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Two Separate Defects Affecting True Naive or Virtual Memory T Cell Precursors Combine To Reduce Naive T Cell Responses with Aging

Kristin R. Renkema, Gang Li, Angela Wu, Megan J. Smithey, and Janko Nikolich-Zugich

Infectious diseases remain among the leading causes of morbidity and mortality in older adults. T cells, critical for defense against intracellular pathogens, are profoundly affected by age (reviewed in Refs. 1, 2). Importantly, differences in the composition and maintenance of the T cell pool in mice are observed with aging in the absence of immunization (reviewed in Ref. 3). These changes result from an incompletely understood interplay of 1) reduced naive T cell production caused by thymic involution; 2) lifetime use of the existing naive T cells to respond to infections, including persistent latent infections; and 3) homeostatic mechanisms that normally attempt to balance and maintain T cell pools, but toward the end of life often distort an already reduced and diminished naive T cell pool (4, 5). Functional consequences of these changes for immune defense remain to be fully elucidated.

A diverse TCR repertoire is important for optimal protective responses to a variety of pathogens; holes in the TCR repertoire can result in reduced, absent, or ineffective immune responses (reviewed in Refs. 6, 7). The TCR repertoire becomes constricted with aging, but the extent, mechanisms, target populations, and the consequences for immune defense of this constriction remain unclear. Decreased thymic output requires naive CD8 T cells to rely upon homeostatic mechanisms to maintain the peripheral T cell pool, which may be particularly important in humans (8), and we understand relatively little about how the homeostatic mechanisms may change with aging.

We have reported that aging leads to >70% reduction of Ag-specific T cell precursors in unimmunized old mice, and that many of the remaining Ag-specific cells acquire a central memory–like CD44hiCD62LloCD11ahiCD127hiCD122hi phenotype and the immediate responsiveness to TCR ligation by IFN-γ secretion (9). Moreover, some of these precursors were preferentially maintained and survived, and then dominated the response to infection in old mice (9). Cells of the corresponding phenotype in adult mice were named “virtual memory” (VM) cells and were shown to respond to stimulation by superior proliferation and effector function compared with naive T cells in young animals (10). Because these cells persisted in germ-free adult mice (10) and responded briskly to IL-7 and IL-15, the authors concluded that they likely are generated/maintained by homeostatic cytokines.

In this study, we examined the rules guiding long-term maintenance of naive cells and the emergence of VM cells in unimmunized old mice. Naive Ag-specific precursors are very rare in unimmunized mice and are generally further reduced with aging to as few as a few 10s per animal, severely limiting experimental analysis. We therefore initially used TCR transgenic (TCRTg) mice, which provide abundant copies of a single clone of naive T cells and were shown to respond to stimulation by superior proliferation and effector function compared with naive T cells in young animals (10). Because these cells persisted in germ-free adult mice (10) and responded briskly to IL-7 and IL-15, the authors concluded that they likely are generated/maintained by homeostatic cytokines.

In this study, we demonstrated that unimmunized TCR transgenic (TCRTg) mice also undergo massive VM effects in both old wild-type and old TCRTg mice; that is, old VM, but not old true naive, T cells exhibited blunted TCR-mediated, but not IL-15–mediated, proliferation. This selective proliferative senescence correlated with increased apoptosis in old VM cells in response to peptide, but decreased apoptosis in response to homeostatic cytokines IL-7 and IL-15. Our results identify TCR as the key factor in differential maintenance and function of Ag-specific precursors in unimmunized mice with aging, and they demonstrate that two separate age-related defects—drastic reduction in true naive T cell precursors and impaired proliferative capacity of their VM cousins—combine to reduce naive T cell responses with aging.

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Abbreviations used in this article: EM, effector memory; KO, knockout; MFI, mean fluorescence intensity; rm, recombinant murine; TCRTg, TCR transgenic; TNA, true naive (CD44hiCD62Llo) precursor in unimmunized mice; TRAV, TCR V variable; VM, virtual memory (CD44hiCD62Llo) precursor in unimmunized mice; wt, wild-type.

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exposure to cognate Ag) CD8 T cells, which are differentially maintained with aging.

Materials and Methods

Mice

Mice were bred and maintained in the animal facility at the University of Arizona, and experiments were conducted under guidelines and approval of the Institutional Animal Care and Use Committee of the University of Arizona. B6.OT-I-Rag-knockout (KO) × B6.Ly5.1-F1, B6.P14.Rag-KO × B6.Thy.1.1 F1, and B6.gBT-I-Rag-KO × B6.Thy.1.1 F1 mice were bred from the stocks of B6.OT-I-Rag-KO (11), B6.OT-II (12), B6.Ly5.1, and B6.PL mice, purchased from Tacomic, the National Cancer Institute, and The Jackson Laboratory, respectively, and from P14 (13) and gBT-I (14) stocks provided to us by Dr. H.P. Pircher via Dr. J.A. Frelinger and by Dr. F.R. Carbone, respectively. Old (18–23 mo) C57BL/6 (B6) mice were purchased from the National Institute on Aging, and adult (12 wk) B6 mice were purchased from The Jackson Laboratory. Mice with large spleens or other obvious abnormalities (e.g., tumors) were excluded from the study. Survival bleeds were performed retro-orbitally.

Flow cytometry

Samples were prepared as previously described (15). We used fluorochrome-conjugated Ab clones: CD8α (53-6.7), CD4 (RM-4), CD45 (IM7), CD62L (MEL-14), Vβ3.1/5.2 (MR9-4), Vvo2 (B20.1), IFN-γ (XM1.2), CD3 (17A2), TCRβ (H57-597), CD5 (53-7.3), PD-1 (29F.1A12), LAG3 (C9B7W), CD122 (TM-β1), IL-4R (mIL4R-M1), phospho-ser139-H2AX (2F3), and CD49d (R1-2), purchased from eBioscience, Invitrogen, BD Biosciences, or BioLegend. Samples were analyzed on a four-laser custom Fortessa cytometer (BD Immunocytometry Systems, Sunnyvale, CA) using the FACSDiva acquisition (BD Immunocytometry Systems) and FlowJo (Tree Star, Ashland, OR) analysis software. For intracellular cytokine staining, samples were stimulated and prepared exactly as previously described (15).

Cell sorting and culture

Prior to sorting, CD8 T cells were enriched by magnetic separation (Miltenyi Biotec) using an autoMACS Pro. CD44hi VM and CD44lo TNa CD8 T cells were sorted based on CD44 and CD62L expression using a BD FACSaria. The sorted cells were stained with CFSE as described (15). The cells were cultured at 8 × 10^5 cells/ml in RPMI 1640 complete medium plus 100 U/ml recombinant murine (rm)IL-2 (eBioscience), with either 10−7 M SINPIFKL peptide or 10 ng/ml rmIL-12 (R&D Systems) and 10 ng/ml rmIL-18 (eBioscience) for 5 d. Alternatively, cells were cultured with 10 ng/ml IL-7 (eBioscience) and 100 ng/ml IL-15 (eBioscience). For polyclonal stimulation, cells were cultured at 8 × 10^5 cells/ml in RPMI 1640 complete medium 1:1 with anti-CD3/anti-CD28 immobilized on beads (Miltenyi Biotech) and 100 U/ml rmIL-2 (eBioscience). On each day of analysis, cells were Live/Dead stained (Invitrogen) according to the manufacturer’s instructions, stained with surface Abs, fixed/permeabilized with Foxp3 Fix/Perm, and stained with intracellular Ab. For cell cycle analysis, cells were stained with Vybrant DyeCycle Orange (Life Technologies) at 1.25 × 10^5 mM for 60 min at room temperature and analyzed immediately. Cells were enumerated using CountBright absolute counting beads (LifeTechnologies) according to the manufacturer’s instructions.

TCRα PCR

Five thousand CD44hi and CD44lo cells were sorted from OT-I mice as described above. RNA isolation was performed by standard chloroform/isopropanol purification. For CDNA synthesis, 15 μl containing 10 μl RNA (500 ng–5 μg), 1.5 μl oligo(dT)20, 1.5 μl 10 mM dNTP, and 2 μl H2O was incubated at 65°C for 5 min and then at 4°C for 2 min. Three microliters 10× PCR buffer, 6 μl 25 mM MgCl2, 0.15 μl 0.1 M DTT, 1.5 μl RNaseOut (Life Technologies), and 1.5 μl SuperScript III reverse transcriptase (Life Technologies) were added and incubated at 50°C for 50 min and then at 85°C for 5 min. RNaseH (1.5 μl; Life Technologies) was added and the mixture was incubated at 37°C for 20 min. TCR α variable (TRAV) primers and PCR reaction were as in Kedel and Mescher (16) using Platinum Taq (Life Technologies). Each primer was used separately at 5 μM final concentration. PCR conditions were 95°C for 5 sec followed by 40 cycles at 95°C for 20 s, 56°C for 20 s, and 72°C for 20 s. PCR products were visualized on a 1.8% agarose gel, and band intensities were calculated using Quantity One software (Bio-Rad). Bands were enumerated by blind scoring by three independent examiners, who scored the presence or absence of bands.

Enrichment of tetramer+ CD8 T cells from wt B6 mice

The tetramer-enrichment protocol was performed exactly as in Rudd et al. (9) using the spleen, inguinal, cervical, and axillary lymph nodes harvested from individual mice.

Statistical analysis

Following the analysis of flow cytometry data using FlowJo software, GraphPad Prism was used for statistical analysis (unpaired Student t test, paired Student t test, linear regression and best fit line, and one-way ANOVA with a Bonferroni posttest). A p value <0.05 was considered statistically significant.

Results

Profound age-related conversion into VM cells in TCRtg CD8 T cells from unimmunized mice

We examined phenotypic changes with aging in a single, large clone of unimmunized CD8 T cells using OT-I Rag+ mice, expressing a transgenic TCR specific for OVA257-264: H-2Kb (11). Blood from 10-, 12-, 18-, and 22-mo-old unimmunized OT-I mice contained progressively increased CD44hi T cell fraction compared with 2- to 4-mo-old mice (Fig. 1A), and this was paralleled by an increase in IFN-γ-producing cells responding to the cognate peptide (Fig. 1B). Large variability was observed in VM conversion in individual 10- to 12-mo-old mice (blood CD8CD44hi, 10–100%). However, with advanced age (in the rare OT-I mice surviving to 18–22 mo) we found uniform and high representation of CD44hi cells (>75%; Fig. 1A). In the experiments below we often used mice younger than the National Institute on Aging–defined age cutoff of 18 mo, because 1) OT-I mice, in our colony, have a median lifespan of 15–16 mo (not shown; colony B6 mice, 26–30 mo), which renders OT-I mice >18-mo-old extremely scarce; 2) 10- to 14-mo-old OT-I mice showed a range of VM conversion from <20 to >70%, allowing us to analyze the results of functional and other assays in individual animals and test whether the VM conversion correlated with functional responsiveness (Fig. 1C); finally, 3) interventions to improve immunity would be implemented not only in old age, but likely also, if not preferentially, in middle-aged individuals that still exhibit reasonable immune function. Importantly, all of our key observations were reproduced in bona fide old TCRtg and/or wt mice.

VM cells were reported to be of central memory (CM) CD44hi 62Lhi phenotype (9). Likewise, CD44hi cells in TCRtg mice were overwhelmingly CD62Lhi (Fig. 1D), and CD44hi 62b0 effector memory (EM) cells did not significantly accumulate with age (Fig. 1E). Indeed, frequency of VM, but not EM, cells tightly correlated with functional responsiveness (Fig. 1C); finally, 3) interventions to improve immunity would be implemented not only in old age, but likely also, if not preferentially, in middle-aged individuals that still exhibit reasonable immune function. Importantly, all of our key observations were reproduced in bona fide old TCRtg and/or wt mice. The tetrramerefficiency protocol was performed exactly as in Rudd et al. (9) using the spleen, inguinal, cervical, and axillary lymph nodes harvested from individual mice.
experiments also in the spleen; for 18- to 22-mo-old mice, zero of one experiment showed lymphopenia in blood or spleen). Collectively, these data show that TCRTg VM cells expressing the CM phenotype increase with age in absolute terms.

VM cells in both adult (10) and old (9) mice exhibit strong immediate effector function, unlike their TNa CD44lo counterparts. We found that the age of OT-I CD8 T cells and the CD44lo phenotype correlated tightly with the acquisition of immediate cytokine production in these cognate Ag-inexperienced cells upon peptide stimulation (Fig. 1C). These results show that with aging TCRTg T cells exhibit generalized increased phenotypic and functional conversion into VM cells, similar to T cell precursors in aging wt mice (9).

Cytokine receptor expression in VM cells of TCRTg mice with aging

Even in unimmunized wt or germ-free adult mice, 10–20% CD8 cells convert into the CD44hi phenotype, likely due to stimulation by IL-7 during the neonatal period in the periphery (10, 17) and stimulation by NK-T cell–derived IL-4 during the neonatal period in the peripheral lymph nodes. To assess whether TCRTg VM cells show signs of differential cytokine maintenance, we examined expression of CD122, CD127, and IL-4R on TCRTg VM cells. We found variable expression of the IL-4R on VM cells in TCRTg mice, and the expression of CD127 (IL-7Rα–chain) did not reproducibly correlate to the VM phenotype (not shown). By contrast, 14-mo-old OT-I mice expressed low levels of CD122 (Fig. 2D; 8- to 14-mo-old samples in (A) and (B), (D) OT-I mice gain VM (CD44hi CD62Llo) and not EM (CD44hi CD62lo) cells with age. (E) Percentage of CD44hi (α4 integrin) CD8 T cells for splenic VM, EM, and CD44lo 14-mo-old OT-I populations. Populations indicated on the y-axis were gated on CD5+CD8+CD4– cells. Data are shown as means ± SEM. (A–D) n = 10–31 for 2, 4, 10, and 12 mo; n = 3–4 for 18 and 22 mo. Data were combined from three experiments. (E) n = 5, representative of two experiments. ***p < 0.001 by (A, B, E) one-way ANOVA with Bonferroni posttest and (D) unpaired Student t test.

Loss of the original TCRTg specificity in aged TCRTg T cells is critical for age-related VM conversion

Naive precursors in young mice rely upon trophic signals from both TCR (recognition of self pMHC ligands) and homeostatic cytokines for maintenance and survival (19). These parameters may change with time: the self or environmental pMHC universe (e.g., food Ag, normal flora-derived Ag) could evolve with aging owing to differential colonization, aberrant gene expression, and other factors; likewise, homeostatic cytokine availability, T cell repertoire, abundance (particularly loss of naive T cells (4, 5), and cellular responsiveness to key homeostatic cytokines (9) could all be altered by aging.

We evaluated the expression of TCRTg chains relative to the acquisition of the VM phenotype. Transgenic TCRββ was stably expressed between 4 and 18 mo of age (not shown). Young (4 mo old, Supplemental Fig. 2A) OT-I CD8 T cells were almost exclusively (>99.8%) VαZβ and exhibited a relatively low (<20%) and stable fraction of CD44hi cells (Fig. 1). However, older OT-I mice contained a significant fraction of VαZβ and VαZβ populations (Supplemental Fig. 2A). Loss of Vα2 was accompanied by retention of overall TCR expression (pan-TCRβ staining, Supplemental Fig. 2A), suggesting that these CD8 T cells expressed another TCRα-chain and another potential TCR specificity, a scenario that physiologically occurs on up to ~30% of human and 15% murine T cells (20–22). Importantly, the VαZβ phenotype correlated significantly with the CD44hi phenotype in 12-mo-old OT-I mice (Supplemental Fig. 2C).

To examine directly the role of TCRα replacement, we sorted CD44hi (TNa) and VM cells from mice of different ages in the presence or the absence of the Rag recombinase and performed PCR amplification of different TRAV gene rearrangements. As expected, 14-mo-old OT-I Rag-KO mice exhibited a dominant TRAV14 band (encoding the TCRβ2 protein) and very few other bands (Fig. 2A); importantly, there was no difference in the number of bands in Rag-KO mice regardless of the age or the CD44 phenotype (Fig. 2D), indicating that these mice, as expected, cannot rearrange endogenous TRAV genes (bands on the gels that are appearing in a few TRAV families are artifacts of the PCR). Moreover, we found comparable low levels of endogenous
TCRα rearrangements in 3-mo-old VM samples, suggesting that young adult VM cells also do not exhibit pronounced endogenous rearrangements (Fig. 2B), in accordance with the flow cytometry analysis of protein expression (Supplemental Fig. 2). TNa cells from 14-mo-old OT-I mice also did not carry more rearrangements compared with their younger counterparts (Fig. 2C, top). In contrast, VM cells at 14 mo of age contained rearrangements in many different TRAV families (see example in Fig. 4C, bottom). When quantified across groups of animals (by blind examination of three independent examiners) VM cells from old Rag-sufficient OT-I mice exhibited significantly increased expression of alternative TCRα families when compared with all other populations analyzed (Fig. 2D).

To examine whether the presence of secondary TRAV rearrangements impacted the frequency of VM cells across the lifespan, we aged different TCRTg strains on a Rag-KO background. Up to 19 mo of age, the three different TCRTg Rag-KO mouse strains examined—OT-I, P14 (23), and gBT-I (14)—did not downregulate the Vα2 chain and also maintained a small (<5%) VM population that did not increase with age (Fig. 2E, filled bars), consistent with the results of Haluszczak et al. (10). This sharply contrasted with progressive, age-related VM accumulation in Rag+ OT-I mice, reaching >50% of all cells (Fig. 2E, open bars). Therefore, we conclude that secondary TCR rearrangements and, therefore, the TCR-mediated signals are essential for the age-related dominance of VM CD8 cells with, but are not necessary for the generation of, neonatal VM cells in TCRTg mice.

**Proliferative potential of VM cells declines with age in response to TCR but not to cytokine stimulation**

We next examined functional responses of VM CD8 T cells with aging. Because VM cells exhibit increased expression of homeostatic cytokine receptors (Supplemental Fig. 1), we tested whether these cells were more sensitive to IL-7/IL-15. Sorted, >98% pure VM (CD44hi) and CD44lo CD8 T cells from adult and old OT-I Rag+ mice were labeled with CFSE and cultured with IL-7/IL-15 for up to 7 d. At both 5 d (not shown) and 7 d of culture (Fig. 3) VM (CD44hi) CD8 T cells from both 3- and 14-mo-old mice proliferated better than did their TNa (CD44lo) counterparts based on percentages (Fig. 3A, 3B) and counts (not shown) of cells reaching fourth division and above. Moreover, compared with their 3-mo-old counterparts, both VM (Fig. 3C) and TNa (Fig. 3D) cells from 14-mo-old mice exhibited significantly higher propensity to divide four or more times in response to IL-7 and IL-15. Therefore, VM OT-I cells exhibit increased sensitivity to homeostatic cytokines, which increased with age and could contribute to their age-related accumulation.

Our results (Fig. 2) indicated that VM cell phenotype and accumulation in old mice must be driven/maintained by TCR signals.
During a lifetime, even low-level proliferation/turnover could exert cumulative effects on old T cells. To examine functional impact of this interaction upon TCR-driven functions, sorted >98% pure VM and TNa cells from 3- and 14-mo-old OT-I mice were stimulated in vitro with the cognate SIINFEKL peptide, or with IL-12 plus IL-18, to elicit IFN-γ secretion and proliferation. An increased proportion of VM OT-I T cells rapidly produced IFN-γ in response to both types of stimulation, compared with TNa counterparts (Fig. 4A, 4B), as was seen in WT VM cells (9, 10). Moreover, aging again accentuated this phenotype, because 14-mo-old VM cells produced IFN-γ at a much higher frequency (~30%) compared with their 3-mo-old counterparts (5–7%; Fig. 4B).

As seen in young adult VM cells from wt mice (10), young adult TCRTg VM CD8 T cells exhibited significantly enhanced proliferation when compared with TNa cells at both day 3 (not shown) and day 5 of culture (Fig. 4C). Importantly, 14-mo-old (Fig. 4D) or older (not shown) VM cells exhibited delayed and reduced entry into advanced cell divisions, and these cells were clearly outperformed by old TNa cells in proliferation on both days 3 (not shown) and 5 after stimulation (Fig. 4D). Collectively, these results suggest that aging leads to selective proliferative impairment; old OT-I VM cells proliferate more than did their TNa counterparts in response to homeostatic cytokines (likely explaining their gradual dominance with time) but are less capable of proliferating in response to cognate peptide (TCR) stimulation. To assess whether the proliferation difference between bulk adult and old naive T cells may be due to differential behavior of TNa and VM cells, we directly compared adult and old VM (Fig. 4E) and TNa cells and found no significant differences between adult and old TNa cells (Fig. 4F). We were surprised that old TNa cells proliferated similarly to their adult counterparts. However, the current literature never distinguished between VM and TNa cells when comparing proliferation between adult and old T cells (24–27).

**Old VM cells exhibit decreased viability when responding to peptide, but increased viability when responding to homeostatic cytokines**

We next sought to address why old VM cells did not proliferate as extensively as did old TNa cells by examining cell cycle progression and survival in the course of proliferation. In response to peptide stimulation, we saw no difference in the ability of VM cells to enter into cell cycle or to cross to different phases of the cycle when compared with TNa cells (Fig. 5A). However, when we measured viability, we found increased cell death in old VM cells when compared with TNa cells (Fig. 5B, 5C), which corresponded to very few live VM cells by day 3 (Fig. 5D). In contrast, we found that VM cells stimulated with IL-7 and IL-15 exhibited superior survival and reduced cell death when compared with TNa cells (Fig. 5E), which resulted in increased cell numbers (Fig. 5F). These results strongly support our findings in Fig. 3, suggesting that survival advantage in response to cytokines and proliferative capacity in response to antigenic peptide may be dissociated in old VM cells.

**Old VM cells proliferate extensively to polyclonal stimulation**

We further investigated proliferative characteristics of the old VM cells in response to cognate TCR stimulation in relationship to their in vivo accumulation. These cells are more sensitive to homeostatic cytokines IL-7 and IL-15 (Fig. 3) and have increased expression of CD122 (Supplemental Fig. 1). Because alternative TCRs may also play a role (Fig. 2), we were interested in whether old VM cells could proliferate to polyclonal stimulation. We stimulated sorted splenic old OT-I Rag+ VM and TNa CD8 T cells with anti-CD3 and anti-CD28 and found old VM cells proliferated similarly to TNa cells (Fig. 6A), and in fact had significantly increased frequency of cells in later cell divisions. This is likely due to increased viability; that is, we found that old VM cells stimulated with anti-CD3 and anti-CD28 exhibit increased viability compared with VM cells stimulated with peptide (Fig. 6B).

**Old precursors from unimmunized wt mice exhibit the same VM conversion and CD122 upregulation seen in TCRTg precursors**

One could argue that many of the above observations could be an artifact of the OT-I TCRTg model. To assess whether the above findings from TCRTg mice also extended to wt mice, we isolated different Ag-specific CD8 T cells from unimmunized adult (2–3 mo of age) and old (18–23 mo of age) mice using the tetramer enrichment method (28) as in our prior work (9). As expected (9), the frequency of CD44hi VM CD8 T cell precursors specific for B8R (poxyvirus immunodominant epitope) (29), OVA, and the West Nile virus NS4b (30) increased with age (9) (Fig. 7A). Importantly, with age, all three types of precursors also exhibited significantly higher...
Expression of the IL-2/IL-15Rβ-chain (CD122; Fig. 7B), consistent with findings from aged OT-I mice (Supplemental Fig. 1). We have also found that old wt VM cells proliferate worse than TNa counterparts both in vitro and in vivo (K.R. Renkema, G. Li, M.J. Smithey, and J. Nikolich-Zugich, manuscript in preparation). Collectively, these results demonstrate the essential similarities found between the TCRTg and wt old VM T cell precursors.

Expression of inhibitory receptors on TCRTg CD8 VM cells with aging

Several additional mechanisms could contribute to cellular proliferative senescence, including exhaustion/anergy; an age-dependent increase in inhibitory and exhaustion receptors on CD8 T cells was reported (reviewed in Ref. 31), even on naive precursors in unimmunized mice (32). Although we observed a significant increase in the frequency of both PD-1+ (Supplemental Fig. 3A, 3C) and LAG3+ (Supplemental Fig. 3B) OT-I T cells at 14 mo of age compared with adults (Supplemental Fig. 3A, 3B), the representation of cells expressing these markers remained modest (>20% in OT-I 14-mo-old mice, with the exception of a single outlier, Supplemental Fig. 3A; and also in Tet+ 20-mo-old wt CD8 T cells, Supplemental Fig. 3C). There were no significant changes in the expression of 2B4 on these cells from 2 to 14 mo of age or on wt T cells up to 24 mo of age (not shown). We conclude that these inhibitory markers are unlikely to be the sole (or perhaps even the main) explanation for the proliferation defects in aging VM precursors, particularly because we have seen them expressed exclusively on the EM (CD44hi62Lhi) and not the CM/VM (CD44hi62Llo) fraction of CD44hi CD8 T cell precursors (not shown). They could, however, contribute to the overall reduction in proliferative capacity in old VM precursors.

Discussion

In this study, we made four discoveries germane to our understanding of aging and homeostatic regulation of naive T cell pools and their function. First, we found that TCR signals are key to VM conversion of TCRTg cells with aging, because fixing the original TCRTg specificity on the Rag-KO background prevented accumulation of VM cells with aging. This significantly strengthens and molecularly defines the idea (9) that there is TCR-based selection in the naive and VM T cell pool with aging. Second, we found that IL-15R (CD122) was increased with aging in both TCRTg and wt VM cells, and that this resulted in strong proliferation of such cells to IL-15, which overshadowed their TNa counterparts, providing a mechanism for their preferential outgrowth over TNa cells. Third, whereas the response to homeostatic cytokines was robust, old VM T cell precursors exhibited selective replicative impairment in response to TCR signals relative to TNa counterparts. Fourth, we found that this impairment was associated to increased apoptosis specifically in response to peptide stimulation, but decreased apoptosis in response to homeostatic cytokines in old VM compared with old TNa cells. All this provides strong evidence that the naive T cell compartment in aging fails to respond properly to challenge due to two different intrinsic defects: 1) drastic loss of relatively proliferatively intact TNa precursors, and 2) selective functional proliferative impairment of VM T cell precursors responding to TCR stimulation.

Our present results, in the context of data from the literature (9, 10, 17, 18, 32), shed new light on long-term maintenance of T cell precursor pools with aging. Collectively, there is evidence that there may be at least four distinct pools of naive (indicating lack of prior contact with cognate Ag) CD8 T cell precursors in old mice and...
that their maintenance with age is differentially regulated. The TNa CD44hi/CD8+precursors are severely depleted by the process of aging (9, 32), suggesting that either the key maintenance factors for these cells are insufficient in old animals, or that age-related lymphopenia drives these cells to convert to VM cells, or both. However, these cells retain much of the proliferative function in middle-aged (12 to 14 mo old) TCRTg mice (this study) and in old wt mice (K.R. Renkema, G. Li, M.J. Smithie and J. Nikolich-Zugich, manuscript in preparation). The IL-4-dependent innate memory cells (18, 33) arise in response to IL-4 produced by NK-T cells and are prominent in BALB/c but not B6 thymi. IL-4 deficiency in young B6 mice reduces by ~30–40% the Ag-specific precursor pool (17). Therefore, the IL-4 innate memory cells contribute to the peripheral pool in B6 mice. Their functional properties in adult and old mice remain to be elucidated, although we did not notice a significant increase in IL-4R+ cells with aging (not shown). These cells are absent in TCRTg mice and therefore were not the subject of the present study. TCRTg Rag-/- mice in our study had a small (<5%) age-insensitive population of VM cells that are most analogous to the cells that expand during the neonatal period in the periphery (17). These VM cells showed to be independent of IL-4 (17) and are most likely expanded by IL-7 immediately upon egress into an empty periphery of a neonate (10, 17). We provisionally name these cells IL-7VM. Of interest, whereas the overall percentage and number of VM cells in TCRTg Rag-/- mice increased with aging, this was not the case with the IL-7VM cells in Rag-/- mice.

That led us to conclude that the original specificities of the TCRTg receptors analyzed in this study were not conducive to age-related VM accumulation on their own, and that VM cells in TCRTg mice depend on the secondary rearrangements that produce other TCR specificities. T cells expressing dual TCRs could be reactive to alternate Ags (34, 35). These cells, perhaps due to self-reactivity, gut flora, or other environmental Ag reactivity or cross-reactivity (36), exhibited the VM phenotype, and we call them here aging-related VM cells. Finally, whereas ~50% of 10- to 14-mo-old OT-I T cells are CD44hi (Fig. 1A), only ~7–15% are Vα2hi or Vα2lo (Supplemental Fig. 1), begging an explanation of why many of the remaining Vα2hi cells still convert to VM. Because very little VM conversion occurs in TCRTg Rag+ mice, we speculate that many of the Vα2hi cells also may express dual TCRs. Some have used the endogenous Vα2 to replace the transgenic Vα2 chain (the mAb used does not distinguish between these), whereas the others would express the secondary, endogenous Vα at levels below flow cytometry detection, but still sufficient to provide the VM-converting signal over time. Single-cell analysis will be necessary to address these possibilities. Of importance, our preliminary analysis suggests that secondary rearrangements also play a role in VM conversion in precursors isolated from unimmunized wt mice (K.R. Renkema and J. Nikolich-Zugich, unpublished observations).

Recently, it was shown that in young adult B6 mice deficient in secondary rearrangements (the TCRα+/- genotype) VM cells persist at the same frequency as in wt mice (~16% of the total naive Ag-specific precursors) (17). We would predict that upon aging such mice may exhibit reduced VM accumulation, but again sequencing of TRAV in a polyclonal TCRα+/- and TCRα-/-
setting will be needed to establish whether dual TCRαs rearrangements are necessary for VM accumulation in the polyclonal TCR repertoire.

In initial publications it was suggested that aging may not adversely affect TCRα T cells; that is, old TCRα T cells were found to be functionally comparable to adult T cells in a variety of strains (37–39) using bulk T cell functional assays. This stood in contrast to old T cells from wt mice, which have long been known to exhibit defects in proliferation and differentiation (40–42; reviewed in Ref. 31). These results can be now explained by the likely confounding effect of massive contamination with functionally mature VM T cells, which have not been separated and separately tested in these studies, and which would account for robust immediate effector function, as well as by the presence of sufficient numbers of TNa cells (due to the artifact of extremely high precursor frequencies in TCRα T mice), which would robustly proliferate in bulk assays.

Perhaps most importantly, our results show that VM conversion is not innocuous for an aging precursor. Whereas in youth VM cells exhibit functionally robust proliferation (10), by middle age their proliferative ability declines (this study) and is even worse in old wt mice (K.R. Renkema, G. Li, M.J. Smithey and J. Nikolich-Zugich, manuscript in preparation). Replicative senescence in vitro occurs owing to a finite number of cell divisions (10–60, depending on species and cell type) and eventual cell cycle arrest (43). In vivo existence and relevance of replicative senescence in lymphocytes remain controversial. In this study, we described a “selective” replicative impairment in old VM CD8 T cells, which exhibit robust proliferation in response to homeostatic cytokines (IL-7 plus IL-15) and can rapidly produce cytokines upon cognate peptide or inflammatory cytokine (IL-12 plus IL-18) activation, but proliferate significantly worse compared with their CD4+ TNA counterparts when activated with cognate peptide. Selective survival differences in response to peptide stimulation and to IL-7/IL-15 homeostatic cytokines appear to underlie these observations, but additional work will be necessary to mechanistically dissect their signaling basis. The other potential culprit for blunted proliferative responses, the accumulation of inhibitory receptors, showed less impressive differences between VM and TNa cells, albeit its functional relevance remains to be tested. Reversing blunted lymphocyte responses with aging remains an implicit goal of this field, and its relevance remains to be tested. Reversing blunted lymphocyte responses with aging remains an implicit goal of this field, and its relevance remains to be tested. Reversing blunted lymphocyte responses with aging remains an implicit goal of this field, and its relevance remains to be tested. Reversing blunted lymphocyte responses with aging remains an implicit goal of this field, and its relevance remains to be tested.

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Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. *J. Exp. Med.* 201: 95–104.


