than others, even when these areas express similar levels of I-E^k. Thus, all I-E^k-bearing cells do not bind equally well to G35. To determine which cell types express the G35-reactive peptide-MHC complexes, we performed ultrastructural analyses of thymic sections. These analyses demonstrated that G35 reactivity is associated with the membranes of all three morphologically-defined thymic APC types, including cortical epithelium, medullary epithelium, and dendritic cells (Fig. 5 B). Staining is most intense on dendritic cells, intermediate on cortical epithelium, and weak but detectable on medullary epithelium. Thus, although G35 staining is not restricted to a particular APC subset, expression of the relevant complexes may be enhanced in cortical epithelial cells as compared with medullary epithelial cells.

To further examine the heterogeneity of G35 reactivity among thymic stromal cells and to confirm that the G35 epitope was expressed by class II-positive cells in the thymus, we used four-color flow cytometry. Immortalized B cells from I-E^k-positive mice (CH27 cells), cells from whole thymuses, and thymic stromal cell cultures were analyzed for expression of the 14.4.4, CD3, B220, and G35

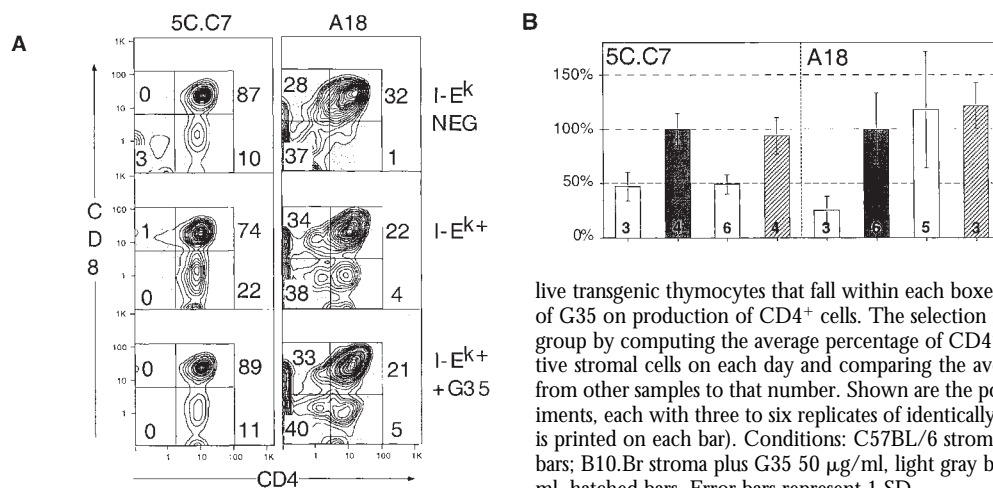


Figure 4. Inhibition of positive selection by G35 is specific for the MCC-I-E^k-restricted TCR. (A) Immature thymocytes from 5C.C7/B10.Br or A18/AKR/J TCR transgenic mice were cultured with thymic stromal cells from C57BL/6 (top) B10.Br (middle) or AKR/J (bottom) mice. Numbers on plots refer to the percentage of total

live transgenic thymocytes that fall within each boxed population. (B) Analysis of the effects of G35 on production of CD4⁺ cells. The selection index was generated for each treatment group by computing the average percentage of CD4⁺8⁻/CD4⁺8⁺ for untreated, I-E^k-positive stromal cells on each day and comparing the average percentage of CD4⁺8⁻/CD4⁺8⁺ from other samples to that number. Shown are the pooled results of two independent experiments, each with three to six replicates of identically treated stromal cells (the exact number is printed on each bar). Conditions: C57BL/6 stroma, white bars; B10.Br stroma, dark gray bars; B10.Br stroma plus G35 50 µg/ml, light gray bars; and B10.Br stroma plus D4 50 µg/ml, hatched bars. Error bars represent 1 SD.

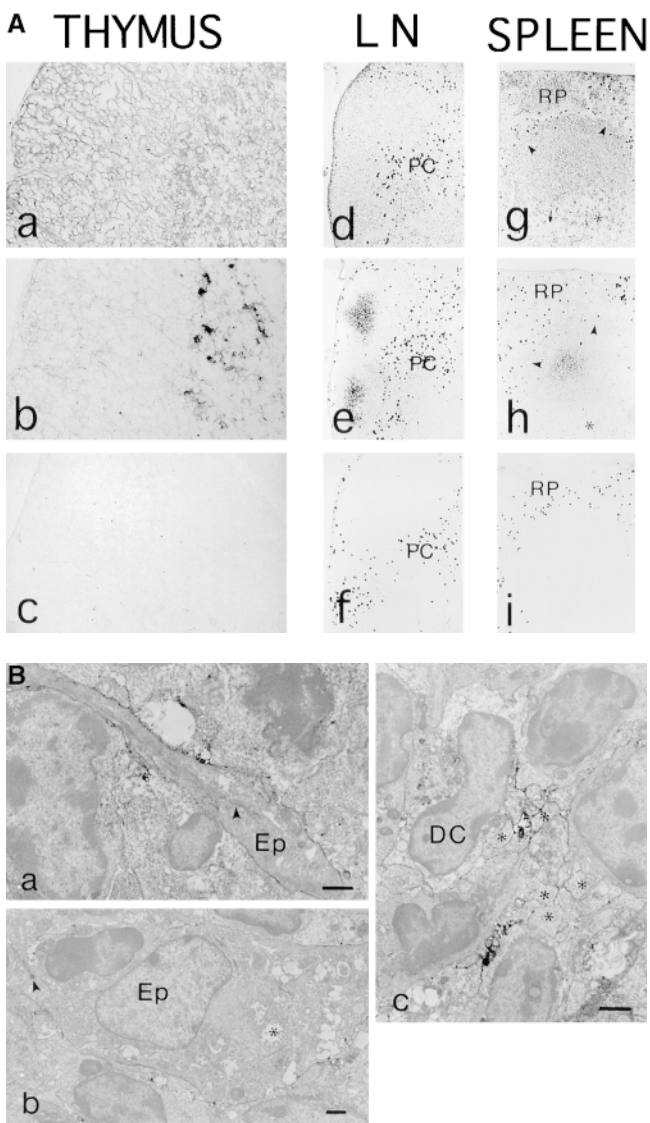


Figure 5. G35 reactivity with thymus and peripheral lymphoid tissues. (A) Frozen sections of thymus (a–c), lymph node (d–f), and spleen (g–i) were labeled with 14.4.4S (a, d, g), G35 (b, e, h), D4 (c), or an irrelevant mAb (f, i). PC, T cell-dependent paracortical area; RP, red pulp and white pulp. Original magnification: a–i, $\times 45$ –55. (B) Ultrastructural analysis of G35 reactivity with thymus tissue. Labeling was associated with (a) cortical epithelium, (b) medullary epithelium (desmosome indicated by arrowhead, characteristic cystic structures indicated by asterisk), and (c) dendritic cells (characteristic bullate processes indicated by asterisks). Original magnification: a, $\times 8,400$; b, $\times 4,950$; c, $\times 8,050$.

or D4 epitopes. Importantly, the I-E^k-expressing B cell line CH27, which expresses a diverse array of peptides, exhibits no G35 reactivity. Thus G35 cannot react with all endogenously produced I-E^k complexes (Fig. 6 a). In contrast, when these cells are incubated overnight with MCC peptide, all of the cells exhibit strong G35 reactivity (Fig. 6 a). These staining levels were used to assess the percentage of cells from other tissues that could be recognized by G35. Approximately 20% of freshly isolated class II-positive thymic cells from B10.Br but not C57BL/6 mice express the

G35 determinant (Fig. 6 c). Close to 40% of I-E^k-positive cells from this particular thymic stromal cell culture bind specifically to the G35 antibody (Fig. 6 b). Neither G35 nor D4 labels CD3⁺ cells and D4 fails to stain any of the thymic stromal cells (data not shown). Thus, a subset of thymic and peripheral I-E^k-positive cells are preferentially labeled by G35. Because G35 stains only a subset of cells that all express equivalent levels of I-E^k, it must preferentially label a subset of peptide-MHC complexes that are unevenly distributed on different cells. In addition, the low affinity of this antibody for MCC-I-E^k complexes (with respect to the similar antibody D4) and the weak overall staining of these tissues suggest that nonspecific binding of G35 to all thymic I-E^k-peptide complexes is unlikely to be the mechanism for the TCR-specific blockade of thymic differentiation.

Purification of I-E^k-bound Peptides. To directly compare the peptides recognized by G35 with those bound to all I-E^k molecules in the thymus, we isolated thymuses from 327 B10.Br and B10.A (a related I-E^k-positive congenic strain) mice. Using a modified version of established MHC purification protocols (34–36), we isolated thymic I-E^k molecules that were bound by G35 or by the peptide-nonspecific antibody 14.4.4. Two G35 columns were run in series and the unbound lysate was then passed over the 14.4.4 column. Western blotting was used to determine the amount of MHC present in each fraction. The first G35 column bound ~ 670 ng (11 pmol) of I-E^k, whereas the second G35 column yielded no detectable MHC (Fig. 7 A). The pooled fractions from the 14.4.4 column bound to ~ 7 μ g of I-E^k (Fig. 7 A). Thus although both G35 and 14.4.4 can recognize the endogenous peptide-MHC complexes displayed on thymic APCs, G35 is less efficient at removing these complexes from thymic extracts. These data argue that G35 recognizes a finite and unique subset of thymic I-E^k-peptide complexes.

To confirm that G35 recognizes a unique subset of thymic I-E^k-peptide complexes, and to estimate the peptide diversity in this preparation, we analyzed the peptides eluted from the thymic I-E^k by HPLC. Equivalent amounts of G35, 14.4.4, or a blank solution containing the

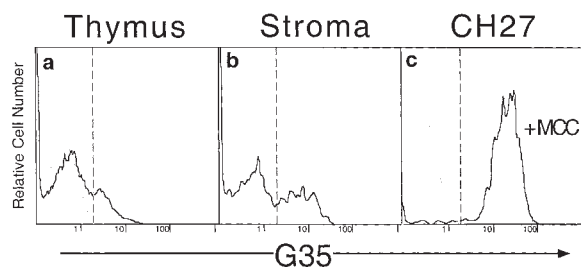


Figure 6. G35 recognizes endogenous I-E^k-peptide complexes on a subset of thymic APCs. Levels of G35 expression on live, large CD3⁺-14.4.4⁺ cells. (a) Cells from B10.Br (black line) or C57BL/6 (gray line) thymuses. (b) The staining of a stromal cell culture derived from B10.Br mice. (c) G35 expression on CH27 B cells pulsed overnight with 10 μ M MCC 88–103 peptide (black line) or PBS (gray line). The percentage of cells with fluorescence greater than the intercept of the dashed lines was <2% in negative controls, 20% in a, 41% in b, and 97% in c.

carrier peptide and protease inhibitor leupeptin were separated on a C18 column with a linear water/acetonitrile gradient in 0.1% TFA. The portion of the gradient in which peptides are known to elute is displayed in Fig. 7 B. Comparison of the G35 and 14.4.4 chromatograms reveals the presence of several peaks that are unique to each sample. For example, at 17.37 min, a triplet peak unique to the G35 sample appears, whereas the 14.4.4 sample has a singlet at 18 min. The peptide-rich region between 20 and 25 min is extremely different in the G35 and 14.4.4 samples as well. The complexity of the 14.4.4 trace resembles the multiplex smears observed in other studies of 14.4.4-reactive I-E^k where sequencing of the peptides from these peaks reveals a diverse spectrum of peptide species (34, 36). In contrast, the number of peptide-like peaks in the G35 column is reduced, although more complex than would be expected for a single peptide species. Thus, G35 recognizes a unique, multipolypeptide subset of the thymic I-E^k-peptide complexes that is required for the positive selection of 5C.C7 CD4⁺ T cells.

Epitope Map of G35. Because the binding of G35 to this subset of thymic MHC-peptide complexes blocks positive selection, the characteristics and degeneracy of G35 recognition are interesting with respect to the range of endogenous peptides that mediate the positive selection of this TCR specificity. To assess the tolerance of G35 to subtle changes in peptide sequence, we performed an ELISA to measure antibody reactivity with I-E^k bound to each of a complete replacement set of monosubstitutions of the MCC epitope (27).

G35 recognition is more degenerate than the recognition by the 5C.C7 TCR, although less so if partial agonis-

tic or antagonistic reactivities are also taken into consideration (Fig. 8). These observations suggest that although G35 cannot recognize most I-E^k-binding peptides, it may be able to efficiently recognize nearly every peptide-I-E^k complex to which 5C.C7 can respond. G35 may recognize other complexes that the 5C.C7 TCR can bind to only weakly as well as those with which 5C.C7 fails to interact. Although the G35 antibody is tolerant to alterations in the NH₂ terminus of MHC-bound peptide, its specificity is very similar to the 5C.C7 TCR at the COOH terminus, particularly at residues 101 and 102. It is difficult to estimate the fraction of bound peptides that can be recognized by G35, but consideration of the restriction at peptide positions 98, 99, 101, and 102 that are not involved in I-E^k binding (14, 11, 3, and 5 residues of 20 tolerated, respectively) suggests a figure in the range of 1–2%. In addition, the variants that can stimulate the 5C.C7 T cell clone to produce IL-3 (including residues that antagonize stimulation by MCC complexes) are quite similar to those seen by G35, especially at the residues 101 and 102. These results suggest that the endogenous peptide antigens recognized by G35 must be very similar to MCC, particularly at the COOH-terminal residues.

Discussion

In summary, the G35 antibody blocks the positive selection of thymocytes expressing the 5C.C7 TCR but does not affect the differentiation of other I-E^k-restricted thymocytes. The distribution of G35 staining in the thymus and in the peripheral tissues shows that the antibody differentially recognizes certain thymic I-E^k-peptide complexes.

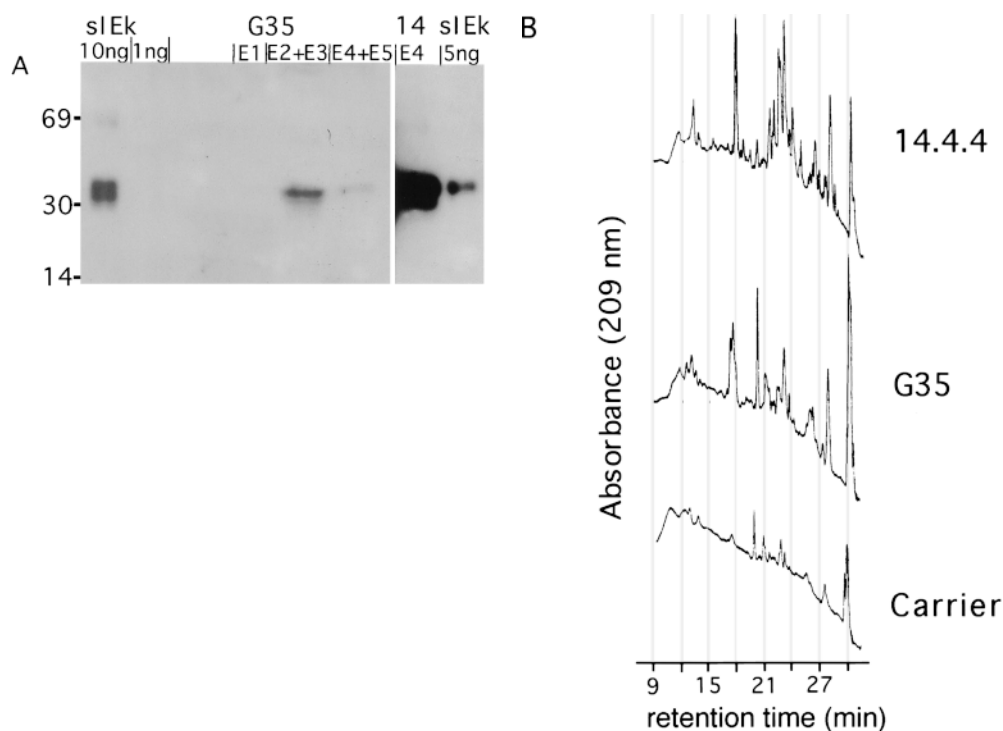


Figure 7. Purification of thymic I-E^k peptide complexes. (A) Western blot of 12% acrylamide gel with polyclonal rabbit anti-I-E^k antisera. 1/60 of each fraction (denoted E1–5) from the first G35 column, the 14.4.4 column or the indicated amount of soluble I-E^k (sIEk) was run in each lane as noted on the figure. (B) HPLC chromatograms of the second fraction of the peptide eluates (in carrier solution) from the G35 or 14.4.4 columns, as noted. Carrier is 10 ng/ml leupeptin in 0.2 N acetic acid.

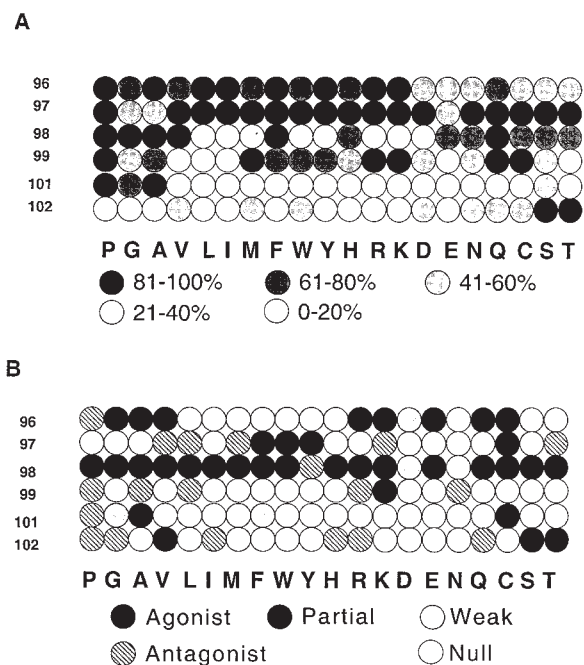


Figure 8. G35 sensitivity to I-E^k bound to single substitution peptide variants. Each circle represents a different peptide; as keyed, the number on the left indicates the amino acid that was replaced and the letters indicate which amino acid was substituted in each position. Replacement amino acids are ordered by rough chemical similarity. Only residues that do not affect MHC binding were analyzed. (A) Recognition by mAb G35. Black circles represent from 81 to 100% of G35 reactivity to MCC-I-E^k complex activity and each shade of gray represents a 20% decrease in activity as calculated by estimating the concentration of G35 necessary to achieve the half maximal signal from the WT MCC-peptide complexes. (B) Interaction with 5C.C7 TCR. Gray circles represent peptides that produce detectable levels of IL-3 when used to stimulate a 5C.C7-bearing cell line as indicated. Hatched circles, peptides that antagonize the stimulation of the cell line by WT MCC peptide-I-E^k complexes; white circles, peptides that did not antagonize or stimulate the 5C.C7 cell line.

Biochemical isolation of these complexes shows that they comprise a finite and unique subset of total thymic I-E^k-peptide complexes. We conclude that this antibody blocks thymocyte development by binding to the endogenous complexes that are required for positive selection of this particular MCC-reactive T cell clone.

Two additional conclusions may be inferred from these data. First, the fact that another I-E^k-reactive T cell clone (A18) and most nontransgenic CD4⁺ thymocytes develop normally in the presence of G35 suggests that different TCR specificities are selected on different subsets of thymic peptide-MHC complexes. Second, the recognition properties of G35 indicate that endogenous, positively selecting peptides are likely to be structurally related to the peptides that can stimulate mature T cells in the periphery.

What is the relationship between the endogenous peptide-MHC complexes that normally mediate the positive selection of a class II MHC-restricted receptor and those that can activate it in the periphery? The G35 data suggest that sequence similarity may play a role, at least in class II MHC-mediated positive selection. In the class I system, however, peptide sequence may be less instructive. In the

experiments of Hogquist et al. (16) and Hu et al. (17), peptides were purified from class I MHC molecules derived from thymic epithelial or other cell lines and screened for their ability to positively select TCR transgenic thymocytes in vitro. Surprisingly, these peptides did not resemble the nominal peptide antigens for the receptors they studied. However, these endogenous peptides are likely to be different from the peptides that allow positive selection to occur in a normal animal for two reasons. First, because the class I MHC molecules expressed by the thymic APCs are improperly folded due to the mutations that facilitate peptide loading, they are expressed at artificially low levels. Second, to rescue selection, the peptides must be added to the cultures at extremely high concentrations that are unlikely to be approximated by the endogenous peptide expression in these cells. In fact, studies of APCs have shown that thousands of different peptides are typically expressed by a given cell type and that even the most abundant peptides typically do not comprise more than a few percent of the total (34–36). Thus, the findings using G35 may be more representative of the normal case in which peptide sequence and/or structure do play an important role in determining the consequences of TCR-peptide-MHC interactions in the thymus.

Although we do not yet know the exact sequences of the peptides that G35 recognizes, we do have an estimate of their abundance and diversity. The HPLC analyses of G35- and 14.4.4 reactive peptide-MHC complexes reveal that although G35 peptides are likely to be less diverse than 14.4.4 they still contain a number of different species (Fig. 7). The 10-fold difference in yield between the two columns suggests that G35-reactive peptides do not comprise more than 10% of the total peptides displayed on the cell surface. In fact, of the several predominantly displayed peptides identified in previous studies (34), none interact with G35 when bound to I-E^k (Baldwin, K.K., unpublished data). Thus, G35 probably interacts with peptides that are less abundant in the thymus than those previously identified.

These data not only underscore the potential importance of peptide sequence in thymic selection, they also provide some insight into the possible role of different cell types in these events. Several lines of evidence have suggested that radiation-resistant cortical thymic epithelial cells are required for positive selection (37–44) and that thymic medullary epithelium and bone marrow-derived cells induce negative selection far more efficiently than do cortical epithelial cells (45–50). However, in some experimental systems, cultured thymic epithelial cells can induce deletion (51–57), whereas some fibroblast-derived cells may mediate inefficient positive selection (58–60). One proposed difficulty in interpreting these results stems from the possibility that the pool of endogenous peptides displayed on the surface of these thymic APCs may have been different in the various experiments. Other sequence-level investigations of peptide expression in different tissues have not found significant differences between tissues or between cell types in the thymus (34). However, these analyses are technically

very difficult and can quantify only the most abundant peptides bound to a particular MHC molecule in a given cell-type preparation. Based on these data, it is formally possible that thymic cortical epithelial cells express a small amount of a special peptide that permits them to induce positive selection. Alternatively, these cells may have another feature that predisposes them to induce positive rather than negative selection.

Our data suggest that special thymic peptides are not responsible for the unique ability of cortical epithelial cells to induce positive selection. If there were such a special peptide, G35 should block positive selection of all T cells, which it does not. In addition, G35 staining should be most intense in the thymic cortex. Instead, G35 staining is stronger in the thymic medulla than in the cortex, even when normalized for the overall MHC expression level. Despite this increased staining, we have not detected any negative selection of 5C.C7 thymocytes in the thymus. Thus, either the peptides that G35 recognizes in the medulla differ from those in the cortex, or cortical cells have peptide-independent unique qualities that permit efficient positive selection. However, without medullary and cortical cell-specific peptide sequence, we cannot distinguish between these alternatives.

Where else are these potentially weakly stimulatory complexes expressed? Staining of the peripheral lymphoid tissues with G35 indicates that these endogenous peptide-MHC complexes are found in both the spleen and lymph nodes. In addition, the peripheral staining varies in different regions of the same lymph node or spleen section even when relative levels of class II MHC are taken into account (Fig. 5). Thus, different APCs can express different peptide-MHC complexes even when they are closely associated in a single tissue. Although tissue-specific expression patterns have been noted previously (29, 61, 62), our data are among the first to suggest that the peptides that are responsible for positive selection in the thymus may also be expressed by cells in the periphery at similar (or higher) levels. In addition, recent reports from animals that lack peripheral MHC molecules have suggested that signaling induced by self-peptide-MHC complexes is required for maintenance of peripheral T cell pools (64–67). Therefore, it is possible that the complexes recognized by G35 in the periphery serve this function for mature 5C.C7 T cells. If so, additional studies using G35 might also provide clues about the identity, affinity for TCR, and localization of such complexes.

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