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Failure of Rearranged TCR Transgenes to Prevent Age-Associated Thymic Involution

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After puberty, the thymus undergoes a dramatic loss in volume, in weight and in the number of thymocytes, a phenomenon termed age-associated thymic involution. Recently, it was reported that age-associated thymic involution did not occur in mice expressing a rearranged transgenic (Tg) TCRαβ receptor. This finding implied that an age-associated defect in TCR rearrangement was the major, if not the only, cause for thymic involution. Here, we examined thymic involution in three other widely used MHC class I-restricted TCRαβ Tg mouse strains and compared it with that in non-Tg mice. In all three TCRαβ Tg strains, as in control mice, thymocyte numbers were reduced by ~90% between 2 and 24 mo of age. The presence or absence of the selecting MHC molecules did not alter this age-associated cell loss. Our results indicate that the expression of a rearranged TCR alone cannot, by itself, prevent thymic involution. Consequently, other presently unknown factors must also contribute to this phenomenon. The Journal of Immunology, 1999, 163: 4262–4268.

Thymic involution during aging reflects the loss of organ mass and cellularity and is the most dramatic age-associated change in the immune system. The decline in thymocyte numbers is associated with a decrease in T cell output from the thymus and precedes the decreased T cell diversity and decline of the T cell function in elderly humans and old mice (reviewed in Refs. 1 and 2). The functional decline manifests itself through reduced T cell responses to mitogens, superantigens, and Ags that are accompanied by blunted biochemical parameters of T cell activation (3–7). The major consequence of immune senescence to the elderly is their increased susceptibility to infectious diseases (8).

Early studies of thymic involution suggested a role of pituitary and gonadal hormones in regulating thymocyte numbers (9, 10). Indeed, surgical and pharmacological castration prevented thymic involution, whereas hormonal treatment either prevented or accelerated (11–15) involution, depending upon the hormone administered. Other experiments suggested that during aging the generation of T cell precursors in the bone marrow (16, 17) and their differentiation in an aging thymus (18, 19) were both defective. At present, there is no consensus on whether a single age-associated defect or multiple defects contribute to thymic involution.

Recently, Aspinall (20) reported that old mice transgenic (Tg)3 for a rearranged TCR did not undergo age-associated thymic involution (20). This observation was interpreted to mean that a defect in T cell rearrangement must be responsible for thymic involution in old mice, and that this defect can be bypassed by the introduction of a rearranged TCR, similar to the situation in Rag−/− mice, in which the introduction of rearranged TCR and B cell receptor genes could rescue T and B cell development, respectively (21, 22).

We now report data on thymic involution in three other well-studied TCRTg mouse strains. In these mice, thymocyte numbers were found to be reduced by 90% between 2 and 24 mo of age, paralleling the cell loss in non-Tg mice; in addition, the kinetics of thymic involution, followed more precisely in one of the three strains, were indistinguishable from those in normal mice. Furthermore, the presence of positively selecting MHC molecules did not alter the course of involution, indicating that factors other than the presence of a rearranged TCR and its MHC-selecting elements must be responsible for the cell loss during thymic involution.

Materials and Methods

Animals

TCRTg 2C (23), H-Y (24), and OT-1 (25) mice were obtained from Drs. D. Y. Loh (Roche Research Laboratories, Nutley, NJ), H. von Boehmer (Hopital Necker, Paris, France) and H.-S. Teh (University of British Columbia, Vancouver, Canada), and W. R. Carbome (Monash University, Melbourne, Australia) and F. R. Heath (Walter and Eliza Hall Institute, Melbourne, Australia), respectively. They were maintained by breeding to C57BL/6N (B6) mice (National Cancer Institute, Frederick, MD) and were housed in the specific pathogen-free facilities at the Memorial Sloan-Kettering Cancer Center (MSKCC). Age-matched mice of either sex, backcrossed to B6 for ≥12 generations, were used in all experiments. To vary the dose of the positively selecting MHC molecule, 2C and OT-1 mice were also bred to B10.A(4R) mice (National Cancer Institute), and the F1 offspring were used for the analysis or backcrossed to the 4R parents to obtain homozygous H-2Kb progeny. Typing for the transgenes was performed by flow cytometric (FCM) analysis of PBLs using TCRTg

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3 Abbreviations used in this paper: Tg, transgenic; DN, CD8− double-negative thymocytes; DP, CD8+ CD4+ double-positive thymocytes; SP, single-positive thymocytes; TCRtg, mice transgenic for the TCRβ encoding genes; B6, C57BL/6N; FCM, flow cytometric; SI, stimulation index; AFU, arbitrary fluorescence units.
α-chain-specific mAbs (23, 26). Typing for the MHC molecules was performed using the H-2Kb- and H-2Kd-specific mAbs AF6,88,5 and 16.3.22 (American Type Culture Collection, Manassas, VA) and FCM.

Cell preparation and FCM analysis
A single-cell suspension of thymocytes was prepared by forcing the tissue through a steel screen using the rubber end of a syringe plunger. Thymocytes were resuspended in HBSS, and live cells were counted by trypan blue exclusion. CD4+8- double-negative (DN) cells were obtained by two rounds of mAb plus C+−mediated depletion using 3.155 (anti-CD8, rat IgM) and 2B6 (anti-CD4, rat IgM) ascites. Cells were washed, resuspended, and stained as indicated. The purity of DN cells was always >95%. For the FCM analysis, thymocytes were incubated with directly conjugated mAbs for 30 min at 4°C, washed, and analyzed by FCM using a FACScan instrument (Becton Dickinson, Mountain View, CA) and CellQuest 3.1 software. Tricolor-conjugated anti-CD4, FITC-conjugated anti-CD8 and H57–597 (anti-TCR CB) mAbs, as well as PE-labeled streptavidin were purchased from CalTag (South San Francisco, CA). The mAbs 1B2 and T3.70, specific for the 2C and H-Y clonotypic determinants, respectively, were purified from ascites and biotinylated in our laboratory. The PE-conjugated Vα2 mAb was obtained from Pharmingen (San Diego, CA).

Proliferation assay
All experiments were performed in RPMI 1640 medium containing 7.5% (v/v) FBS, supplements, and antibiotics, prepared by the Media Core Facility of the MSKCC (RP 7.5 (27)). Briefly, 5 × 106 splenocytes from TCRTg OT-1, 2C, and H-Y mice were resuspended in HBSS, and live cells were counted by trypan blue exclusion. CD4+8- double-negative (DN) cells were obtained by two rounds of mAb plus C−mediated depletion using 3.155 (anti-CD8, rat IgM) and 2B6 (anti-CD4, rat IgM) ascites. Cells were washed, resuspended, and stained as indicated. The purity of DN cells was always >95%. For the FCM analysis, thymocytes were incubated with directly conjugated mAbs for 30 min at 4°C, washed, and analyzed by FCM using a FACScan instrument (Becton Dickinson, Mountain View, CA) and CellQuest 3.1 software. Tricolor-conjugated anti-CD4, FITC-conjugated anti-CD8 and H57–597 (anti-TCR CB) mAbs, as well as PE-labeled streptavidin were purchased from CalTag (South San Francisco, CA). The mAbs 1B2 and T3.70, specific for the 2C and H-Y clonotypic determinants, respectively, were purified from ascites and biotinylated in our laboratory. The PE-conjugated Vα2 mAb was obtained from Pharmingen (San Diego, CA).

Results and Discussion
To test the influence of the expression of a Tg TCR on thymic cellularity, three mouse strains bearing rearranged TRC-encoded genes (2C (specific for p2Ca + Ld) (23), H-Y (H-Y Ag + Dd) (24), and OT-1 (OVA257-264 + Kk) (25)) as well as their non-Tg counterparts were maintained in our colony for ≤30 mo of age. The number and phenotype of thymocytes was examined at 8–10 wk (the time of young adulthood) and at 3, 6, 9, 12, 18, 24, and 30 mo. TCRTg mice also allowed us to test the influence of positive selection on the age-associated thymic involution, an issue previously not examined in detail. To that effect, we analyzed thymocyte numbers in young and aging Tg or littermate animals of the B6 strain (two copies of the positively selecting MHC molecules) and compared them with those in (B6 × B10.A(4R))F1 (a single copy of the H-2Kb molecule that selects OT-1 and 2C thymocytes) and B10.A(4R) mice (nonselecting for 2C and OT-1 TCRs). Finally, we tested T cell function in aged TCRTg mice and compared it with the non-Tg counterparts.

Thymocyte numbers in aged TCRTg and non-Tg mice
Fig. 1A shows thymic cellularity in young normal and TCRTg mice. As described previously, all young TCRTg mice used in this study exhibited a reduction in thymocyte numbers compared with the wild-type young counterparts (23–25). Of interest, the number of thymocytes in 24-mo-old normal mice was only ~7.4% of that in 8- to 10-wk-old mice (Fig. 1B). Although the thymocyte numbers were lower in 8- to 10-wk-old TCRTg mice than in non-Tg counterparts, 24-mo-old TCRTg mice still exhibited an 88–95% decrease in thymocyte numbers (88% for 2C, 94% for OT-1, and 95% for H-Y). No improvement in the thymic cellularity of aging mice was observed in any of the three TCRTg strains at 24 (Figs. 1 and 2) or 18 (Fig. 2) mo of age. These results differed from those reported by Aspinall (20), who reported only a 25–35% reduction of thymic cellularity between 3 and 20 mo of age in F5 TCRTg mice and no difference in cellularity between 3 and 12 mo of age. These results further suggested that the presence of a rearranged TCR does not, by itself, prevent thymic involution. Aspinall did not analyze mice as old as 24 mo, and a distant possibility remained that the Tg TCR protected against thymic involution, but that this effect faded between 20 and 24 mo of age. Therefore, we were prompted to analyze mice ranging between 3 and 12 mo of age. For this analysis, we selected OT-1 mice, whose TCR does not adversely interact with the self MHC molecules in the course of T cell development. The results shown in Fig. 1C clearly show that this Tg TCR did not prevent age-associated thymic involution at any timepoint examined.

Influence of selecting MHC molecules on age-associated thymic involution in TCRTg mice
The effect of intrathymic positive and negative selection on age-associated thymic involution has not been examined so far, and the TCRTg model was well suited to address this issue. For example, it was possible that in the three TCRTg strains studied here, positively selecting interactions between the Tg TCR and the MHC molecules reduced the number of thymocytes by forcing an efficient transition of CD8+4+ double-positive (DP) cells into CD8+ single-positive (SP) ones. If this interaction, on the other hand, was less efficient in the F5 mice (28) studied by Aspinall (as suggested by the CD4/CD8 profiles in these mice; Ref. 28), such a discrepancy could explain the difference between the obtained results. To investigate whether the dosage and the presence of the selecting MHC molecule may influence thymic involution in TCRTg mice, we introduced the TCR transgenes into (B6 × B10.A(4R))F1 mice.
(K\textsuperscript{b}K\textsuperscript{b}), which carry only one copy of the selecting molecule, and into homozygous B10.A(4R) mice (K\textsuperscript{b}K\textsuperscript{b}), which fail to positively select thymocytes bearing 2C and OT-1 TCRs, and compared their age-associated thymic involution to that of homozygous K\textsuperscript{b}K\textsuperscript{b} TCRTg mice. In our hands, age-associated thymic involution was observed regardless of whether the Tg TCR was positively selected in an H-2K\textsuperscript{b} homozygous environment, was not selected at all (Fig. 2B), or was selected by only one copy of the selecting MHC molecule (data not shown). Therefore, intrathymic interactions of the TCR and its selecting ligand did not influence the dynamics of thymocyte loss in aging.

Thymocyte subsets in aging TCRTg mice

Thymocytes can be divided into four main developmentally related populations according to the expression of CD4 and CD8. Among these, the CD8\textsuperscript{+} CD4\textsuperscript{−} DN fraction (2–4\% of all thymocytes) contains the precursors of other subsets (29). These cells yield the major DP population (75–85\%), which subsequently develops into CD8\textsuperscript{−} CD4\textsuperscript{+} (3–6\%) and CD8\textsuperscript{+} CD4\textsuperscript{−} (6–12\%) SP cells (30). MHC class I-restricted TCRTg mice, by contrast, have fewer DP cells and more CD8 SP cells, as a result of increased positive selection (31). Of note, this distribution of the four populations remained constant with age in both non-TCRTg and TCRTg mice (data not shown).

DN cells can be further subdivided into four sequential developmental stages according to the expression of CD44 and CD25...
Commitment to the T cell lineage occurs at stages 2 through 3, with the rearrangement of Tcr-b genes. Aspinall (20) reported that the numbers of the stage 1 (pre-rearrangement) cells did not change with aging, whereas the numbers of the other three subsets decreased with age. Consequently, the proportional representation of CD44+CD25− cells increased in senescence. Based on this finding, Aspinall (20) hypothesized that the block in TCR rearrangement did not allow development of cells beyond stage 1, and that the introduction of the rearranged Tg TCR bypassed this block. However, lesions of T cell rearrangement do not arrest T cell development at stage 1, but rather at stage 3 of development (21, 34), and a possibility remained that the accumulation in this subset reflected an increase of the cells similar in phenotype to the stage 1 cells, which did not reflect a problem in rearrangement. Our analysis of the normal young and aged DN thymocytes confirmed the findings of Aspinall regarding the accumulation of the stage 1 cells. However, this accumulation was not consistent in the TCRTg mice examined here, and did not correlate with age (e.g., note the discrepancy in Fig. 3, B and C, between low numbers of these cells at 24 mo compared with 9 mo). A consistent dominance of the CD44+CD25− cells (previously demonstrated to be terminally differentiated nonprecursor cells in TCRTg mice (35)) was observed regardless of age in these mice (Fig. 3). These findings show that age-associated thymic involution does not necessarily correlate with the alterations in the DN subsets, and caution against the simplistic conclusion that the alterations in the DN subsets must reflect disturbances of the Tcr rearrangement. For example, they could very well reflect disturbances of T cell commitment or some other process.

Functional responsiveness of young and old TCRTg cells

Finally, we compared the response of young and old TCRTg T cells to cognate Ags. To that effect, young and old H-Y, OT-1, and 2C TCRTg animals were stimulated with the corresponding cognate Ags (B6 male splenocytes, OVA-8-coated B6 splenocytes, and BALB/c splenocytes, respectively; female B6 spleen cells were used as controls in all three cases), and their T cell proliferation was compared. To facilitate comparison, results were expressed as the percentage of the response of the young animals (which was taken as 100%), measured as the SI, as described in Materials and Methods. Non-Tg mice that had been stimulated by immobilized Abs against TCR/CD3 showed an age-associated reduction in the proliferative response (Fig. 4A). Likewise, OT-1 and H-Y TCRTg T cells exhibited an age-associated hyporesponsiveness to cognate Ags compared with the young counterparts (Fig. 4, B and C). A similar reduction was also observed in a preliminary experiment with the 2C mice (data not shown). In certain old mice, a reduction of the percentage of TCRTg+ cells (moderately to severely) and of TCR expression intensity (moderately) was observed, compared with the young controls (see the percentages above the histograms in Fig. 4, and the arbitrary fluorescence units (AFU) in the legend to this figure). In others, however, this percentage was essentially identical with the young controls (compare the histograms on the far left (young control) with those on the far left (old mouse no. 3) in the H-Y and OT-1 panels), yet the old TCRTg cells responded less vigorously.

To compare more directly the functional capacity of TCRTg and normal cells, we stimulated both with the same reagent (anti-CD3 mAb), and have normalized data to the number of T cells bearing the TCR, over the course of aging between 3 and 12 mo. (This approach, rather than cell sorting, was chosen so as to avoid cross-linking the TCR and the consequent disturbance of TCR signal-
age-dependent hyporesponsiveness was observed starting as early as 6 mo of age. These results are similar to the observations of Linton et al. (36) in a different TCRTg system. We conclude that the presence of the TCR transgene does not prevent the age-associated loss of T cell function.

Concluding remarks

In the past decade, a vast body of knowledge on T cell development has been obtained using the TCRαβTg mouse lines H-Y, 2C, and OT-1. The above results demonstrate that the expression of any of these three Tg TCRs at the surface of thymocytes does not prevent the age-associated decline in thymocyte numbers or the decline in the proliferative responses of T cells in old mice. Furthermore, a preliminary study using a fourth TCRTg strain, 6.5, bearing the TCR specific for the influenza hemagglutinin peptide 110–120 on the BALB/c background, revealed a progressive and unimpeded thymic involution with age (8 wk = 6.0 × 10^6 thymocytes; 12 mo = 8 × 10^6; 16 mo = 2 × 10^6 thymocytes).

At present, it is not clear why our results differ from those of Aspinall (20) but it is likely that a complex explanation, rather than
only the expression of a TCR, must account for the discrepancy. Aspinall used a TCR transgene encoding an influenza nucleoprotein-specific TCR (F5, Ref. 28). It is possible to speculate on the possible reasons for the discrepancy along at least two lines. One source of the discrepancy between the two sets of results could lie in a difference in the affinity of the various TCRs for their MHC-selecting elements. Some of the TCRtg strains, including 2C, exhibit thymic profiles that are consistent with partial negative selection (Fig. 2A), with an overall paucity of DP cells (Ref. 23 and data not shown), possibly owing to a very high affinity for the intrathymic ligands. Indeed, 2C is capable of weakly reacting to the self peptide dEV-8:H-2Kb complex, and this interaction could cause partial negative selection (37). If all of our TCRtgs expressed similar reactivity, and the F5 did not, perhaps the results we observed reflected negative selection, rather than true age-associated thymic involution. We consider this explanation unlikely, given that H-Y and, in particular, OT-1 thymocytes show no signs of negative selection in B6 mice. Moreover, the removal of H-2Kb molecule (which may cause partial negative selection of 2C) did not prevent the age-associated involution in aged 2C mice.

Another difference between the TCRtg strains used in our study and the one used by Aspinall is seen in the regulatory elements driving the transgenes. Of the six DNA constructs encoding TCR α- and β-chains that were used to generate the TCRtg mice examined by us, five are the genomic constructs, driven by the endogenous regulatory elements; only the OT-1 Vα is composed of a CDNA under the control of an Ig enhancer and the H-2Kb promoter (23–25). All three receptors are overexpressed early in the development (Refs. 35, 38, 39; H.D.L. and J. N-Z., unpublished observations). By contrast, the F5 TCR constructs were both cDNAs expressed under the control of the CD2 promoter minicassette. Although this could cause a slightly delayed expression of the F5 chains compared with the TCRtgs used in our study, this is apparently not the case, and the F5 is expressed at an early DN stage (Ref. 28; D. Kioussis, unpublished observations). At present, it is difficult to exclude the possibility that subtle differences in transgene expression levels or timing or other unknown differences inherent to the Tg systems may have influenced the different results obtained in the two studies. Furthermore, it is also possible that the differences in the genetic background account for the observed differences. This possibility is considered rather unlikely, because B6 (our three strains) and B10 (the F5 mice) substrains differ only at a very limited number of minor H loci and are considered very close.

Importantly, regardless of the exact reasons for the difference between our results and those of Aspinall (20), the basic conclusions of our study remain that the expression of the TCRtg cannot singlehandedly prevent thymic involution. Therefore, our results do not support the idea that impaired TCR rearrangement is the only or the most important cause of thymic involution. Indeed, even if the cellularity of the young TCRtg thymi was lower than that of the non-Tg littermates in our system, the TCRtg thymi underwent a similar course and extent of involution, with a severe cell loss at 24 mo, regardless of the presence of the rearranged and expressed TCR. To unveil the role of other factors involved in thymic involution, it will be of interest to examine the expansion and involution of homogenous TCRtg precursor populations injected into young and old environments, as well as to examine the aged TCR knock-in mice of all of the above strains side-by-side.

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