Brief Definitive Report

Both Intrathymic and Peripheral Selection Modulate the Differential Expression of $V_\beta 5$ among $CD4^+$ and $CD8^+$ T Cells

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Summary

Murine T cells expressing $V_\beta 5$ are characterized by (*a*) intrathymic deletion in the presence of I-E and products of endogenous mouse mammary tumor viruses, and (*b*) a greater representation in $CD8^+$ relative to $CD4^+$ peripheral T cells, thought to be due to more efficient intrathymic positive selection on class I rather than class II major histocompatibility complex antigens. We have engineered mice that are transgenic for a rearranged gene encoding a $V_\beta 5^+$ β chain of the T cell receptor for antigen. Deletion is not predicted in I-E^- $V_\beta 5^+$ transgenic mice, and until the age of 2 wk, the CD4/CD8 ratio of peripheral T cells is >3:1 and indistinguishable between transgenic and nontransgenic mice. Transgenic mice then show a rapid, age-dependent decline in the ratio of $CD4^+$ to $CD8^+$ T cells in the lymphoid periphery, reaching a low of 1:10 by 7 mo of age. Furthermore, the percent of peripheral $CD4^+$ cells that express the transgene drops with age, reaching a low of about 60% at 7 mo, while the percent of $CD8^+$ cells that express $V_\beta 5$ remains greater than 95% at all ages. The lymphoid periphery is implicated in this selection against $CD4^+$ $V_\beta 5^+$ T cells as it occurs more rapidly in thymectomized transgenic mice, and can be delayed in mice whose peripheral T cells are replaced by recent thymic emigrants after depletion by in vivo treatment with anti-Thy-1 antibodies. These results indicate that the relative expression of $V_\beta 5$ in T cell subsets can be influenced not only intrathymically in I-E^+ $V_\beta 5^+$ transgenic mice, but also by events in the periphery, in the absence of I-E expression.

Although helper and cytotoxic T cells differ in function, accessory molecule expression, MHC restriction specificity, and antigen recognition, they express largely overlapping V_α and V_β TCR genes. One apparent exception to this rule is the observation that $V_\beta 5$ expression is lower among $CD4^+$ compared with $CD8^+$ T cells in mice that do not express I-E (1, 2). Thus, in an adult C57BL/6 mouse, ~10–12% of $CD8^+$ peripheral T cells are $V_\beta 5^+$, compared with only 3–5% of $CD4^+$ peripheral T cells. This skewed V_β expression has been documented for both functional members of the $V_\beta 5$ family, $V_\beta 5.1$ and $V_\beta 5.2$ (2), and has been taken to mean that regardless of the coexpressed α chain, cells expressing $V_\beta 5^+$ TCR are more efficiently positively selected on class I than on class II MHC (2).

Other studies have indicated that $V_\beta 5.1^+$ and $V_\beta 5.2^+$ cells are deleted intrathymically in mice that express I-E molecules and a "cotolerogen," originally named Etc-1 (3). Intrathymic deletion in this (2) and many other cases (see reference 4 for review) results in the elimination of cells of both $CD4^+$ and $CD8^+$ phenotypes expressing high levels of TCR, suggesting the signal for deletion is delivered to a thymocyte population bearing both accessory molecules. It is now known that Etc-1 encodes an endogenous mammary tumor provirus (Mtv), Mtv-9, and that the open reading frame of the 3′ long terminal repeat of this defective viral integrant encodes the "self"-determinant that deletes $V_\beta 5^+$ cells in I-E^+ mice (5, 6). A second endogenous retroviral integrant, Mtv-6, has recently been implicated in I-E-mediated deletion of $V_\beta 5.1^+$ and $V_\beta 5.2^+$ TCR (7).

To study the expression of $V_\beta 5$ in T cells in I-E^+ and I-E^- mice and the relationship between $V_\beta 5$ expression and CD4 and CD8 positivity, we engineered mice transgenic for a rearranged $V_\beta 5.2$ β chain of the TCR (8). Our work illustrates that chronic selective forces can operate on mature T cells after they have left the thymus.

Materials and Methods

Mice. $V_\beta 5^+$ transgenic mice were derived on a congenic C57BL/6 background (H-$2K^{b+}$, I-E^-, Mtv-6^-, Mtv-9^+) by injection of a rearranged genomic β chain gene from the $CD8^+$ cytotoxic T cell clone B3, specific for chicken OVA and H-$2K^b$ (9).

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This β chain gene consists of $V_\beta 5.2/D_\beta 2/J_\beta 2.6$ and includes the endogenous promoter and enhancer. Two lines of independently derived mice were used throughout these studies, both carrying 5–10 copies of the transgene, and both transmitting the transgenes in a Mendelian fashion (8). $V_\beta 5^+$ transgenic mice were maintained as heterozygotes by crossing with C57BL/6 females purchased from The Jackson Laboratory (Bar Harbor, ME). Weanlings were screened for transgene expression in PBL using a mAb specific for $V_\beta 5.1$ and $V_\beta 5.2$ (10). Control nontransgenic mice were offspring from these same matings. I-$E^+V_\beta 5^+$ transgenic mice were derived by mating heterozygous $V_\beta 5$ transgenic mice with line 107–1 (11), a line of E_α transgenic mice demonstrating a normal pattern of transgene expression. PBL from offspring were screened for cell surface expression of both $V_\beta 5$ and I-E.

Surgery. Mice were anesthetized with tri-bromoethanol and either their thymuses were removed by suction or their cervical lymph nodes were removed by dissection. Wounds were closed by suture or wound clips.

Cell Separation. PBL were isolated by water lysis of whole heparinized blood. Cortisone-resistant thymocytes were removed from animals 2 d after intraperitoneal injection of 5 mg of hydrocortisone acetate in ethanol and water. Care was taken to remove the thymus free of neighboring lymph nodes. Lymph node cells were derived from inguinal, brachial, axial, cervical, and mesenteric lymph nodes.

Antibodies and In Vivo T Cell Depletion. MR9-4, a murine IgG mAb specific for $V_\beta 5.1$ and $V_\beta 5.2$ (10), and H7-597, an Armenian hamster antibody specific for the murine TCR β chain (12), were purified from ascites (MR9-4) or culture supernatant (H7-597) over protein G columns and directly conjugated with fluorescein as described (13). PE-conjugated anti-mouse IgG1 was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Fluorescein-conjugated anti-mouse I-E (clone AMS-16), PE-conjugated anti-CD4, and FITC- and PE-conjugated anti-CD8 were purchased from PharMingen (San Diego, CA). T24, a rat anti-Thy-1 mAb (14), was used as unpurified ascites for the in vivo elimination of peripheral T cells. In vivo titration of T24 was performed by intraperitoneal injection of graded doses of unpurified ascites once or twice into C57BL/6 mice. The efficacy of T cell elimination was judged by analysis of lymph node cells removed 3–5 d after the last injection. Cells were stained with anti-T cell reagents (anti-Thy-1, anti-CD4, anti-CD8, H7-597) and analyzed by flow cytometry. Cells were also assayed for their ability to proliferate to the T cell mitogen Con A. A >10-fold decrease in the number and proliferative capacity of T cells from injected mice was achieved using the following protocol. Animals of the indicated ages were prebled, injected intraperitoneally with 100 μl of unpurified T24 ascites, rested 1 d, given a second injection of 100 μl of ascites, and bled again 5 d later to test the effectiveness of the treatment. In each experiment, thymectomized transgenic and nontransgenic animals were also treated to test the dependence of T cell recovery on a functional thymus.

Antibody Staining and Cell Analysis. From 0.3 to 1.0 $\times$ 10^6 cells were incubated on ice with the appropriate dilution of antibody for 20 min in HBSS with 1% BSA in a total volume of 100 μl, washed with 1.5 ml, and incubated under the same conditions with each subsequent stage. After the final wash, cells were resuspended in 0.5 ml PBS and analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) using Lysis II or Consort 30 software. With the exception of the rare subpopulations, 10^4 live gated cells were analyzed in each case. Data are presented on a logarithmic scale.

Results and Discussion

The CD4/CD8 Ratio in T Cells from Young $V_\beta 5$ Transgenic Mice Is Indistinguishable from That in Young Nontransgenic Animals. The preferential expression of $V_\beta 5$ among $CD8^+$ relative to $CD4^+$ peripheral T cells in I-E^- strains of mice led us to analyze the ratio of $CD4^+$ to $CD8^+$ T cells in $V_\beta 5^+$ TCR transgenic mice. Not surprisingly, while the CD4/CD8 ratio in PBL from 12-wk-old nontransgenic animals is ~3, that of PBL from age-matched transgenic littermates is <0.4 (Fig. 1 *a*). These differences are equally apparent in peripheral T cells from spleen and lymph node (data not shown) as well as PBL. In contrast to these data from adult animals, in pups from 5 d to 2 wk of age, the ratio of CD4 to CD8 expression in spleen cells from transgenic and nontransgenic animals is indistinguishable. The CD4/CD8 ratio in the eight transgenic and eight nontransgenic 5-d-old pups examined was 6.3 and 7.1, respectively. At 7 d, two pups of each type were examined, with an average ratio of 5.6 for transgenic and 5.7 for nontransgenic. The average ratios for 13-d-old pups were 1.8 (for seven transgenic pups) and 2.3 for (three nontransgenic pups), with the disparity between transgenic and nontransgenic mice increasing thereafter. Aside from the initial drop in the ratio of CD4 to CD8 expression during the first few weeks after birth, the mean ratio in nontransgenic mice is relatively constant, at ~2–3:1. In contrast, transgenic mice are characterized by high CD4/CD8 ratios in young animals that drop >30-fold to a low of 0.1 at ~7 mo of age (Fig. 1 *a*). The age-dependent decline in the ratio of CD4/CD8 expression seems to be a result of a decrease in the number of $CD4^+$ T cells rather than an increase in the number of $CD8^+$ T cells. The number of $CD8^+$ lymph node cells is relatively constant between age-matched transgenic and nontransgenic mice, while the number of $CD4^+$ cells is much lower in the transgenic animals (data not shown).

The $CD4^+$ Population of Peripheral T Cells in Transgenic Animals Shows a Marked, Age-related Decrease in $V_\beta 5$ Expression, while the $CD8^+$ Population Does Not. We analyzed the expression of $V_\beta 5$ in $CD4^+$ and $CD8^+$ T cells in transgenic animals from the ages of 5 d to 45 wk (Fig. 1 *b*). In animals 2 mo of age and younger, >95% of both $CD4^+$ and $CD8^+$ populations of peripheral T cells are $V_\beta 5^+$ by surface staining. As these animals age, there is no detectable change in the transgene expression in $CD8^+$ T cells; however, there is a marked decrease in the percent of $CD4^+$ cells that express the transgene. By 6 mo of age, as few as 60% of $CD4^+$ peripheral T cells from transgenic animals are $V_\beta 5^+$ (Fig. 1 *b*). Most of the remaining ~40% of $CD4^+$ cells express a TCR α/β, as judged by bright staining with H7-597 (data not shown).

The Age-dependent Loss of $CD4^+V_\beta 5^+$ Cells Occurs in the Lymphoid Periphery and Not in the Thymus. To determine whether the selection against $V_\beta 5$ expression among $CD4^+$ T cells occurs intrathymically, we first analyzed thymocyte subpopulations for signs of deletion of $V_\beta 5^+CD4^+$ cells in I-E^- and I-E^+ TCR transgenic mice. From the data shown

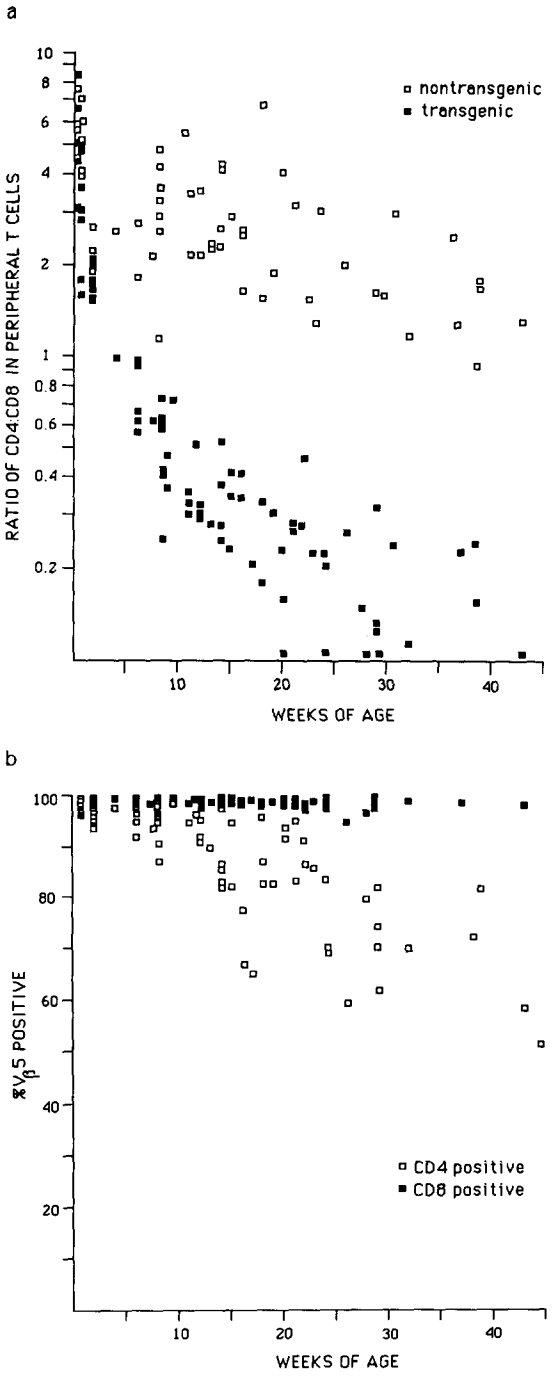

Figure 1. In $V_\beta5$ transgenic mice, both the CD4/CD8 ratio and the percent of $CD4^+$ PBL that are also $V_\beta5^+$ decline with age. (*a*) Relative CD4 and CD8 expression in peripheral T cells from transgenic and nontransgenic mice. Splenocytes from 5-, 7-, and 13-d-old pups and PBL from 4–45-wk-old $V_\beta5$ transgenic and nontransgenic animals were stained with FITC-MR9-4 and anti-CD4-PE or anti-CD8-PE and analyzed with a FACScan®. (*b*) Transgene expression in $CD4^+$ and $CD8^+$ peripheral T cells. Cells from transgenic mice collected and stained as in *a* were gated for PE^+ ($CD4^+$ or $CD8^+$) and then analyzed for $V_\beta5$ expression. At least 2,000 PE^+ events were analyzed from pups 5, 7, and 13 d of age; 10^4 gated events were analyzed for samples from older animals.

in Fig. 2 *a*, it is clear that the mature, $V_\beta5^{high}$ population of thymocytes is deleted in $I\text{-}E^+V_\beta5^+Mtv\text{-}9^+$ transgenic mice but not in their $I\text{-}E^-$ counterparts. These data are in agreement with previous work showing that in mice expressing both Mtv-9 and I-E, deletion of $V_\beta5^+$ cells occurs in the thymus, at the time of transition from the TCR^{dull}, $CD4^+CD8^+$ to the TCR^{high}, $CD4^+$ or $CD8^+$ stage (2). The incomplete, slow deletion of $CD4^+V_\beta5^+$ cells in $I\text{-}E^-$ transgenic animals does not occur at this same stage of development.

Furthermore, the CD4/CD8 ratio of "mature" cortisone-resistant thymocytes pooled from two 33-wk-old transgenic mice is 1.9, similar to the value of 2.4 obtained from a pool of two age-matched nontransgenic animals (Fig. 2 *b*). In contrast, lymph node cells from untreated littermates showed the expected decrease in the ratio of CD4/CD8 in the transgenic (0.2) compared with the nontransgenic animal (1.4). In 33-wk-old transgenic animals, 97% of $CD4^+CD8^-$ thymocytes are $V_\beta5^+$, while only 79% of $CD4^+$ lymph node cells are $V_\beta5^+$ (data not shown).

We next sought direct evidence that the lymphoid periphery influences the percent of $CD4^+$ transgene-expressing T cells. We analyzed both the CD4/CD8 ratio and $V_\beta5$ expression in transgenic animals that had been thymectomized at 6 wk of age. By analyzing these parameters at increasing times after thymectomy, we can observe what happens when peripheral T cells "age," in the absence of recent thymic emigrants. In adult thymectomized animals, the percent of $CD4^+$ PBL that express the transgene decreases dramatically, reaching a low of <40%, compared with a low of 70% for age-matched nonthymectomized control transgenics (Fig. 3 *a*). As in nonthymectomized animals, the percent of $CD8^+$ cells that are $V_\beta5^+$ remains uniformly high, at >95% (data not shown). Thus, thymectomy appears to accelerate the deletion of $CD4^+V_\beta5^+$ T cells, while having no effect on $V_\beta5$ expression in $CD8^+$ cells.

In the last series of experiments, peripheral T cells were depleted in 28–32-wk-old nonthymectomized transgenic animals by in vivo treatment with anti-Thy-1 mAbs. This protocol eliminates T cells from the spleen and lymph nodes, while causing minimal diminution in the size of the thymus (15, and data not shown). Before anti-Thy-1 injection, PBL from the transgenic animals showed the expected low CD4/CD8 ratio (0.10–0.13) with 20–30% of $CD4^+$ cells expressing a TCR β chain gene other than $V_\beta5$ (Fig. 3 *b*, and data not shown). Analysis of PBL 5 d after the final injection of anti-Thy-1 revealed that an average of only 7% of the original number of $CD4^+$ or $CD8^+$ T cell populations survived the anti-Thy-1-mediated elimination. Over the course of the next 7–11 wk, the animals were allowed to replenish their populations of peripheral T cells, to determine whether replacing "old" peripheral T cells with recent thymic emigrants can reverse the depletion of transgene-expressing $CD4^+$ peripheral T cells. Only $H7\text{–}597^+$ ($TCR\ \alpha/\beta^+$) cells were included in these analyses, as the percent of $Thy\text{-}1^+$ cells that are also $H7\text{–}597^+$ varied considerably from mouse to mouse (data not shown). Depletion of peripheral T cells reversed

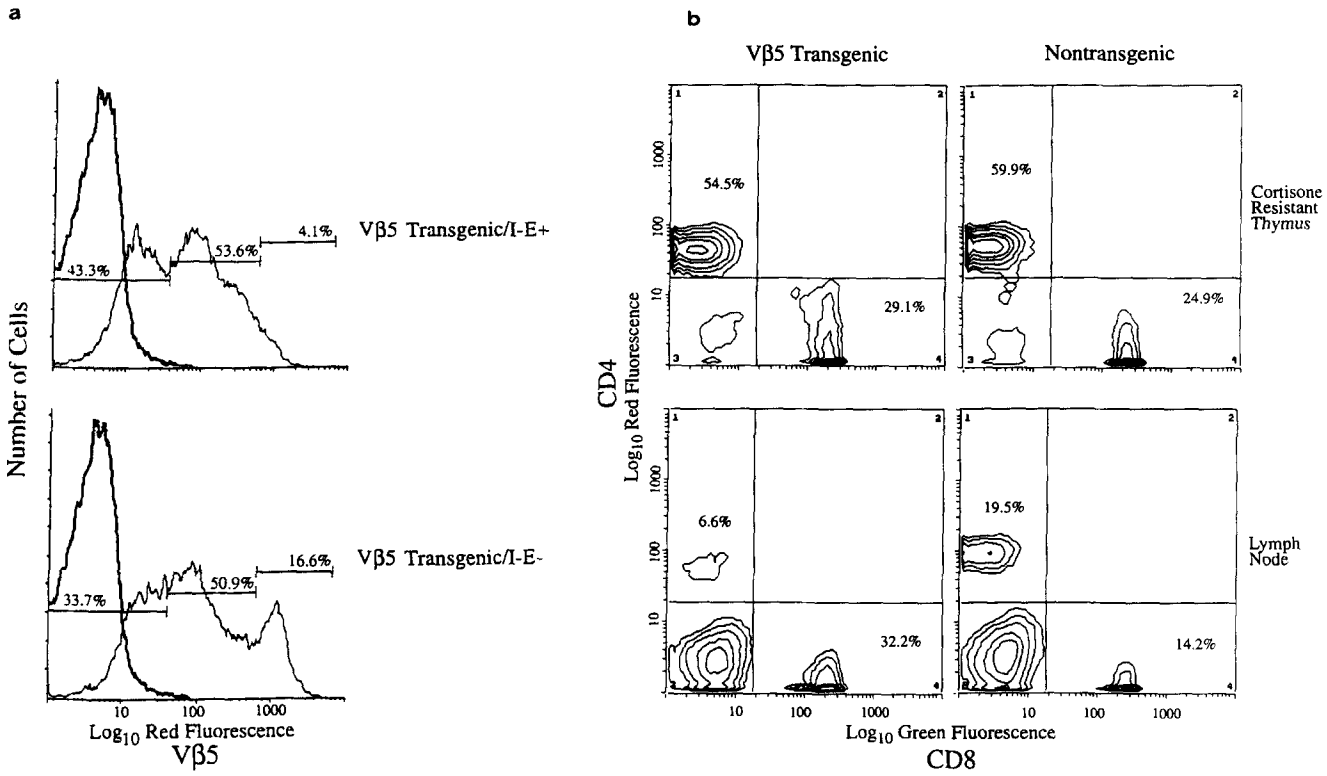

Figure 2. I-E$^-$Vβ5 transgenic mice do not delete CD4$^+$ Vβ5$^+$ cells intrathymically. (*a*) Thymocytes from 5-wk-old I-E$^+$ (*top*) and I-E$^-$ (*bottom*) Mtv-9$^+$ Vβ5$^+$ transgenic mice were stained with MR9-4 followed by PE-conjugated anti-mouse IgG1 and analyzed by FACScan®. Markers delineate the TCR^{high}, TCR^{med}, and TCR$^-$ subpopulations. (*b*) Cortisone-resistant thymocytes pooled from two 33-wk-old transgenic or nontransgenic mice and lymph node cells from untreated littermates were stained with anti-CD8-FITC and anti-CD4-PE.

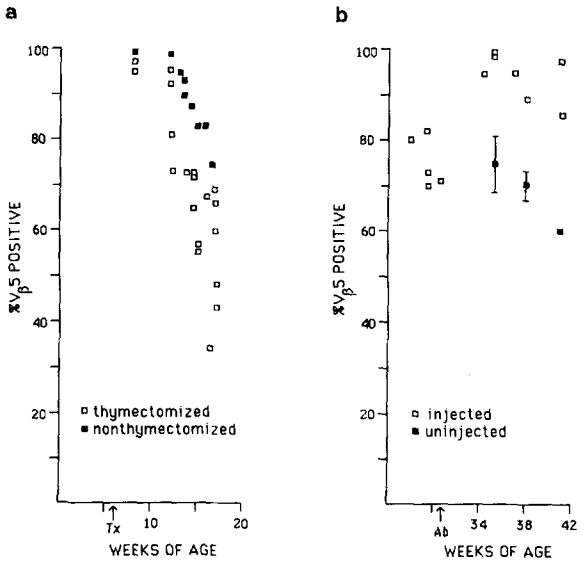

Figure 3. The loss of CD4$^+$Vβ5$^+$ PBL is accelerated by thymectomy and delayed by temporary elimination of peripheral T cells. (*a*) Mice were thymectomized at 6 wk of age, bled at various times postthymectomy, and PBL stained with FITC-MR9-4 and anti-CD4-PE. The arrow above *Tx* indicates the time of thymectomy. (*b*) Transgenic mice 28–32 wk of age were prebled and injected twice with anti-Thy-1 antibodies. PBL or lymph node cells were stained with anti-CD4-PE and either FITC-MR9-4 or FITC-H7-597. PE$^+$ cells were analyzed for FITC staining. Values are corrected for the percent of Thy-1$^+$ cells in each animal that were also H7-597$^+$, to eliminate TCR$^-$ Thy-1$^+$ cells from consideration. Values at 36 and 38 wk for uninjected animals represent the average ± SD from four and three age-matched animals, respectively. All other points are derived from individual animals. The arrow above *Ab* indicates the time of antibody injection.

the age-dependent loss of CD4$^+$Vβ5$^+$ cells, leaving injected mice with a population of H7-597$^+$ peripheral T cells that were >90% positive for Vβ5 expression (Fig. 3 *b*). The ratio of CD4/CD8 expression also increased markedly, reaching an average of 1.6 at 8.5 wk postinjection (data not shown). No change in Vβ5 expression was detectable in the CD8$^+$ population of peripheral T cells from injected mice (data not shown).

What Causes the Decline in the CD4/CD8 Ratio and the Gradual Loss of CD4$^+$Vβ5$^+$ Cells in Transgenic Mice? The driving force for both is most likely a selection against CD4$^+$Vβ5$^+$ cells, resulting in a rapid, early decline in the CD4/CD8 ratio of peripheral T cells and the eventual appearance of CD4$^+$Vβ5$^-$ cells after expansion of a small number of preexisting CD4$^+$ cells that do not express a Vβ5$^+$ TCR. This newly expanded T cell population is H7-597$^+$, indicating surface expression of a TCR α/β (data not shown). Previous analyses of TCR β chain transgenic mice (16) and mice transgenic for both α and β chain genes

of the TCR (17) have led to the suggestion that while allelic exclusion of α chain genes is leaky, that of β chain genes is nearly absolute. Our finding that up to 40% of peripheral $CD4^+$ T cells in old but otherwise unmanipulated β chain transgenic animals can express endogenously rearranged β chain genes is indicative of the power of the peripheral selection we have analyzed. Use of endogenous α and β chain genes has recently been documented in TCR α/β transgenic mice bearing inappropriate MHC molecules (18).

The selection against $CD4^+V_\beta5^+$ cells is not an idiosyncracy of the particular transgene we have chosen to study, since both $V_\beta5.1$ and $V_\beta5.2$ expression decline with age in $CD4^+$ but not $CD8^+$ peripheral T cells from nontransgenic animals (data not shown). In vivo anti-Thy-1 treatment implicates the lymphoid periphery in this selection, just as in transgenic mice (data not shown).

Why then are the $CD4^+V_\beta5^+$ cells selected against? Our unpublished observations that expression of the memory cell marker Pgp-1 (19) is high among $CD4^+V_\beta5^+$ T cells in transgenic mice, and that these cells are mildly anergic to signals through their TCR, have suggested that $CD4^+V_\beta5^+$ cells may be stimulated by their antigen, rendered anergic, and then disappear (20). One candidate antigen is Mtv-9, already well documented to delete $V_\beta5^+$ thymocytes in $I\text{-}E^+$ animals (5–7, and Fig. 2 *a*). Perhaps in the absence of I-E, Mtv-9 no longer mediates intrathymic deletion (of both $CD4^+$ and $CD8^+$ single-positive cells) but instead causes the progressive, peripheral deletion of $CD4^+$ cells that we see. We are currently deriving $I\text{-}E^-$Mtv-6/9$^-V_\beta5^+$ transgenic mice to test this hypothesis.

Can Selection against $CD4^+V_\beta5^+$ Peripheral Cells Wholly Account for the Skewed Expression of $V_\beta5$ among $CD8^+$ Relative to $CD4^+$ Peripheral Cells? If this skewed expression is due solely to the weak positive selection of $V_\beta5^+$ TCR on class II relative to class I MHC, one would predict that this skewing would be stable, without regard for the age of the animal in question. Our results add another level of complexity to this picture, but do not rule out the notion of the enhanced positive selection of $V_\beta5^+$ TCR on class I. Even as early as day 5 of neonatal life, $V_\beta5$ expression is skewed toward the $CD8^+$ population of peripheral T cells in nontransgenic mice (data not shown). Furthermore, in 11- and 33-wk-old nontransgenic animals there is an enhanced expression of $V_\beta5$ in hydrocortisone-resistant $CD8^+$ relative to $CD4^+$ thymocytes ($\sim$15% vs. 5%; data not shown). These data are in agreement with the notion that class II is inefficient at positively selecting $V_\beta5^+$ TCR intrathymically, and suggest that class II and some peripherally expressed antigen also serve to modulate the TCR repertoire of mature T cells. This latter means of manipulating the expressed repertoire of TCR can cause profound alterations in the population of T cells available during the lifetime of the animal.

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