Molecular, Cellular, and Antigen Requirements for Development of Age-Associated T Cell Clonal Expansions In Vivo

Ilhem Messaoudi, Jessica Warner, Dragana Nikolich-Zugich, Miranda Fischer and Janko Nikolich-Zugich

J Immunol 2006; 176:301-308; doi: 10.4049/jimmunol.176.1.301
http://www.jimmunol.org/content/176/1/301

References This article cites 43 articles, 26 of which you can access for free at: http://www.jimmunol.org/content/176/1/301.full#ref-list-1

Subscription Information about subscribing to J Immunol is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Molecular, Cellular, and Antigen Requirements for Development of Age-Associated T Cell Clonal Expansions In Vivo

Ilhem Messaoudi, Jessica Warner, Dragana Nikolich-Žugich, Miranda Fischer, and Janko Nikolich-Žugich

T cell aging manifests itself both at the cellular (cell-autonomous defects in signaling) and at the population (age-related dysregulation of T cell homeostasis) levels. A prominent contributor to the latter is the appearance of T cell clonal expansions (TCE), with a potential to impair immune defense. In this study, we investigated molecular, cellular, and Ag requirements for TCE development. Of the mutant mice tested, old animals lacking MHC class I exhibited 7-fold fewer TCE than controls, with a 7-fold reduction in TCE. By contrast, animals lacking only one of the MHC class I molecules (Kb or Db), or IL-7R, or devoid of T cell renewal via adult thymectomy, all exhibited significant increases in TCE incidence. This increase directly correlated to lymphopenia, increased CD8 T cell turnover and an accumulation of memory-phenotype T cells. These data suggested that homeostatic cell division in the CD8 compartment enhances the formation of TCE. Repeated immunization with peptide/adjuvant did not result in an increase in Ag-specific TCE; however, adjuvant alone increased TCE incidence. In these experiments, therefore, nonspecific and/or homeostatic proliferation was more efficient in generating TCE in mice than repeated Ag-driven stimulation, suggesting that many, if not most, TCE in specific pathogen-free laboratory mice may be Ag-independent. The Journal of Immunology, 2006, 176: 301–308.

Aging of the organism is marked by primary cellular changes that reflect either loss or change of function. Consequently, these alterations trigger secondary (and, possibly, tertiary), downstream changes, which represent an attempt to partially restore and/or maintain structure and function, and/or to adapt to the initial changes. This paradigm is applicable to most organs and organ systems (1–3). Age-associated changes, then, represent a sum of primary and secondary/tertiary, downstream changes, presenting a unique challenge to not only identify the sum of all age-related changes, but to also delineate the most critical ones and to dissect the primary or compensatory nature of these.

In light of the above paradigm, T cell aging brings about a very profound change at the population level—the decline in naive T cell numbers. This loss is caused by the simultaneous primary effects of decreased naive T cell production due to the involution of the thymus (which itself appears to be a complex process caused by diminished supply of early T cell progenitors and to the aging of thymic stroma) and their increased consumption due to lifelong encounters with new antigenic challenges and with persistent, chronic pathogens (reviewed in Refs. 4–7). Both processes also lead to an increase in memory (and memory-phenotype) T cells and in reduction of diversity of T cell repertoire (8). Many facets of this complex network of changes, however, remain poorly understood.

Perhaps the most remarkable change associated with the reduced TCR repertoire diversity in aging is the appearance of age-associated T cell clonal expansions (TCE) (9–12). These abnormal cells were so far detected in every mammalian species tested and are overwhelmingly of the CD8 + phenotype. Each expanded population belongs to a single clone of expanded cells, and can take up to 80–90% of the murine and up to 20–30% of the human CD8 pool. Their biology has recently been extensively reviewed (13), and they can be divided into the following: 1) those believed to be responding, to and are stimulated by, chronic, persistent infections (particularly CMV and other herpesviruses); and 2) those that are not acutely engaged in an immune response and have gained a level of Ag- and homeostasis-independence, most likely by virtue of mutation in survival/homeostasis response pathway(s). To distinguish between them, we will call them AR-TCE (Ag-responding TCE) and AI-TCE (Ag-independent TCE). The above distinction is both conceptual and operational, and is based primarily on the absence of acute activation markers on the AI-, but not on AR-TCE. However, at this point, Ag dependency of these two categories, whereas strongly suggestive, is still not definitively established. Neither is the ontogenic relationship between these two TCE types. In that regard, three scenarios can be envisioned: 1) AI-TCE and AR-TCE are independent of each other, so that the former arise via Ag-independent induction of cell division (homeostatic, bystander, etc.), whereas the latter arise in response to, and are continuously maintained by, persisting Ag; 2) AI-TCE
arise from AR-TCR following repeated Ag stimulation, and become Ag-independent by a stochastic mutation during the Ag-induced division; and 3) both “2” and “3”) can occur, and therefore AI-TCE can arise both from AR-TCE and directly from naive or memory cells induced to proliferate in an Ag-independent manner. To begin to distinguish between the above possibilities, we initiated experiments to investigate what cellular and molecular conditions may impact development of AI-TCE. The common underlying theme behind the conditions tested suggests that functional CD8 cells must exist, and must be driven to proliferate, to generate AI-TCE. Our data demonstrate that, in B6 mice, AI-TCE are efficiently generated in the absence of overt Ag stimulation, by homeostatic and/or nonspecific proliferation. By contrast, neither AR-TCE nor AR-derived AI-TCE were generated by repeated intermittent Ag stimulation in our hands. These results are discussed with regard to the existing models of TCE generation.

Materials and Methods

Mice

C57BL/6 (B6) mice of indicated ages and of both sexes were purchased from the National Institute on Aging breeding colony (Harlan). MHC class I- (14), II- (15), CD8+ (16), and IL-7r-KO (knockout, carrying targeted disruption of indicated molecule) (17) mice were obtained from The Jackson Laboratory and were aged at the Vaccine and Gene Therapy Institute vivarium. H-2Kb- and D- KO mice (18) were generously provided by Dr. Francois Lemonnier (Pasteur Institute, Paris, France). Adult control animals used in these experiments were 3–6 mo old, and the age of old animals used is indicated within each experiment. All mutant/transgenic (Tg) animals were backcrossed onto the B6 background for a minimum of 12 generations. Moreover, the animals used in all experiments were born and/or housed in the specific pathogen-free block, and were negative for specific viral and pathogen titers throughout the study. Old animals were subjected at the time of sacrifice to thorough clinical examination and necropsy, and those exhibiting signs of poor health or tumors were excluded from the analysis.

Single-chain TCRα Tg mice, carrying the Vα2 transgene specific for the HSV-1 glycoprotein B (gB)498–505 (SSIEFARL), bound to H-2Kb, and named gBT-1.tg, were derived by outcrossing the gBT-1.tg Tg mice (19) and selecting for progeny expressing only the TCRA transgene, and were kindly provided by Dr. F. Carbone (Melbourne University, Melbourne, Australia). Single-chain TCRRβ Tg mice, carrying the Vβ8.2 Tg specific for the same peptide-MHC combination, and named gBT-1β, were produced in our laboratory, by amplifying the BV8S1-BD1-BJ1S2 segment from the cDNA of the CTL clone BMHC12 (20) using high-fidelity PFU polymerase (Stratagene) and the following primers containing the full Nru restriction site: forward 5'- CGT CGC GAC TTA CCT ATT ACC AAA AGC CTG GTC CCT G; reverse 5’- CGT CGC GAC TTA CCT ATT ACC AAA AGC CTG GTC CCT G; reverse 5’- CGT CGC GAC ATG GCC TGC AGA CTC TGG TTT GG. The obtained PCR product was subcloned into pCDNA3.1 using the TA cloning kit (Invitrogen Life Technologies). Following sequence verification, one clone was selected for large-scale production and purification of the insert, which was then re-cloned into the eukaryotic TCR expression vector (21) kindly provided by Dr. S. J. Anderson (Northwestern University, Chicago, IL), containing the endogenous TCRβ regulatory elements. The original VDJ fragment was removed following Nru I digestion, the ends were repaired by Klenow enzyme treatment (Promega), phosphatase treated (Shrimp Alkaline Phosphatase; Promega), and the BMHC12 TCRRβ VDJ fragment inserted into the vector. The vector was linearized and injected into fertilized C57BL/6 (B6) HeJ) F2 blastocysts at the Memorial Sloan-Kettering Cancer Center’s Transgenic Core Facility (New York, NY). Founders were identified by Southern blot, confirmed by FACS staining, and backcrossed onto C57BL/6 mice for >14 generations to establish the gBT-1β Tg line.

CDR3 length analysis

The PCR conditions and the primers were described previously (12). CDR3 length polymorphism profiles of Vβ families not containing TCE exhibited comparable diversity to that shown for the Vβ2 family (Fig. 1, top and middle). When single peaks, suggestive of TCE, were detected, the PCR products were sequenced exactly as detailed previously, revealing a single readable sequence in each case.

Flow cytfluorometry (FCM)

Abs directed against CD8, CD4, BrdU, CD127 (anti-IL-7rα-chain), CD44, and CD122 (anti-IL-2 and IL-15 common β-chain) were purchased from BD Pharmingen. All samples were acquired on either FACS-Calibur (BD Pharmingen) or FACS LSRII (BD Pharmingen). Data files were analyzed using FlowJo (Tree Star).

Thymectomy

Thymectomy was performed on animals under isoflurane anesthesia, as described previously (22), with the exception that vacuum suction was applied to remove the organ. The effectiveness of thymectomy was verified at necropsy, and only fully thymectomized animals were included in this study. In initial studies, we found no difference between control unmanipulated and sham-ATX (adult thymectomy) mice, and therefore we used the former as a control group in most experiments.

Immunization with peptide and adjuvant

Mice received either emulsified adjuvant TiterMax alone (TM; Sigma-Aldrich) or adjuvant with 10 μM HSV-1 gB498–505 peptide (SSIEFARL; Sigma-Aldrich) as an intradermal injection in the footpad, exactly as described previously (23). Injections were conducted at monthly intervals, starting at 2 mo of age, for six injections; and then every other month until 18 mo, for a total of eight injections. The presence of TCE was evaluated at 18 mo following hemisplenectomy, by FCM. For BrdU labeling experiments, groups of three mice received injections with TM, treated with BrdU as described below, and the number of labeled cells were analyzed on days 3 and 7 in the popliteal (draining) lymph nodes (LN) and spleens.

BrdU labeling

Mice were given BrdU in drinking water at 0.8 mg/ml as described elsewhere (24) for 7 days. BrdU incorporation was measured in PBMC as well as LN and splenocytes on day 7 by FCM using a kit from BD Pharmingen as per the manufacturer’s recommendation.
Statistical evaluation

Statistical comparisons were performed using Fisher’s exact test. χ² test was also performed (data not shown) and confirmed the significance results obtained by the Fisher test.

Results

Defining the phenotype of AI-TCE

Previous publications from this and other laboratories (10, 12, 25, 26) have described many of the features of AI-TCE in rodents; however, no phenotypic signature is available to date that would definitively separate TCE from other memory-type T cells. In this study, we used four-parameter analysis, followed by molecular confirmation of CDR3 length diversity to achieve this task. Initial screening for TCE was performed by FCM staining using mAb against the 13 detectable TCRVβ proteins (Vβ2–14), and mice in which expression of any of these proteins exceeded 3 SD over the mean values were identified as TCE-carrying animals and analyzed further. In those animals, expanded TCRVβ⁺CD8⁺ cells were further stained and AI-TCE reliably identified by high and homogeneous expression levels of CD122 (IL-2/15Rβ chain) and CD44 at the surface (Fig. 1, upper panels). Importantly, these TCR did not express any of the acute activation markers in mice, including CD69 and CD25, and were also CD62Lhigh (I. Messaoudi and J. Nikolich-Zugich, unpublished observations). Corroboration of the clonal nature of these TCE was obtained by CD3 length analysis, performed as in Ref. 12, showing that a single peak characterizes TCE cells (Vβ2⁺ in the mouse depicted in Fig. 1, top panel). By contrast, whereas the non-TCE cells in this same mouse (Vβ2⁻ in the mouse depicted in Fig. 1, middle panels) and the cells from another, non-TCE bearing mouse that were of the Vβ2⁻CD8⁻ phenotype exhibited heterogenous expression of CD44 and CD122 (Fig. 1, lower panels), and their CD44highCD122high cells exhibited polyclonal CD3 profiles representative of normal TCR repertoire diversity. (Unfortunately, we found that this combination of markers was very useful to define TCE well after the beginning of this long-term study; therefore, many animals analyzed between 1999 and 2003 were not subjected to this staining.) Historically, we were always (in >500 animals analyzed so far over the last 9 years) able to confirm that a single peak corresponds to a single clone of cells, and therefore the experiments in this study mostly focused upon the large, mAb-detectable TCE followed by CD3 length analysis, without confirmatory sequencing.

CD8 T cell lineage development and AI-TCE generation

Because all large TCE tend to be of the CD8 phenotype, we asked whether deficiencies in molecules important for development of CD8 and CD4 T cells would impact TCE onset. To that end, mouse strains deficient in MHC or coreceptor molecules were aged to 24 mo, sacrificed, and examined for the presence of TCE. Data from several cohorts of animals purchased from either the NIA or from National Cancer Institute and aged in our colony indicates that 30–40% of 20- to 24-mo-old B6 animals develop AI-TCE (although variability between different animal cohorts is often found at that age), and that this incidence invariably further increases with age (12). Indeed, in experiments shown in Fig. 2, 25 of 64 analyzed B6 animals (39%) had detectable AI-TCE, and, consistent with previously published results (10, 12), were exclusively of CD8⁺ phenotype (Fig. 1). Although it is presently unclear why CD8 cells almost exclusively generate TCE, it is known that upon stimulation CD8⁺ T cells activate more vigorous cellular proliferation programs compared with CD4 cells (27–29), and it is likely that such differences over the lifetime would account for differential AI-TCE formation from CD8 and CD4 cells.

Class I deficiency causes drastic disturbances in generation of CD8⁺ T cells (14), and it was perhaps not surprising that in the absence of these molecules TCE incidence was reduced by >7-fold (only 1 of 15, or 6.6% of animals of class I-KO animals developed AI-TCE at 24 mo; Fig. 2A). These AI-TCE were of the CD4 phenotype, consistent with the notion that under rare circumstances CD4 cells can give rise to TCE as well (30). We further examined the impact of CD8 coreceptor loss on the frequency of TCE formation, and found that these animals develop AI-TCE less frequently than wild-type (wt) animals (with suggestive, but not statistically significant, reduction of 45%). We can conclude from these results that the presence of CD8 may be important, but is not absolutely essential, for TCE formation. Of note, expanded AI-TCE in the CD8-KO animals exhibited high expression of CD122, which is characteristic of CD8 memory T cells and atypical of CD4 memory T cells (Fig. 2B), suggesting that either these cells may belong to the CD8 lineage even in the absence of the coreceptor (31, 32), or that high levels of CD122 expression may be a consequence of the AI-TCE phenotype. Analysis of other lineage markers would be necessary to resolve this issue. MHC class II-deficient animals exhibited a similar, <2-fold reduction in AI-TCE incidence, which was suggestive but not statistically significant, suggesting that CD4 help, like the CD8 coreceptor, is not indispensable for TCE generation. Finally, we tested the effects of absence of one of the MHC class I molecules (H-2Kb or Db) on TCE formation. In these animals, the CD8 pool is reduced by 50%, consistent with the idea that the number of CD8 cells is formed by an equal contribution from cells positively selected and maintained on H-2Kb and H-2Db (Ref. 18 and Fig. 3A). At 20–24 mo of life,
AI-TCE formation as a function of homeostatic proliferation

In our previous study, we performed transfers of TCE and of non-TCE-containing spleen cells into Rag-KO animals (12). In these experiments, we found that the original AI-TCE could rarely be found in recipients 6 mo after transfer, and that, instead, new TCE were detectable. Most remarkably, such TCE could be readily obtained from young donor cells as well as old. We hypothesized that increased homeostatic proliferation in the donor T cell pool resulted in increased appearance of AI-TCE, and that repeated cell division facilitates transformation of certain clones into AI-TCE (12). It is now clear that the bulk of this proliferation upon transfer into Rag-KO animals occurs in response to intestinal microbial flora (33), and is, therefore, de facto Ag-driven. However, the above finding that H-2Kb- and H-2Dd-KO mice exhibited higher TCE incidence appeared consistent with our original hypothesis; if each MHC class I allele selects and maintains a specific and separate pool of CD8 T cells, the absence of one allele would result in relative lymphopenia (Fig. 3A), which should in turn lead to enhanced homeostatic proliferation and a consequent increase in AI-TCE formation. To test this experimentally, we measured the turnover of T cells in adult H-2Kb- and H-2Dd-KO mice, and found it to be significantly increased compared with control B6 animals for CD4, and, in particular, for CD8 cells (Fig. 3B). Consistent with our hypothesis, the percentage of CD44hi/CD122hi cells was also elevated (Fig. 3C). Clearly, such differences in homeostatic regulation between wt and mutant mice over the life of an animal would be further amplified and exaggerated, providing potential for TCE formation.

To further link the homeostatic turnover to AI-TCE formation, we investigated other models where T cell proliferation should be increased as a consequence of T cell subset imbalance and/or these animals contained, on the average, more TCE than age-matched controls (Fig. 2A; 11 of 22, or 50%), suggesting that the absence of one MHC class I molecule somehow predisposes for TCE development. Although these results did not achieve statistical significance, we could detect TCE in these animals unusually early—at 15 mo (4 of 14 animals or 29%, Table I), at which point the results were highly significant. Taken together, the above data strongly suggest that the presence of MHC class I greatly facilitates TCE development, that neither CD8 nor class II are essential, and that the absence of one of the MHC class I molecules results in higher TCE incidence.

Table I. TCE incidence in old B6 and mutant animals

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>TCE Incidence at 15 mo (%)</th>
<th>Significance compared to B6</th>
<th>TCE Incidence at 20–24 mo (%)</th>
<th>Significance compared to B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>0/64 (0%)</td>
<td>N/A</td>
<td>25/64 (39%)</td>
<td>N/A</td>
</tr>
<tr>
<td>β2-M-KO</td>
<td>Nd</td>
<td>N/A</td>
<td>1/15 (7%)</td>
<td>0.016</td>
</tr>
<tr>
<td>Class II-KO</td>
<td>Nd</td>
<td>N/A</td>
<td>2/10 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>CD8α-KO</td>
<td>Nd</td>
<td>N/A</td>
<td>3/15 (20%)</td>
<td>NS</td>
</tr>
<tr>
<td>Kb- or Db-KO</td>
<td>4/14 (29%)</td>
<td>0.001</td>
<td>11/22 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>ATX</td>
<td>11/27 (41%)</td>
<td>&gt;0.001</td>
<td>27/45 (60%)</td>
<td>0.035</td>
</tr>
<tr>
<td>IL-7R-KO</td>
<td>6/9 (67%)</td>
<td>&gt;0.001</td>
<td>7/8 (87%)*</td>
<td>0.019</td>
</tr>
<tr>
<td>TM-repeatedly immunized</td>
<td>2/7 (29%)</td>
<td>0.008</td>
<td>9/18 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>TCRβ-Tg</td>
<td>Nd</td>
<td>N/A</td>
<td>3/11 (27%)</td>
<td>NS</td>
</tr>
<tr>
<td>TCRα-Tg</td>
<td>Nd</td>
<td>N/A</td>
<td>2/3 (67%)</td>
<td>Nd</td>
</tr>
</tbody>
</table>

*Boldfaced are the statistically significant p values.

Incidence was determined by anti-TCRVβ mAb staining, as detailed in Materials and Methods, and, due to the incomplete coverage of the TCR repertoire, the numbers presented could underestimate the TCE frequencies. N/A: Not applicable; Nd, not done.

*p values were determined by Fisher’s exact test.

*IL-7R−/− were analyzed at 18 mo of age since their mortality rate increased dramatically after this age.

These animals were screened with the available anti-TCRV mAb (six available), which cover only about 20–25% of the TCR repertoire and the underestimation factor therefore likely larger than in other cases.

*No statistical analysis was performed due to a small number of animals.
maintenance. We first performed an extensive analysis of thymectomized mice. ATX prevents renewal of naive T cell compartment and is expected to result in increased compensatory proliferation and turnover of the naive T cells already present in the periphery (24). Moreover, such homeostatic proliferation is known to alter cell surface phenotype of naive T cells and to induce expression of several markers typically associated with memory cells (34, 35). We indeed found that T cell turnover was increased in ATX animals, as evidenced by increased incorporation of BrdU in vivo, which was again particularly pronounced in CD8 cells (Fig. 4A). Similarly, these animals contained increased percentages of memory-phenotype cells as judged by the expression of CD44 (Fig. 4B). In concert with our hypothesis, the incidence of TCE in these animals with age was more than twice that observed in control mice, reaching >90% in 24-mo-old animals, and the onset of TCE occurred much earlier than in the control animals. Indeed, whereas we can rarely detect any TCE before 18 mo of age, and whereas animals at 15 mo almost never contain TCE in control B6 animals, we could readily detect AI-TCE in 37% of ATX animals as young as 11 mo (Fig. 4C), and in 41% of animals at 15 mo (Table I). All of these values were significantly different compared with age-matched wt controls. An alternative explanation in this study could be that thymectomy may not merely lead to increased proliferation of T cells to compensate for the loss of naive cell production, but also remove regulatory populations important for control of homeostasis. To address this possibility, we investigated the percentages of CD25+ T regulatory cells, and found them not to differ between control and ATX animals with aging (difference <10%; data not shown). These results strongly imply that increased homeostatic proliferation of T cells in ATX animals leads to increased TCE formation.

The next model used was even more exaggerated with regard to homeostatic proliferation. Animals deficient in IL-7R (17) exhibit defects in early T cell development, affecting chiefly the CD4+CD8− double-negative 2 stage of intrathymic differentiation (36). The block in development is not absolute, however, and those cells that escape to the immature single-positive and double-positive stage can complete maturation and populate the periphery.

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** T cell homeostasis and phenotype in adult ATX animals and the incidence of age-related TCE. Animals were thymectomized at 6–8 wk of age and were aged subsequently. A, In vivo BrdU incorporation by adult CD4 and CD8 spleen cells from wt and ATX mice after 7 days of administration of BrdU in drinking water. Mean values ± SD from 3 animals/group are shown. Similar results were obtained in blood and LN. B, Representative CD44/CD122 profile of wt and ATX KO CD8+ cells of one B6 and one ATX animal. C, TCE incidence in the blood as a function of age in wt and ATX mice. Each time point contains 33–60 ATX animals and 30 B6 animals analyzed.

![Figure 5](http://www.jimmunol.org/)  
**FIGURE 5.** T cell homeostasis and phenotype in adult IL-7R-KO animals and the incidence of age-related TCE. A, In vivo BrdU incorporation by adult CD4 and CD8 spleen cells from wt and IL-7R-KO mice after 7 days of administration of BrdU in drinking water. Mean values ± SD from 3 animals/group are shown. B, Representative CD44/CD122 profile of wt and IL-7R-KO CD8+ cells of one B6 and one IL-7R-KO animal. C, TCE incidence in the blood as a function of age in wt and IL-7R-KO mice. Each time point contains 8–11 ATX animals and 30 B6 animals analyzed.
The peripheral compartment is heavily lymphopenic, and the existing T cells in adult mice exhibit intense homeostatic proliferation (Fig. 5A) and are nearly universally of the memory phenotype (Fig. 5B). As a consequence, even at 10 mo, these animals often exhibited AI-TCE, and the incidence of AI-TCE in IL-7R-KO animals reached 85–100% starting at 18 mo of age (Fig. 5C). These results were highly statistically significant (Table I), further lending support to our hypothesis that homeostatic proliferation critically contributes to TCE formation in specific pathogen-free mice.

TCR specificity, nonspecific proliferation, and AI-TCE formation

Having established that increased homeostatic proliferation can result in AI-TCE formation, we sought to test the role of Ag stimulation in TCE formation. The goal was to test whether repeated Ag stimulation can induce AR-TCE, as well as AI-TCE. To that effect, we used HSV-1, which in B6 mice elicits a vigorous CTL response. This response is almost entirely (>95% of CTL activity) directed against the immunodominant epitope gB-8p (gB495–502, SSIEFARL) (37). gB-8p-specific CTLs predominantly use Vβ10 (50–70%) and Vβ8 (20–25%) TCRs (38). Therefore, we reasoned that if repeated Ag stimulation can generate AR-TCE, and, perhaps, AI-TCE, we should be able to observe an increase in the percentage use of these two Vβ families following restimulation. Repeated immunization with the above immunodominant epitope using peptide emulsified in adjuvant (23) and started at 2 mo of age (8 injections total; see Materials and Methods) generated the expected Vβ10 and Vβ8 responses, but these responses did not appear to increase in frequency, compared with acutely immunized animals (9.8 ± 2.1% Tetramer⁺ CD8⁺ cells with repeated immunization, as opposed to 8.9 ± 2.7% upon single immunization). However, other TCE, bearing TCRs that used other Vβ segment families, appeared at an increased frequency (Fig. 6A). This increase was a direct function of adjuvant stimulation, because indistinguishable TCE frequencies were found in the groups treated with adjuvant alone (7 of 18 with peptide/adjuvant, 7 of 19 with adjuvant alone). The effect of adjuvant could be observed even when treatment was started relatively late in life, at 15 mo (data not shown), and again correlated to the adjuvant-induced T cell proliferation (Fig. 6B; adult animals). The increase in TCE incidence after adjuvant treatment at 18 and 26 mo, although clear, was not statistically significant with the numbers of mice analyzed;
however, analysis at 15 mo showed an early onset of TCE in these animals, but not in control B6 mice, which was statistically significant (Table I). We conclude that AI-TCE can be efficiently generated in response to homeostatic and/or nonspecific stimulation, whereas stimulation with Ag appears to give rise to these expansions less frequently under the conditions tested here.

We further addressed the role of TCR specificity in AI-TCE generation by imposing partial restriction upon the T cell repertoire by fixing TCRα or β parts of the receptor, using two strains of single-chain TCR Tg mice, where either TCRα or TCRβ were specific for the immunodominant HSV-1 gB peptide SSIEFARL (gB-8p), bound to H-2Kb. These lines, named gBT-1.3α and gBT-1.βNY, respectively, express the Tg on most, if not all, of their cells (Ref. 19 and Fig. 7), and a subset of their cells (up to 0.2% in gBT-1.α and up to 4% in gBT-1.βNY) is specific for the original peptide MHC combination even in the absence of any prior stimulation (data not shown), reinforcing the notion that the TCR repertoire in such animals is restricted.

These animals were then aged, and the incidence of TCE was examined. Both types of mice exhibited AI-TCE; 2 of 3 of analyzed TCRαTg and 3 of 11 TCRβTg animals exhibited AI-TCE by FCM analysis (Table I). In the case of TCRβTg, this analysis only encompassed the five available anti-TCRα mAb and therefore is likely to yield an underestimate of the actual TCE incidence. Analysis of larger animal numbers will be necessary to determine whether there is a statistically significant increase of AI-TCE incidence in the above TCRTg animals. Regardless, we can conclude from this data that full diversity of TCR repertoire is not necessary for the formation of AI-TCE, consistent with the idea that they dominantly arise in an Ag-independent manner.

Discussion
The above experiments have probed some of the germane issues related to the loss of homeostatic controls and reduction of diversity in the old age. Several salient points can be drawn from the presented results. First, development of functional CD8 lineage T cells is conducive to TCE formation, and our data add to the evidence that CD4 lineage T cells rarely produce large TCE. Although the requirement for class I was very strong, the presence of CD8 and MHC class II was not obligatory. The effect of MHC class II (modulation of TCE frequency by 50%), if significant, is probably indirect, because we cannot detect large CD4+ TCE in wt, MHC II-sufficient mice. This is consistent with the possibility that some TCE or TCE precursors benefit from experiencing CD4 T cell help at some point in their life span, but that other TCE can emerge and survive entirely independently from that help. This is consistent with the main conclusion of this work, which is that Ag-independent, homeostatic and/or bystander proliferation is an important, if not critical, contributor to the formation of TCE in mice. Other lines of evidence presented herein that support this contention were obtained from three different models (Kb-o or D8-KO mice, ATX mice, and IL-7R-KO mice). In all three strains, there is an age-related increase in TCE. In all three, CD8+ T cells turn over vigorously (and at higher rates than CD4+ T cells) and enter the memory compartment at increased rates, even in the young adult animals, due to increased homeostatic proliferation.

The incidence of these disturbances was significantly increased in all three models compared with wt B6 controls at 15 or at both 15 and 20–24 mo of age. We believe that the analysis of larger animal numbers would substantiate significant differences at latter time points, because at all times these models showed higher TCE incidence, even when statistical significance was not reached. It is possible that in one or more of these models, targeted disruption or experimental manipulation leads to loss of regulatory cells and/or mechanisms, and that this loss, rather than increased turnover, is responsible to TCE onset. Although we cannot formally exclude this possibility, we have ruled out its most obvious case, that CD25+ regulatory cells are differentially affected by ATX. Moreover, it is not clear how these three disparate models would all experience disturbances in the mechanism that controls homeostasis, other than the obvious and demonstrated fact that they have increased T cell turnover. We therefore believe that increased turnover in all three models best explains and is consistent with increased TCE incidence. That would be true even if the turnover is secondary to the loss of regulatory mechanisms.

Furthermore, repeated immunization experiments showed that repeated adjuvant administration facilitated the appearance of AI-TCE, but that peptide/adjuvant could not elicit AR-TCE, directed against the antigenic peptide. In this case, direct stimulation of cell turnover results in increased TCE incidence, suggesting that increased turnover is primary to the memory phenotype of cells turning over, rather than vice versa, and further strengthening our hypothesis. We believe that the number of divisions is the key determining factor in the onset of AI-TCE. In that regard, it is important to note that peptide (a nonreplicating stimulus) must be inducing a fairly limited number of divisions in a small subset of Ag-specific CD8+ T cells, as compared with the effects of a non-specific adjuvant (depo stimulation of many CD8 clones) or the effects of homeostatic proliferation. Additional experiments using chronic persistent viruses, which in humans have been implicated in age-related dysregulation of clonal homeostasis (39–42) as well as in shorter life span (43), are in progress to more stringently address the role of continuous Ag stimulation in the possible formation of AR-TCE, and in the possible emergence of AI-TCE from AR-TCE.

Acknowledgments
We thank Byung Park for statistical analysis; James Brien for help with figure preparation; and the members of the Nikolich laboratory for helpful discussion.

Disclosures
The authors have no financial conflict of interest.

References


17. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky,

Generation of mutant mice lacking surface expression of CD4 or CD8 gene
20. Messaoudi, I., J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
dent mice.

dent mice.

eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
gle-positive thymocytes requires the thymus to produce long-lived, functional T

antigen encounter triggers a developmental program in naive cells. Nat. Immuno-
malignant clonal expansions of CD8+ memory T cells in aged individuals. Immuno-
al-205: 170–189.

mision of a disrupted β2-microglobulin gene procured by homologous re-

pletion of CD4+ T cells in major histocompatibility complex class II-deficient

eration of mutant mice lacking surface expression of CD4 or CD8 gene

17. Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky,
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional

25. Messaoudi, I., L. J. LeMaoult, B. M. Metzner, M. J. Miley, D. H. Fremont, and
determination in vivo priming of murine CTLs by optimal MHC class I-restricted peptides
sin-
eallelic exclusion of a TCR β-chain minilocus occurs in the absence of a functional